

In vivo detection of Sonic hedgehog (Shh) signaling during postnatal myelination and
after acute and chronic demyelination

by

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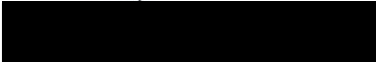
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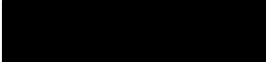
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DEDICATION

I dedicate this thesis to my parents, Luz Maria Martinez and Eugenio Sanchez. Thank you for showing me the meaning of hard work and for always supporting my career goals.

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ABSTRACT

In vivo detection of Sonic hedgehog (Shh) signaling during postnatal myelination and after acute and chronic demyelination

Maria A. Sanchez, Ph.D, 2018

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Multiple sclerosis is a demyelinating disease characterized by progressive disability from axonal damage and inefficient remyelination in chronic disease stages. Our studies tested Sonic hedgehog (Shh) as a therapeutic target to promote remyelination. Shh modulates embryonic and postnatal myelination and regulates neural stem cells (NSC) in adults. We used inducible genetic fate labeling to detect in vivo activation of canonical *Shh* signaling. *Shh*^{CreERT2} or *Gli1*^{CreERT2} mice were crossed to reporter mice to fate label cells actively transcribing *Shh* or *Gli1*, an effective readout of canonical Shh signaling. Tamoxifen administration on postnatal days 6-9 (P6-9) in *Shh*^{CreERT2} crosses identified neurons as the major source of Shh synthesis. In *Gli1*^{CreERT2} crosses, tamoxifen from P6-9 fate-labeled cells that populated the corpus callosum (CC) and expressed oligodendrocyte lineage cells markers for progenitors and mature oligodendrocytes. *Gli1* fate-labeled cells were also found in the ventricular-subventricular zone, a germinal site for NSCs. *Gli1* fate-labeled cells that survived to adulthood proliferated and differentiated into mature oligodendrocytes in response to acute and chronic

demyelination with cuprizone. Delaying tamoxifen administration until P14-17 revealed a drastic reduction of *Shh* and *Gli1* expressing cells. Interestingly, cells fate-labeled for *Gli1* from P14-17 or in adulthood rarely populated the healthy CC. Tamoxifen was next administered to adult *Shh*^{CreERT2} and *Gli1*^{CreERT2} mice during the course of demyelination and remyelination. *Gli1* fate-labeled astrocytes began to localize within the CC after chronic demyelination but not after acute demyelination. Surprisingly, *Shh* expression was not detected in activated microglia, reactive astrocytes, or other CC cells within acute or chronic lesions. Finally, a Shh agonist (SAG) was microinjected directly into the chronically demyelinated CC. SAG increased proliferation and enhanced remyelination but did not induce *Gli1* expression in CC cells, indicating that SAG promoted remyelination through non-canonical Shh signaling. These studies demonstrate that canonical Shh signaling is active during myelination and that these postnatal cells seed the CC as a source of remyelinating cells in adulthood. In contrast, canonical Shh signaling is not active in the adult CC during homeostasis, demyelination, or remyelination. Importantly, non-canonical Shh signaling may be a therapeutic target to promote remyelination after chronic demyelination.

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CHAPTER 1: Introduction

STATEMENT OF RESEARCH GAP

Multiple sclerosis (MS) is a demyelinating autoimmune disease that affects approximately 2.3 million people worldwide. Prolonged or repeated episodes of demyelination in MS lead to axonal loss and long term disability (29). Remyelination of viable axons can occur in some cases but remyelination is limited in chronic disease stages (92). Available treatments are immunosuppressant drugs that effectively reduce demyelinating episodes and slow disease progression, but these treatments are ineffective in progressive disease stages. Therefore, therapeutic treatments to enhance remyelination are necessary to prevent progressive disability. However, myelination of axons is a complex multistep process regulated by multiple signaling pathways (79). Understanding how these pathways are regulated during different stages of myelination and remyelination can provide valuable information on how to promote remyelination in chronic disease stages.

