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# The Role of Wnt Signaling in Oligodendrocyte Development

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## Abstract

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Judith Grinspan, Ph.D.

The developmental regulation of the central nervous system is established through a complex network of signaling factors that control the timing of cell generation, specification, migration, and differentiation. Oligodendrocytes, the myelinating cells of the central nervous system, originate during embryonic stages in ventricular zones and are responsive to many of these inductive and inhibitory signals as they migrate, mature, and interact with axons. The specific timing and regulation of these actions are critical to axonal conductance. Many signals that promote oligodendrocyte specification and differentiation are active ventrally, and more recent studies have examined the less well known actions of several inhibitory dorsal signaling factors, among these the Bone morphogenic proteins (BMPs) and Wnt signaling factors.

I investigated the role of the canonical Wnt signaling pathway in oligodendrocyte development, both *in vivo* and *in vitro*. In primary oligodendrocyte precursor culture systems, canonical Wnt activity inhibits the differentiation of precursors, maintaining them in the precursor stage without causing a change in proliferation, cell death, or cell fate determination. Following these studies, I generated mice with a gain of function Wnt signaling mutation targeted to cells of oligodendroglial lineage. These mice had significant delays in myelin forming cell development early in development, but seemed to recover upon reaching adulthood. Together, these findings indicate that Wnt activity is sufficient to delay oligodendrocyte maturation *in vivo* and *in vivo*.

I also investigated the interaction between the Wnt and BMP signaling pathways during oligodendrocyte development. Using pharmacological and genetic paradigms, I found that when the canonical BMP signaling pathway is abrogated, neither BMP nor Wnt manipulation has any effect on oligodendrocyte differentiation in culture. In contrast, blocking the canonical Wnt signaling pathway does not limit the activities of BMP, suggesting that the effect of Wnt signaling on oligodendrocyte development is dependent on BMP signaling, but not vice versa. These two facets of my thesis offer insight into the signaling mechanisms regulating the timing of oligodendrocyte development, and could have implications for the treatment of demyelinating disorders and central nervous system injury.



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# THE ROLE OF WNT SIGNALING IN OLIGODENDROCYTE DEVELOPMENT

Keith A. Feigenson

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## Dedication

I dedicate my thesis to my family, who provided the love and support, even at the hardest times, which enabled me to complete this work.

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## Abstract

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I investigated the role of the canonical Wnt signaling pathway in oligodendrocyte development, both *in vivo* and *in vitro*. In primary oligodendrocyte precursor culture systems, canonical Wnt activity inhibits the differentiation of precursors, maintaining them in the precursor stage without causing a change in proliferation, cell death, or cell fate determination. Following these studies, I generated mice with a gain of function Wnt signaling mutation targeted to cells of oligodendroglial lineage. These mice had significant delays in myelin forming cell development early in development, but seemed

to recover upon reaching adulthood. Together, these findings indicate that Wnt activity is sufficient to delay oligodendrocyte maturation *in vivo* and *in vivo*.

I also investigated the interaction between the Wnt and BMP signaling pathways during oligodendrocyte development. Using pharmacological and genetic paradigms, I found that when the canonical BMP signaling pathway is abrogated, neither BMP nor Wnt manipulation has any effect on oligodendrocyte differentiation in culture. In contrast, blocking the canonical Wnt signaling pathway does not limit the activities of BMP, suggesting that the effect of Wnt signaling on oligodendrocyte development is dependent on BMP signaling, but not vice versa. These two facets of my thesis offer insight into the signaling mechanisms regulating the timing of oligodendrocyte development, and could have implications for the treatment of demyelinating disorders and central nervous system injury.

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## Introduction

### Thesis goals and rationale

Oligodendrocyte (OL) development is regulated by inhibitive and inductive signaling factors in the local environment. The generation of oligodendrocyte precursor cells (OPCs) in ventral regions and the relatively late appearance of specific populations of OLs in dorsal regions of the neural tube have long suggested these areas contain inhibitors of OL maturation and specification, originally shown by Wada et al. (Wada et al., 2000). One group of signaling molecules present in the dorsal neural tubes is the Wnt family of secreted factors, which are well known to have effects on many developmental and cellular processes, including proliferation, specification, and patterning. For these reasons, the Wnts are attractive candidates to be inhibitors of OL maturation.

Early experiments showed that Wnt signaling inhibited OL differentiation *in vitro*, but the extent of this inhibition was unknown, as was the specific mechanism of action (Shimizu et al., 2005; Kim et al., 2008). The first aim of my thesis was to investigate to what extent gain and loss of function Wnt signaling affected OL development *in vivo* and *in vitro*. My second aim was to analyze the interaction between Wnt signaling and the other major family of dorsal signaling factors, the bone morphogenic proteins (BMPs). Taken together, the results of my thesis experiment identify a role for Wnt in central nervous system (CNS) myelination.

### Oligodendrocyte background

Myelin composes the insulating, fatty membrane that wraps axons and allows neural signals to be conducted quickly and efficiently. In the vertebrate CNS, myelin is

synthesized by OLs as part of their plasma membrane (Bunge, 1968). Damage to the myelin sheath results in improper signal communication, leading to axonal degeneration and impaired neural functioning (Waxman et al., 1991). White matter regions of the CNS are composed largely of axons and myelin and are disturbed in a number of disorders, generally associated with three types of pathology: autoimmune disorders (such has multiple sclerosis), genetic disorders (such as Pelizaeus Merzbacher Disease), and injury (such as periventricular leukomalacia). Recently, alterations in myelin or white matter have been associated with other diseases and disorders, such as schizophrenia, bipolar disorder, and autism spectrum disorder (Volpe, 2001; Herbert et al., 2003; Barley et al., 2009; Mahon et al., 2010). Understanding the regulatory components that generate mature OLs under normal developmental conditions is crucial to forming treatments for such disorders.

Oligodendrocyte precursor cells (OPCs) originate in the pMN domain of the ventral spinal cord as early as E12.5 in rodents (Warf et al., 1991; Pringle and Richardson, 1993; Ono et al., 1995). Later in development, small populations of OPCs are generated in dorsal regions of the spinal cord, hindbrain, and telencephalon (Cameron-Curry and LeDouarin, 1995). After generation, OPCs migrate radially and dorsally until they come into contact with nascent axons, during which time they are exposed to a spectrum of signaling factors, influencing their migration, proliferation, differentiation, and axonal interaction. More signals contribute to the process of wrapping the myelin sheath around axons, promoting survival, negotiating appropriate positions, and binding membrane proteins (Lubetzki et al., 1993; Colognato, 2002; Chan et al., 2004; Piaton et al., 2010). Each mature OL is capable of myelinating segments of up to

40 distinct axons. Myelination is not complete until several weeks after birth in rodents and up to 30 years in humans (Lajtha et al., 1977).

Two steps characterize OL development: specification of multipotent neural precursor cells to the oligodendroglial lineage and the ensuing differentiation of these cells into fully functional OLs. In both cases, transcription factors and growth factors play large roles as both activators and repressors of fate induction (Fig 1.1). Initial transplantation and injury studies indicated that the notochord contains OPC inductors, such as Sonic Hedgehog (Shh), a major ventralizing signal generated in the notochord and floorplate (Echelard et al., 1993; Roelink et al., 1994; Roelink et al., 1995; Trousse et al., 1995; Orentas and Miller, 1996; Pringle et al., 1996; Maier and Miller, 1997). Shh signaling induces multipotent neuroepithelial cells to commit to the motor neuron or OPC lineage in a concentration and timing specific manner, depending on a combination of subsequently active transcription factors (Roelink et al., 1994; Roelink et al., 1995; Pringle et al., 1996; Richardson et al., 1997; Orentas et al., 1999; Kessaris et al., 2001). Ensuing expression of Olig1/2 and Neurogenin-1/2 promotes the formation of motor neurons. Neurogenins are subsequently downregulated, allowing for the ventrally expressed Nkx2.2 to expand dorsally into the pMN, interact with Olig1/2, and specify OPCs (Zhou et al., 2001; Zhou and Anderson, 2002). Even at the precursor stage, however, cells retain some measure of plasticity, as they can be converted to astrocytes with BMP4 application *in vitro* (Mabie et al., 1997; Grinspan et al., 2000a).

After specification, OPCs are exposed to multiple signals that control the specific times at which they migrate, proliferate, and differentiate (Fig. 1.2). Initial signals that promote proliferation and survival include fibroblast growth factors (FGFs),

neuregulin-1, platelet-derived growth factor A (PDGFA), CXCL1, neurotrophin-3 (NT3), and retinoic acids (Noble et al., 1988; Richardson et al., 1988; Noll and Miller, 1994; Barres et al., 1994a; Vartanian et al., 1999; Wu et al., 2000). OPCs at this stage appear bipolar and express several identifying antigens, many of them receptors for growth factors, such as the monoclonal antibody A2B5, platelet-derived growth factor alpha receptor (PDGFRα), and ErbB (Raff, 1989; Pringle et al., 1992; Lemke, 1996).

Differentiation only begins once OPCs have exited the cell cycle, and there is evidence that OPCs have a default set of proliferative cycles before this can occur (Barres and Raff, 1994; Ibarrola et al., 1996; Tang et al., 2000). Premature removal of growth factors, addition of thyroid hormone (T4), and activation of retinoic acid receptors, however, can induce differentiation (Raff et al., 1990; Barres et al., 1994; Billon et al., 2002). Many transcription factors are intricately and combinatorially involved in the differentiation process, including Olig1/2 (Zhou et al., 2001; Zhou and Anderson, 2002; Xin et al., 2005; Ligon et al., 2006; Maire et al., 2009), ASCL1/MASH1 (Parras et al., 2004; Battiste et al., 2007; Kim et al., 2007), several members of the Sox family (Stolt, 2002; Stolt et al., 2003; Finzsch et al., 2008; Kuspert et al., 2010), Pax6 (Mizuguchi et al., 2001), Nkx6.1, Nkx6.2 (Cai et al., 2005), and Nkx2.2 (Qi et al., 2001; Vallstedt et al., 2005). Upon differentiation, cells extend processes, contact neurons, and express myelin proteins, such as myelin basic protein (MBP), proteoplipid protein (PLP), and lastly myelin oligodendrocyte glycoprotein (MOG).

A major event in the developmental progression of maturing cells is the activity of histone deacetylases (HDACs), recruited by such factors as YY1 to the promoters of inhibitory factors. Histone deacetylation promotes differentiation in precursors that have

exited the cycle, and plasticity can be induced by their inhibition (Marin-Husstege et al., 2002; Shen et al., 2005; He et al., 2007; Lyssiotis et al., 2007). HDACs bind repressive transcription factors, such as inhibitor of DNA binding proteins (IDs) and Hes family members, which inhibit bHLH transcription factors, such as Nkx2.2 and Sox10, known to promote myelin producing genes (Kondo and Raff, 2000; Wang et al., 2001a; Gokhan et al., 2005; Liu et al., 2006; Marin-Husstege et al., 2006). These interactions illustrate how HDACs can directly enhance differentiation by blocking the activity of inhibitory factors (Gokhan et al., 2005; Li et al., 2007).

In contrast to ventral regions of the developing CNS, dorsal regions contain secreted signals that are inhibitory toward OPC differentiation and antagonistic to Shh (Wada et al., 2000). Notable among them are the Wnt and BMP signaling families. Other dorsal factors include Notch (Wang et al., 1998; Genoud, 2002; Park and Appel, 2003), Lingo-1 (Mi et al., 2005; Lee et al., 2007), and p57kip2 (Kremer et al., 2009). These signals are important regulators of OL development, preventing premature migration, impaired differentiation, and abnormal OL to neuron ratios.

**Wnt Signaling**

The Wnts are a large family of secreted glycoproteins that serve as signaling molecules in many different processes throughout embryogenesis. They are evolutionarily conserved across many different species, including *C. elegans* and *D. melanogaster*. In the developing mammalian system, there are 19 members of the Wnt family, and they are involved in processes encompassing embryonic patterning, cell proliferation, cell migration, cell specification, and cell differentiation, with stage- and

context- specific effects (Dorsky et al., 1998; Patapoutian and Reichardt, 2000; Coyle-Rink et al., 2002; Megason and McMahon, 2002; Braun et al., 2003; Zechner et al., 2003; Hirabayashi et al., 2004; Bonner et al., 2008). Wnt signaling has been implicated in cancers, degenerative diseases, injury, and other post developmental contexts. The best understood Wnt signaling system is the canonical pathway (Fig. 1.3). In canonical Wnt signaling, the Wnt ligand binds to a two-part membrane receptor composed of the 7-transmembrane Frizzled (Fz) and low-density lipoprotein receptor-related protein (LRP5/6). This interaction initiates a signaling cascade, beginning with phosphorylation Disheveled (Dvl) and then of the LRP5/6 cytoplasmic tail (Tamai et al., 2004; Davidson et al., 2005; Zeng et al., 2005). This in turn binds axin and recruits it away from a destruction complex. Without Wnt stimulation, the destruction complex is normally composed of glycogen synthase 3 (GSK3), casein kinase I (CKI), adenomatosis polyposis coli (APC), and axin, among several other molecules (Itoh et al., 1998; Kishida et al., 1999; Peters et al., 1999). One of its primary functions is to bind β-catenin, an essential effector of canonical Wnt signaling, facilitating its phosphorylation (Liu et al., 2002). Under these circumstances, β-catenin is marked by the destruction complex for ubiquitination and subsequent degradation in the proteosome, limiting its cytosolic half-life (Rubinfeld et al., 1996; Yost et al., 1996; Yamamoto et al., 1999; van Noort et al., 2002). When canonical Wnt signaling is activated, however, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it displaces Groucho in the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription complex (Behrens et al., 1996; Molenaar et al., 1996; Cavallo et al., 1998; Kramps et al., 2002; Hoffmans et al., 2005; Stadeli and Basler, 2005; Arce et al., 2006; Mieszczanek et al., 2008). This conformation

change alters the TCF complex from that of a transcriptional silencer of Wnt target genes to an activator of the same targets (Stadeli et al., 2006; Willert and Jones, 2006; Parker et al., 2008; Ye et al., 2009).

Traditionally, Wnts have been classified as having either 'canonical' or 'non-canonical' activities. While Wnt activity can induce action that is independent of β-catenin, there is growing evidence that the pathways are complexly intertwined, and even non-canonical pathways can interact with arms of the canonical pathways (van Amerongen and Nusse, 2009). The first of these pathways is the Wnt/jun N-terminal kinase (JNK) pathway, which regulates planar cell polarity, and is involved in processes of cell motility, such as convergent-extension, migration, neurulation, and gastrulation (Djiane et al., 2000; Wallingford et al., 2000; Habas et al., 2001; Choi and Han, 2002; Habas et al., 2003). The other significant pathway mediates calcium release and activating protein kinase C (PKC), affecting such processes as cell adhesion and gastrulation (Slusarski et al., 1997; Kuhl et al., 2000; Sheldahl et al., 2003).

The roles of Wnt/β-catenin signaling concerning specification and proliferation are largely stage and temporally specific (Yu et al., 2008). Wnt proteins are expressed both dorsally and ventrally in cells of the developing spinal cord (Parr et al., 1993; Hollyday et al., 1995). In conjunction with other patterning transcription and signaling factors, canonical Wnts confer dorsal identities to spinal cord progenitors and contribute to signaling gradients in the developing spinal cord through antagonistic regulation of Shh activity (Yu et al., 2008; Joksimovic et al., 2009). Specifically, Wnt/TCF signaling is a key enhancer of Gli3, a repressor of Shh signaling (Alvarez-Medina et al., 2008). The pMN domain, from which motor neurons and most OPCs are generated, is established by

the mutually restrictive actions of Pax6 and Nkx2.2, while Pax6 restricts the expansion of sFRP2, a secreted inhibitor of canonical Wnt signaling (Lei et al., 2006). In addition to their patterning effects, canonical Wnts are necessary for the proliferation of dorsal neurons and their stem cell precursors (Chenn and Walsh, 2002; Megason and McMahon, 2002; Muroyama et al., 2002; Zechner et al., 2003; Ahn et al., 2008), and canonical Wnt downregulation is important for the specification of neural stem cells (Kunke et al., 2009).

**The effects of Wnt signaling on oligodendrocytes**

Several recent studies have investigated the direct role of canonical Wnt signaling in OL development. Shimizu et al. (2005) initially determined that canonical Wnt signaling inhibited the differentiation of OPCs into mature cells in spinal cord explants, and Kim et al. (2008) showed similar effects in zebrafish (Shimizu et al., 1997; Kim et al., 2008). My studies and those of several others (Fancy et al., 2009; Feigenson et al., 2009; Ye et al., 2009) showed that canonical Wnt signaling is sufficient to inhibit OPC differentiation both *in vivo* and *in vitro*. In culture systems, activating the canonical Wnt pathway inhibits precursor cells from maturing, even with concurrent application of differentiating factors. When canonical Wnt signaling is constitutively activated *in vivo*, delays in both developmental myelination and remyelination after injury are observed (Fancy et al., 2009; Feigenson et al., 2009; Ye et al., 2009). In telencephalon-derived precursors, Wnt signaling prevents specification of neural precursor cells into OPCs, but increases proliferation of already specified OPCs (Langseth et al., 2010).

This regulation of maturation is believed to signal through the TCF transcriptional complex (Fancy et al., 2009; Fu et al., 2009). TCF is expressed by cells of OL lineage embryonically through early postnatal development, at which point it begins to be downregulated, but can be reactivated during white matter injury (Fancy et al., 2009). It is therefore unlikely to be involved in specification of OPCs, but remains an important factor in their growth and maturation. TCF functions as a repressor of canonical Wnt signaling genes until it binds with the β-catenin complex, at which point those same genes are transcribed. A major switch in OPC lineage occurs when HDACs bind to, and subsequently repress, the TCF complex, mediating the switch from precursor to myelinating adult cell (He et al., 2007; Ye et al., 2009).

The method by which canonical Wnt signaling affects OL development is under exploration. Canonical Wnt signaling directly activates ID proteins in several other cell types (Rockman et al., 2001; Memezawa et al., 2007) and recent studies have showed similar effects in OPCs (Ye et al., 2009; Feigenson et al., submitted for publication). This may be accompanied by a downregulation of transcription factors that induce differentiation, such as Olig2 (Samanta and Kessler, 2004). Canonical Wnt signaling also works to control the dorsal boundary of Nkx2.2, a critical transcription factor for OPC differentiation (Yu et al., 2008). Taken together, these studies represent a growing body of work suggesting an inhibitory role of canonical Wnt signaling in development, but the extent to which it is involved remains a target of investigation.

**BMP Signaling**

One of the major dorsal signaling groups that inhibit OPC differentiation is the bone morphogenic protein (BMP) family. The 20 individual BMPs are secreted proteins and members of the transforming growth factor-β (TGFβ) signaling factor family. They have many functions throughout development in a variety of systems, affecting cell development in relation to patterning, proliferation, specification, differentiation, and apoptosis, (Liem et al., 1995; Liem et al., 2000; Wine-Lee et al., 2004; See and Grinspan, 2009). Canonical signaling is initiated when BMPs bind to a dimerized serine-threonine receptor composed of the BMPRI and BMPRII membrane receptors (Fig. 1.4). Upon binding, the Type II receptor phosphorylates the Type I receptor, which subsequently phosphorylates receptor Smads 1, 5, or 8. This cascade initiates a complex with the co-Smad, Smad4, allowing it to translocate to the nucleus and induce transcription (Wrana et al., 1994; Zhang and Miller, 1996; Kretzschmar et al., 1997). The Type I BMP receptor has two sub-classes in OL lineage cells, BMPR1A and BMPR1B, which can compensate for one another in dorsal spinal cord patterning (Wine-Lee et al., 2004). BMP signaling can activate non-canonical pathways as well, independent of Smad phosphorylation, predominantly through activation of STAT proteins (Rajan et al., 2003).

The role of BMP signaling at various stages in OL lineage has been well studied. BMP treatment can induce astrogliogenesis in cultures of OL pre-progenitors and OPCs in culture systems (Gross et al., 1996; Mabie et al., 1997; Grinspan et al., 2000a), indicating that BMPs can shift the specification and differentiation of multipotent cells towards the astrocyte lineage instead of towards the OL lineage. BMP treatment of mature cells *in vitro* does not alter their lineage pattern, but prevents the expression of

myelin proteins (See et al., 2004). *In vivo*, the effects are more complicated. Genetic BMP overexpression decreases the number of mature OLs and increases the number of astrocytes in mouse and chick models (Mekki-Dauriac et al., 2002; Gomes et al., 2003). Genetic ablation of BMP signaling, however, decreases both the number of total astrocytes and OLs during development (See et al., 2007), suggesting that oligodendrogliogenesis may require a dose dependent amount of BMP signaling.

BMPs regulate OL development through control of intrinsic transcription factors and CNS patterning. BMPs can limit the influence of Shh and its effectors (Bastida et al., 2009), which are known inducers of OL specification and subsequent differentiation. BMP treatment increases ID2/4 *in vitro* (Cheng et al., 2007); IDs interact with basic helix-loop-helix (bHLH) transcription factors and block their ability to bind DNA (Norton et al., 1998; Hollnagel et al., 1999). In OPCs, IDs can bind Olig1/2, which localizes to cytoplasm the after BMP treatment, inhibiting differentiation (Wang et al., 2001b; Samanta and Kessler, 2004).

**The interactions between the BMP and Wnt signaling pathways**

Members of the Wnt and BMP families are present in the developing CNS. They are both present in dorsal spinal cord during embryonic development, and they have similar patterning behaviors: Ectopic expression will dorsalize the neural tube, and ablation will have ventralizing effects (Nguyen et al., 2000; Muroyama et al., 2002; Timmer et al., 2002; Zechner et al., 2007; Alvarez-Medina et al., 2008). These studies indicate that in some instances Wnt and BMP signaling can have functionally complimentary, redundant, or interacting effects during CNS development. How they

may interact during OL development, however, has not been extensively investigated, and may be quite complicated based on a number of examples in the literature.

The relationship between the Wnts and BMPs varies extensively depending on context. Wnt signaling acts upstream of the BMP pathway during neurogenesis and astrogliogenesis (Kasai et al., 2005), limb mesenchyme development (Hill et al., 2006), and tooth development (Liu et al., 2008a). The BMP pathway, however, acts upstream of Wnt signaling in neural crest delamination (Burstyn-Cohen et al., 2004), keratinocyte development (Yang et al., 2006), and dorsal/ventral patterning (Chesnutt et al., 2004; Zechner et al., 2007). In other contexts Wnt and BMP signaling can be directly antagonistic, such as in neuroepithelial cell development (Ille et al., 2007), aspects of gene regulation in anterior-posterior patterning (Gomez-Skarmeta et al., 2001), muscle positioning (Bonafede et al., 2006), intestinal stem cell proliferation (He et al., 2004) and osteoblast development (Kamiya et al., 2008b; Kamiya et al., 2008a; Honda et al., 2010).

Adding further complexity, the interaction between the two pathways is often indirect and multifaceted. In some systems, such as during aspects of limb bud and apical ectodermal ridge formation, the BMP and Wnt pathways can complexly regulate each other through parallel signaling and feedback systems (Soshnikova et al., 2003; Villacorte et al., 2010). In myoblast differentiation, β-catenin interacts with Smads to suppress BMP activity, while BMP coordinately upregulates β-catenin activated TCF transcription. In the development of anterior visceral endoderm, BMPs simultaneously upregulate levels of canonical Wnts and the secreted Wnt inhibitor, Dikkopf-1 (Dkk-1, Miura et al., 2010).

Some of the earliest identified relationships between canonical Wnt and BMP signaling involved synergistic activation of downstream transcription factors (Crease et al., 1998; Labbe et al., 2000; Nishita et al., 2000; Letamendia et al., 2001; Theil et al., 2002; Hussein et al., 2003). This occurs because some genes have Smad and TCF binding sites in close proximity, such as *Emx2* (Theil et al., 2002), *Msx2* (Willert et al., 2002; Hussein et al., 2003), *c-myc* (Hu and Rosenblum, 2005), and *Xtwin* (Labbe et al., 2000; Nishita et al., 2000; Letamendia et al., 2001). These genes can be activated independently by stimulating either BMP or Wnt signaling, but are significantly more responsive with concurrent activation of both pathways. Additionally, Wnt signaling can prolong the activity of BMP signaling by inhibiting phosphorylated-Smad degradation (Fuentealba et al., 2007), and they can increase neurogenesis, subsequently leading to neuronal production of BMPs (Kasai et al., 2005). Given all of the interactions between Wnt and BMP signaling in a wide variety of developmental and cellular processes, I hypothesize that they interact in OL development as well.

**Significance**

OLs are the myelin producing cells of the CNS. Axonal myelination is critical for the rapid and efficient transmission of neural signals, and demyelination results in axonal damage, impaired cognition, and impaired motor control, characteristics common to a variety of disorders. The damage varies depending on the type and extent of the injury, but spontaneous recovery can occur after demyelinating events, evidence that the adult CNS has the potential to generate new myelin (Shields et al., 1999; Murtie et al., 2005b; Patrikios et al., 2006). Adult progenitor cells are actively respond to many of these

events, but their ability to differentiate into functionally mature cells varies according to age, injury location and duration, size of progenitor pools, and active signaling factors (Shields et al., 1999; Chari and Blakemore, 2002; Mason et al., 2004; Tatsumi et al., 2008; Islam et al., 2009). Treating these disorders will depend on a number of elements, especially the manipulation of OPCs and local environments to facilitate cellular differentiation, axon recovery, and myelination.

Part of the challenge in treating such conditions is the complex signaling environment of the CNS during injury. Many of the same signals involved in developmental systems are involved in regulating injury response, including FGF, PDGFA, BMPs, and Wnts (Armstrong et al., 2002; Murtie et al., 2005b; Zhang et al., 2009; Cate et al., 2010; White et al., 2010). BMPs are upregulated in aspects of CNS injury and demyelinating models (Setoguchi et al., 2001; Setoguchi et al., 2004; Ara et al., 2008; See and Grinspan, 2009; Cate et al., 2010; Jablonska et al., 2010), and can play roles in preventing remyelination and axon regeneration. In parallel, several recent studies have also found that members and effectors of the Wnt family are upregulated in similar paradigms, and may have similarly inhibitory effects on recovery (Liu et al., 2008b; Fancy et al., 2009; Miyashita et al., 2009; White et al., 2010). There is, however, evidence that Wnt3a can facilitate spinal cord recovery by enhancing stem cell differentiation into neurons (Yin et al., 2008), illustrating the complexity involved in this line of *in vivo* treatment

If these signaling pathways can be appropriately manipulated, both endogenous and transplanted progenitor cells can be directed down specific developmental lineages to aid in recovery. This approach would be an end unto itself, but it would also provide

insight into the internal mechanisms controlling cellular differentiation. Understanding the grand sum of these interactions would be invaluable for combating demyelinating events.

The body of work in my dissertation concerns the canonical Wnt signaling pathway, which is active in CNS development and injury. My experiments have shown that ectopically activated Wnt signaling will delay the progression of OPCs to mature OLs, and that this interaction is dependent on the BMP signaling pathway. These findings have direct relevance for understanding the complex environment of signaling factors that regulate cell fate and differentiation in OL lineage cells.

**Significance**

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If these signaling pathways can be appropriately manipulated, both endogenous and transplanted progenitor cells can be directed down specific developmental lineages to aid in recovery. This approach would be an end unto itself, but it would also provide insight into the internal mechanisms controlling cellular differentiation. Understanding the grand sum of these interactions would be invaluable for combating demyelinating events.

The body of work in my dissertation concerns the canonical Wnt signaling pathway, which is active in CNS development and injury. My experiments have shown that ectopically activated Wnt signaling will delay the progression of OPCs to mature

OLs, and that this interaction is dependent on the BMP signaling pathway. These findings have direct relevance for understanding the complex environment of signaling factors that regulate cell fate and differentiation in OL lineage cells.

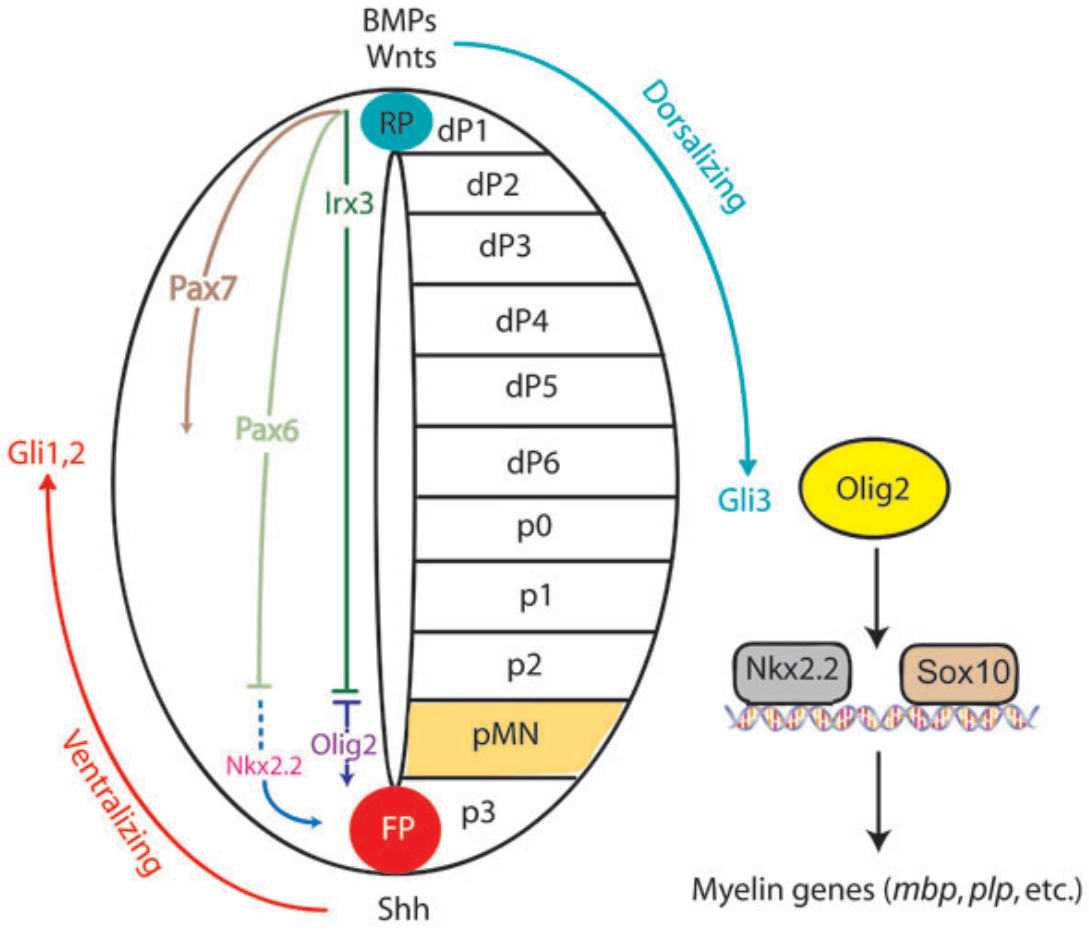


**Fig 1.1 Interactions of signaling factors in the developing spinal cord regulate oligodendrocyte maturation**

Patterns of signaling factors are established during spinal cord development after the formation of the notochord and floorplate, where Shh is secreted. A gradient of Shh induces transcription factors in a concentration dependent manner, originally through induction of activating Gli1/2. In contrast, dorsal spinal cord contains signals such as members of the BMP and Wnt signaling families, which establish signaling gradients contrary to those of Shh. Wnt activates repressive Gli3, limiting the dorsal influence of Shh. From these opposing concentrations of signals, transcription factors are expressed in

regionally specific areas. They are kept from expanding via mutually repressive dorsal to ventral signaling, demarcating areas from which specified populations of cells arise. OLs and motor neurons are generated in the pMN domain, identified by expression of Olig1/2 transcription factors. Motor neurons are generated with concurrent expression of Neurogenins, and as development proceeds, Neurogenins are downregulated and Nkx2.2, initially expressed near the floorplate in P3, expands dorsally into the pMN. Cells that simultaneously express Nkx2.2 and Olig2 are specified as OPCs.

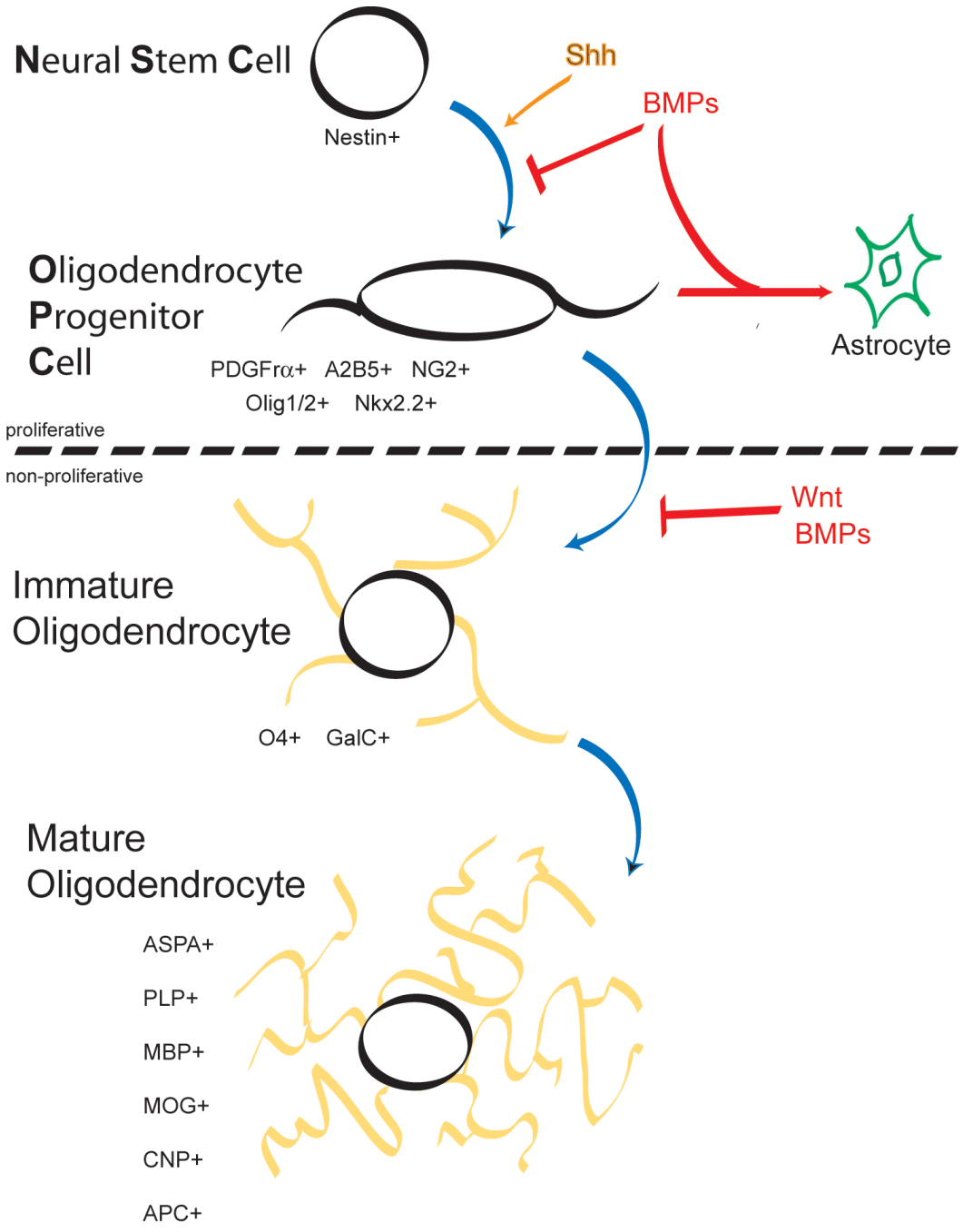


**Fig 1.2: Oligodendrocytes progress through several stages before reaching maturity**

Nestin expressing multipotent neuroepithelial cells in the ventricular zone of the ventral spinal cord begin to appear around E9 in rodents. Inductive signals, primarily Shh, promote their proliferation and specification into OPCs, which begins at E12.5. These cells are characterized by bipolar processes and the expression of PDGFRα, A2B5,

NG2, Nkx2.2, and Olig1/2. Prohibitive signals in the dorsal spinal cord, such as BMP, prevent this progression, instead directing precursor cells to the astrocyte lineage. Following concurrent expression of transcription factors Olig2, Sox10, and Nkx2.2, OPCs differentiate into mature OLs. At this stage, cells have exited the cell cycle, begin to extend multiple processes, and express O4 and GalC. Between the OPC and immature OL stage, dorsal inhibitory factors can prevent further differentiation, maintaining cells in a precursor state. At the final mature OL stage, cells have extended multiple complex processes while expressing myelin proteins, including PLP, MBP, MOG, ASPA, and APC. Cells at this stage are competent to myelinate axons.

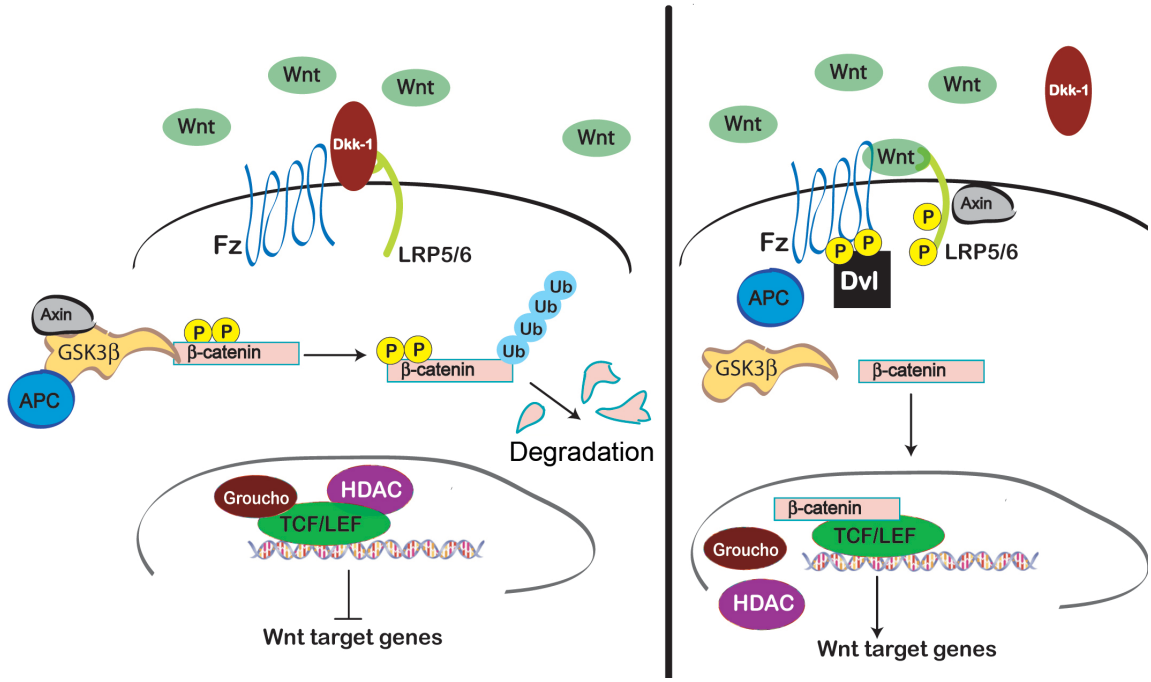


**Fig 1.3: Canonical Wnt signals through β-catenin**

Canonical Wnt signaling initiates a cascade of signaling events that result in β-catenin translocation into the nucleus. (Left) In the unbound state, Wnt proteins have not interacted with the membrane receptors Fz and LRP5/6. This occurs when there are few secreted Wnts or many Wnt antagonists present in the extracellular environment. Examples of antagonists include extracellular sFRP proteins that bind secreted Wnts, or Dkk-1, which binds LRP5/6. Under these circumstances, a destruction complex composed of GSK3β, APC, and Axin, among other molecules, phosphorylates β-catenin. This leads to its ubiquitination and subsequent degradation in the proteosome. Wnt binding to Fz and LRP5/6 leads to the phosphorylation of Dvl and subsequent phosphorylation of LRP5/6, recruiting Axin away from the destruction complex and rendering it inactive. β-catenin can no longer be phosphorylated, accumulates in the cytoplasm, and translocates to the nucleus. There, it displaces Groucho and HDACs from

the TCF/LEF transcription complex, which is altered from a repressor to an activator of Wnt target genes.

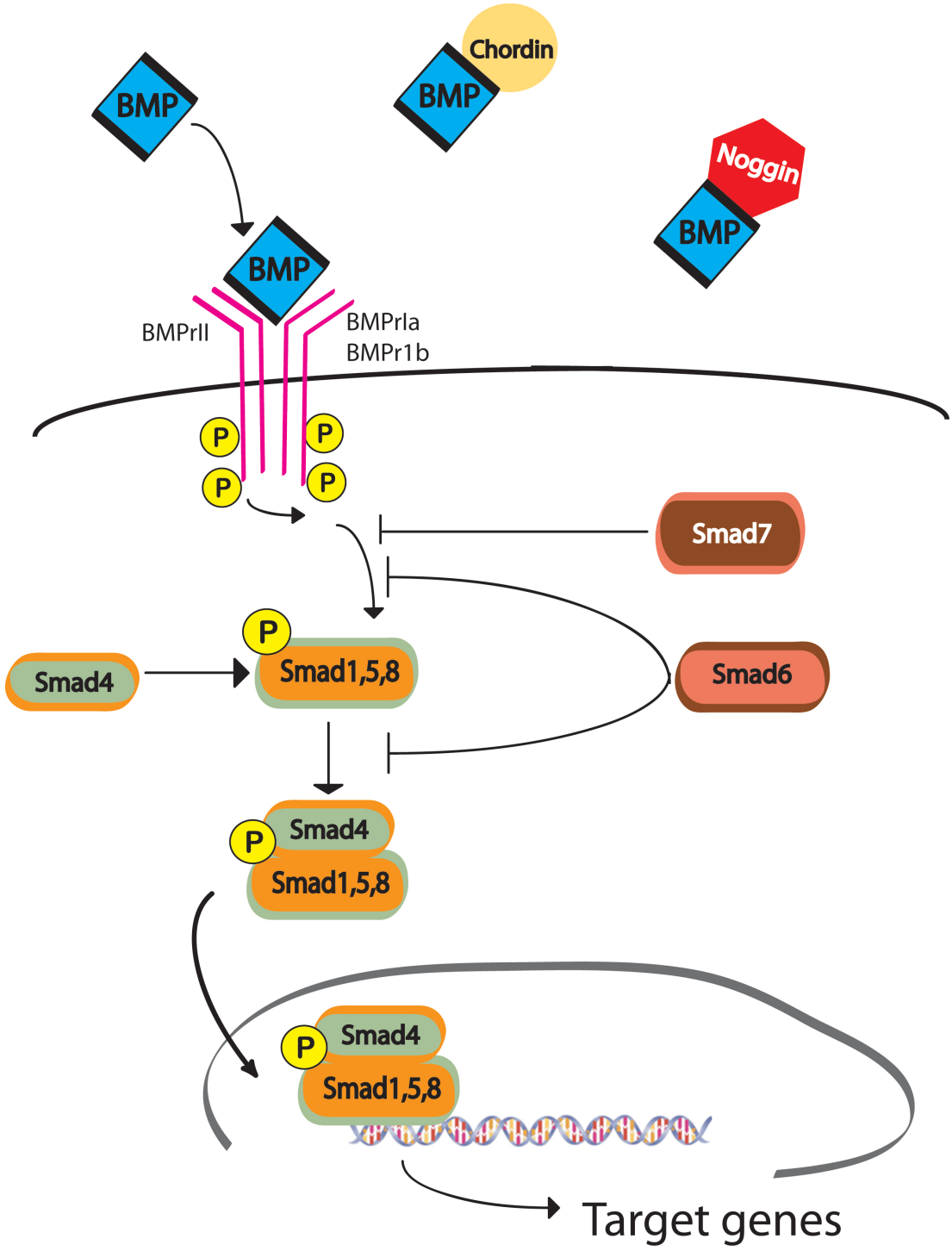


**Fig 1.4: The canonical BMP signaling pathway**

BMPs signal by binding to the BMPR type I and II heterodimers. Upon binding, the BMPRII receptor phosphorylates the BMPRIA/B receptor. This cascade initiates phosphorylation of receptor Smads 1/5/8, allowing them to form a complex with the co-Smad4. This complex can then translocate into the nucleus and activate transcription of BMP target genes. This signaling pathway can be inhibited extracellularly by the action of secreted proteins, such as Noggin and Chordin, which bind extracellular BMP proteins. Inhibitory Smads 6/7 act intracellularly to prevent the phosphorylation of receptor Smads and the formation of the Smad4 complex.

## Chapter 2

## Wnt signaling is sufficient to perturb oligodendrocyte maturation

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**Abstract**

The development of oligodendrocytes, the myelinating cells of the central nervous system, is temporally and spatially controlled by local signaling factors acting as inducers or inhibitors. Dorsal spinal cord tissue has been shown to contain inhibitors of oligodendrogliogenesis, although their identity is not completely known. We have studied the actions of one family of dorsal signaling molecules, the Wnts, on oligodendrocyte development. Using tissue culture models, we have shown that canonical Wnt activity through β-catenin activation inhibits oligodendrocyte maturation, independently of precursor proliferation, cell death, or diversion to an alternate cell fate. Mice in which Wnt/β-catenin signaling was constitutively activated in cells of the oligodendrocyte lineage had equal numbers of oligodendrocyte precursors relative to control littermates, but delayed appearance of mature oligodendrocytes, myelin protein, and myelinated axons during development, although these differences largely disappeared by adulthood. These results indicate that activating the Wnt/β-catenin pathway delays the development of myelinating oligodendrocytes.

## Introduction

Myelin, the lipid membrane that ensheathes axons, is necessary for the rapid and effective conduction of neural signals. In the central nervous system, it is synthesized by oligodendrocytes (OLs). These specialized glial cells arise in ventricular zones as oligodendrocyte precursors (OPCs) and progress through several stages before myelinating axons (Pringle and Richardson, 1993; Ono et al., 1995). During this period, OPCs are exposed to a variety of environmental signaling factors that can be inductive or inhibitive of developmental processes, including differentiation (Miller, 2002). Although much of OL development is well characterized, the factors that affect OL differentiation and the mechanisms by which they operate are less well understood.

In the spinal cord, the majority of OPCs originate in the ventral ventricular zone beginning at 12.5 dpc. The specification of these precursors is controlled by floor plate signals in the ventral spinal cord, notably sonic hedgehog (Shh), which induces expression of transcription factors necessary for the stages of OL development (Lu et al., 2000; Zhou et al., 2000; Zhou and Anderson, 2002). OPCs then migrate from the subventricular zone to populate the spinal cord, eventually contacting axons and expressing myelin proteins. Recent evidence suggests, however, that there are populations of dorsally generated OLs, although their contribution to the general pool may be small and late (Cai et al., 2005; Vallstedt et al., 2005; Kessaris et al., 2006). The predominantly ventral origin of OPCs is thought to result, in part, because of specific OL inhibitors present in the roof plate and adjacent structures (Wada et al., 2000). These dorsal tissue areas contain several families of signaling factors with overlapping

expression patterns that may repress multiple aspects of OL development (Wine-Lee et al., 2004).

One of these families, the bone morphogenic proteins (BMP), has been extensively studied in this regard. Treatment of OPC cell cultures with BMP4 or BMP4 overexpression *in vivo* induces astrogliogenesis at the expense of OL differentiation (Grinspan et al., 2000b; Mekki-Dauriac et al., 2002; Gomes et al., 2003; Miller et al., 2004; See et al., 2004). BMPs, however, may not be the only family of factors that dorsalize the neural tube to affect OL development. BMPs interact with other signaling molecules, among them the Wnts, to regulate dorsal-ventral patterning and other developmental processes (Soshnikova et al., 2003; Wine-Lee et al., 2004; Ille et al., 2007; Zechner et al., 2007). One example is upregulating the Wnt signaling pathway in neural precursors cells increases the expression of BMPs, which may coordinately regulate OL differentiation (Kasai et al., 2005). The extent of their interaction relating to signaling in OL development, however, is not well understood.

In the developing mammalian system, Wnt signaling is involved in regulating embryonic patterning, cell proliferation, migration, specification, and differentiation, and has stage and context specific effects (Dorsky et al., 1998; Patapoutian and Reichardt, 2000; Coyle-Rink et al., 2002; Braun et al., 2003; Hirabayashi et al., 2004; Karim et al., 2004; Kalani et al., 2008). Wnt signaling is active during central nervous system development, in dorsal spinal cord from E9.5-E12.5 (Wine-Lee et al., 2004; Shimizu et al., 2005), and in the subventricular zone of the developing CNS at E14.5 (Kalani et al., 2008). In the canonical Wnt pathway, Wnt binds with LRP5/6 to frizzled (Fz) receptors which, through a signal cascade, results in the dephosphorylation and thereby

accumulation of β-catenin in the cytoplasm (Mi and Johnson, 2005). Ordinarily, β-catenin is sequestered in a complex with GSK3β, APC, and axin, marking it for degradation. Dephosphorylated β-catenin collects in the cytoplasm and translocates to the nucleus, where it activates TCF-Lef transcription factors (Wodarz and Nusse, 1998; Staal, 2002). GSK3β inhibitors, such as LiCl, prevent phosphorylation of β-catenin, allowing nuclear translocation and initiation of the signaling cascade. The role of Wnt signaling during OL development has not been extensively investigated. Recent studies have demonstrated that Wnt signaling has inhibitory effects on OPC differentiation in mouse spinal cord explants and in culture and in zebrafish (Shimizu et al., 2005; Kim et al., 2008). We have investigated the role of Wnt/β-catenin signaling on OL maturation in primary cultures of OPCs (Grinspan et al., 2000b) and in a mutant mouse in which β-catenin is constitutively active.

Using the Cre-Lox system, we created a mutant mouse that expresses constitutively active β-catenin in CNP expressing cells. The ubiquitin binding site on the β-catenin gene is flanked by LoxP sites, so upon Cre induced recombination, the resulting transgene expresses a β-catenin protein that is not targeted for degradation and remains perpetually transcriptionally active. CNP is present in cells of the OL lineage and is essential for axonal survival; hence its expression is preserved throughout life (Lappe-Siefke et al., 2003). Using this approach, we can study the effects of constitutively active canonical Wnt signaling in all cells of OL lineage.

In normal rat OPC cultures, the addition of Wnt3a significantly decreases the number of immature and mature OLs generated during differentiation. Similarly, cultures prepared from *Cnp-Cre* mice in which β-catenin was constitutively activated show a

marked reduction in numbers of immature and mature OLs relative to cultures taken from control littermates. Spinal cord sections show significant decreases in mature OLs and myelin proteins at postnatal stages, and, although less pronounced, this decrease persists until adulthood. Similarly, semi-thin preparations of myelin show fewer myelinated axons at postnatal stages, although this effect recovers by adulthood. These findings indicate that constitutively activated canonical Wnt signaling delays myelination by inhibiting the differentiation of OPCs, and that this signaling pathway is a regulator of OL development.

**Results**

Wnt3a inhibits oligodendrocyte differentiation via the canonical Wnt signaling pathway

To determine whether Wnt3a signaling affects the progression of the OL lineage in culture, we generated purified primary OPC cultures from rat and mouse forebrain, grew them on 100mm dishes or 12mm coverslips, and then removed growth factors and added differentiation medium (DM) when the cells became confluent. After 3 days in DM, 20% of the cells had differentiated and could be labeled with antibody to galactocerebroside (GalC), an early marker of differentiation (Fig. 2.1A, B). Eighty-six percent of the cells labeled with the A2B5 antibody, indicating that some cells remained OPCs, and other cells still expressed both markers (Fig. 2.1A, B). Addition of 50 ng/ml Wnt3a to the coverslips concurrently with DM, however, resulted in 36% fewer GalC+ cells than controls at 3 days ($p < 0.05$), while the number of A2B5+ cells was comparable to controls (Fig. 2.1A, B). This reduction in the numbers of differentiating cells was

confirmed by western blots on protein from OPC cultures, which showed less MBP expression at 5 days in Wnt3a treated conditions relative to controls (Fig. 2.1C).

OPCs are multi-potent and the failure to differentiate could suggest that Wnt3a signaling switches cells to a different neural lineage. To determine whether neurons were generated by Wnt3a treatment, we labeled with an antibody to Tuj1 and found only occasional (less than 1%) TuJ1+ cells in both control and treated cultures (data not shown). Because inhibition of differentiation in OLs can be associated with the expression of glial fibrillary acidic protein (GFAP) and generation of "type II" astrocytes (Raff et al., 1983), we labeled treated cells with an antibody to GFAP after 3 days treatment with Wnt3a. Our cultures contain a small percentage (< 5%) GFAP+ type 1 astrocytes that remain after immunopurification. The number of GFAP+ cells in the Wnt3a treated cultures was not significantly different from controls (Fig. 2.1D).

To determine whether the decrease in differentiated cells reflected altered proliferation of precursors, we labeled cells with BrdU for 3, 6, and 24 hours, but observed no differences between the treated and control cells (Fig. 2.1E). To determine whether the decrease in differentiated cells reflected increased apoptosis, we performed the TUNEL assay for cell death and observed no change in the number of TUNEL+ cells (Fig. 2.1F).

The canonical Wnt signaling pathway is mediated through β-catenin, but there are non-canonical pathways as well (Zhou et al., 2000; Zhou and Anderson, 2002; Veeman et al., 2003; Montcouquiol et al., 2006). To determine whether the canonical signaling pathway was activated in our system, we labeled OPCs in culture for β-catenin, which, when transcriptionally active, translocates to the nucleus. After 6 hours of Wnt3a

treatment, β-catenin was observed in the nuclei of cells double labeled with A2B5 (Fig. 2.2A). An increase in total β-catenin was also observed on western blots of protein from OPC cultures treated with Wnt3a, relative to protein from control cultures (Fig. 2.2B). To determine whether the inhibitory effect of Wnt3a on OPC differentiation operated via the canonical pathway, cultures were treated concurrently with Wnt3a and Dickopf-1 (Dkk-1, 100 ng/ml), a canonical Wnt signaling inhibitor that prevents signaling through the β-catenin pathway (Bafico et al., 2001; Semenov et al., 2001), and differentiation was rescued (Fig. 2.2C). Additionally, treatment with 10 μM SB216763, a GSK3β inhibitor that activates constitutively active β-catenin, also decreased OL differentiation by 32% relative to controls ($p < 0.055$; $n = 3$). Our results indicate that activating canonical Wnt signaling *in vitro* through β-catenin inhibits the differentiation of OPCs to immature OLs.

Constitutively active β-catenin signaling delays oligodendrocyte development *in vivo*

To determine the effect of Wnt signaling *in vivo*, we used the LoxP/Cre approach to constitutively activate β-catenin signaling in transgenic mice. In these experiments, we use the *Cnp-Cre* transgenic strain to direct Cre-mediated DNA rearrangements to in oligodendroglial lineage. To ensure that this strain targets specifically cells of OL lineage, we crossed the *Cnp-Cre* mice with *Rosa26* reporter mice which express LacZ upon Cre mediated recombination (Soriano, 1999). Expression of LacZ therefore, marks cells that express *Cnp-Cre* or in the subsequent lineage of cells that inherit the rearranged DNA. Spinal cord sections from P5 offspring were double immunolabeled with antibody directed against β-galactosidase and either PDGFRα or APC, which label OPCs or mature OLs, respectively (Fig. 2.3A, B). Cells expressing β-galactosidase were

consistently colabeled with both PDGFRα and APC, indicating that Cre-mediated rearrangements are found in most cells of OL lineage.

To activate ß-catenin specifically in the oligodendrocyte lineage, we intercrossed *Cnp-Cre* strain with a pedigree containing the β-catenin$^{floxedexon3/floxedexon3}$ allele. Upon Cre-mediated DNA rearrangement, exon 3 of the β-catenin gene is excised, resulting in a constitutively active form of ß-catenin. Robust and highly penetrant Cre-mediated rearrangement of this β-catenin$^{floxedexon3/floxedexon3}$ allele has been observed with numerous Cre strains (Lappe-Siefke et al., 2003; Rasband et al., 2005; Edgar et al., 2009). From these matings, we would expect half of the resulting offspring to express the activated form of β-catenin (referred to as *β-Cat-CA* mice). Although significantly smaller than control littermates, viable mutant animals were born in the expected Mendelian ratios.

*β-Cat-CA* mice and littermate controls were sacrificed at specific time points and spinal cord sections were analyzed for OL lineage development and myelination. At birth, myelin proteins, such as proteolipid protein (PLP) and myelin basic protein (MBP), were largely concentrated in ventral spinal cord (Fig. 2.4A, B). At P1, we observed 32% fewer PLP+ cells in ventral spinal cord of *β-Cat-CA* mice than in control littermates ($p < 0.05$, Fig. 2.4G). Western blots confirmed this effect, showing a decrease in MBP expression from forebrain tissue taken from mutant mice and control littermates (Fig. 2.4H).

To address the possibility that the decrease in maturing OLs could reflect an alteration in cell fate such that OPCs became astrocytes or neurons, we immunolabeled for GFAP and NeuN. In the same areas of ventral spinal cord, there were no significant differences in numbers of astrocytes, labeled with GFAP (Fig. 2.4C, D, I) or neurons,

labeled with NeuN (data not shown). To examine whether the decrease in mature OLs was reflected in altered numbers of precursors, we labeled P1 spinal cord sections with antibody to PDGFRα or NG2, both of which stain OPCs (Fig. 2.4E, F). There was no difference in numbers of positive cells between the normal and mutant sections (Fig 4. J).