MULTIPLE SCLEROSIS

MS is considered a multifactorial disease with genetic and environmental predispositions (42). Over the past few decades, an increased susceptibility to MS has been detected in women. The increased susceptibility is thought to be related to increased exposure to environmental risk factors including rate of smoking in women (85). Other environmental factors associated with the disease include exposure to viral and bacterial agents, low levels of vitamin D and exposure to UV radiation (42). Several gene alleles have also been associated with higher incidence of MS. Additionally, risk of developing

MS is higher in relatives of MS patients and the risk increases with increased relatedness (85).

The clinical presentation of MS varies depending on which axons become demyelinated. Some of the most common clinical presentations involve visual disturbances, numbness, tingling, hemifacial spasms and coordination deficits, among others (68). Due to the variability in clinical presentation, diagnosing MS typically requires a neurological exam as well as imaging and laboratory studies. The current guidelines for MS enable earlier diagnosis based on neuroimaging evidence of demyelination (42; 90). Proper diagnosis is essential to determine prognosis and treatment.

There are four different types or classifications of MS: clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS). CIS is considered an early stage of the disease and can be given a MS diagnosis if in addition to clinical symptoms, magnetic resonance imaging shows inflammation and demyelinating lesions in the central nervous system (90). CIS can progress to RRMS which accounts for approximately 85% of the cases (30). RRMS is characterized by alternating episodes of neurological disability and recovery. The majority of patients initially diagnosed with RRMS eventually reach a SPMS stage of the disease, in which patients experience progressive neurological decline. Additionally, PPMS is diagnosed in 10-15% of patients who have a disease course characterized by steady neurological decline without episodes of recovery (30). Steady neurological decline in both PPMS and SPMS is attributed primarily to the cumulative loss of axons and inefficient remyelination of viable axons (29; 30; 59).

The pathological features in MS lesions can vary drastically between patients (65). There are, however, some features considered more common in acute disease stages versus chronic disease stages and vice-versa. Acute demyelinating lesions are typically characterized by breakdown of the blood-brain barrier (BBB), high levels of inflammatory cells, demyelination, reactive astrogliosis and axonal damage (30). Macrophages in acute demyelinating lesions typically contain myelin byproducts, indicating that these cells are actively contributing to demyelination. These lesions are referred to as active demyelinating lesions. Although active demyelinating lesions can also be found in chronic disease stages, most demyelinating lesions in chronic stages have low levels of inflammation. Chronic lesions in which macrophages are present but do not contain myelin debris are referred to as silent demyelinating lesions (64). Silent lesions are also typically depleted of oligodendrocytes while active lesions usually have ongoing remyelination (59; 65). The mechanisms of demyelination and axonal damage might also be different in acute versus chronic disease stages. In acute disease stages, axon loss correlates with the presence of immune cells suggesting that the inflammatory profile mediating demyelination is also toxic for axons (30). In chronic disease stages, demyelination and axonal loss continue in the absence of new inflammatory lesions. Therefore, apoptosis of oligodendrocyte and inefficient remyelination of denuded axons are thought to be the mechanisms mediating ongoing demyelination and axon loss in chronic disease stages (29; 30; 64).

There is no cure for MS. Current available treatments are immune modifying agents that can slow the progression of the disease by reducing the probability of an episode of demyelination in RRMS patients (42). However, most available treatments

have not proven beneficial for patients in chronic disease stages. Therefore, treatments focused on increasing remyelination are needed to prevent loss of viable axons and subsequent neurological decline. Inefficient remyelination in MS has been attributed to reduced proliferation and/or recruitment of oligodendrocyte progenitor (OP) cells to demyelinated lesions as well as to the presence of inhibitory signals preventing OP cell differentiation (Fig. 1) (59).