To determine whether there were any changes in cell proliferation, we labeled ventral spinal cord with phospho-histone 3 and Ki67, but did not observe any differences between *β-Cat-CA* mice and control littermates (Supplemental Figure 2.1A-C). Because OLs are highly susceptible to cell death in the absence of survival cues, especially as they begin to mature, we assayed ventral spinal cord sections with TUNEL to observe if the decrease in differentiated OLs resulted from increased cell death. We did not observe any differences in TUNEL labeling between *β-Cat-CA* mice and control littermates (Supplemental Figure 2.1D).

At P7, we observed the greatest difference in the number of cells expressing myelin proteins between normal and *β-Cat-CA* mice (Fig. 2.5A, B). We observed 38% fewer cells labeling with antibody to PLP in P7 *β-Cat-CA* mice compared to control littermates in ventral spinal cord ($p < 0.05$; Fig. 2.5G). In the same areas, mice at this stage do not have significant differences in staining of PDGFRα, NG2, GFAP, NeuN, and cleaved-caspase 3 (data not shown), or in labeling with phospho-histone 3, Ki67, or TUNEL (Supplemental Figure 2.1A-D). Western blots performed on protein taken from forebrain of P7 mice showed a decrease in expression of MBP in *β-Cat-CA* mice relative to control littermates (Fig 5. H).

After myelination has progressed for two weeks or more, it becomes difficult to label individual cells labeled with antibody to PLP because of the overlapping

arrangement of OLs and their processes. To label OLs at this age, we used anti-aspartoacylase (ASPA), which specifically targets OL cell bodies (Hershfield et al., 2006). At P21, *β-Cat-CA* mice have 19% fewer ASPA+ cells in white matter of ventral spinal cord than control littermates ($p < 0.05$, Fig. 2.5C, D, I). The number of ASPA+ OLs in ventral and dorsal regions decreased equally between mutants and control littermates, indicating that the β-catenin mutation affects OLs in both regions of the spinal cord in a similar manner. At 10 weeks, *β-Cat-CA* mice have 13% fewer ASPA positive cells in white matter of ventral spinal cord compared to control littermates ($p < 0.05$, Fig. 2.5E, F, J). At both of these time points, there is no significant differences between *β-Cat-CA* mice and control littermates in the number of cells labeling with antibody to PDGFRα, NG2, GFAP, NeuN, phospho-histone 3, and anti-cleaved-caspase 3 (data not shown). These results indicate that constitutively activated canonical Wnt signaling in cells of OL lineage inhibits or delays OL maturation, but that this delay is overcome in adulthood.

Semi thin sections demonstrate a decrease in myelinated axons during development following constitutively active signaling by β-catenin.

To characterize the effect of activating the canonical Wnt signaling pathway on myelination, we counted myelinated axons in semi thin sections from spinal cord and sciatic nerves of *β-Cat-CA* and control pups (P7, Fig. 2.6A-F) and adult mice (Fig. 2.6G-L). In comparable sections of P7 ventral spinal cord, we observed a 44% decrease in myelinated axons in *β-Cat-CA* mice when compared to control littermates ($p < 0.05$, Fig. 2.6M). Cross sections of tibial sciatic nerve had a 27% reduction in myelinated axons in

*β-Cat-CA* compared to control littermates; this difference only trended towards significance ($p < 0.07$, Fig. 2.6M). In contrast, there were no discernable differences in the number of myelinated axons in adult optic nerve (data not shown), ventral spinal cord, or sciatic nerve (Fig. 2.6M). These results further indicate that there is a delay in myelinating cell development in *β-Cat-CA* mice relative to control littermates. Since CNP expression in the nervous system is limited to OLs and Schwann cells (Lappe-Siefke et al., 2003; Rasband et al., 2005; Edgar et al., 2009), these are likely to be cell-autonomous effects.

Cultures of OPCs from *β-Cat-CA* mice contain fewer differentiated oligodendrocytes than controls

To determine if the effects of the constitutively activated β-catenin mutation were cell autonomous, we generated primary cultures of OPCs from *β-Cat-CA* mice, grew them in dishes, and differentiated them as described previously, comparing them to cultures taken from control littermates. After 3 days in DM, there were 60% fewer cells labeled with GalC in mutant cultures compared to controls ($p < 0.05$), indicating a large reduction in differentiating cells (Fig. 2.7A, B).

Because this reduction in immature OLs could have been caused by a change in the number of precursors, we labeled cells with A2B5 and did not observe significant differences (Fig 7. A, B). Similarly, there were no significant changes in the number of GFAP, BrdU, or TUNEL labeled cells, indicating that the number of astrocytes, proliferating cells, and cells undergoing apoptosis, respectively, were unchanged (Fig. 2.7C-E). These results indicate that constitutively activated canonical Wnt signaling

inhibits differentiation *in vitro* in a cell autonomous manner, and that this is a direct result of inhibition of differentiation and does not result from a change in the number of precursors, astrocytes, proliferating cells, or cells undergoing apoptosis.

**Discussion**

We have investigated the role of canonical Wnt signaling during OL development both *in vitro*, using cultures from normal rats and from mice in which a dominant stable form of β-catenin is active, and *in vivo*, examining spinal cords from these same mutant mice. We showed a significant inhibition of OPC differentiation in cultures with activated canonical Wnt signaling. In spinal cord sections, we observed a decrease in numbers of OLs and myelin protein early in postnatal development of *β-Cat-CA* mutant mice, relative to control littermates. This difference, however, became progressively less pronounced as the mice reached adulthood. Similarly, we observed fewer myelinated axons in the CNS and PNS of mutant pups compared to controls, but the difference largely disappeared in adults.

During spinal cord development, OPCs are generated from E12.5 through birth in ventral progenitor zones. After specification, they migrate into other areas of spinal cord, mature, and, upon contact with axons, begin the myelination process. Throughout this period, they are exposed to a variety of internal and external signals that regulate their proliferation, specification, and differentiation. Dorsal regions are the latest to be populated by OPCs and are thought to contain inhibitors of OPC specification and differentiation (Wada et al., 2000).

Canonical Wnt signaling plays a variety of roles in the developing nervous system. Members of the Wnt family that signal through the canonical Wnt pathway, notably Wnt1 and Wnt3a, are found in the developing dorsal spinal cord during the onset of neural and glial specification (Hollyday et al., 1995; Megason and McMahon, 2002; Wine-Lee et al., 2004; Shimizu et al., 2005). Canonical Wnt signaling through the stabilization of β-catenin is involved in neural precursor proliferation in both spinal cord (Megason and McMahon, 2002; Chenn and Walsh, 2003) and brain (Gulacsi and Anderson, 2008), while also helping to pattern developing spinal cord (Muroyama et al., 2002; Zechner et al., 2007). Wnt3a is known to inhibit Shh signaling to regulate the spatial and temporal specification of neurons and glial cells throughout neural tube (Lei et al., 2006), spinal cord (Yu et al., 2008), and hindbrain development (Joksimovic et al., 2009). Using a cell autonomous approach, we have shown that canonical Wnt signaling inhibits and delays OL differentiation. Our data agrees with that of Shimizu et al. (2005) in mouse explants and Kim et al. (2008) in zebrafish. In zebrafish, however, the Wnt receptor Fz8a is required for the proliferation and organization of radial glial cells, which indirectly controls OPC specification (Shimizu et al., 2005; Kim et al., 2008). Following specification, canonical Wnt signaling could be preventing premature OPC differentiation to help control the temporal and spatial patterning of myelination in the developing spinal cord.

How does canonical Wnt signaling control OL differentiation? A lack of mature OLs can result from a decrease in the size of the progenitor pool, increased apoptosis among cells of OL lineage, a change in specification of OPCs, or the prevention of OPCs from undergoing differentiation into mature adult cells. Canonical Wnt signaling could

limit the number of precursors available, thereby decreasing the total potential amount of adult OLs. Our data show this is unlikely, as canonical Wnt signaling does not decrease the total pool of precursors, as seen by A2B5 labeling *in vitro* and PDGFRα and NG2 labeling *in vivo*. Proliferation among precursors could be another factor: decreased proliferation could limit the total pool size of the OL population. Our data show, however, no change in markers of cell proliferation, as Wnt3a treated OPCs *in vitro* do not have significant differences in BrdU labeling relative to controls and proliferation appears to be unchanged in the *β-Cat-CA* mouse, as measured by Ki67 and phospho-histone 3 labeling. The pool of OPCs could also be reduced by increasing the number of cells that undergo fate switching to astrocytes or neurons, at the expense of OLs. Our results show, however, no change in the number of cells labeled with GFAP or Tuj1. Finally, OLs could be decreased by apoptosis of OPCs, or more likely immature OLs, but we have shown no increase in TUNEL labeling *in vitro* or *in vivo*.

Taken together, our results suggest the possibility that canonical Wnt signaling prevents cells already committed to the OL lineage from undergoing the maturation stages needed to become myelin-producing OLs. In this model, cells in the precursor stage would remain progenitors without increasing their rate of proliferation, apoptosis, or adoption of other cell fates. This has been seen in other genetically altered mice in which transcription factors or receptors involved in differentiation have been disregulated, thus inhibiting differentiation. Examples include elimination of signaling through BMP type 1 receptors, genetic deletions of the transcription factors Olig1, Nkx2.2, and Sox10, and increased Sox4 expression (Qi et al., 2001; Lu et al., 2002; Stolt et al., 2002; See et al., 2004; Potzner et al.,

2007). In all of these genetic models, differentiation is inhibited without a concurrent change in precursor number.

Myelination in the *β-Cat-CA* mice eventually reaches relatively normal levels, showing a small deficit (12%) in OLs and normal numbers of myelinating axons in the adult. Mechanisms for this could include a constant slower rate of precursor differentiation until nearly full myelination is achieved, or a decrease in the importance of β-catenin signaling for inhibition of OL differentiation in the adult, thus making it more permissive. In addition, more immature OLs are produced in normal development than are needed for myelination and are pruned by apoptosis (Barres et al., 1992a). In our mutant mouse, if not all OLs were inhibited from differentiating by β-catenin activation, less cell death in the differentiating population would slowly restore the number of mature OLs to normal levels.

The interactions between elements downstream of β-catenin in the canonical Wnt signaling pathway and factors known to be involved in OL differentiation, which might account for the lack of OL maturation, have not been directly explored. There is, however, precedence for their potential interactions. Tcf4, a downstream transcription factor of active β-catenin, is known to repress the expression of the transcription factor Nkx2.2 during development of the ventral spinal cord (Lei et al., 2006). Nkx2.2 is one of several transcription factors, along with Olig1/2 and Sox10, which are necessary for OL differentiation. Direct interactions between β-catenin, downstream effectors, and these factors need to be pursued.

It is possible that stabilizing β-catenin alters the interaction of OPCs with their environment in the spinal cord, making it difficult to identify the cell autonomous effects of Wnt signaling OL lineage cells. For this reason, we cultured cells from mutant and

control mice and analyzed their differentiation capacity after 3 days in DM. Our observations mimicked the application of exogenous Wnt3a to control cultures. While some cells expressed GalC in both mutant and control cultures, there were far fewer in cells from mutant cultures. These findings support the idea that canonical Wnt signaling partially inhibits or delays OPC differentiation, while not completely preventing it.

The later appearance of OLs in dorsal regions is an essential part of spinal cord patterning. Inhibitory signals in these areas likely play important roles in preventing ventrally generated OPCs from migrating dorsally and subsequently differentiating. Recent studies have found that there may be dorsally generated, albeit significantly smaller, populations of OPCs, although their contribution to the total OL pool is difficult to determine and may be small (Cai et al., 2005; Vallstedt et al., 2005; Kessaris et al., 2006). While no clear differences between these OPC populations have been observed, it stands to reason that their responses to environmental cues could vary, allowing for distinct temporal, spatial, and functional patterns. Because Wnt signaling greatly reduces the proportion of differentiated cells at certain time points, and appears to prevent differentiation in some cells and not others, it is possible that there are differences in threshold responses within OPC populations. This could contribute to the varied responses of some populations of OPCs to the gradient of signaling factors established in the spinal cord, helping to control their temporal and spatial development. We likely observed a universal reduction in lieu of regional differences in the *β-Cat-CA* mouse because β-catenin is artificially activated in all OLs.

Understanding the inductive and repressive signals that guide OL development is important in generating strategies to more effectively manipulate OL specification,

growth, survival, and maturation, to promote regeneration and remyelination following CNS disease or injury. We have shown that activated canonical Wnt signaling is sufficient to inhibit the differentiation of OLs *in vivo* and *in vitro*, and that this effect is transitory in the developing CNS.

**Methods**

Cell culture generation and treatment

All experiments were performed in accordance with the guidelines set forth by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee. To generate cultures of purified OPCs from newborn rats, a mixed population of cells was harvested from Sprague-Dawley rats and seeded on 100 mm Petri dishes in serum-containing medium as previously described (See et al., 2004). After 24 hours, the cell cultures were switched to a serum free growth medium, containing Neurobasal medium (Invitrogen) with B27 supplement (1:50; Life Technologies), 10 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN), 2 ng/ml platelet-derived growth factor (R & D), and 1 ng/ml neurotrophin-3 (Peprotech, Rocky Hill, NJ).

Cultures were purified by immunopanning 6 to 8 days after isolation as previously described (Grinspan et al., 2000b). Briefly, the cells were sequentially seeded on two changes of dishes coated with antibody to RAN-2 to remove type 1 astrocytes, meningeal cells, and microglia (Barres et al., 1992a), and then onto dishes coated with the A2B5 antibody to collect OPCs. Following trypsinization, the cells were seeded in one 75 sq cm flask in the serum free growth medium described above. Within three days, these cells were confluent and could be subcultured into polylysine-coated flasks, 12mm polylysine–

coated coverslips for immunofluorescence, or 100mm polylysine-coated Petri dishes for western blotting. These cells could be passaged 3-4 times.

To differentiate precursors into immature OLs, growth medium was removed from cultures, and cells were fed with "differentiation medium" (DM), consisting of 50% DMEM, 50% Ham's F12 with 50 μg/ml transferrin, 5 μg/ml putresine, 3 ng/ml progesterone, 2.6 ng/ml selenium, 12.5 μg/ml insulin, 0.4 μg/ml T4, 0.3% glucose, 2mM glutamine, 10 ng/ml and biotin. Signaling molecules were added to some cultures, including Wnt3a (50 ng/ml, R & D), Dkk-1 (100 ng/ml, R & D), and SB216763 (10 μM, Biomol International), and cells were immunolabeled after 3 days.

To establish cultures of mouse OLs, a mixed population of cells was isolated from forebrains of normal and *CNP-Cre* β-catenin constitutively active *(β-Cat-CA*) mice using the same methods and media as described above for rat OL isolation. Individual mouse brains were cultured separately until mice were genotyped by PCR of tail DNA, at which point cultures from mice of identical genotypes were then combined. Cultures were purified using a gentle modified washdown procedure. Four ml of Hanks Buffered Salt Solution without Mg+ and Ca+ was drawn up into a Pasteur pipette and was vigorously ejected at the cell monolayer at an angle. Small, round process-bearing cells (OPCs) detached, leaving a population of cells adherent to the plastic (GFAP+ astrocytes). The plate was tilted and the non-adherent cells plus the Hank's medium were drawn up into the pipette and the procedure was repeated several times. The detached cells were collected in the Hank's medium, triturated several times with the Pasteur pipette, and centrifuged at 100g for 5 minutes. The pellet, at 90-95% OPCs, was plated on polylysine-coated vessels.

Immunofluorescence

Cells on coverslips were processed for detection of specific antigens as described previously (Grinspan and Franceschini, 1995; See et al., 2004). Antibody pairs used for cell cultures were A2B5 (undiluted, hybridoma supernatant, ATCC, (Eisenbarth et al., 1979) with goat anti-mouse IgM, anti-Galactocerebroside (GalC, R-mAb, hybridoma supernatant (Ranscht et al., 1982), with goat anti-mouse IgG3, anti-PLP (AA3, hybridoma supernatant, gift of Dr. Alex Gow, Wayne State), anti-GFAP (hybridoma supernatant, gift of Virginia Lee, University of Pennsylvania) with goat-anti rat IgG, anti-Tuj1 (1:50, Chemicon/Millipore, Billerica, MA ) with goat anti-mouse IgG, and anti-β-catenin (1:1000, Transduction laboratories) with goat-anti mouse IgG1. To quantify cell proliferation, cells were treated with a pulse of 10 μM BrdU. Three, 6, or 24 hours later, cells were fixed with 70% ethanol in 50 mM glycine buffer for 20 minutes on ice, labeled with anti-BrdU antibody (1:10, Roche Applied Science), followed by anti-mouse-fluorescein IgG. To determine cell death, coverslips were labeled with anti-cleaved caspase antibody (1:200, Cell Signaling) followed by goat anti-rabbit IgG. The TUNEL assay to determine apoptosis was also performed as previously described (Grinspan et al., 1998).

To count cells expressing antigens in culture, antigen+ and DAPI+ cells were counted in 10 fields in each of 2 coverslips from at least 3 separate preparations of cells using a Leica DM6000B fluorescence microscope at 63x magnification. Approximately 1000 total cells were counted per condition.

Western Blotting

Tissue sections from P1, P7, P21, and adult mouse spinal cord and brain were dissected, sonicated for 30s in cold lysis buffer (containing 25 mM Tris, pH 7.6, 1mM $MgCl_2$, 1 mM EDTA, 1% TritonX-100, 1% SDS, 1 mM PMSF, 50 µg/ml antipain, 2 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A), centrifuged, and the supernatants preserved. For cells in culture, cells were harvested in lysis buffer with a cell scraper (Corning Inc), passaged 10x through a 22g syringe, rotated on ice for 30 minutes, and spun at 14,000g for 15 minutes. Amount of protein in each sample was determined by the BCA method (Pierce Chemical, Rockford, IL). Samples were be denatured by boiling in sample buffer containing β–mercaptoethanol, separated on 15% or 8% acrylamide gels, and transferred to immobilon-P membranes (Millipore). Membranes were incubated with appropriate primary antibodies (MBP, 1:500, and β-catenin, 1:1000) followed by the appropriate horseradish peroxidase-conjugated anti-rat IgG secondary antibody (1:100), and imaged using ECL reagents (Amersham, Piscataway, NJ) and hyperfilm (Amersham). Blots were stripped and reprobed with GAPDH (1:2,500, Chemicon International) as a loading control for protein quantification.

Generation of *Cnp-Cre* β-catenin constitutively active mice

Constitutively activated β-catenin (*β-Cat-CA*) mice were generated by crossing heterozygous *Cnp-Cre* mice (obtained from Dr. Klaus Nave, Max Planck Institute, Germany, (Lappe-Siefke et al., 2003) with β-catenin$^{floxedexon3/floxedexon3}$ mice (obtained from Dr. Mark Taketo, Kyoto University, Japan). Half of the offspring were heterozygous for *CNP-Cre* and floxed β-catenin, the other half were heterozygous for

floxed β-catenin, showed no phenotype, and used as controls. PCR analyses of tail genomic DNA were used to identify all mouse genotypes. *β-Cat-CA* mice survive to adulthood but are smaller than control littermates.

Immunohistochemistry

To prepare sections of normal and mutant spinal cords, mice were sacrificed at P1, P7, P21, and adulthood according to established protocols (See et al., 2007). Mice at P1 and P7 were decapitated, spinal cord sections were removed and fixed in 4% paraformaldehyde for 1 hr, placed in 30% sucrose overnight, and embedded in OCT (Sakura Finetek). Adult and P21 mice were perfused with 0.9% saline and 4% paraformaldehyde, then spinal cord sections were removed, fixed in 4% paraformaldehyde overnight, washed in phospho-buffered saline (PBS), placed in 30% sucrose overnight, and embedded in OCT. Frozen sections were cut on a Leica cryostat at 12μm. To label OL lineage cells and astrocytes, slides were washed PBS, incubated in block with 20% fetal bovine serum, 2% bovine serum albumin, and 0.1% triton in PBS for 20 minutes to 1 hour, washed in PBS, incubated overnight at 4°C in primary antibody, washed in PBS, and then incubated in the appropriate secondary antibody for 30 minutes (all secondaries were diluted 1:100 and purchased from Jackson Immunoresearch Laboratories), washed in PBS, and then mounted on slides with mounting medium containing DAPI (Vector Laboratories).

The antibody pairs used to label mature OLs *in vivo* were anti-PLP (1:2) with goat anti-rat IgG, anti-MBP (1:2, rat hybridoma supernatant, gift of Virginia Lee, University of Pennsylvania) with goat anti-rat IgG, and anti-aspartoacylase (ASPA, dilution, gift of

Dr James Garbern, Wayne State University) with goat anti-rabbit IgG, and anti-CC1 (1:20, Calbiochem) with goat anti-mouse IgG. Antibody pairs used to label OPCs were anti-PDGFRα (1:250, BD Biosciences Pharmigen) with goat anti-rat IgG and anti-NG2 (1:100, Chemicon) with goat anti-rabbit IgG. Astrocytes were labeled with anti-GFAP (1:100) with goat- anti-rat IgG, and neurons were labeled with anti-NeuN (1:100, Chemicon) with goat anti-mouse IgG.

To characterize changes in cell proliferation between the control and mutant animals, we employed both the mitosis marker phosphorylated-histone H3 (PH3, 1:250, Upstate technology) and antibody to the proliferation protein Ki-67 (1:00, Vector labs), as described above, both with goat anti-rabbit IgG (1:100).

Cell death was measured using an anti-cleaved-caspase 3 antibody (1:200, Cell signaling) or the TUNEL method as previously described. Detection of cell death was combined with immunophenoptyping of OLs using the NG2 antibody.

To count cells from frozen sections of all mice, normal and mutant animals from at least three litters were used. For P1 mice, digital images were taken at from three cervical spinal cord sections per animal, counting 6 40x fields of white matter per section. For P7 animals, digital images were taken at 40x from 4 white matter and 4 grey matter sections. For adult animals, composite images were accumulated at 10x to count all cells in the white matter of hemisections.

Semi thin sections

Adult mice and P7 mice were sacrificed and processed for semi-thin sections according to established protocols (Arroyo et al., 1998; Scherer et al., 1998). Three

control and mutant mice were anesthetized at each age, perfused with PBS followed by 2% paraformaldehyde and 2% gluteraldehyde in 0.1M phosphate buffer. The spinal cord, sciatic nerves, and optic nerves were removed and placed in fixative overnight. They were post fixed in 4% $OsO_4$ in 0.1M phosphate buffer for 2 hours, rinsed in 0.1M phosphate buffer, and dehydrated in ascending concentrations of ethanol. Tissue was then embedded in ascending concentrations of epoxy and semi-thin sections were stained with toluidine blue. Images were recorded at 20x, 40x, and 63x magnification. For each animal, myelinated axons were counted in 2 ROIs of 50 x 150μm (in P7 mice) or 100 x 250μm (adult mice) in ventral spinal cord, and in an entire cross section of sciatic nerve.

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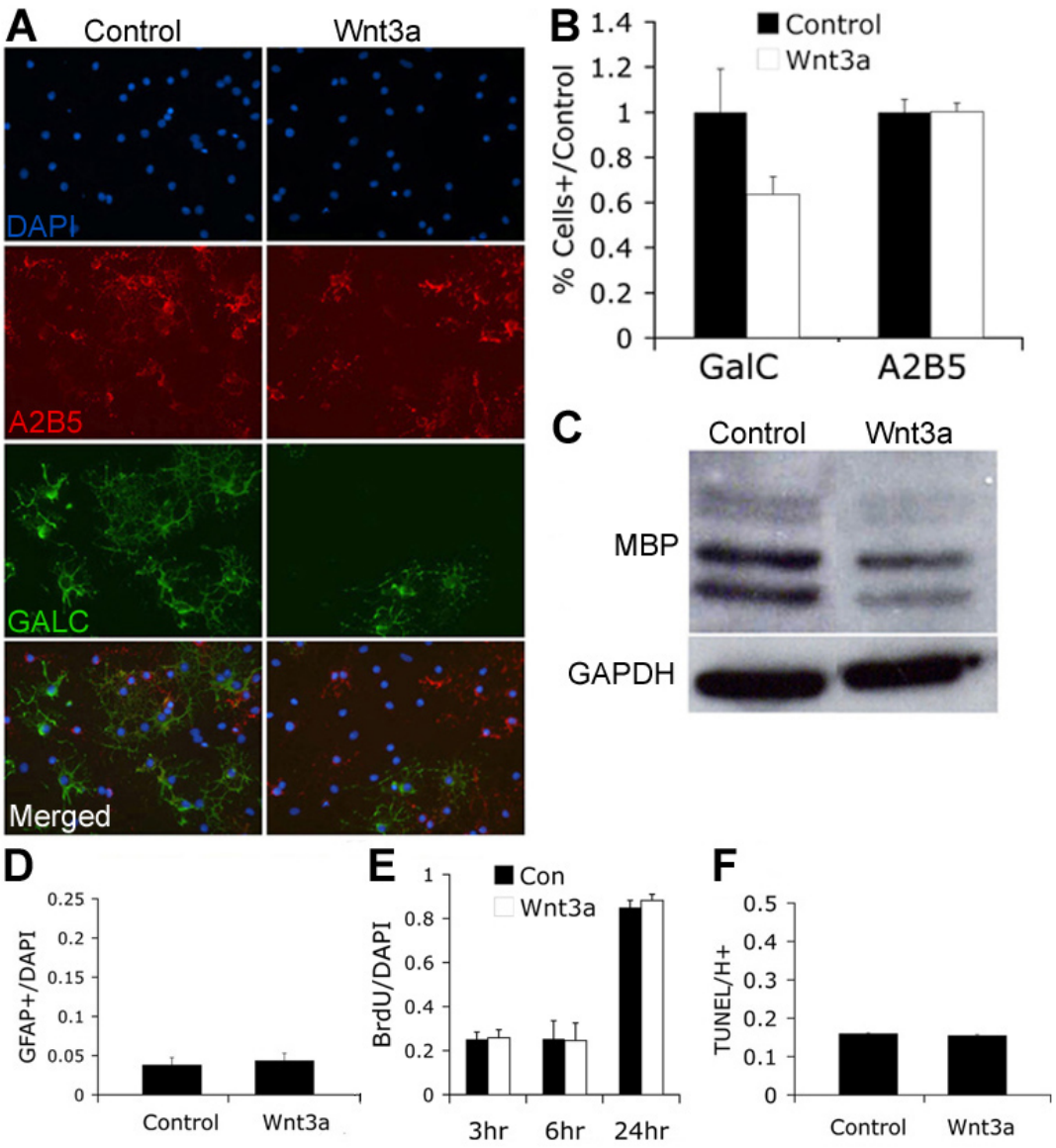



**Figure 2.1: Wnt signaling inhibits OPC differentiation *in vitro* and does not promote the generation of astrocytes, nor alter cell death or proliferation.**

Rat OPC cultures were placed in differentiation medium with 50 ng/ml Wnt3a and processed for immunofluorescence. (A, B) Following 3 days of treatment, cells were labeled with the A2B5 antibody to identify OPCs and antibody to GalC to identify

immature or mature OLs. The antibody positive cells and DAPI positive nuclei were counted and expressed as percent of control. Cultures treated with Wnt3a showed a 40% reduction in GalC labeled cells compared to controls after 3 days in DM ($n = 8$, $p < 0.05$), while there was no statistically significant change in the number of A2B5 labeled cells ($n = 5$). (C) Western blots performed on protein from cells cultured with DM or DM plus Wnt3a for 5 days were probed with antibody to MBP. GAPDH is used as a loading control. Wnt3a treated cells showed a decrease in MBP expression relative to controls. (D) Cultures treated with Wnt3a for 3 days showed no statistically significant alteration from controls in number of GFAP+ astrocytes ($n = 9$). (E) Proliferation was unchanged between treated cultures and controls as measured by cells labeling with BrdU after 3 ($n = 8$), 6 ($n = 3$), and 24 hours ($n = 3$). (F) The number of apoptotic cells was not altered in the Wnt3a treated cultures after 3 days as measured by the TUNEL assay ($n = 3$).

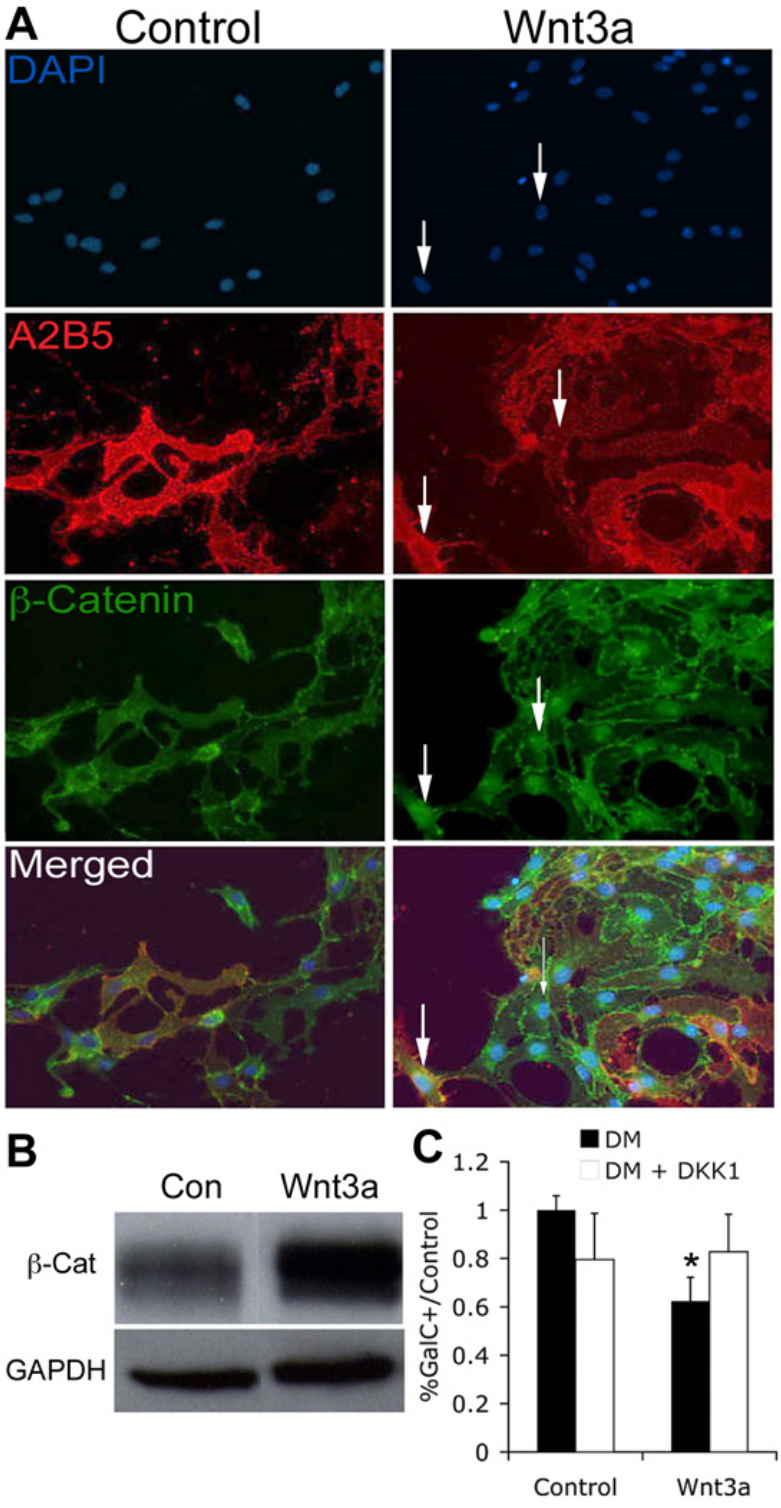



**Figure 2.2: Wnt3a Treatment inhibits oligodendrocyte differentiation through activation of the canonical β-catenin signaling pathway.**

(A) Rat OPC cultures were double labeled with antibodies to A2B5 and β-catenin 6 hours after treatment with Wnt3a. β-catenin labeling appeared in the nuclei of A2B5+, Wnt3a-treated cells, but not controls (indicated by the white arrows). (B) Western blots were performed on protein from rat OPC cultures 24 hours after Wnt3a treatment, using antibodies to β-catenin and GAPDH as the loading control. Protein taken from cells treated with Wnt3a showed an increase in β-catenin staining relative to controls (n = 3). (C) Cultures were placed in differentiation medium and treated with Wnt3a or Wnt3a plus the canonical Wnt signaling inhibitor Dkk-1. Cells treated with Wnt3a alone showed a 38% reduction in GalC staining relative to controls ($p < 0.05$, n = 3), whereas cells treated with Wnt3a and Dkk-1 did not have statistically different numbers of GalC+ cells compared to controls.

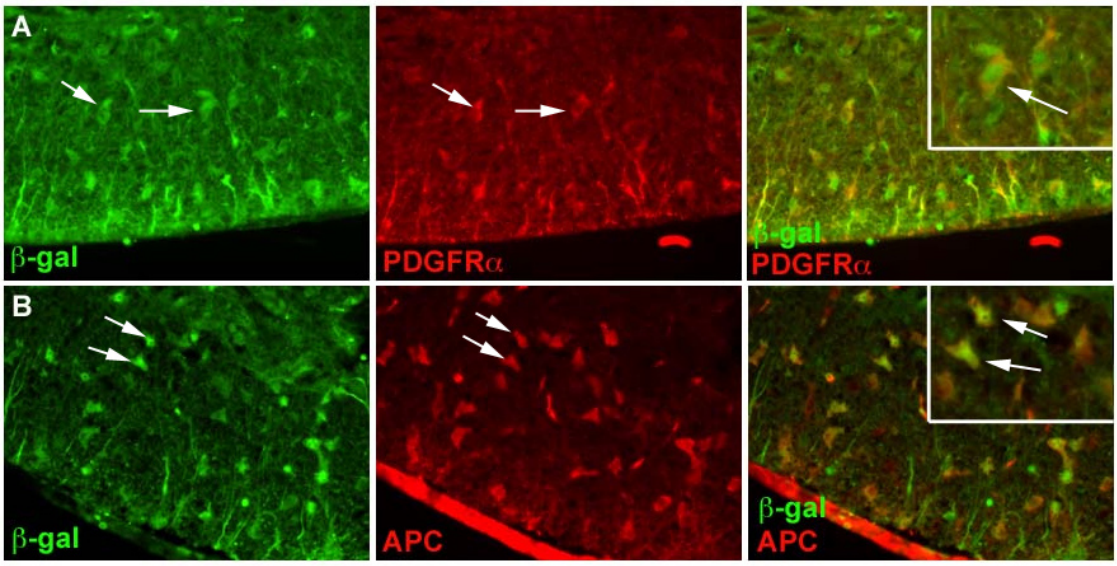



**Figure 2.3: *Cnp-Cre* is expressed in both OPCs and mature oligodendrocytes.**

*Cnp-Cre* mice were mated with Rosa26 mice. β-galactosidase expression in cells of these animals indicates somatic recombination of the Rosa26 gene. Animals were sacrificed at P5 and spinal cords were sectioned. (A) Photomicrographs taken at 40x magnification showing PDGFRα+ OPCs also labeling with antibody to β-galactosidase. Inset is at 63x magnification; white arrows indicate double-labeled cells. (B) Photomicrographs taken at 40x magnification depicting cells double labeling with APC, which identifies mature OLs, and β-galactosidase. Inset is at 63x magnification; white arrows indicate double-labeled cells.

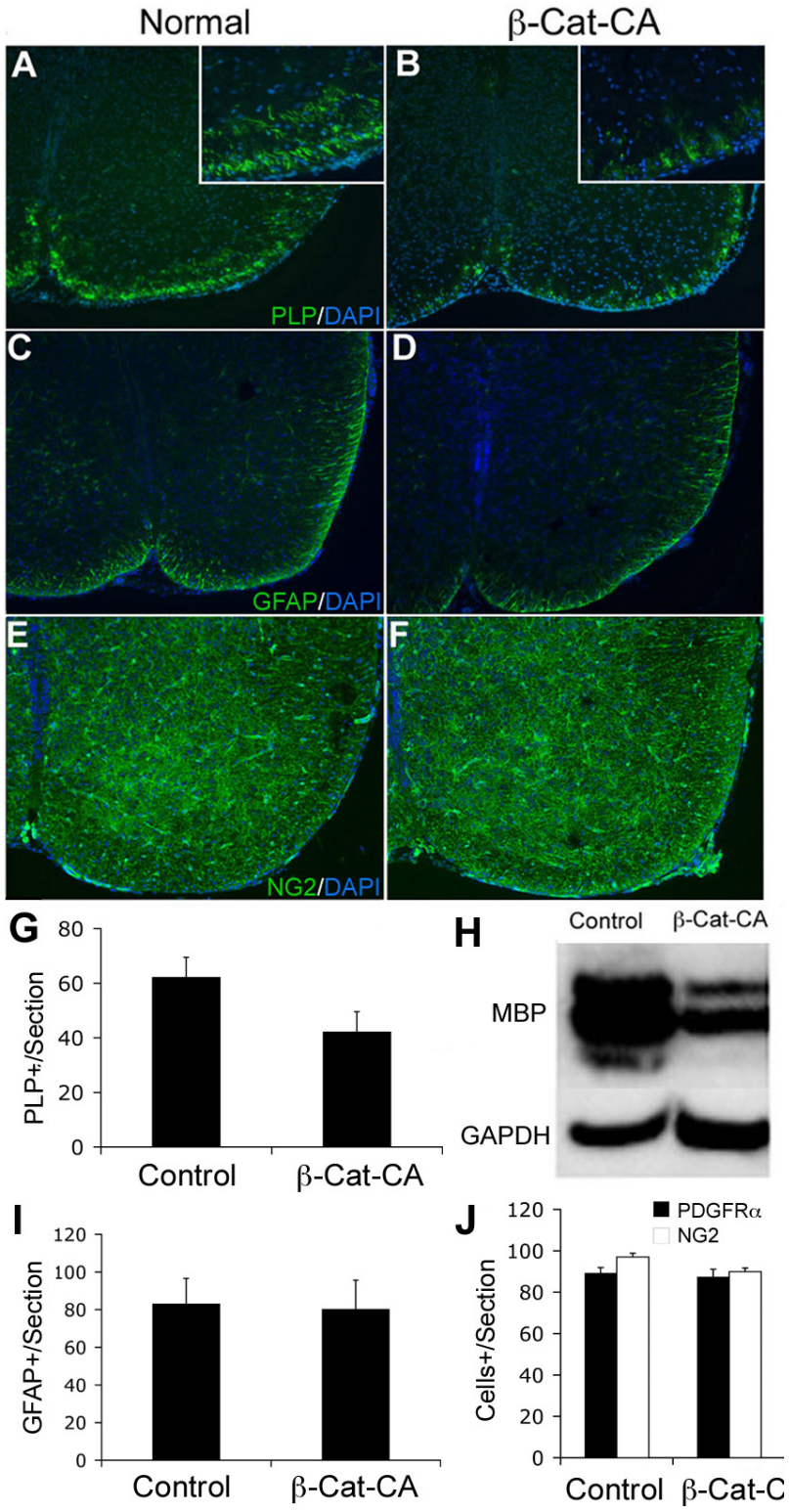



**Figure 2.4: P1 *β-Cat-CA* mice have fewer oligodendrocytes and less myelin protein than control littermates, but the same numbers of OPCs.**

Spinal cords were removed from P1 control and *β-Cat-CA* mice and prepared for cryosectioning and labeling. Cell counts were performed at 40x magnification in 6 fields of white matter, on 3 spinal cord sections per animal from each of 6 control and mutant animals from 3 litters. (A, B) Photomicrographs depicting ventral cervical spinal cord in *β-Cat-CA* mice and control littermates labeled with DAPI and antibody to PLP at 10x magnification, with 40x magnification insets. (C, D) Photomicrographs depicting equal numbers of GFAP+ cells in spinal cord sections of *β-Cat-CA* mice and control littermates. (E, F) Photomicrographs depicting equal numbers of cells labeling with DAPI and antibody to NG2 in *β-Cat-CA* mice and control littermates. (G) Counts of PLP+ cells per section showed a 32% decrease in OLs in *β-Cat-CA* mice relative to controls ($p < 0.05$, $n = 6$). (H) Western blots were performed on protein taken from forebrain from P1 mice, and indicated a decrease in MBP expression in *β-Cat-CA* mice relative to controls, using GAPDH as a loading control ($n = 3$). (I) Counts of GFAP+ cells per section showed no significant difference between mutant mice and controls ($n = 5$). (J) There were no significant differences in the numbers of OPCs between mutant and control spinal cords using both antibody to NG2 ($n = 3$) and PDGFRα ($n = 6$).

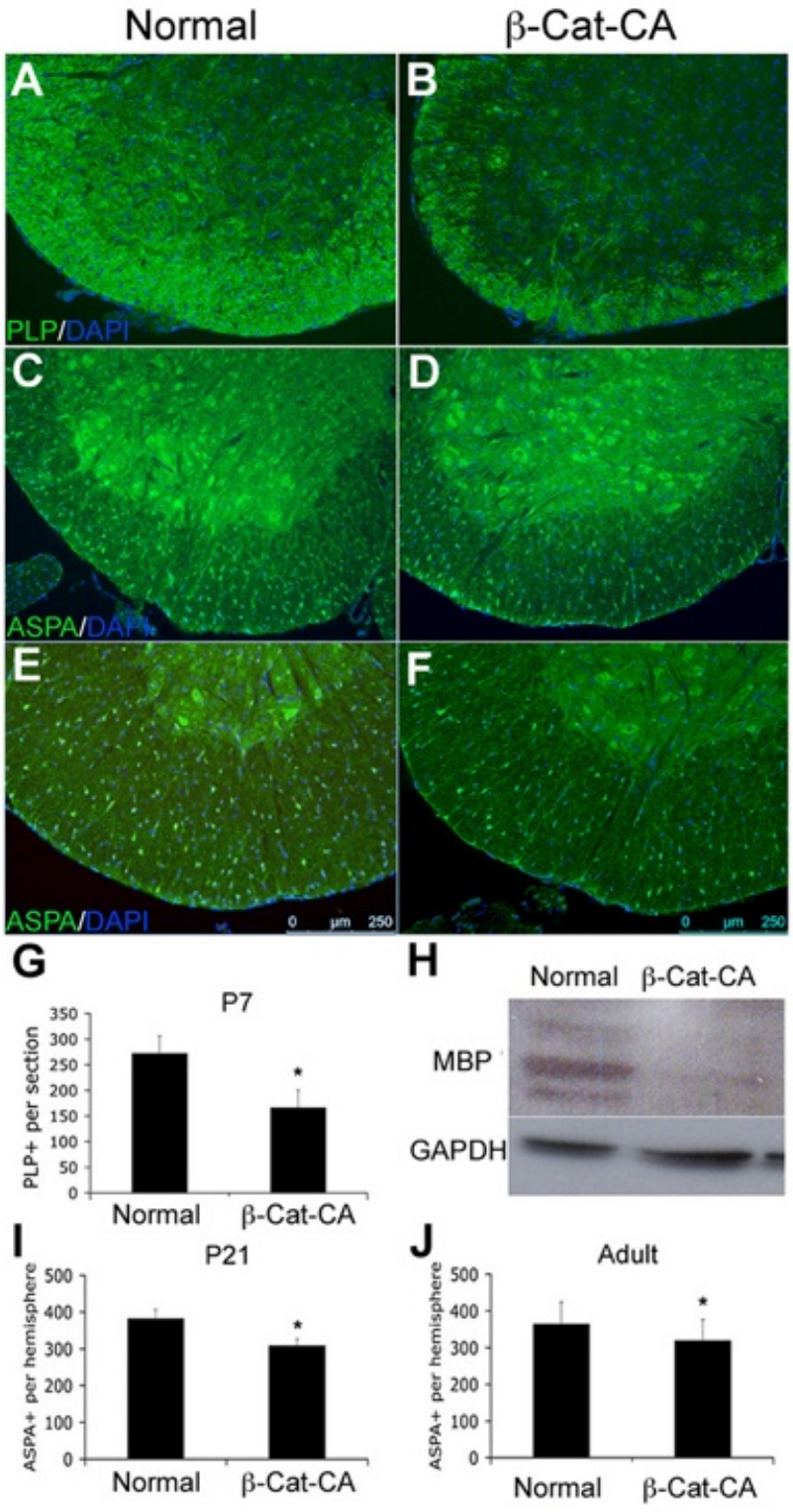



**Figure 2.5: *β-Cat-CA* mice show a decrease in myelin protein expression that becomes less severe with age.**

*β-Cat-CA* and normal mice were sacrificed at P7, P21 and 10 weeks, and spinal cords were prepared for sectioning and immunolabeling. (A, B) Photomicrographs of spinal cord sections of P7 *β-Cat-CA* mice and control littermates labeled with DAPI and antibody to PLP, demonstrating decreased labeling in both the white and grey matter of the mutant cord sections. (C, D) Photomicrographs of spinal cord sections of P7 *β-Cat-CA* mice and control littermates labeled with DAPI and antibody to ASPA, which labels OL cell bodies, demonstrating fewer OLs labeling with ASPA in the white matter. (E, F) Photomicrographs of adult *β-Cat-CA* mice and control littermates showing labeling for ASPA and DAPI. (G) To perform cell counts at P7, PLP+ cells in 8 identical fields were counted at 40x magnification in 3 sections of ventral spinal cord per animal in 6 animals from 3 liters. *β-Cat-CA* mice had 38% fewer PLP+ cells than control littermates ($p < 0.05$, $n = 6$). (H) Western blots were performed on protein taken from forebrain of P7 mice, and indicated a decrease in MBP expression in *β-Cat-CA* mice relative to controls, using GAPDH as a loading control ($n = 3$). (I) At P21, cell counts were performed on the white matter of 3 composite hemisections per animal in 6 animals per 3 litters. There were 19% fewer ASPA+ cells in *β-Cat-CA* mice compared to control littermates ($p < 0.05$, $n = 6$). (J) In adults, cell counts were also performed on the white matter of 3 composite hemisections per animal in 3 animals in 6 liters. There were 12% fewer ASPA+ cells in *β-Cat-CA* mice compared to control littermates ($p < 0.05$, $n = 8$).

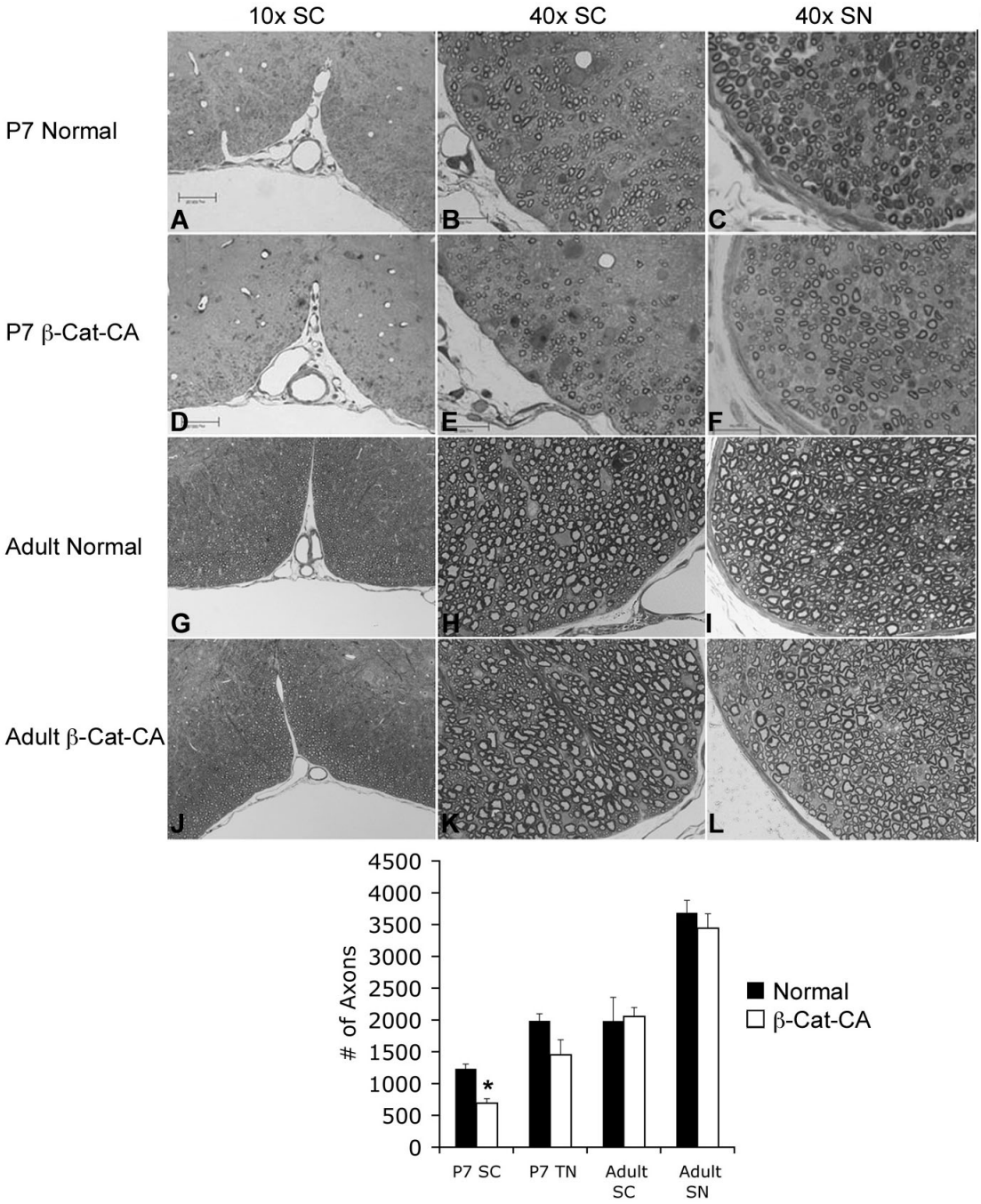



**Figure 2.6: *β-Cat-CA* mice have fewer myelinated axons than control littermates at P7, but not at adulthood.**

*β-Cat-CA* and normal mice were sacrificed at P7 or adulthood, then spinal cord and sciatic nerve were removed and prepared for semi-thin sections as described in the

methods. Images were taken at 10x and 40x magnification, and myelinated axons were counted in 2 regions of interest of 50 x 150μm (at P7) or 100 x 250μm (in adults) in ventral spinal cord, or in complete cross sections of sciatic nerve. (A, B) Images taken at 10x and 40x magnification of ventral spinal cord demonstrating 44% more myelinated axons ($p < 0.05$, $n = 3$) in normal mice than in *β-Cat-CA* littermates (D, E). (C) Images taken at 40x magnification of tibeal nerve demonstrating 27% more myelinated axons ($p < 0.07$, $n = 3$) in normal mice than in *β-Cat-CA* mice littermates (F). (G, H) Images taken at 10x and 40x magnification of ventral spinal cord showing similar numbers of myelinated axons in adult normal mice and *β-Cat-CA* littermates (J, K). (I) Images taken at 40x magnification of sciatic nerve showing similar numbers of myelinated axons in normal mice and *β-Cat-CA* littermates (L). (M) Graph showing counts of the number of myelinated axons in ventral spinal cord and tibeal nerve of P7 mice and ventral spinal cord and sciatic nerve of adult mice.

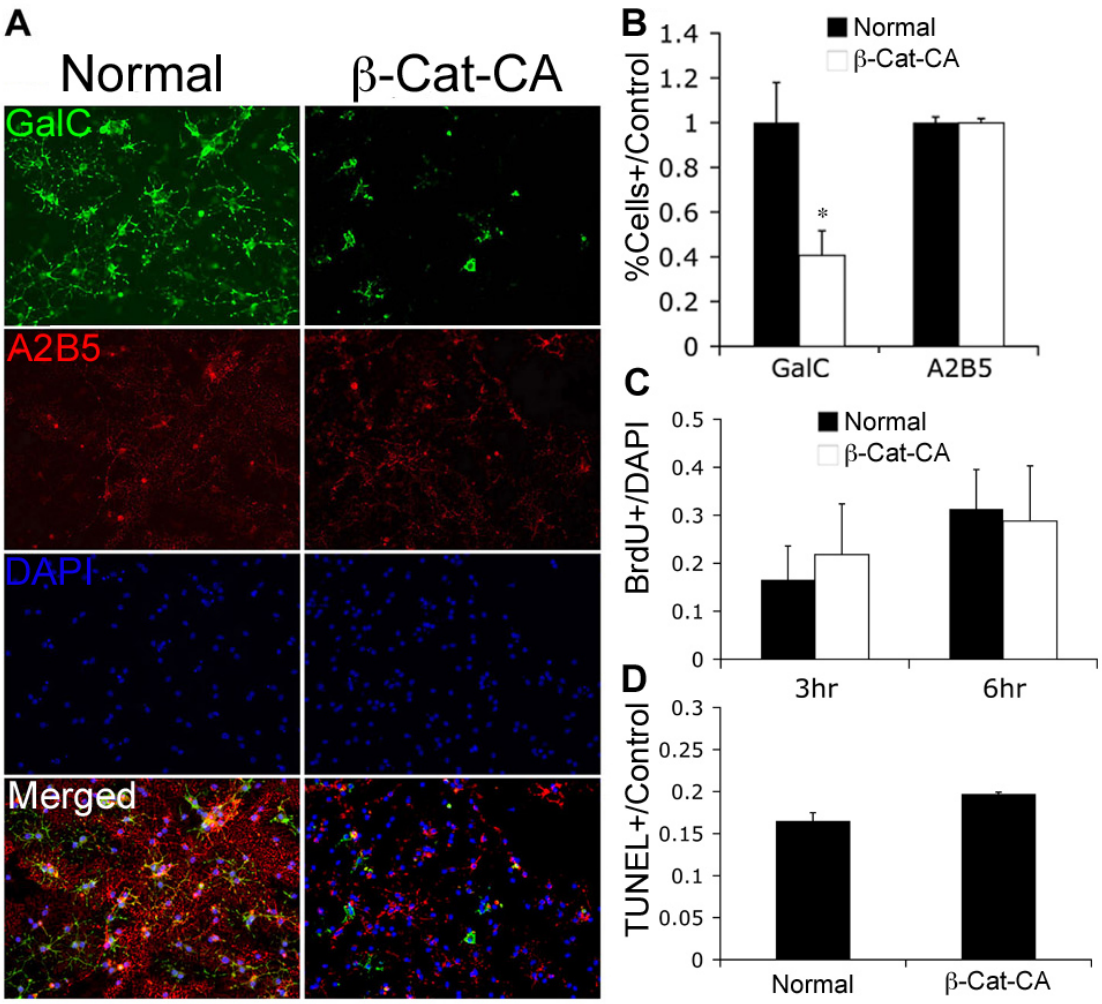
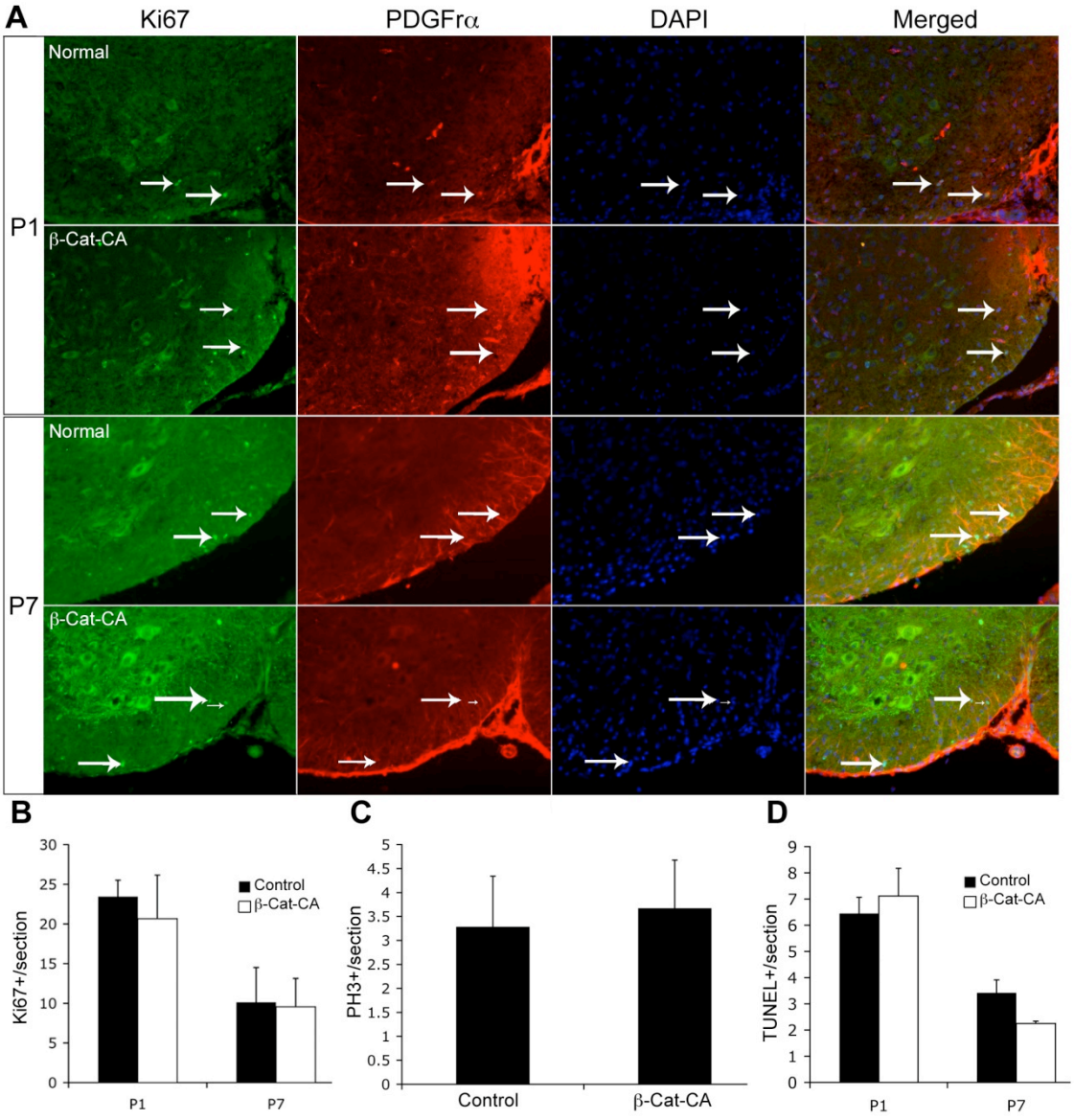



**Figure 2.7: OPCs from *β-Cat-CA* mice differentiate poorly in culture.**

OPC cultures were generated from forebrain of normal and *β-Cat-CA* mice. Cultures were then placed in DM and were immunolabeled after 3 days. (A) Photomicrographs of cultures after 3 days in DM. Cells were labeled with antibodies to GalC and A2B5. (B) Cells from *β-Cat-CA* cultures had 60% fewer GalC+ cells relative to control cultures after 3 days in DM ($p < 0.05$, $n = 5$), but maintained similar levels of DAPI and A2B5 ($n = 5$). (C) After 3 days in DM, there was no significant difference in

the number of GFAP labeled cells. (D) After 3 and 6 hours, there was no significant difference in the number of BrdU labeled cells in mutant cultures relative to controls (n = 3). (E) Mutant cultures did not have significantly different numbers of TUNEL labeled cells relative to controls (n = 3).