Figure 1

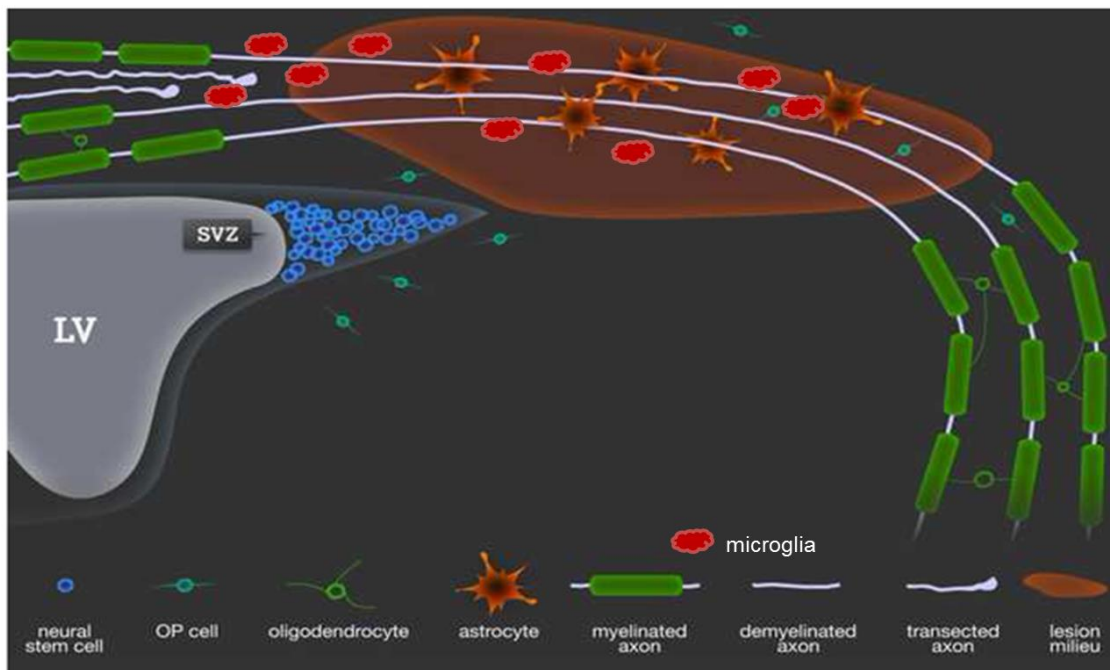


Figure 1: Factors contributing to limited repair after chronic demyelination.

Schematic shows several factors contributing to limited repair in chronic demyelinating lesions. Axon loss contributes to progressive disability in MS. Extensive loss of myelinating oligodendrocytes contributes to episodes of neurological dysfunction. Depletion of oligodendrocyte progenitor (OP) cells and lack of recruitment of neural stem cells from ventricular-subventricular zone (SVZ) limits remyelination. Inhibitory factors released by astrocytes and microglia can inhibit differentiation of OP cells into myelinating cells. Schematic modified from Gallo and Armstrong, 2008 (40).

In vitro studies and studies in pre-clinical models of demyelination have identified several therapeutic targets to promote remyelination (48). Recently, two interventions (Opicinumab and Clemastine fumarate) known to promote OP cell differentiation were tested in phase 2 clinical trials. Opicinumab is a monoclonal antibody against Lingo-1, a known inhibitor of oligodendrocyte progenitor (OP) cell differentiation (74). Clemastine fumarate is an antihistamine that competes with histamine for receptor binding; histamine binding prevents OP cell differentiation, so antihistamines can promote OP cell differentiation (45; 48). Opicinumab and Clemastine fumarate both improved signal transmission after visual evoked potentials suggesting that these interventions were able to promote remyelination along the optic nerve in MS patients (45; 61). However, in a follow up phase 2b clinical trial, Opicinumab failed to meet its primary outcome measure of improved disability based on the results from the Expanded Disability Status Scale test (74; 97). The lack of improvement in the follow up study might be due to the fact that remyelination requires both availability of OP cells and their differentiation into myelinating oligodendrocytes, and Opicinumab only regulates the latter. Treatment options that can regulate more than one aspect of the myelination process may have a greater potential to improve remyelination and recovery.

DEVELOPMENTAL MYELINATION

Understanding the mechanisms that regulate myelination during development can be useful to identify therapeutic targets for remyelination. Myelination of the central nervous system (CNS) is a multistep process regulated by multiple signaling molecules essential for the formation of functional neuronal circuits (13; 79). The myelination process begins with the induction of neural stem cells into oligodendrocyte lineage cells.

Further modulation is then needed to achieve proliferation, migration and differentiation of OP cells. In mammals, the process of myelination begins during embryonic development and continues through adulthood (12; 43).