**Supplemental Figure 2.1: Spinal cord sections from *β-Cat-CA* mice and control mice did not contain statistically different numbers of proliferating cells or cells undergoing apoptosis.**

*β-Cat-CA* mice and normal littermates were sacrificed at P1 and P7, processed for sectioning, and labeled with markers for OPCs, proliferation, and apoptosis. (A) Photomicrographs of ventral spinal cord sections from *β-Cat-CA* mice and control littermates at P1 and P7. Sections were labeled with PDGFRα, Ki67, and DAPI, and

taken at 63x magnification. White arrows indicate cells colabeling with PDGFRα and Ki67. (B) Cell counts show no statistical difference in the number of Ki67+ cells from spinal cords of *β-Cat-CA* mice and control littermates at P1 and P7 (n=3). (C) There was also no statistically significant differences in the number of PH3+ cells in spinal cord sections taken from *β-Cat-CA* mice and control littermates at P1 (n=3). (D) There was no statistical difference in the number of TUNEL+ cells in spinal cord sections taken from *β-Cat-CA* mice and control littermates at P1 and P7 (n=4).

## Chapter 3

# Canonical Wnt signaling requires the BMP pathway to inhibit oligodendrocyte maturation

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**Short running title:** Wnts and BMPs interact to regulate OL development

**Key words:** Wnt, BMP, oligodendrocytes, development, glia, myelin

**Abbreviations:** *β-Cat-Null*, β-catenin loss of function mutant mice; BMP, bone morphogenic protein; BrdU, Bromodeoxyuridine; CNS, central nervous system CNP, 2'3' cyclic nucleotide 3'-phosphodiesterase; DM, differentiation medium; Dkk-1, Dikkopf-1; DKO, double knockout; GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; Histone deacetylase, HDAC; IHC, immunohistochemistry; ID, inhibitor of differentiation; KO, knockout; MBP, myelin basic protein; OL, oligodendrocyte; OPC, oligodendrocyte precursors; PCR, polymerase chain reaction; PLP, proteolipid protein; Shh, sonic hedgehog

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**Abstract**

We examine the relationship between the canonical Wnt and BMP pathways during oligodendrocyte development *in vitro*. The regulation of oligodendrocyte development, from precursor to mature myelinating cell, is controlled by a variety of inhibitory and inductive signaling factors. The dorsal spinal cord contains signals that inhibit oligodendrocyte development, including members of the Wnt and BMP signaling pathways. Wnts and BMPs have overlapping temporal activity and similar inhibitory effects on oligodendrocyte differentiation. In addition, both pathways feature prominently in many developmental processes and during demyelinating events, and they are known to interact in complex inductive, inhibitive, and synergistic manners in many developing systems. The interaction between BMP and Wnt signaling in oligodendrocyte development, however, has not been extensively explored. We use pharmacological and genetic paradigms to show that both Wnt3a and BMP4 will inhibit oligodendrocyte differentiation *in vitro*. We also show that when the canonical BMP signaling pathway is blocked, neither Wnt3a nor BMP4 have inhibitory effects on oligodendrocyte differentiation. In contrast, abrogating the Wnt signaling pathway does not alter the actions of BMP4 treatment. Our results indicate that the BMP signaling pathway is necessary for the canonical Wnt signaling pathway to exert its effects on oligodendrocyte development, but not vice versa, suggesting that Wnt signals upstream of BMP.

## Introduction

Myelin is an essential physiological structure, allowing for the rapid and effective conductance of neural signals. Demyelination, which occurs in disorders such as multiple sclerosis, severely impairs neuronal communication, resulting in functional deficits and axonal degeneration (Trapp et al., 1998; Lappe-Siefke et al., 2003; Edgar and Garbern, 2004). Because several factors involved in regulating myelination during development are also involved in demyelinating disorders, understanding their actions is crucial to designing treatments or therapies (Setoguchi et al., 2001; Armstrong et al., 2002; Liu et al., 2008b; Zhang et al., 2009; Cate et al., 2010).

Oligodendrocytes (OLs) are the myelinating cells of the central nervous system (CNS). OLs are generated through a series of specific developmental stages (Pringle and Richardson, 1993; Ono et al., 1995), during which they are exposed to a range of signaling factors that can be inductive or inhibitory, extracellular or intracellular (Miller, 2002). OL precursor cells (OPCs) originate in ventricular zones at E12.5 in the rodent CNS and migrate dorsally and radially, expressing markers such as A2B5, NG2, and PDGFRα. Once differentiation begins, OPCs progress to immature OLs, generating processes and expressing galactocerebroside (GalC). After contacting neurons, OPCs will begin to extend processes, express myelin proteins, including proteolipid protein (PLP), myelin basic protein (MBP), and 2'-3' cyclic nucleotide 3'-phosphodiesterase (CNP), then myelinate axons (Grinspan, 2002; Miller, 2002). While the signals that influence this development are beginning to be characterized, the extent of their interactions remains to be fully explored.

Initially, signals in the ventral spinal cord, especially sonic hedgehog (Shh), induce expression of transcription factors essential for OL specification and development, including Olig1 and Olig2 (Lu et al., 2000; Zhou et al., 2000; Zhou and Anderson, 2002). In contrast, signals emanating from the roofplate in dorsal spinal cord can inhibit this development (Wada et al., 2000), possibly to control the exact times when these cells reach and myelinate dorsal regions. There are, however, dorsal populations of OPCs whose generation is Shh independent, although their overall contributions appear to be limited and their distinct functions are unknown (Cai et al., 2005; Kasai et al., 2005; Vallstedt et al., 2005; Kessaris et al., 2006).

Two families of dorsal signaling factors, the bone morphogenic proteins (BMPs) and the Wnts, have been shown to exert inhibitory effects on OPC differentiation. BMPs are members of the TGFβ signaling family, and they have many roles in the developing nervous system involving embryonic patterning, cell proliferation, specification, differentiation, and apoptosis (Liem et al., 1995; Mehler et al., 1997; Liem et al., 2000; Wine-Lee et al., 2004; See and Grinspan, 2009 for review). BMP has been more extensively investigated with regard to OL development, exerting time and stage specific effects. BMPs drastically inhibit OPC differentiation into mature OLs, instead promoting astrogliogenesis (Grinspan et al., 2000a; Miller et al., 2004; Samanta and Kessler, 2004; See et al., 2004; Cheng et al., 2007).

The Wnts are involved in many of the same roles as BMPs in the nervous system, including embryonic patterning, cell specification, proliferation, migration, and differentiation (Dorsky et al., 1998; Coyle-Rink et al., 2002; Braun et al., 2003; Hirabayashi et al., 2004; Karim et al., 2004; Kalani et al., 2008; Ulloa and Marti, 2010

for review). Recent studies have investigated the influence of canonical Wnt signaling on OL development, both *in vitro* and *in vivo*, indicating that the Wnts and BMPs have similar effects. In spinal cord explants and OPC cultures, ectopic Wnt3a application inhibits the differentiation of OPCs into mature cells (Shimizu et al., 2005; Kim et al., 2008; Fancy et al., 2009; Feigenson et al., 2009; Ye et al., 2009). Constitutively-activated Wnt signaling in cells of OL lineage results in an early developmental decrease in mature OLs. This effect diminishes as the mice age, however, suggesting that the importance of Wnt signaling may vary over the course of OL development (Fancy et al., 2009; Feigenson et al., 2009).

The interaction of BMPs and Wnts is well documented in a variety of different systems, their expression is spatially and temporally similar, and they are functionally involved in many of the same processes. Their relationship, however, varies based on tissue and developmental timeline: depending on context, they can directly or indirectly be inductive, antagonistic, or synergistic (Soshnikova et al., 2003; Guo and Wang, 2009; Itasaki and Hoppler, 2010 for review). There is evidence that Wnt signaling prevents oligodendroglial specification at the neural stem cell stage by upregulating BMPs through neurogenesis (Kasai et al., 2005), but how these two pathways may interact in OL development has not been explored in detail. An understanding of the interaction of these pathways is an important avenue for developing treatments and therapies for demyelinating events. BMPs are upregulated in CNS injury and models of demyelinating disease and can inhibit remyelination (Setoguchi et al., 2001; Setoguchi et al., 2004; See and Grinspan, 2009; Cate et al., 2010; Jablonska et al., 2010). In parallel, several recent studies have also found that members and effectors of the Wnt family are upregulated in

similar paradigms, and may have similar inhibitory effects on recovery (Liu et al., 2008b; Fancy et al., 2009; Miyashita et al., 2009; White et al., 2010).

Because Wnts and BMPs have similar sites of origin and effects on OLs during development and demyelinating events, we hypothesized that these two signaling factors mutually regulate OL development. To explore this interaction, we have employed effectors and antagonists of both canonical Wnt and BMP signaling on both mutant and normal OPC cultures. We find that while both BMP4 and Wnt3a inhibit the differentiation of OPCs, when the BMP pathway is blocked - by either chemical or genetic means - the effects of both Wnt3a and BMP4 are abrogated. In contrast, the effect of BMP4 on OPC differentiation is not altered when the canonical Wnt signaling pathway is blocked. Our results indicate that the BMP pathway is necessary for canonical Wnt signaling to inhibit OPC differentiation.

**Results**

Wnt and BMP inhibit oligodendrocyte differentiation

To observe the effects of Wnt3a and BMP on OL development *in vitro*, we examined highly purified primary cultures of OPCs from mouse or rat brain. OPCs were grown to confluency on coverslips or dishes, subsequently, growth factors were removed and differentiation medium (DM) was added with or without BMP4 (50ng/ml) or Wnt3a (50ng/ml). After 3 days in DM, cells were immunostained with antibodies against GalC (a marker of early differentiation) and A2B5 (which labels OPCs). In control conditions, 26% of rat cells expressed GalC, and 67% labeled with A2B5, as observed in previous studies (Feigenson et al., 2009). In contrast, BMP4 treatment almost completely

eliminated GalC labeling while maintaining comparable levels of A2B5 labeling (Fig. 3.1A, C). Wnt3a treatment reduced GalC labeling by 33% ($p < 0.05$), in keeping with the 30-40% decrease we have observed previously (Feigenson et al., 2009), and also did not have a significant effect on the amount of cells labeling with A2B5 (Fig. 3.1A, C).

To determine whether BMP4 or Wnt3a affected the number of astrocytes, we immunolabeled cells with antibodies directed against glial fibrillary acidic protein (GFAP). Control cultures and cultures treated with Wnt3a contained fewer than 5% astrocytes, while in BMP4 treated cultures the number of astrocytes increased 6.5-fold ($p < 0.05$, Fig. 3.1B, D). We have previously shown that neither BMP4 nor Wnt3a significantly affected the number of cells undergoing apoptosis, proliferation, or differentiation into neurons (Grinspan et al., 2000a; Feigenson et al., 2009). Thus, BMP4 completely inhibited OPC differentiation and induced astrogliogenesis, while Wnt3a partially inhibited OPC differentiation without promoting astrocyte differentiation.

A BMP inhibitor blocks the effects of both BMP4 and Wnt3a signaling but a Wnt inhibitor blocks only Wnt3a signaling.

To determine whether there is a relationship between the Wnt and BMP signaling pathways, pharmacological inhibitors of both pathways were added to our treatment paradigm. Noggin (500ng/ml), an inhibitor of canonical BMP signaling, was added to OPC cultures along with DM, 50ng/ml BMP4, or 50ng/ml Wnt3a. Noggin treatment alone did not alter the number of GalC+ cells relative to control conditions. Noggin added with BMP4 completely blocked the effect of BMP4, and the number of GalC+ cells was not significantly different from DM treatment alone (Fig. 3.2A). Similarly,

when Noggin was added along with Wnt3a, the number of GalC+ cells was the same as in the untreated control condition, indicating that blocking the BMP signaling pathway blocks the inhibitory actions of BMP4 and Wnt3a on OPC differentiation *in vitro* (Fig. 3.2A).

To determine whether blocking the canonical Wnt signaling pathway could block BMP signaling, we treated OPC cultures with Dikkopf-1 (Dkk-1), an inhibitor of canonical Wnt signaling. Dkk-1 treatment (100ng/ml) alone did not alter the number of GalC+ cells relative to control conditions. When used concurrently with Wnt3a, however, Dkk-1 blocked the inhibitory effect of Wnt3a. In contrast, BMP4 completely inhibited GalC expression in cells even when treated concurrently with Dkk-1 (Fig. 3.2B), indicating that blocking the canonical Wnt signaling pathway does not block the BMP signaling pathway *in vitro*. These results suggest that BMP4 does not signal upstream of the canonical Wnt pathway, and that the BMP pathway is not dependent on the Wnt signaling pathway.

Genetic ablation of the BMP signaling pathway blocks the effects of BMP4 and Wnt3a

We next used a genetic model to help determine the relationship between BMP4 and Wnt3a and confirm our pharmacological results. We cultured OPCs from single BMPR1 double receptor knockout mice (*Bmpr1 DKO*) in which both BMPR1A and BMPR1B have been functionally inactivated in the neural tube by E10.5 using the *Bcre-32* transgenic allele (Wine-Lee et al., 2004; See et al., 2007). When the two type I membrane receptors, 1A and 1B, are both deleted, BMP signaling is effectively eliminated (Wine-Lee et al., 2004). The BMP signaling pathway is described in detail in

Chapter 1. OPCs cultured from the animals are able to differentiate, do not translocate Smad to the nucleus, and do not respond to BMP treatment (See et al., 2007).

We generated cultures from these double knockout animals as well as from single knockouts (*Bmpr1b KO*, *Bmpr1a KO*), and normal littermates. We then treated these cultures with DM alone or DM containing BMP4 or Wnt3a. OPCs in the *Bmpr1 DKO* cultures differentiated to the extent of the normal cells even in the presence of BMP4, as we have previously shown (See et al., 2007). When *Bmpr1 DKO* OPC cultures were treated with Wnt3a, the number of GalC+ cells was not significantly different from control conditions (Fig. 3.3A). BMP4 completely eliminated GalC expression in normal mouse OPC cultures after 3 days in DM, and Wnt3a reduced the number of GalC+ cells by 41%. The number of A2B5+ cells did not vary from control conditions, replicating our previous findings in control cultures. These results were further replicated in OPC cultures from *Bmpr1a KO* and *Bmpr1b KO* mice (Fig. 3.3A), indicating that when the BMP signaling pathway is completely abrogated, the canonical Wnt signaling pathway is similarly prevented from inhibiting OPC differentiation. Furthermore, when either *Bmpr1a* or *Bmpr1b* is individually deleted, the other type I receptor can compensate to maintain the integrity of the BMP signaling pathway and allow it to influence OL development.

Genetic inhibition of the canonical Wnt signaling pathway blocks the effect of Wnt3a, but not that of BMP4, on oligodendrocyte differentiation

To observe the effectiveness of canonical Wnt signaling and canonical BMP signaling on OL development when the Wnt signaling pathway has been effectively

eliminated, we employed mutant mice with a conditional knockout of the nuclear effector of canonical Wnt signaling, β-catenin *(β-Cat-Null*). *Cnp-Cre* mice were bred to a line containing floxed sites surrounding exons 3-6 on the β-catenin gene (Huelsken et al., 2001; Lappe-Siefke et al., 2003; Feigenson et al., 2009). Cre mediated recombination results in no functional β-catenin protein, preventing the activation of canonical Wnt signaling. The Wnt pathway is described in detail in Chapter 1. In these mutant mice, the Wnt signaling pathway is inhibited in all cells of OL lineage by preventing active β-catenin from being produced.

OPC cultures were generated from mutants and normal littermates at P1 and treated with DM with or without BMP4 or Wnt3a. Wnt3a did not change the number of GalC-expressing cells relative to DM in the *β-Cat-Null* OPC cultures, indicating that canonical Wnt signaling was effectively eliminated from OPCs (Fig. 3.3B). In contrast, BMP4 completely eliminated expression of GalC in treated cells. These results indicate that blocking the canonical Wnt signaling pathway prevents the inhibitory effect of Wnt3a on OL development, but not that of BMP4, and that the BMP signaling pathway is not upstream of the Wnt signaling pathway or dependent on the Wnt signaling pathway in this system.

BMP4, but not Wnt3a, increases phosphorylated Smad levels

To see if Wnt3a directly upregulates the canonical BMP signaling pathway, we treated cultured OPCs with BMP4 and Wnt3a and looked for increases of phosphorylated Smad 1/5/8. When cultures were treated with BMP4, phosphorylated-Smad levels increased after 30 minutes post treatment, as observed by Western blots (Fig. 3.4A). In

contrast, Wnt3a did not noticeably increase Smad levels after the same amount of time or at longer time points, up to 48 hours post treatment (data not shown), demonstrating that Wnt3a does not directly upregulate BMP signaling.

BMP4 and Wnt3a do not decrease OPC differentiation in a combinatorial manner

To determine whether the effects of BMP4 and Wnt3a were additive, we treated OPCs with DM containing 50ng/ml Wnt3a and 10ng/ml BMP4, both of which individually reduce the number of GalC+ cells by approximately 40% relative to control conditions. We also treated cells with each growth factor individually and with 50ng/ml BMP4 as a positive control. There were no significant decreases in GalC expression, however, in the combined treatment condition when compared to individual treatments with 10ng/ml BMP4 or 50ng/ml Wnt3a (Fig. 3.5). This is consistent with the interpretation that the same downstream pathway is used by both signaling factors to inhibit OL maturation.

Wnt3a and BMP4 target *Id2* and Olig2

We used quantitative real-time PCR to determine the effects Wnt and BMP treatment on OPC development. Treating cultures with Wnt3a or BMP4 decreased *Mbp* transcript levels after 48 hours, mirroring our previous results (Fig. 3.6A). ID proteins are known inhibitors of differentiation, and previous studies have revealed that they are targets of BMP and Wnt signaling (Samanta and Kessler, 2004; Ye et al., 2009). When assaying for *Id2* mRNA, both Wnt3a and BMP4 significantly increased transcript levels

(Fig. 3.6B). These results indicate that Wnt3a and BMP4 can target a key effector of OL differentiation.

ID2 prevents the nuclear localization of Olig2 after stimulation with BMP4, thus inhibiting BMP signaling (Samanta and Kessler, 2004). We examined the localization of Olig2 after treatment with either BMP4 or Wnt3a. After 2 days of treatment, we observed a significant decrease in the total number of cells in which Olig2 was clearly visible colocalized with cell nuclei in both BMP4 and Wnt3a treated cultures. There was a 55% reduction in Olig2 and DAPI colabeling in BMP4 treated cultures and a 19% reduction in Wnt3a treated cultures relative to controls ($p < 0.05$, Fig. 3.7A, B). These results further indicate that BMP4 and Wnt3a are targeting common factors in OPC differentiation.

**Discussion**

We have analyzed the relationship between canonical Wnt and BMP signaling in OL development, and our results indicate that both pathways rely on a fully functional BMP signaling pathway. In primary OPC cultures, BMP4 by itself almost completely eliminates OPC differentiation and significantly increases the number of astrocytes. Wnt3a by itself decreases OPC differentiation by 30-40% without a corresponding increase in astrocyte generation. Blocking the BMP signaling pathway, however, prevents the effects of both factors. In contrast, blocking the canonical Wnt signaling pathway was able to counter the effect of Wnt3a on OPC differentiation, but not that of BMP4.

The BMP and Wnt pathways have many contextually and temporally dependent interactions throughout development (Itasaki and Hoppler, 2010). In the developing

spinal cord, overexpression of either signaling factor alone induces a dorsalized phenotype, whereas their removal produces ventralized phenotypes, indicating that both signals may have functionally similar, interacting, or redundant effects (Nguyen et al., 2000; Muroyama et al., 2002; Timmer et al., 2002; Zechner et al., 2007; Alvarez-Medina et al., 2008). In some developmental systems, the BMP pathway upregulates or acts upstream of Wnt signaling, such as in neural crest delamination (Burstyn-Cohen et al., 2004), keratinocyte development (Yang et al., 2006), and dorsal/ventral patterning (Zechner et al., 2007). Conversely, Wnt signaling can act upstream or upregulate the BMP pathway, such as during neurogenesis and astrogliogenesis (Kasai et al., 2005), limb mesenchyme development (Hill et al., 2006), and tooth development (Liu et al., 2008a). The downstream effectors of both signaling pathways can also interact in synergistic manners to regulate transcription (Labbe et al., 2000; Nishita et al., 2000; Letamendia et al., 2001; Theil et al., 2002; Hussein et al., 2003), and canonical Wnt signaling can prolong the activity of the BMP pathway (Fuentealba et al., 2007). In other contexts, Wnt and BMP signaling can be directly antagonistic, such as in neuroepithelial cell development (Ille et al., 2007), aspects of neural development (Gomez-Skarmeta et al., 2001), muscle positioning (Bonafede et al., 2006), and osteoblast development (Kamiya et al., 2008b; Kamiya et al., 2008a; Honda et al., 2010). In some systems, such as during aspects of limb bud and apical ectodermal ridge formation, the BMP and Wnt pathways can complexly regulate each other through parallel signaling and feedback systems (Soshnikova et al., 2003; Villacorte et al., 2010).

Our results clearly demonstrate that the BMP signaling pathway is essential for the Wnt signaling pathway to inhibit OPC differentiation. Treatment of cultures with

Wnt3a does not upregulate phosphorylated Smad levels, however, indicating that Wnt signaling does not directly upregulate canonical BMP signaling. The possibility remains, however, that Wnt signaling may directly activate the BMP signaling pathway, but at levels below detection in Smad assays. Some targets of Smads have TCF/LEF binding sites in close proximity, indicating that the two pathways have synergistic activity among their downstream effectors, including *Emx2* (Theil et al., 2002), *Msx2* (Willert et al., 2002; Hussein et al., 2003), *c-myc* (Hu and Rosenblum, 2005), and *Xtwin* (Labbe et al., 2000; Nishita et al., 2000; Letamendia et al., 2001). In some of these instances, activation of either pathway results in downstream activity, but the activity is significantly increased upon concurrent activation of both BMP and Wnt signaling. In our system, BMP and Wnt activity could independently activate signaling, but blocking BMP would completely prevent both pathways from having any effect. Furthermore, while we investigated the canonical BMP and Wnt signaling pathways, both systems have non-canonical pathways that are active in development. Wnt treatment could directly upregulate a non-canonical arm of the BMP signaling pathway, which would not necessarily be observed by Smad phosphorylation.

While both BMP4 and Wnt3a treatment inhibit OPC differentiation and maintain similar levels of A2B5+ cells, only BMP4 directly increases the number of astrocytes in culture. It has been shown that BMP4 may activate various pathways in cells of multipotent neural precursor cells, and that its effects on astrocyte induction may result from non-canonical pathways (Rajan et al., 2003). It is also possible that there is a lower threshold required to induce astrogliogenesis with canonical BMP signaling than with canonical Wnt signaling.

To find at what level BMP4 and Wnt3a may be inhibiting OPC differentiation, we performed Real-Time Quantitative PCR and assayed for *Id2* transcript levels. Both BMP4 and Wnt3a consistently reduced transcript levels of *Mbp*, complementing our IHC results, and also increased levels of *Id2*, in keeping with earlier studies (Samanta and Kessler, 2004; Ye et al., 2009). IDs are actively involved in preventing OPC differentiation and OL myelination, largely by preventing nuclear localization of Olig proteins to keep the timing of differentiation precise and regulated (Kondo and Raff, 2000; Wang et al., 2001a; Samanta and Kessler, 2004; Gokhan et al., 2005; Marin-Husstege et al., 2006; Cheng et al., 2007). We found that both Wnt3a and BMP4 reduced the number of cells colabeled with Olig2 in the nucleus, indicating that both factors were actively preventing the nuclear activity and differentiating capabilities of Olig2, possibly via ID2 induction.

The BMP and Wnt signaling pathways may also interact during epigenetic regulation of OL differentiation. Recent studies have shown that histone deacetylases (HDACs) compete with activated β-catenin to bind with TCF4. Bound to β-catenin, TCF4 acts as a transcriptional repressor of OL differentiation by upregulating such factors as ID2/4. Bound to HDACs, TCF acts as a transcriptional repressor, inhibiting the activity of ID2/4 and upregulating myelin-promoting genes such as *Mbp* (Marin-Husstege et al., 2002; Shen et al., 2005; He et al., 2007; Ye et al., 2009). Our experiments indicate that canonical Wnt signaling depends on the BMP pathway to inhibit OL development; it is possible that the TCF transcriptional complex requires inherent Smad elements or other downstream effectors of BMP signaling to interact with IDs or other inhibitory elements.

Understanding what signals regulate the timing mechanisms OL maturation will facilitate the development of therapies for myelin disorders. BMP upregulation has been

well documented in many types of CNS disease and demyelinating paradigms (See and Grinspan, 2009). Furthermore, several recent studies demonstrate that Wnt signaling is active during white matter injury as well, including aspects of axonal regeneration (Liu et al., 2008b; Miyashita et al., 2009), NG2+ cell proliferation (Orre et al., 2009; White et al., 2010), and remyelination (Fancy et al., 2009). BMPs and Wnts interact in many intricate ways during development, and it is likely they have similar interactions during CNS injury and recovery. It is, therefore, important to understand how these pathways function independently and in relationship to one another. We have shown that the BMP pathway is required for canonical Wnt signaling to inhibit OPC differentiation in cell culture paradigms. Further experiments should investigate this interaction during remyelination events and CNS injury models.

**Experimental Methods**

Cell culture generation and treatment

All experiments were performed in accordance with the guidelines set forth by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee. To generate purified OPC cultures from newborn Sprague-Dawley rats or mixed background mice, forebrain cells were harvested and seeded on 100mm Petri dishes in serum containing medium as previously described (See et al., 2004; Feigenson et al., 2009). After 24 hours, cultures were switched to serum free growth medium containing Neurobasal medium (Invitrogen) with B27 supplement (1:50 Life technologies), 10ng/ml basic fibroblast growth factor (R & D Systems, Minneapolis, MN), 2ng/ml platelet-derived growth factor (R & D), and 1ng/ml neurotrophin-3 (Peprotech, Rocky Hill, NJ).

Rat cultures were purified 6 to 8 days after plating by immunopanning as previously described (Grinspan et al., 2000a). Cells were successively seeded onto two sets of dishes coated with Ran-2 antibody to bind type-1 astrocytes, meningeal cells, and microglia. Cells were then put on dishes plated with A2B5 antibody (undiluted, hybridoma supernatant, ATCC, Rockville, MD, (Eisenbarth et al., 1979)) to bind the OPCs. When cells reached confluency, they were subcultured into polylysine-coated flasks, 12 mm polylysine coated coverslips, 100 mm polylysine coated Petri dishes, or 50 mm polylysine coated Petri dishes. Cells could be passaged 3-4 times. Cultures were considered purified if they contained fewer than 5% astrocytes.

To culture mouse OPCs, cells were harvested from the forebrains of individual animals using the same methods and media as previously above. Individual mouse brains were cultured singularly until genotypes were identified by PCR of tail DNA. At this stage, identically genotyped cultures were combined. OPCs were purified using a gentle modified washdown procedure as previously described (Feigenson et al., 2009). Briefly, 4 ml of Hanks Buffered Salt Solution without Mg+ and Ca+ was drawn into a Pasteur pipette, and then forcefully released on the cell monolayer at an angle. OPCs would detach, leaving any astrocytes adherent to the plate. OPCs and the Hanks medium were then drawn up into the pipette and the procedure could be repeated several times until enough cells were collected. The detached OPCs were collected in the Hank's medium, triturated several times with the Pasteur pipette, and centrifuged at 100 g for 5 minutes. The pellet was then plated onto a polylysine-coated flask. Cultures were considered purified if they contained fewer than 5% astrocytes.

When OPCs were differentiated into mature OLs, growth medium was removed,

and cell cultures were fed with differentiation medium (DM), consisting of 50% DMEM and 50% Ham's F12, with 50μg/ml transferrin, 5μg/ml putresine, 3ng/ml progesterone, 2.5ng/ml selenium, 12.5μg/ml insulin, 0.4μg/ml T4, 0.3% glucose, 2 mM glutamine, and 10ng/ml biotin. Cells were allowed to differentiate for 3 days to examine markers of immature OLs or 5 days to look at markers of mature OLs. Treatment conditions involved application of signaling factors to some cultures; these were 50ng/ml Wnt3a (R & D), 50ng/ml or 10ng/ml BMP4 (R & D), 100ng/ml Dkk-1 (R & D), and 500ng/ml Noggin (R & D).

Immunofluorescence

Immunostaining and preparation of coverslip-plated cells were performed as previously described (Grinspan and Franceschini, 1995; Feigenson et al., 2009). Antibody pairs used to detect surface antibodies were anti-A2B5 with goat anti-mouse IgM, and anti-Galactocerebroside (RmAb GalC, undiluted, hybridoma supernatant, (Ranscht et al., 1982)) with goat-anti-mouse IgG3. Internal antibodies included anti-GFAP (undiluted, hybridoma supernatant, gift of Dr. Virginia Lee, University of Pennsylvania) with goat anti-rat IgG, and anti-Tuj1 (1:50, Chemicon/Millipore, Billerica, MA) with goat anti-mouse IgG. To label Olig2 (1:500, Millipore, paired with goat anti-rabbit IgG), cells were blocked for 10 minutes in 0.3% triton after fixing with 4% paraformaldehyde. To quantify apoptosis, the TUNEL assay was used as previously described (Grinspan et al., 1998). To label cell proliferation, cells were treated with BrdU and cell counts were performed as previously described (Feigenson et al., 2009). For all cell counts, positively labeled cells and DAPI+ cells were counted in 10 fields in each of

2 coverslips from each of at least 3 separate *in vitro* preparations, using a Leica DM6000B fluorescence microscope at 63x magnification. Approximately 3000 cells were counted per experimental data point.

Western Blots

Rat cells were collected for Western blot analysis as previously described (Feigenson et al., 2009). Cells were treated with 50ng/ml BMP4 or Wnt3a and collected after 30 minutes, 3 hours, or 48 hours. Membranes were incubated with Phosphorylated-Smad (1:3000, Cell Signaling) followed by horseradish peroxidase-conjugated anti-rat IgG secondary antibody (1:100), and imaged using ECL reagents (Amersham, Piscataway, NJ) and hyperfilm (Amersham). Blots were stripped and reprobed with GAPDH (1:2,500, Chemicon International) as a loading control for protein quantification. Secondary antibodies were purchased from Jackson Laboratories.

Generation of mutant mice

The *Bmpr1* double knockout mice (*Bmpr1 DKO*) were generated as previously described (Wine-Lee et al., 2004; See et al., 2007). Lines include a classical BMPR1A knockout (Mishina et al., 1995), a BMPR1A conditional knockout mouse (Ahn et al., 2001; Mishina et al., 2002), a BMPR1B classical knockout mouse (gift from Dr. Karen Lyons, UCLA (Yi et al., 2000), and a *Bcre-32* transgenic mouse. The generation of these mice has been previously described (Wine-Lee et al., 2004; See et al., 2007). Normal littermates have one functional allele of both BMPR1 receptors and do not exhibit any phenotype. All mice were sacrificed at P1 and OPC cultures were successfully harvested.

*β-Cat-Null* mice were made available by crossing β-catenin$^{floxedexon3\text{-}6/floxedexon3\text{-}6}$ mice (made available by Dr. Walter Birchmeier, Max Delbruck Centrum, Germany) with *CNP-Cre* mice, obtained from Dr. Klaus Nave, Max Planck Institute, Germany. Generation of β-catenin$^{floxedexon3\text{-}6/floxedexon3\text{-}6}$ mice has been described previously (Huelsken et al., 2001), as has the generation of *CNP-Cre* mice (Lappe-Siefke et al., 2003). Offspring were established by mating β-catenin$^{floxedexon3\text{-}6/floxedexon3\text{-}6}$; +/+ mice with β-catenin$^{floxedexon3\text{-}6/+}$; CNP/+ mice. β-catenin loss of function mice had the genotype β-catenin$^{floxedexon3\text{-}6/floxedexon3\text{-}6}$; CNP/+.

Real Time PCR

OPC cultures were grown on 50 mm dishes and harvested after 6 or 48 hours post treatment with 50ng/ml Wnt3a or 50ng/ml BMP4, with or without 500ng/ml Noggin. Cells were extracted with Trizol (Molecular Research Center, Inc., Cincinnati, OH). Complementary DNA (cDNA) was reverse transcribed from 2-5μg RNA per treatment group using Superscript III First Strand Synthesis Kit for RT-PCR (Invitrogen #18080044) Primers used were GAPDH, MBP, and ID2 (IDT Technologies). Quantitative Real-Time PCR was performed using SYBR Green (Applied Biosystems) with the MXPro 3000P. Quantification was determined using the SYBR Green with Dissociation Curve method, as per the protocol of the manufacturer. Threshold values of fluorescence, $C_t$ values, were calculated at least three times per cycle, and values relative to control conditions were used to determine treatment differences. Samples were measured in triplicate for each experiment.

Image Acquisition

All images were acquired using a Leica DM6000B fluorescence microscope. All images were mounted using Vectashield (Vector). Images were acquired with a Leica DFC360 Fx camera, using Leica Application Suite 2.1.0 software. Images were evenly enhanced using Adobe Photoshop (Adobe Systems, Mountain View, CA).

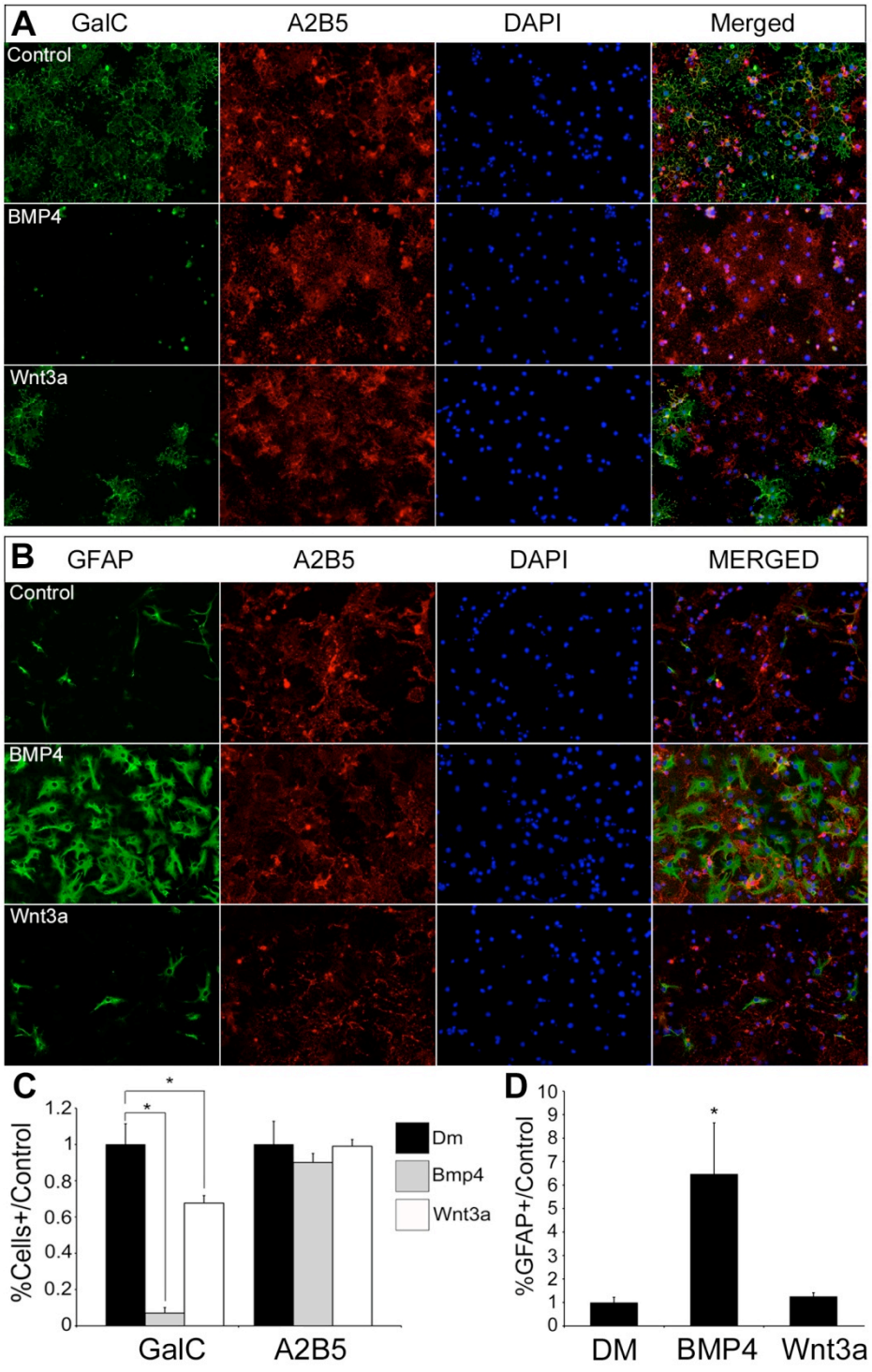


**Figure 3.1: BMP4 and Wnt3a treatment inhibit oligodendrocyte differentiation**

Cultures of rat OPCs were grown to confluence, switched to DM, alone or with BMP4 or Wnt3a, and then immunostained for GalC, GFAP, and A2B5. (A) After 3 days

in DM, many control OPCs expressed GalC and A2B5. BMP4 treated OPCs expressed A2B5 at comparable levels to controls conditions, but almost no cells expressed GalC ($p < 0.01$). Wnt3a treated cells had comparable levels of A2B5 expressing cells relative to control conditions, but a 33% reduction in the number of cells expressing GalC ($p < 0.05$). (B) Control cultures had occasional labeling of GFAP+ cells, whereas 6.5 times as many cells in the BMP4 treatment condition were positively labeled with GFAP antibody ($p < 0.01$). Wnt3a treatment did not noticeably change the number of GFAP+ cells relative to control conditions. (C, D) Bar graphs quantify the number of cells labeled with specific antibodies, measured relative to control conditions, $n = 9$. Images shown were taken at 20x magnification.

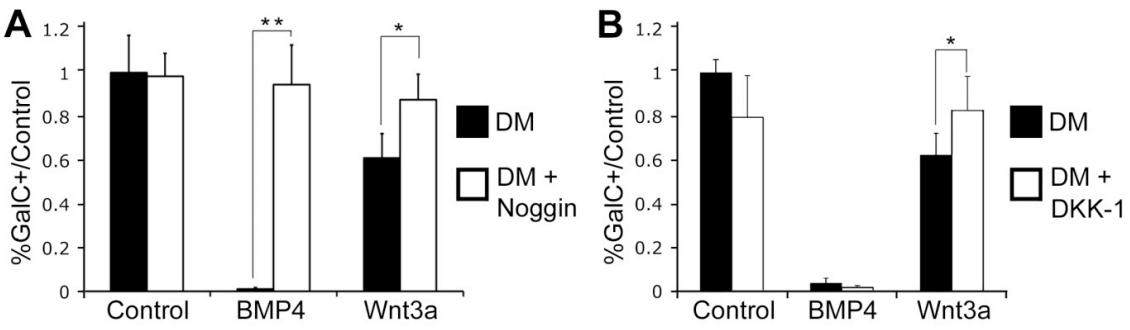


**Figure 3.2: Pharmacological inhibition of the BMP pathway blocks the effects of Wnt3a and BMP4 on oligodendrocyte differentiation**

OPC cultures were placed in DM, alone or with BMP4 or Wnt3a. Noggin or Dkk-1 was added to similarly treated cells. (A) BMP4 treatment eliminated GalC expression ($p < 0.01$), but the addition of Noggin concurrently with BMP4 rescued this effect. Similarly, Wnt3a treatment reduced the number of GalC+ cells by 39% relative to controls ($p < 0.05$), but concurrent Noggin treatment rescued this effect ($n = 7$). (B) Dkk-1 addition abrogated the effect of Wnt3a on the number of GalC+ cells ($p < 0.01$), did not alter the BMP4 mediated elimination of GalC expressing cells ($p < 0.05$, $n = 3$).

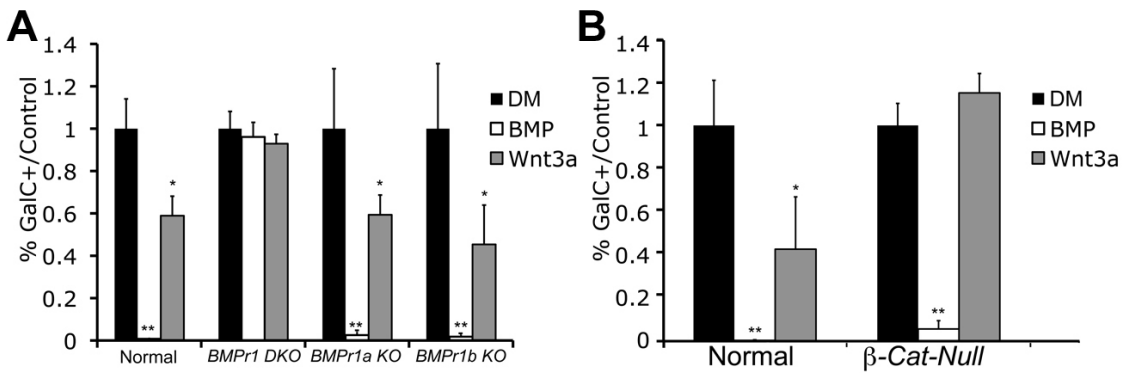


**Figure 3.3: Oligodendrocyte precursor cells from mice lacking the BMP type I receptor do not respond to BMP4 or Wnt3a treatment**

OPC cultures were generated from *Bmpr1 DKO*, *Bmpr1a KO*, *Bmpr1b KO*, and normal mice, grown, and placed in DM. (A) After DM treatment, OPCs from all 3 types of mutant mice exhibited similar numbers of GalC+ cells relative to controls. BMP4 treatment almost completely eliminated GalC+ cells in *Bmpr1a KO*, *Bmpr1b KO*, and normal mouse OPCs ($p < 0.01$), but not in OPC cultures from *Bmpr1 DKO* mice. Similarly, Wnt3a expression decreased GalC+ cells in cultures from *Bmpr1a KO*, *Bmpr1b KO*, and normal mice ($p < 0.05$), but not in those from *Bmpr1 DKO* mice. (B) OPC cultures generated from *β-Cat-Null* mice and normal littermates were treated with BMP4 or Wnt3a. BMP4 almost completely reduced the number of GalC+ cells in both types of cultures ($p < 0.01$). Wnt3a reduced the number of GalC+ cells by 57% in control cultures, but did this effect was not observed in the mutant cultures ($p < 0.05$, $n = 3$).

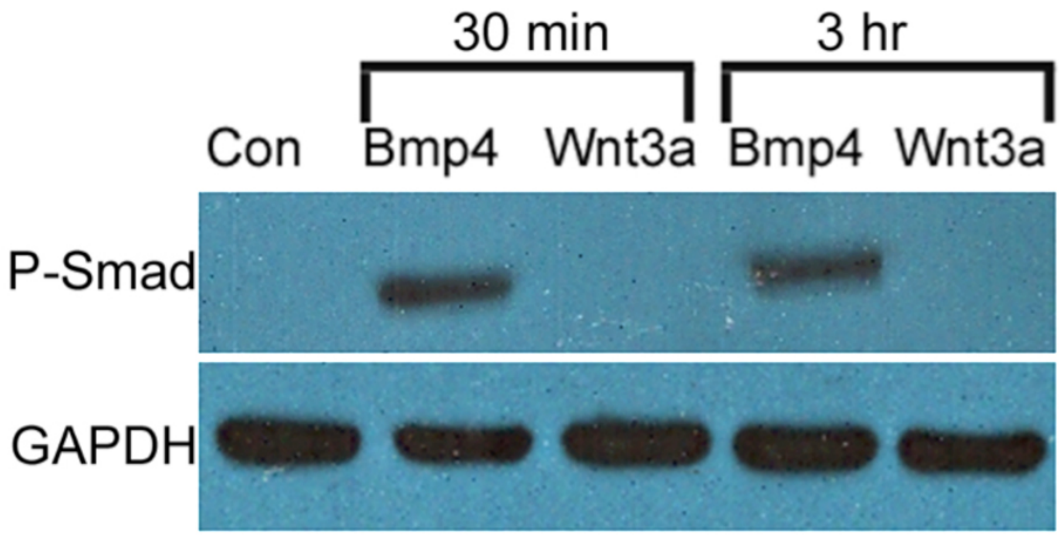


**Figure 3.4: BMP4, but not Wnt3a, increases levels of phosphorylated Smad 1/5/8**

Rat cells were treated with Wnt3a or BMP4, and cells were harvested for protein at 30 minutes and 3 hours. Phosphorylated Smad bands are visible in BMP4 treated conditions but not in Wnt3a or control conditions. GAPDH was used as a loading control (n = 3).

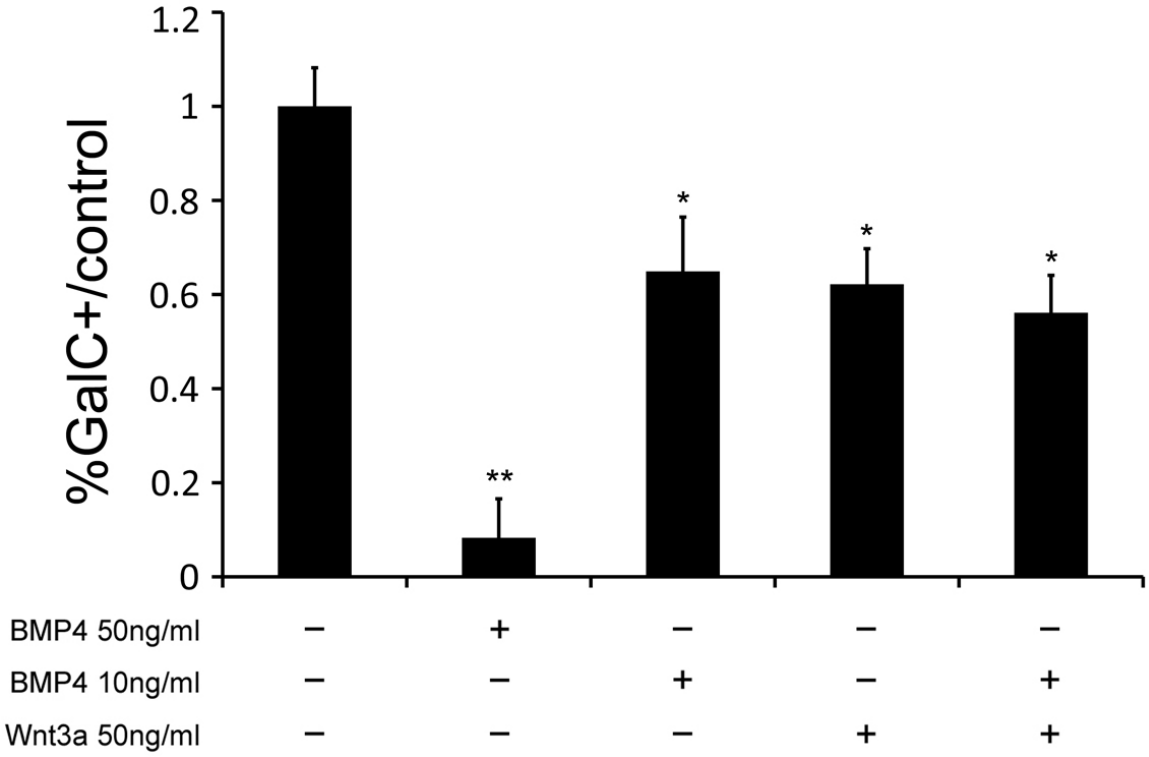


**Figure 3.5: Combined treatment with BMP4 and Wnt3a does not inhibit precursor differentiation greater than individual treatment with BMP4 or Wnt3a**

Rat OPCs were cultured and then placed in DM with BMP4 (50ng/ml), BMP4 (10ng/ml), Wnt3a (50ng/ml), or Wnt3a (50ng/ml) and BMP4 (10ng/ml). BMP4 (50ng/ml) decreased GalC+ cells by 92% relative to control conditions ($p < 0.05$), BMP4 (10ng/ml) decreased OPC differentiation by 35% ($p < 0.05$), Wnt3a 50ng/ml decreased OPC differentiation by 38% ($p < 0.05$), and Wnt3a (50ng/ml) combined with BMP4 (10ng/ml) decreased OPC differentiation by 44% ($p < 0.05$). The difference between the combined addition of BMP4 and Wnt3a was not significantly different from the application of either treatment factor individually ($n = 3$).

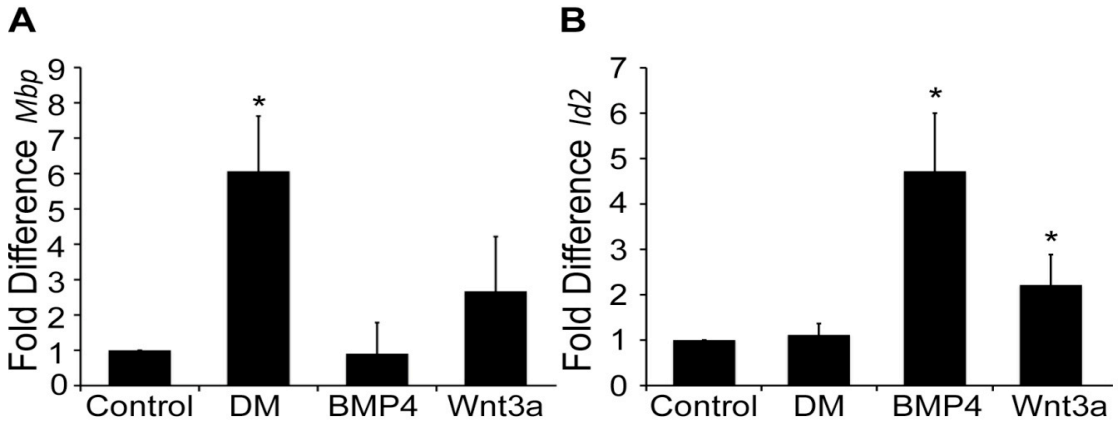


**Figure 3.6: BMP4 and Wnt3a regulate *Id2* and *Mbp* transcript levels**

Rat OPCs treated with Wnt3a or BMP4 and assayed for Q-PCR. (A) After treatment for 48 hours, levels of *Mbp* mRNA were calculated compared to untreated control OPCs. DM treated cells had a 7-fold increase in *Mbp* expression over that of untreated cells and BMP4 treated cells ($p < 0.05$). This expression was also 2.33 fold more than that of Wnt3a treated cells ($p < 0.05$, $n = 4$). (B) After treatment for 6 hours, levels of *Id2* mRNA were calculated compared to untreated control OPCs. BMP4 and Wnt3a treatment increased *Id2* transcript expression 4.7 fold and 2.2 fold, respectively ($p < 0.05$, $n = 7$).

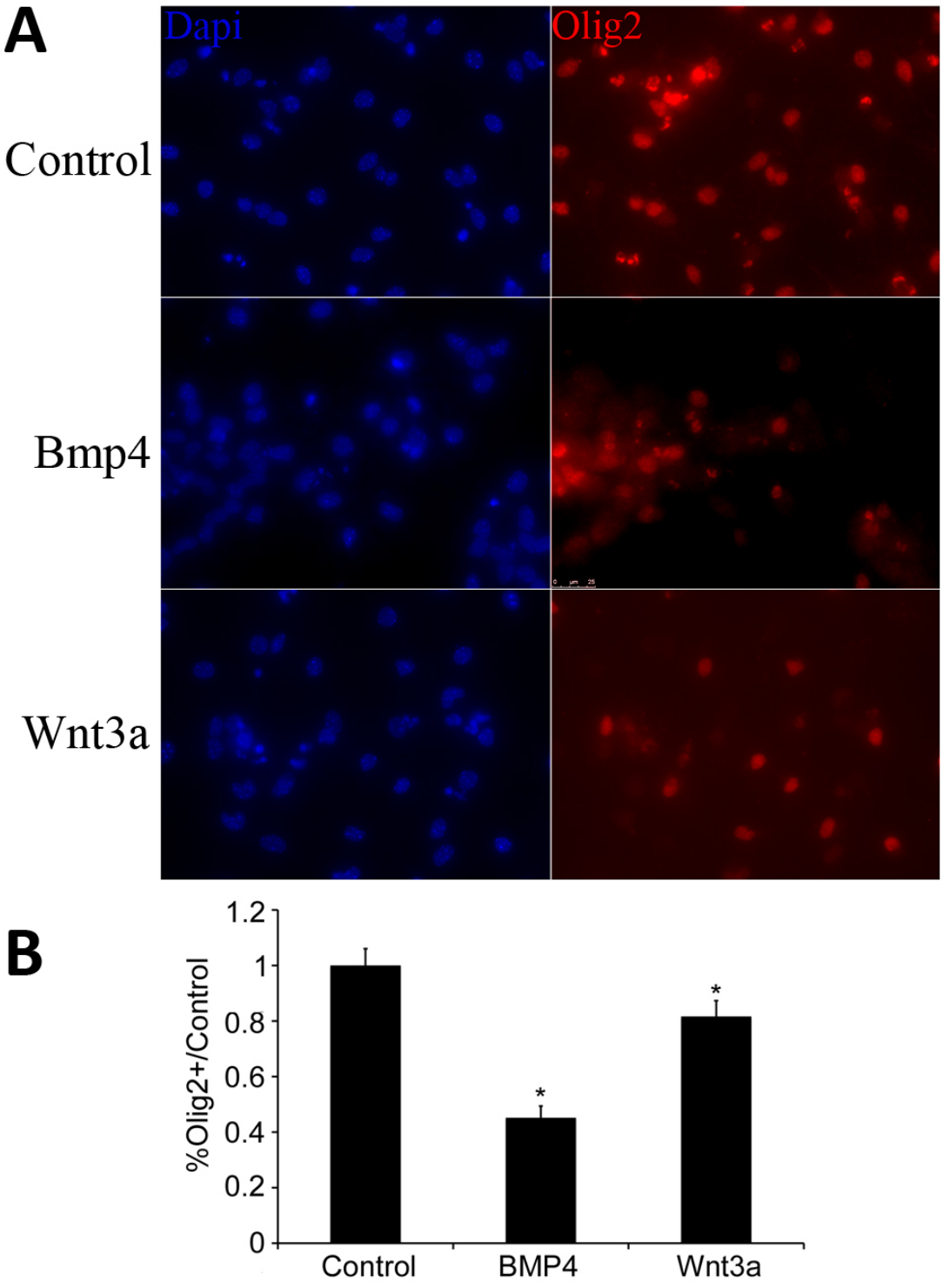


**Figure 3.7: BMP4 and Wnt3a decrease levels of Olig2**

Mouse OPCs were treated with Wnt3a or BMP4. (A) Two days after treatment, cells were labeled for Olig2 and A2B5. (B) Bar graphs quantify the number of Olig2+

cells in each condition relative to control. BMP4 decreased the number of nuclei colabeled with Olig2 by 55% ($p < 0.01$, $n = 5$). Wnt3a decreased the number of nuclei colabeled with Olig2 by 19% ($p < 0.05$, $n = 5$). Images shown were taken at 40x magnification.

## Chapter 4

### Discussion

My experiments have shown that canonical Wnt signaling inhibits the maturation of OPCs, causing developmental delays in axonal myelination *in vivo* (Chapter 2). In contrast, I have also shown that β-catenin inactivation does not significantly regulate OPC maturation *in vivo* (Appendix). Furthermore, I have shown that the inhibitory effects of canonical Wnt signaling are dependent on a functioning BMP pathway (Chapter 3). These results add insight into signaling systems that regulate OL development and also identify a potential role of Wnt signaling following demyelinating injury.

Canonical Wnt signaling is a significant factor during OL differentiation. When treating cultured OPCs with Wnt3a, there is a 30-40% reduction in the number of differentiating cells relative to control conditions. This is not accompanied by a change in the number of precursors, astrocytes, proliferating cells, or cells undergoing apoptosis, indicating that Wnt/β-catenin signaling is specifically preventing precursor cells from undergoing the steps required to become mature cells (Fig. 2.1A-F). These results are consistent with those of other recent studies. Wnt3a treatment significantly, but not completely, inhibited OPC differentiation in OPC cultures and in spinal cord explants (Shimizu et al., 2005). Similarly, transfection of cultured neural progenitor cells with a dominant active form of β-catenin prevented the appearance of fully mature OLs without affecting the number of precursors (Ye et al., 2009).