In mice, the earliest OP cells arise from caudal neuroepithelium at embryonic day 12.5; these cells go on to myelinate different regions of the CNS including the spinal cord and the optic nerve (78; 110). In the developing spinal cord, OP cells are generated in sequential waves from cells in ventral and dorsal regions of the ventricular zone. OP cells derived from ventral and dorsal regions myelinate 90% and 10% of spinal cord respectively (18; 86). Different signaling molecules have been shown to induce the specification of OP cells in ventral and dorsal regions. The Sonic hedgehog (Shh) signaling pathway is essential for the specification and proliferation of OP cells derived from ventral regions of the ventricular zone (63; 86), while dorsally derived OP cells in the spinal cord can be generated in the absence of Shh signaling (18). Specification of OP cells in dorsal regions of the spinal cord is mediated by fibroblast growth factors (FGFs) signaling (21).

The myelination process in the forebrain begins during embryonic development and continues postnatally. OP cells that go on to myelinate the forebrain are also generated in sequential waves from three different regions along the ventricular zone. But in the forebrain, OP cells generated from the most dorsal region of the ventricular zone account for most of the oligodendrocytes found in the postnatal and adult forebrain (23; 58). Interestingly, in the forebrain, Shh signaling is necessary for the specification and proliferation of OP cells derived from both ventral and dorsal regions (104; 106). However, in ventral regions, loss of FGF signaling in the presence of Shh signaling

inhibits OP specification suggesting that both Shh and FGF signaling are required for the generation of OP cells in this region (39).

After OP cell specification there is typically a period in which OP cells divide and migrate out from their site of origin prior to differentiation. During postnatal myelination, several signaling pathways are known to regulate the amplification and migration of OP cells including Shh, FGF2, epidermal growth factor receptor (EGFR) and platelet-derived growth factor-A (PDGF-A) (1; 38; 79; 106). Interestingly, signaling molecules known to enhance OP cell proliferation are also typically inhibitors of OP differentiation and the sole removal of these molecules can initiate OP differentiation (79). Therefore, downregulation of Shh and FGF2 signaling after the amplification phase of OP cells in postnatal myelination might be essential for OP cell differentiation (81; 106).

Given the multiple roles of some of these signaling pathways, it becomes extremely important to consider how timing and lengths of exposure of these molecules affects or contributes to the different stages of the myelination process. It is also important to understand how the different signaling pathways interact with each other to modulate different cell functions. Shh signaling pathways modulate multiple steps of the myelination process but these pathways are also highly regulated (17). Understanding when and how Shh signaling is activated will give us insights on how it contributes to the different stages of myelination and/or remyelination.

SONIC HEDGEHOG SIGNALING PATHWAYS

In mammals, the Shh protein belongs to a family of secreted proteins best known for their role in the patterning of tissues during embryonic development. However, these proteins also play essential roles in the homeostasis of different tissues in adulthood

including the maintenance of neural stem cells in the CNS (11; 17; 66). The Shh protein is synthesized as a larger protein that must undergo autoproteolysis and post translational modification prior to its release for proper function (17; 108). Shh is known to mediate different cell functions through canonical and non-canonical signaling pathways (16; 17).

The canonical Shh signaling pathway is mediated by receptor proteins Patched1 (Ptch1) and Smoothened (Smo), the activation of Gli transcription factors and subsequent expression of target genes. In the absence of Shh ligand, Ptch1 inhibits the translocation of Smo to the primary cilium which blocks a signaling cascade required to activate Gli transcription factors (Fig. 2A). The binding of Shh to Ptch1 leads to removal of Ptch1 from the cell membrane and allows translocation of Smo to the primary cilium (93). Accumulation of Smo in the primary cilium initiates a cascade of events that allows Gli transcription factors to move into the nucleus and initiates the expression of Shh target genes (Fig. 2B). Gli transcription factors activate genes associated with cell cycle progression, pro-survival signals and genes known to regulate Shh signaling including *Hedgehog inhibitory protein (Hhip)*, *Ptch1* and *Gli1* (56). Ptch1 and Hhip are both negative regulators of Shh signaling pathway, while Gli1 is known to amplify Shh signaling (17). Expression of *Gli1* is therefore often used as an indicator of high levels of canonical Shh signaling (2; 10).