The mechanisms responsible for these effects could be explained through epigenetic regulation. OLs must exit the cell cycle to differentiate and additionally require myelin genes to be activated, a process mediated by HDACs (Marin-Husstege et al., 2002; He et al., 2007). Canonical Wnts compete with HDACs to bind TCF, upregulating inhibitory transcription factors, such as ID2/4, at the expense of inductive ones, such as Olig1/2. In this fashion, cells are maintained in precursor states while their proliferative capabilities are unaffected. When cells are not bound with canonical Wnts, TCF forms a complex with HDACs and Groucho to repress the transcription of *Ids*, permitting differentiation. Future experiments will include ID2/4 knockdown using transfected siRNA into OPC cultures: If Wnt3a limits OPC differentiation by restricting Olig proteins to the cytoplasm via ID induction, there should be no inhibitory effect of Wnt3a treatment on transfected OPC cultures. Similarly, transfecting OPCs with constitutively active Olig2 should be able to rescue differentiation in Wnt3a treated cultures.

Driving constitutively active canonical Wnt signaling in OPCs *in vivo* replicated the *in vitro* effects (Fig 2.4-2.6). These *β-Cat-CA* mutant mice were viable, but displayed significantly fewer OLs and less myelin at early ages than normal littermates, although this largely recovered as mice reached adulthood (Fig 4.1). This recovery could occur because OLs are developing at a slower overall rate or because the importance of canonical Wnt signaling in OL development is reduced as the mice age. In the mutants, fewer overall mature OLs were produced at early time points, but normal amounts of myelin could be achieved if OLs undergo relatively fewer apoptosis events. In normal mice, more OLs are produced than are needed, and apoptosis functionally limits the final

number. I did not observe any changes in the number of cells labeled with TUNEL, a marker of apoptosis, but it is possible that this occurs at levels below detection or at time points that were not observed. A more likely reason is the reduced importance of canonical Wnt signaling: Levels of TCF are active throughout CNS development in cells of OL lineage, but their expression decreases after P21 (Fancy et al., 2009). This would lead to a scenario wherein constitutively active β-catenin limits the progression of precursors to mature cells through the actions of TCF, but as TCF itself is removed from the signaling environment those cells can begin differentiating, albeit comparatively later than their normal counterparts.

These results indicate that Wnt/β-catenin signaling is sufficient to disrupt or delay myelination *in vitro* and *in vivo*, but what occurs when canonical Wnt signaling is eliminated? Blocking Wnt signaling pharmacologically prevents its effects *in vitro*, and cells show no abnormal differentiation effects, implying Wnt activity is not required for normal aspects of OPC differentiation (Fig 3.2). *In vivo*, a β-catenin a loss of function mutation targeted to the *Cnp* promoter does not alter OL cell numbers or noticeably impair white matter development, suggesting canonical Wnt signaling is not necessary to regulate OL development *in vivo* (Fig 3.3). Although mutants experience high embryonic and adult lethality, this effect is probably not a result of abnormal OL development. While β-catenin does factor into the multiple aspects of cellular processes outside of TCF signaling, including cell adhesion and migration, it is possible that other signals can compensate at this stage of specification (Huelsken et al., 2000). Similarly, as multiple signals intricately regulate OL development, removing the impeding effects of Wnt/β-

catenin signaling may not result in ectopic or premature differentiation if other signals, such as Notch or the BMPs, can compensate.

Wnt signaling, however, is upregulated during spinal cord injury, and can exacerbate white matter damage in demyelinating models (Fancy et al., 2009, Appendix). While loss of function canonical Wnt signaling may not alter embryonic development, it could affect remyelination. I attempted to test this using a cuprizone white matter injury model on both β-catenin loss of function and β-catenin gain of function mutant mice. The lethality rates, however, were too high to feasibly perform experiments in the loss of function conditions. Future experiments could involve crossing the floxed loss of function mutant mouse to an inducible Cre targeted to similar promoters active in cells of OL lineage. These mutations could then be activated in adult mice, removing from the equation any developmentally lethal effects. Cells lacking β-catenin may be able to remyelinate or differentiate into mature cells more easily if they cannot respond to upregulated levels of Wnt during injury. This would be observed after cuprizone treatment if mutant mice had fewer or less severe white matter lesions, increased numbers of mature OLs in lesioned areas, smaller inflammatory responses, or shorter recovery periods.

My results are important in context with two other recent studies in which Wnt signaling was disrupted in cells of OL lineage *in vivo*. Fancy et al. (2009) used an *Olig2-Cre* mouse line to drive constitutively active Wnt signaling in OLs and observed a postnatal hypomyelination effect that disappeared as animals reached adulthood, very similar to the phenotypes of my β-Cat-Ca mutants (Fig 2.5A-J). These animals also experienced significantly less remyelination following lysolecithin induced injury than

wild type littermates, despite similar numbers of precursors recruited to lesion sites, suggesting Wnt signaling impairs the differentiation of adult OPCs *in vivo.* The mutant mice had a more exacerbated white matter deficit compared to my mutant animals, however: They observed greater than 60% reduction in PLP+ cells at P9, compared to the 39% reduction I observed at P7. This most likely results from the earlier activation of the Olig2 promoter in neural precursor cells compared to the CNP promoter, which is activated in OPCs, leading to more cells expressing the β-catenin mutation (Fancy et al., 2009).

Ye et al. (2009) also drove a constitutively activated β-catenin signaling mutation in cells of OL lineage using an *Olig1-Cre* mouse line. They observed a significant loss of myelin gene expression up to P14 in these mutants, but did not track them to adulthood to observe if the phenotype recovered. The more exaggerated hypomeylination phenotype compared to my mutant animals is most likely again because the Olig1 promoter is active before the CNP promoter in OL lineage. They also targeted the same loss of function β-catenin mutation that I employed to the *Olig1-Cre*, observing ectopic expression of Olig2 and PDGFRα compared to wild type littermates at E12.75, but did not examine later time points (Ye et al., 2009). These results are significant, because my *β-Cat-Null* mice did not have a myelin phenotype at P1, although it is possible that they had an embryonic phenotype that normalized by birth (Fig. Appendix 1.A-C). Future experiments in my lab should investigate whether there is an embryonic phenotype in mutant animals. These results support to the theory that endogenous Wnts prevent ectopic or premature differentiation of OLs in dorsal spinal cord. Ye et al. also investigated the phenotype of a TCF7L2 (TCF4) knockout mouse and observed a complete loss of *Plp*

and *Mbp* expression at E17.5, indicating proper OL development requires TCF4, the downstream transcriptional effector of canonical Wnt signaling. These animals died at P1, however, preventing further study of their adult phenotypes. Taken together, this recent data highlight the importance of the Wnt signaling pathway during OL development *in vivo*.

A further goal of my dissertation was to examine whether canonical Wnt signaling interacts with other dorsal signals, and I specifically examined the BMP signaling pathway because of its similar expression patterns and effects in embryonic development (Chapter 3). Genetic and pharmacological inhibition of canonical BMP signaling not only blocked the effects of BMP4 treatment on OPC cultures, but also that of Wnt3a. In contrast, pharmacological and genetic inhibition of the canonical Wnt signaling pathway effectively blocked the effects of Wnt3a on OPC cultures, but not that of BMP4. This suggests that canonical Wnt signaling requires the BMP pathway to inhibit OPC differentiation, although it is still unknown at what levels the pathways interact (Fig 4.2). The most direct possibility would be if Wnt/β-catenin signaling directly upregulates BMP activity. Wnt3a treatment, however, does not increase levels of phosphorylated-Smad, the downstream effector of BMP signaling, suggesting this is not the manner in which the pathways cooperate (Fig. 3.5).

The next step will therefore be to determine at what specific level the BMP and canonical Wnt pathways interact. There are many examples in the literature of the canonical Wnt and BMP pathways interacting, discussed in detail in Chapter 3. There are also examples of how the Wnt pathway can influence the BMP pathway without overtly upregulating BMP ligand or phosphorylated-Smad. These include mutual regulation of

downstream genes (Nishita et al., 2000; Letamendia et al., 2001), synergistic regulation of transcriptional complexes (Labbe et al., 2000), and maintenance of the BMP signal (Fuentealba et al., 2007).

There are several different experiments that would offer significant insight into the relationship between the BMP and Wnt signaling pathways. Performing microarrays from primary OPC cultures would be the first. After treatment with BMP4, Wnt3a, or a combination of the two factors, the regulation of a number of genes could be analyzed. Instead of looking at genes important for OL differentiation, I would look at effectors of the signaling pathways. This assay would be able to examine multiple components of both pathways at early and late time points. If Wnt3a signals through the BMP pathway without directly upregulating ligands or phosphorylated-Smad levels, there could instead be increased expression of BMP target genes or decreased expression of BMP inhibitors, such as Smurf1, Noggin, or Chordin. If the pathways interact in a synergistic manner, commonly regulated genes would have increased expression when both pathways are activated simultaneously as opposed to individually. Additional experiments would include sequence analysis to determine if TCF and phosphorylated-Smad regulatory sites are located in close proximity on co-regulated genes involved with OL differentiation and myelination. Furthermore, chromatin immunoprecipitation experiments could determine whether Smad and TCF bind to the same target genes, and Co-IPs could be performed to observe whether Smad and TCF directly form regulatory complexes in cells of OL lineage.

Another way to determine the specific nature of BMP and Wnt signaling would be to combine the pedigree of BMP signaling mutant mice with canonical Wnt signaling

mutant mice. If constitutively-activated canonical Wnt signaling mice are crossed with *Bmp1r DKO* mice, the abrogated BMP pathway should be able to rescue the developmental delay in myelination caused by dominant active β-catenin expression. However, the BMP mutant mice die early in embryonic development as a result of the *Brn-32 Cre* being universally expressed in the neural tube (Wine-Lee et al., 2004). For this experiment to be viable, the breeding scheme would have to be reassigned to an OL lineage specific Cre. A similar experiment that would require fewer breeding generations would be to cross the Wnt signaling mutant mice with a Noggin overexpressing mutant mouse (Chalazonitis et al., 2004). These mice overexpress Noggin in neurons, leading to ectopic levels in extracellular regions of the CNS. Blockade of the BMP signaling pathway by Noggin should rescue the myelination delays induced by β-catenin gain of function mutants *in vivo*.

My dissertation has added to the current understanding of the role of canonical Wnt signaling in OL development. I have shown how Wnt signaling interacts *in vitro* and *in vivo* to delay OL maturation, and how this process relies on the BMP signaling pathway *in vitro*. These results indicate how Wnts are involved in cellular development, OL differentiation, and recovery from demyelinating events. Future studies should examine the biochemical relationship between canonical Wnt and BMP signaling and how this relationship could be manipulated when treating white matter injury.

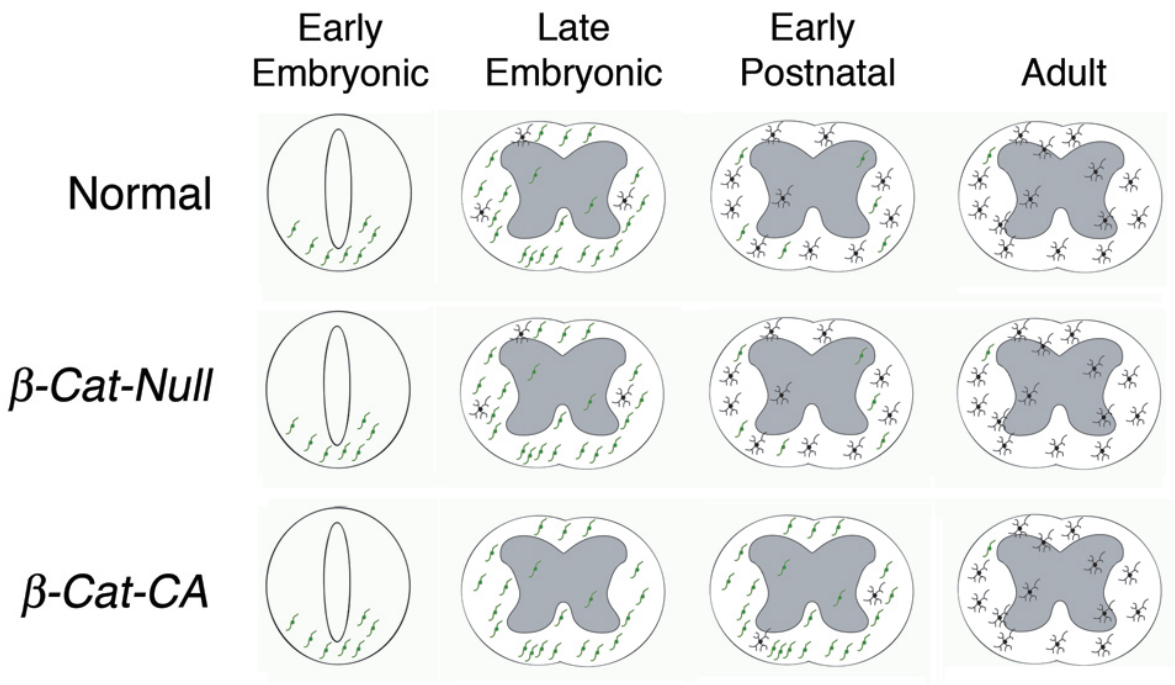


**Fig 4.1: Model of OL development in β-catenin mutant mice**

The time course of spinal cord development is described for three types of mice: Wild type (top row), loss of function Wnt signaling mutant mice *(β-Cat-Null*, middle row), and gain of function Wnt signaling mutant mice *(β-Cat-CA*, bottom row). All mutations are activated by the *Cnp* promoter early in OL lineage. In wild type and *β-Cat-Null* mice, OPCs develop normally in ventral spinal cord, migrating outward and beginning to mature towards the late embryonic stage. Extensive myelination begins at early postnatal stages and is completed by adulthood, at which point OLs are spread throughout the spinal cord, including the grey matter. In *β-Cat-CA* mice, OPCs are generated in ventral spinal cord and migrate outward in similar proportions and at similar

time points to their wild type littermates. Far fewer OPCs, however, differentiate into mature cells at late embryonic and early postnatal stages, although they are sporadically observed. While mutant mice still have fewer mature OLs when they reach 3 months of age, myelination levels are comparable to those of wild type littermates.

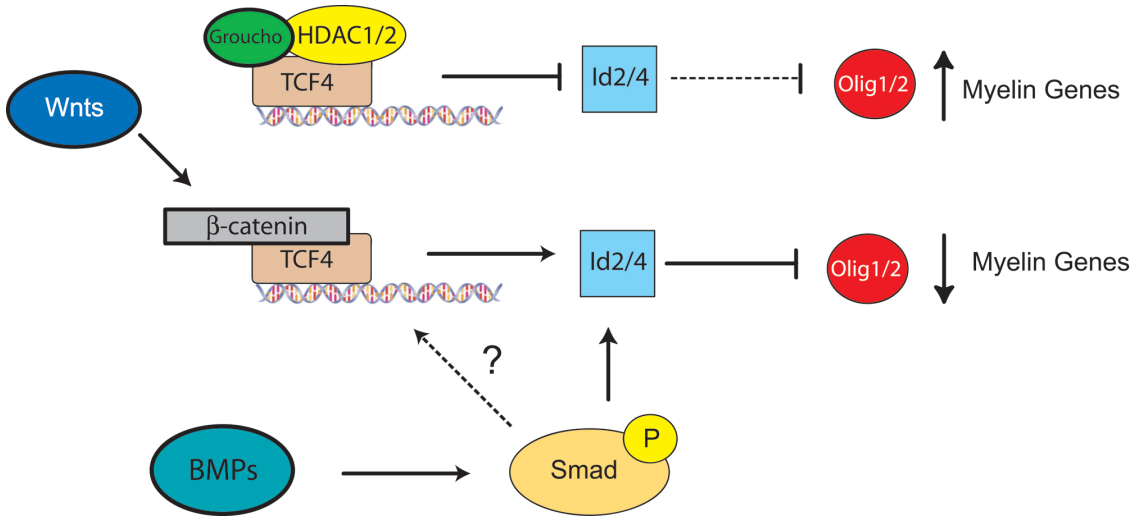


**Fig 4.2: A model by which canonical Wnt and BMP signaling regulated OL development**

TCF4 exists in two regulatory states, complexed with either HDAC1/2 or β-catenin. When bound with Groucho and HDACs, TCF represses Wnt target genes, such as the OL differentiation inhibitors, ID2/4. These transcription factors are thus prevented from binding Olig1/2. In this scenario, Olig1/2 can translocate to the nucleus, promoting the transcription factors Nkx2.2 and Sox10, in turn allowing them to transcribe myelin genes, such as *Mbp* and *Plp*. Upon Wnt stimulation, HDACs and Groucho are replaced in the TCF4 transcription complex by β-catenin, altering its state from transcriptional repressor to transcriptional activator. The resulting upregulation of IDs inhibits differentiation by restricting Olig1/2 to the cytoplasm. BMPs, through the actions of phosphorylated-Smad, independently upregulate ID2/4 levels and inhibit differentiation. It is possible, however, that the TCF4 activating complex requires effectors of the endogenous BMP signaling pathway to induce transcription.

## Appendix

The role of Wnt signaling during white matter injury

This section contains unpublished experiments that will be included in future publications

**Abstract**

Oligodendrocytes (OLs), the myelinating cells of the central nervous system (CNS), undergo defined stages during development before they mature into adult cells. Their development is regulated by a variety of intracellular and extracellular signaling factors that balance levels of proliferation, migration, and differentiation. I have shown that activation of members of one of these families of dorsal signals, Wnts, inhibits OL differentiation *in vitro* and *in vivo*. I next investigated what functional effects canonical Wnt signaling may have on OLs during development or demyelinating injury. I generated loss of function β-catenin signaling mutant mice and examined their development *in vivo*. Mutant animals did not have significant differences in myelination compared to normal littermates. I next generated loss of function and gain of function β-catenin signaling mutant mice and placed them on a cuprizone diet to induce demyelination of the corpus callosum. I found that the loss of function mutant mice had normal myelin phenotypes in control conditions. Preliminary results from cuprizone treated mice indicate that activation of Wnt/β-catenin signaling exacerbates white matter injury. Additional mutants will be necessary to infer significance and to determine whether blocking canonical Wnt signaling ameliorates the effects of injury.

**Introduction**

My thesis has investigated the role of canonical Wnt signaling *in vitro* and *in vivo*. Results indicated that Wnt/β-catenin signaling is sufficient to prevent differentiation in primary culture, and that it delays or prevents OL maturation in mice. Having shown that canonical Wnt signaling was sufficient to inhibit or delay myelination *in vivo*, the next step was to observe if it was also necessary. *β-Cat-Null* mice were generated as described in Chapter 3. These mice have a loss of function mutation driven by the *Cnp* promoter, which is active in cells of OL lineage. Mice were sacrificed in a developmental time course and spinal cord white matter was examined, but the number of mature OLs and amount of PLP expression was not significantly different from that of normal littermates. These findings suggest that a loss of function Wnt signaling mutation targeted to cells of OL lineage does not regulate their maturation *in vivo*.

While a functional requirement for canonical Wnt signaling in OL development was not observed, this pathway could still regulate adult cells during different processes. Several studies have shown evidence that dorsal signaling factors in the developing neural tube, such as BMP and Wnt3a, are upregulated during injury (Liu et al., 2008b; Fancy et al., 2009; Miyashita et al., 2009; White et al., 2010). The cuprizone model of inducing chronic white matter injury was employed to determine whether eliminating functioning β-catenin in OLs could alter injury response *in vivo*. At 6 weeks of age, mice were placed on a diet of normal food combined with 0.3% cuprizone (oxalic acid bis-(cyclohexylidenehydrazide) for a period of 6 weeks to induce demyelinating lesions, according to established protocols (Armstrong et al., 2002; Murtie et al., 2005a).

Cuprizone is a copper **c**helating agent that, when ingested over a period of time, results in chronic loss of OLs and myelin in specific regions of the brain, notably the corpus callosum (Benetti et al., 2010).

The experimental paradigm used on normal animals produced results consistent with the literature. Fewer OLs were observed in corpus callosa after chronic cuprizone treatment, while inflammatory responses and astrogliogenesis were marked by increased numbers of microglia and astrocytes. Initial studies on *β-Cat-CA* mice showed that injury was exacerbated when canonical Wnt signaling is constitutively active. Both gain of function and loss of function mutant mice, however, did not survive the treatment periods with the same frequency as wild type littermates. While the results are promising, to draw conclusions about the role of Wnt signaling will necessitate generating more mutant mice.

**Results and Discussion**

A loss of function β-catenin mutation was targeted to the *Cnp* promoter and the mice (*β-Cat-Null*) were generated according to a breeding scheme described in Chapter 3. OPC cultures were taken from these mice at birth, grown, placed in DM, and treated with Wnt3a. After 3 days, the percentage of GalC+ cells in culture was similar to untreated conditions, indicating that canonical Wnt activity had been functionally eliminated (Fig. 3.3). Mice were viable at P1 and adulthood, but were not born at expected Mendelian ratios, indicating a high rate of prenatal lethality. Mutant mice and wild type littermates that survived were sacrificed at P1 and P21, cryosectioned, and assayed for markers of myelination. At P1, mutant mice had similar numbers of PLP+

cells relative to wild type littermates (Fig. Appendix.1A, B). At P21, mutant mice had similar numbers of ASPA+ cells relative to wild type littermates, suggesting that developmental myelination was unimpaired (Fig. Appendix.1A, C).

While eliminating canonical Wnt signaling in different aspects of the neural system can have dramatic effects (McMahon et al., 1992; Ikeya et al., 1997; Brault et al., 2001; Ye et al., 2009), it does not appear necessary for OL development when targeted to specified OPCs. There could be several different reasons for this effect. The lack of β-catenin in adherens junctions can be compensated with other proteins, negating potential migratory or cell-to-cell adhesion consequences (Huelsken et al., 2000). Similarly, Wnts can have non-canonical roles in OL development that are unaffected by functional elimination of β-catenin. Furthermore, canonical Wnt signaling appears to influence cells that have exited the cell cycle and prevent their differentiation. It is possible that when canonical Wnt signaling is removed other dorsal inhibitory signals, such as Notch or BMP, can compensate in this regard.

These findings indicate that canonical Wnt signaling is not necessary to delay or inhibit myelin formation *in vivo*. They do not, however, preclude a cell autonomous effect of eliminating canonical Wnt signaling in OLs during post-developmental events. Wnt signaling is regulated during demyelinating injury, and it is possible that eliminating β-catenin can alter cellular responses to injury or recovery. Therefore, it is important to examine the functional role of Wnt/β-catenin signaling during white matter injury.

After 6 weeks of cuprizone treatment, mice were either sacrificed or placed on normal food for an additional 3 weeks and then sacrificed. Treated mice were compared to untreated control littermates. After 6 weeks of cuprizone ingestion, corpus callosa of

treated animals had significantly decreased numbers of ASPA+ cells (Fig. Appendix.2A-D, G), and Olig2+ cells (Fig. Appendix.3A-D, G), and PLP expression (Fig. Appendix.4A-F), indicating a decrease in cells of OL lineage. These same regions had increased numbers of GFAP+ (Fig.Appendix.5A-D, G) and Iba1+ cells (Fig. Appendix.6A-D, G), indicating that reactive astrocytes and microglia were increased simultaneously increased. Some mice were allowed to recover after 3 weeks on normal food, allowing time to evaluate how much natural remyelination occurs after injury. These animals had increased numbers of ASPA+ cells (Fig 2E, F, G), Olig2+ cells (Fig. Appendix.3E, F, G), and PLP labeling (Fig. Appendix.4E, F) compared to mice from the 6 week treatment period. The number of GFAP+ cells remained the same in the recovery conditions compared to animals sacrificed at 6 weeks (Fig 5E, F, G), although the number of Iba+ cells appeared to decrease (Fig. Appendix.6E, F, G), indicating that a reactive response is somewhat maintained.

With the cuprizone treatment paradigm employed, the next step was to observe whether gain of function or loss of function canonical Wnt signaling affected the injury response. *β-Cat-CA* mice and normal littermates were generated as previously described (Chapter 1); however, many *β-Cat-CA* mice did not survive the 6 week cuprizone treatment period. Three mutants survived the treatment period and were sacrificed, but the corpus callosa were almost completely degenerated and the cells could not be counted in two of the mutants. Preliminary results from the one mutant in which cells could be counted showed increased degeneration of the corpus callosum when labeling for PLP and Olig2 compared to normal littermates (Fig. Appendix.7A-I). *β-Cat-Null* mice were generated as previously described (Chapter 2), but 75% fewer mutants were born than

expected according to Mendelian ratios, and none survived the 6 week treatment period. These results indicate that canonical Wnt signaling may increase demyelination during injury, but it will require more mutant animals to draw substantial conclusions. In summary, cuprizone treatment induced white matter damage in selected regions of the corpus callosa in treated animals, with an observable recovery when mice were switched back to normal food. This replicates findings observed in the literature (Crawford et al., 2009; Xie et al., 2010). Preliminary analysis of *β-Cat-CA* mice treated with cuprizone indicate that canonical Wnt signaling impairs natural recovery of demyelination or intensifies the injury itself, in keeping with recent studies (Fancy et al., 2009).

Future experiments would involve generating sufficient numbers of Wnt signaling mutant mice, both gain of function and loss of function, to obtain significant results. This will help determine whether blocking canonical Wnt signaling in cells of OL lineage prevents or attenuates white matter damage during injury. If this were the case, Wnt signaling could be exacerbating the initial injury by decreasing survival of OLs and axons. *β-Cat-Null* mice should then have more cells labeled with markers of mature OLs after 6 weeks of cuprizone treatment compared to control littermates. In contrast, *β-Cat-CA* mice should have significantly fewer mature OLs in the same areas. Wnt/β-catenin signaling could also be inhibiting the ability of precursors to proliferate in or migrate to the areas of injury. If this were the case, *β-Cat-Null* mice may have demyelinating lesions after initial cuprizone treatment, but more OPCs would be observed in the regions of interest after 6 weeks of treatment and after the 3 week recovery, whereas *β-Cat-CA* mice should have fewer. Canonical Wnt signaling could also be impairing the ability of OPCs to differentiate into mature, myelinating cells once they reach injured regions. If this were

the case, *β-Cat-Null* mutant mice should have similar levels of precursors in regions of interest compared to control littermates after cuprizone treatment, but after the 3 week recovery, they should have more myelinating OLs. *β-Cat-CA* should have fewer myelinating OLs at the same time points.

**Methods**

Animals and cuprizone treatment

Mutant mice were generated as described previously *(β-Cat-CA*, Chapter 2*; β-Cat-Null*, Chapter 3). For spinal cord anatomy, viable mice were sacrificed along with wild type littermates at P1 and P21, perfused with 4% paraformaldehyde, and processed for cryosectioning and immunolabeling as previously described (Chapter 1).

For cuprizone treatment, mice were genotyped and at 6 weeks of age transferred from a normal diet to one containing 0.3% cuprizone [oxalic bis(cyclohexylidenehydrazide); Adrich, St. Louis, MI] mixed into milled chow (Harlan Teklad). Mice were kept on the diet for 6 weeks and then sacrificed, perfused with 4% paraformaldehyde, and processed for cryosectioning as previously described (Chapter 1). A cohort of mice was removed from the cuprizone diet after 6 weeks, and maintained on a diet of normal chow for 3 weeks, at which point they were sacrificed.

Immunohistochemistry

Sections were processed at 12μm and labeled with various antibodies. To label sections, slides were washed in PBS and incubated in block for 20 minutes with 20% fetal bovine serum, 2% bovine serum albumin, and 0.1% triton in PBS, incubated

overnight at 4° C in primary antibody, washed in PBS, and incubated in the appropriate secondary antibody, diluted 1:100 in PBS. All secondary antibodies were purchased from Jackson Immunoresearch Laboratories. Sections were then washed in PBS and mounted using mounting medium containing DAPI (Vector Laboratories).

Antibody pairs used to label mature OLs *in vivo* were anti-PLP (1:2, AA3, hybridoma supernatant, gift of Dr. Alex Gow, Wayne State) with goat anti-rat IgG and anti-ASPA (1:3000, gift of Dr James Garben, Wayne State) with goat anti-rabbit IgG. Anti-GFAP (1:2, hybridoma supernatant, gift of Virginia Lee, University of Pennsylvania) with goat anti-rat IgG was used to label astrocytes. Iba1 (1:1000, Wako) with goat anti-rabbit IgG was used to label microglia. To label precursors, slides were baked at 37 °C for 20 minutes and then autoclaved at 105 °C for 10 minutes before labeling with Olig2 (1:1000, Millipore) with goat anti-rabbit IgG.

To count spinal cord cells from frozen sections at P1, digital images were taken from 3 cervical spinal cord sections per animal, counting PLP+ cells in 6 40x fields of white matter per section. Two mutant and control animals were processed from each of 2 separate litters. For P21 animals, composite images were accumulated at 10x magnification to count all ASPA+ cells in the white matter of hemisections. One mutant and control animal was used from each of three separate litters. To quantify cells in corpus callosum, 4 200μm x 200μm ROIs were taken at 20x magnification for in each of 3 sections per animal. Rostral corpus callosum was selected as regions of the corpus callosum anterior to the appearance of the lateral ventricles and caudal corpus callosum was selected as regions of the corpus callosum beginning dorsal to the first appearance of the hippocampus. Two litters were used for cuprizone experiments, from the first, 6 wild

type mice were placed on the cuprizone diet for 6 weeks with 2 fed normal diets for the same duration. After treatment, two mice were switched back to normal food for an additional 3 weeks, while the rest were sacrificed. In the second litter, 2 wild type mice were placed on a cuprizone diet, with one placed on normal food for the same duration. Three *β-Cat-CA* mice were placed on the cuprizone diet, with two fed normal diets for the same duration.

All images were acquired using a Leica DM6000B fluorescence microscope, using a Leica DFC360 Fx camera and Leica Application Suite 2.1.0 software.

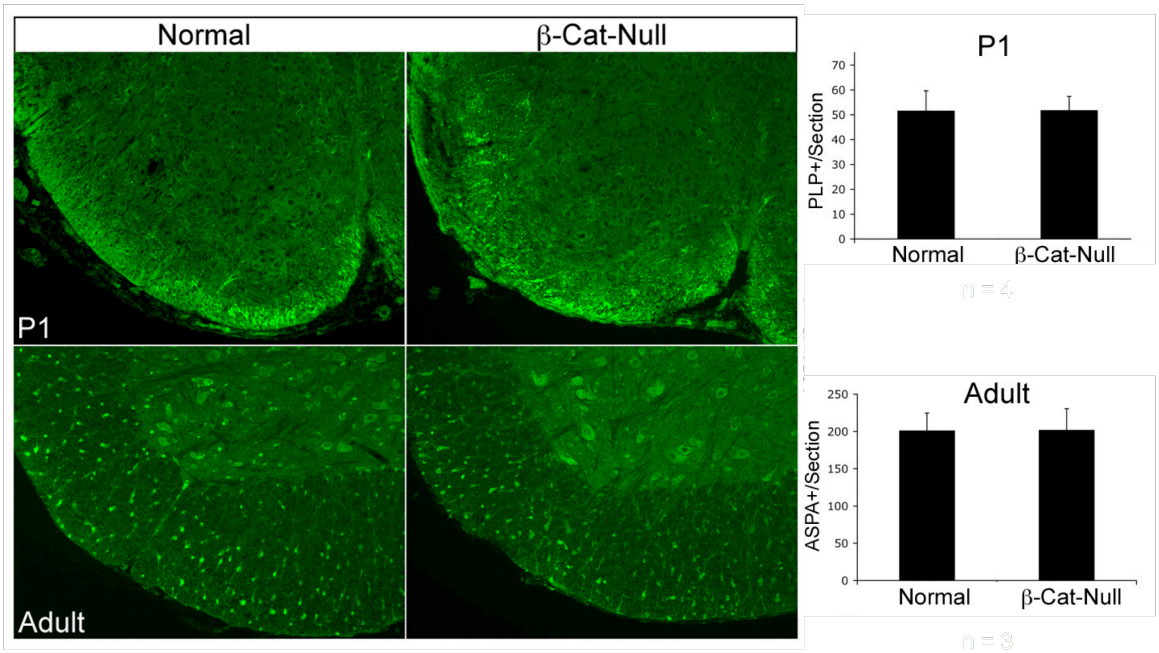


**Fig. Appendix.1: *β-Cat-Null* mice have normal myelin phenotypes**

β-Cat-Null mice and wild type littermates were sacrificed at P1 and P21 and assayed for markers of myelination. (A) The number of PLP+ cells is similar at P1 (top row) in both mutant mice and wild type littermates. The number of ASPA+ OLs was similar at P21 (bottom row) in both mutant mice and wild type littermates. (B) Quantification of PLP+ cells at P1 indicated no significant difference between mutant and normal mice. (C) Quantification of ASPA+ cells at P21 indicated no significant difference between mutant and normal mice. All images were taken at 10x magnification.

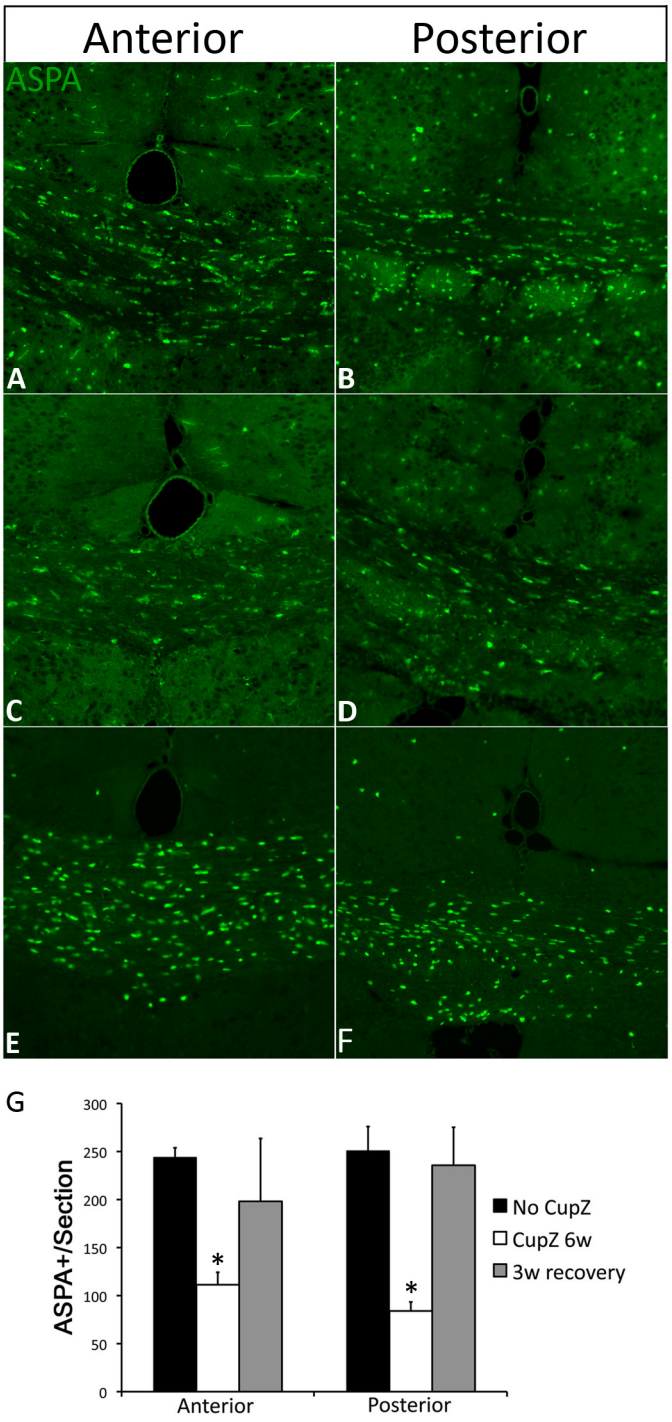


**Fig. Appendix.2: ASPA+ cells decrease after cuprizone treatment**

Mice were fed cuprizone for a period of 6 weeks and then sacrificed or placed on normal chow 3 weeks before being sacrificed. Immunolabeling of ASPA+ cells in

anterior corpus callosa (A) and posterior corpus callosa (B) of wild type mice fed normal diets. ASPA+ cells were decreased by 55% ($p < 0.05$) in cuprizone treated mice in anterior corpus callosa (C) and by 67% ($p < 0.05$) in posterior corpus callosa (D). Mice allowed to recover for three weeks had similar numbers of ASPA+ cells in both anterior (E) and posterior corpus callosa (F) compared to untreated controls. (G) Quantification of ASPA+ cells in anterior and posterior corpus callosa. Controls n = 2; Cuprizone 6 weeks, n = 4; Three week recovery, n = 2. All images were taken at 10x magnification.

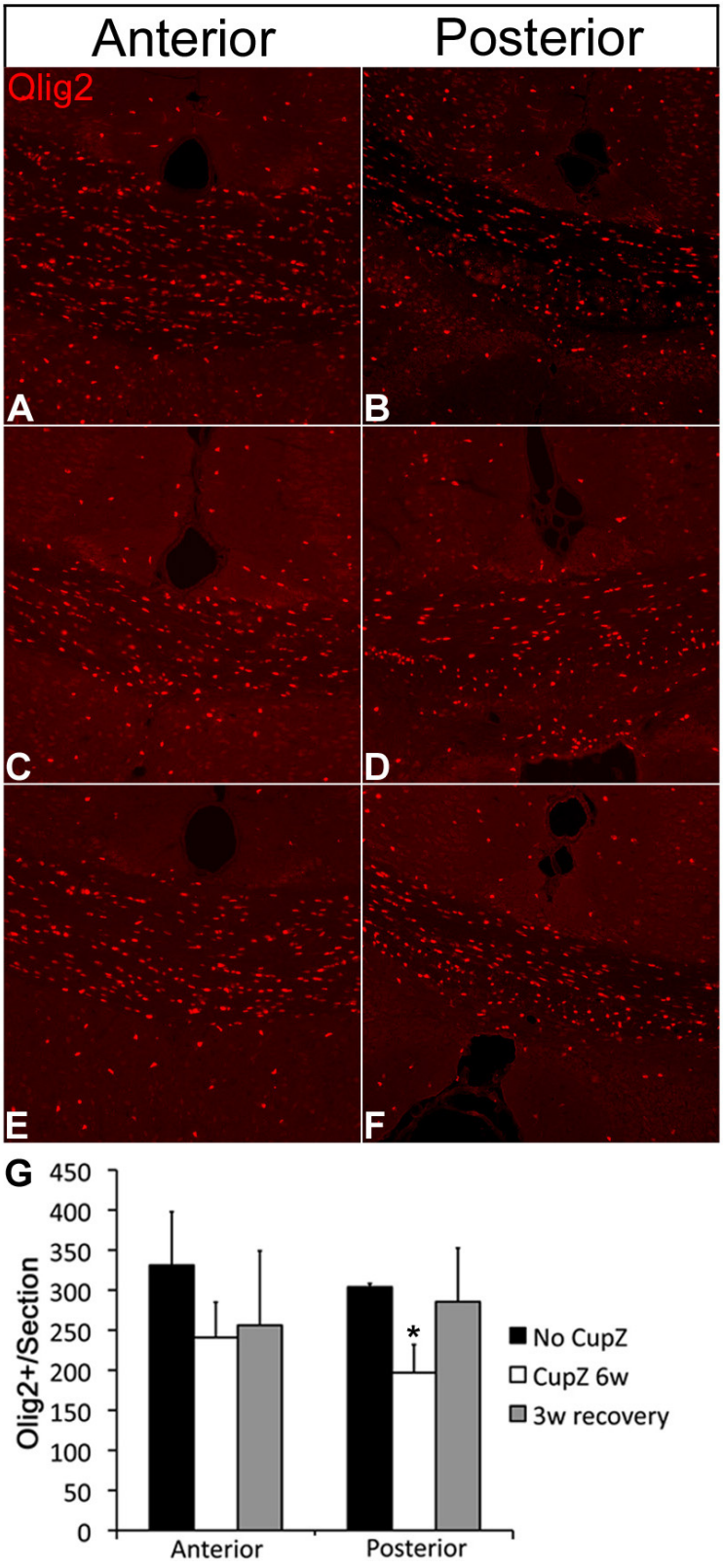


**Fig Appendix.3: Olig2+ cells decrease after cuprizone treatment**

Mice were fed cuprizone for a period of 6 weeks and then sacrificed or placed on normal chow 3 weeks before being sacrificed. Immunolabeling of Olig2+ cells in anterior (A) and posterior corpus callosa (B) of wild type mice fed normal diets. In cuprizone treated animals, Olig2+ cells were decreased in both anterior (C) and posterior corpus callosa (D), with 28% fewer Olig2+ cells in anterior corpus callosa ($p < 0.01$) and 35% fewer in posterior corpus callosa ($p < 0.01$). In mice allowed to recover following 3 weeks of cuprizone treatment, anterior corpus callosa had similar numbers of Olig2+ cells compared to treatment conditions (E), although these levels were not significantly different from non treatment control animals. In posterior corpus callosa (F), the number of Olig2+ cells appeared to return to untreated levels ($p < 0.05$). (G) Quantification of Olig2+ cells in anterior and posterior corpus callosa. Controls, $n = 2$; Cuprizone 6 weeks, $n = 4$; Three week recovery, $n = 3$. All images were taken at 10x magnification.

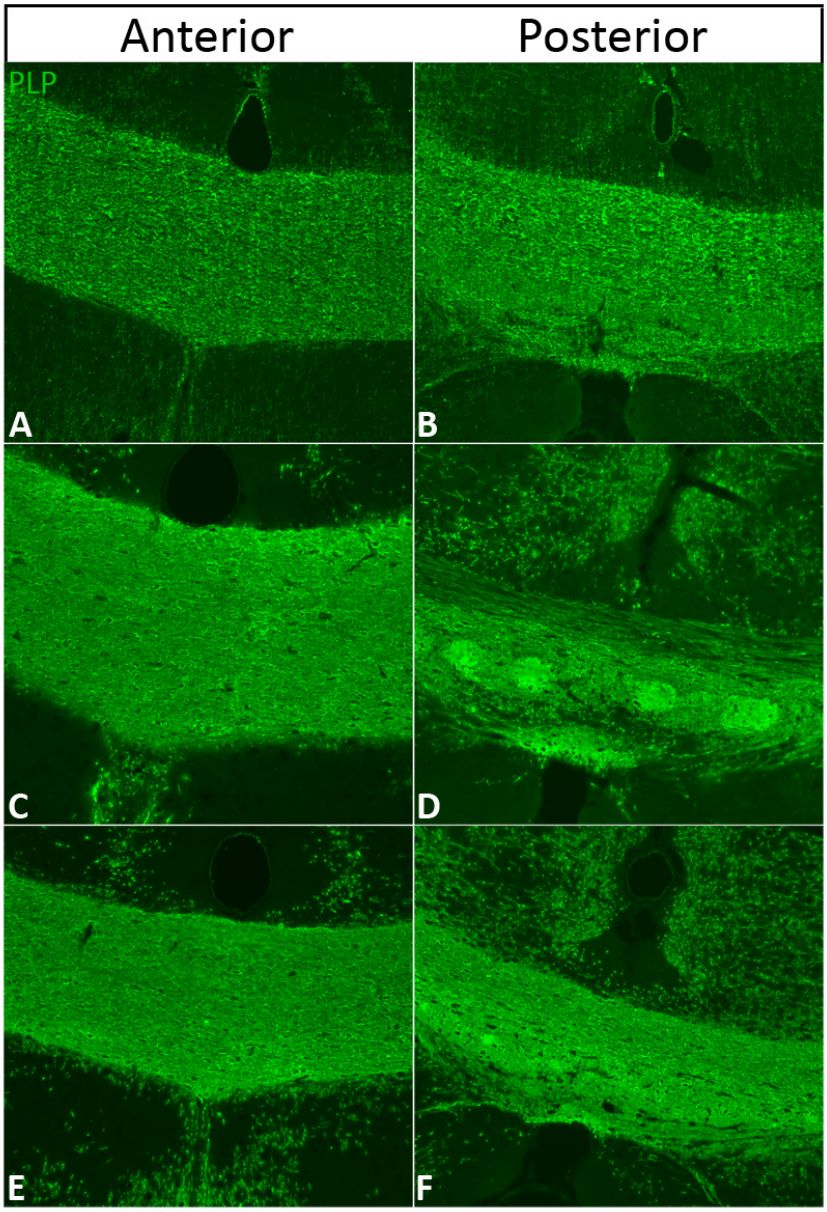


**Fig Appendix.4: PLP labeling decreases in cuprizone treated mice**

Mice were fed cuprizone for a period of 6 weeks and then sacrificed or placed on normal chow 3 weeks before being sacrificed. Immunolabeling of PLP+ cells in anterior (A) and posterior corpus callosa (B) of wild type mice fed normal diets. In mice treated with cuprizone for 6 weeks, PLP expression in anterior corpus callosa (C) appeared similar to untreated controls, while posterior corpus callosa (D) appeared to have less

PLP expression. Mice that were allowed to recover for 3 weeks appeared to recover PLP expression in both anterior (E) and posterior (F) corpus callosa. Images were taken at 10x magnification.

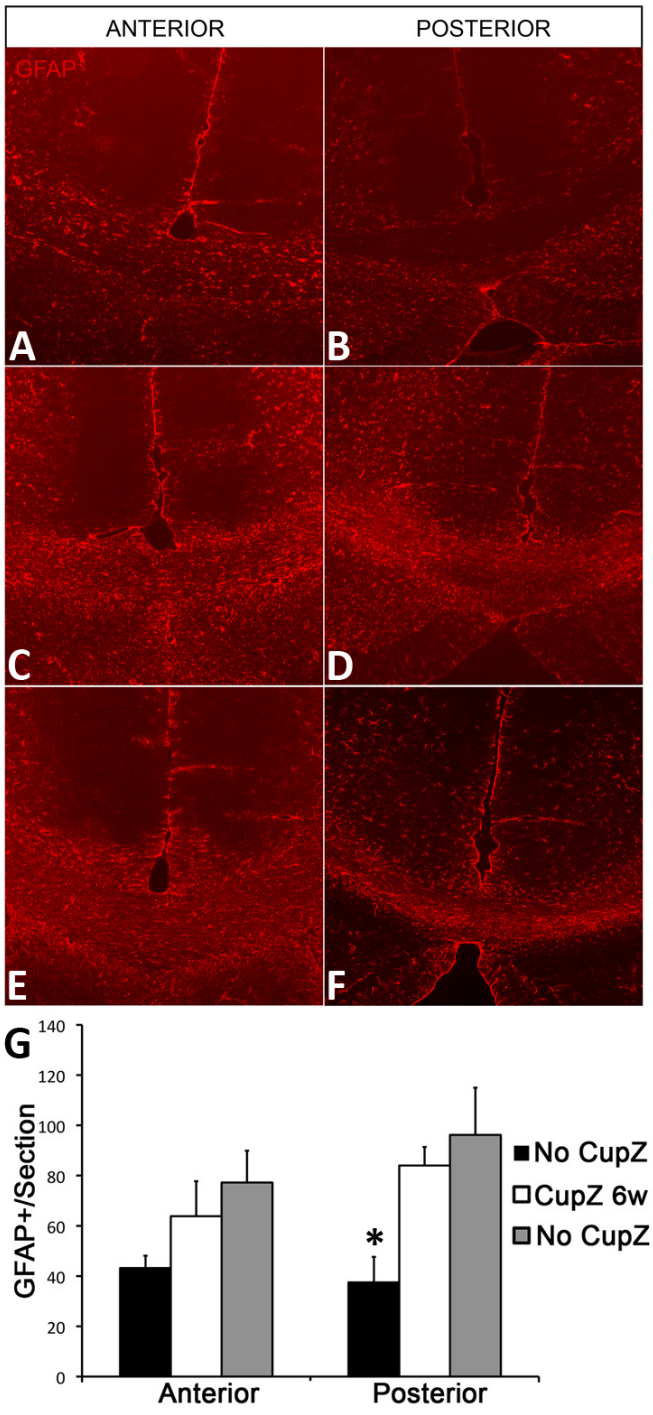


**Fig. Appendix.5: GFAP+ cells increase after cuprizone treatment and are maintained through recovery**

Mice were fed cuprizone for a period of 6 weeks and then sacrificed or placed on normal chow 3 weeks before being sacrificed. Immunolabeling of GFAP+ cells in anterior (A) and posterior corpus callosa (B) of wild type mice fed normal diets. GFAP+ cells were increased in both anterior (C) and posterior corpus callosa (D), with a 2.25 fold increase in GFAP labeled cells in cuprizone treated animals when compared to untreated control animals in posterior corpus callosa ($p < 0.01$). The difference in anterior corpus callosa trended towards significance. GFAP+ cells remained higher in the corpus callosa of mice allowed to recover for 3 weeks after cuprizone treatment in anterior corpus callosum (E) and posterior corpus callosum, in which condition the effect was significant ($p < 0.05$). (G) Quantification of GFAP counted cells in anterior and posterior corpus callosa. Controls, n = 2; Cuprizone 6 weeks, n = 4; Three week recovery, n = 3. Images are taken at 10x magnification.

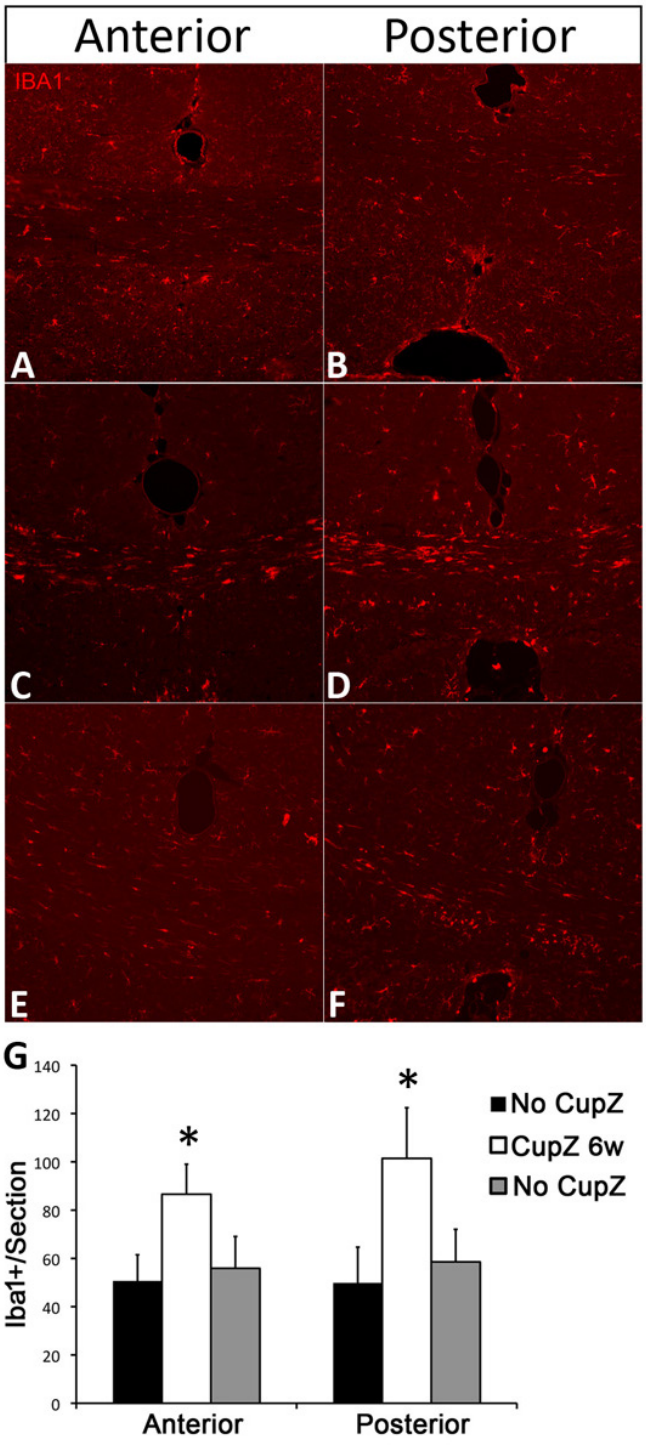


**Fig. Appendix.6: Iba+ cells increase after cuprizone treatment, but return to control levels after recovery**

Mice were fed cuprizone for a period of 6 weeks and then sacrificed or placed on normal chow 3 weeks before being sacrificed. Immunolabeling of Iba+ cells, representing microglia, in the anterior (A) and posterior (B) corpus callosa of wild type mice fed normal diets. In the corpus callosa of mice treated with cuprizone for 6 weeks, Iba+ cells were increased 1.7 fold ($p < 0.05$) in anterior corpus callosa (C) and 2 fold ($p < 0.05$) in posterior corpus callosa (D). Mice allowed to recover for 3 weeks post cuprizone treatment showed similar numbers of Iba+ cells compared to control littermates in both anterior (E) and posterior (F) corpus callosum ($p < 0.05$). (G) Quantification of the number of Iba+ cells. Untreated controls, n = 2; Cuprizone 6 weeks, n = 3; Three week recovery, n = 3. Images were taken at 10x magnification.

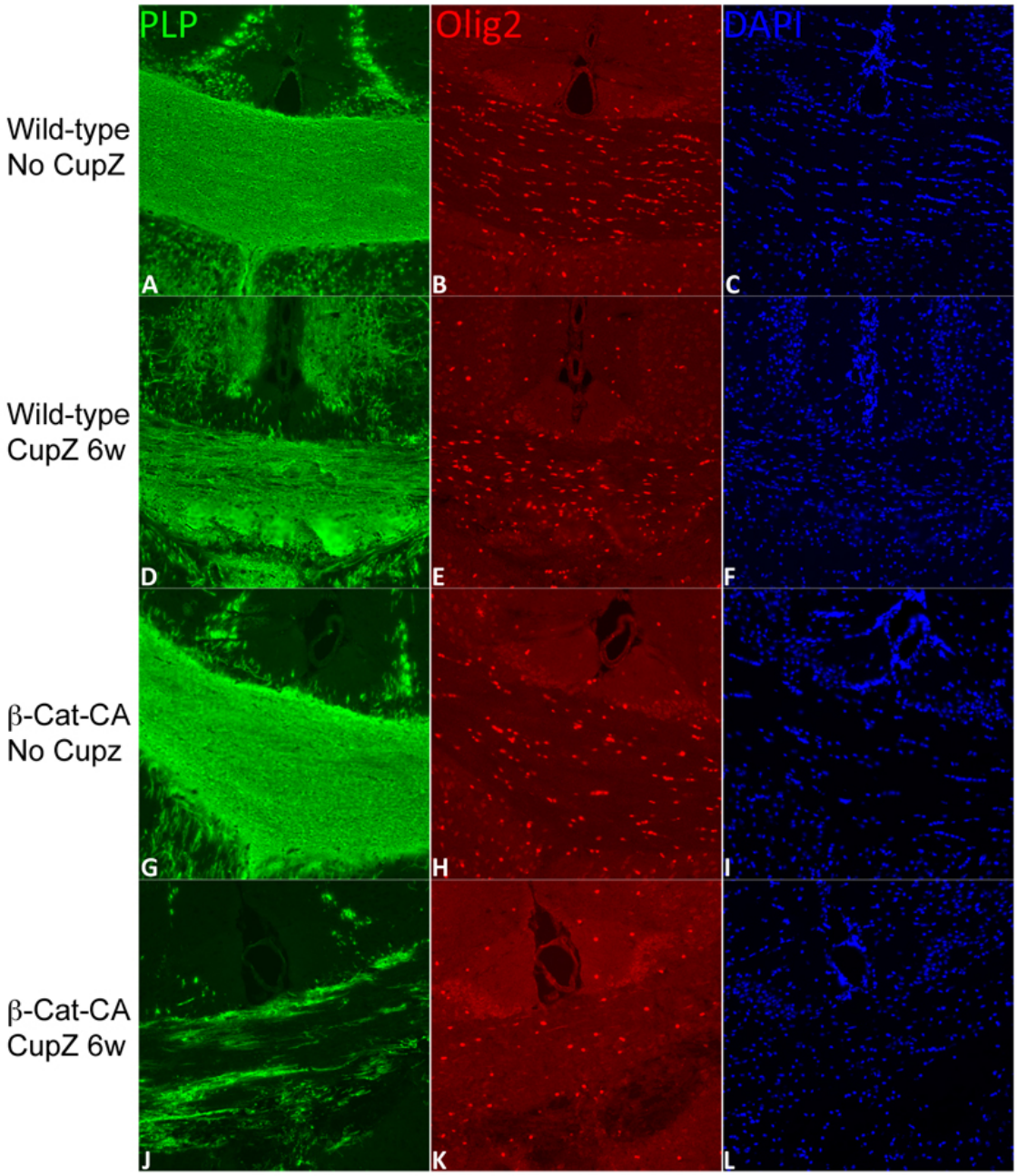


**Fig. Appendix.7: *β-Cat-CA* mice have increased white matter damage after cuprizone treatment relative to wild type littermates**

*β-Cat-CA* mice and wild type littermates were fed cuprizone for a period of 6 weeks and then sacrificed or allowed to recover for 3 weeks before being sacrificed. (A-C) Rostral corpus callosum of a wild type mouse fed a normal diet and labeled for PLP,

Olig2, and DAPI. (D-F) Rostral corpus callosum of a wild type mouse treated with cuprizone for 6 weeks; a loss of PLP and Olig2 can be observed. (G-I) Rostral corpus callosum of a *β-Cat-CA* mouse fed a normal diet; myelination appears similar to that of wild type mice. (J-L) Rostral corpus callosum of one *β-Cat-CA* mice treated with cuprizone for 6 weeks; extensive loss of PLP and Olig2 can be observed relative to its control littermate. All images were taken at 10x magnification.

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