Figure 2

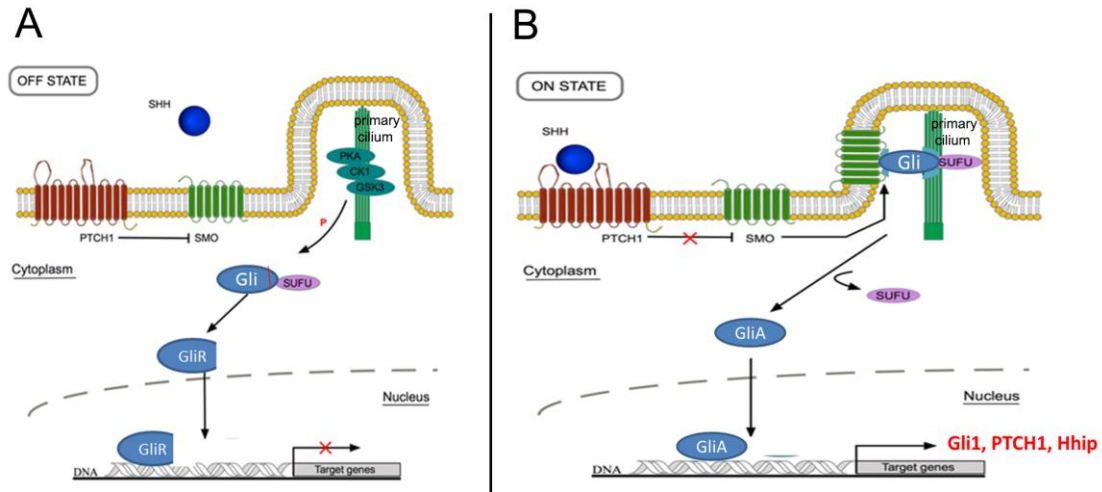


Figure 2: Simplified canonical Sonic hedgehog (Shh) signaling pathway.

Canonical Shh signaling is mediated by receptors Patched1 (PTCH1) and Smoothened (SMO) and Gli transcription factors. Prior to Shh ligand binding to receptor PTCH1 (off state), PTCH1 inhibits SMO translocation to the primary cilium and prevents activation of Gli transcription factors. In the absence of Shh ligand, inhibitory protein SUFU binds Gli transcription factors and blocks them from entering the nucleus. Gli transcription factors in the cytoplasm also get phosphorylated and cleaved into repressor structures (GliR) to prevent the expression of Shh target genes (A). Upon Shh binding to PTCH1 (on state), SMO is translocated to the primary cilium where it mediates release of activated Gli (GliA) transcription factors from SUFU. GliA can then enter the nucleus and induce expression of Shh target genes which include Gli1, PTCH1 and Hhip (B). Modified from Moura et al., 2016 (80).

In addition to the canonical Shh signaling pathway, there are two other known mechanisms by which Shh regulates cell functions: Type I and Type II non-canonical signaling pathways (Fig. 3). Type I non-canonical Shh signaling pathway is mediated by receptor Ptc1 and does not involve the activation of co-receptor Smo or the activation of Gli transcription factors (16). In type I non-canonical Shh signaling, Ptc1 negatively regulates canonical Shh signaling by inducing apoptosis and preventing cell cycle progression through its interaction with caspases and cyclin proteins (Fig. 3A-B) (16). Type II non-canonical signaling pathway requires receptors Ptc1 and Smo but does not

require the activation of Gli transcription factors. Type II non-canonical Shh signaling regulates cell migration and axonal guidance through the modulation of cytoskeleton rearrangements. Shh role in cytoskeleton remodeling does not require Smo to be translocated to primary cilium (Fig. 3C). In fact, preventing Smo translocation to the primary cilium enhances Shh chemotaxis properties (14). Importantly, in cells lacking primary cilium, Smo agonists can be used to induce cell migration indicating that Smo can be activated anywhere in the cell independently of Shh and Ptch1 binding. These studies also indicate that Smo can mediate different cell functions depending on where it is located in the cell (14; 16). Based on our current understanding of the Shh signaling pathways, it appears that both canonical and non-canonical pathways are needed for different stages of myelination. However further analysis is needed to determine if and how Shh signaling is regulated during demyelination and remyelination.

Figure 3

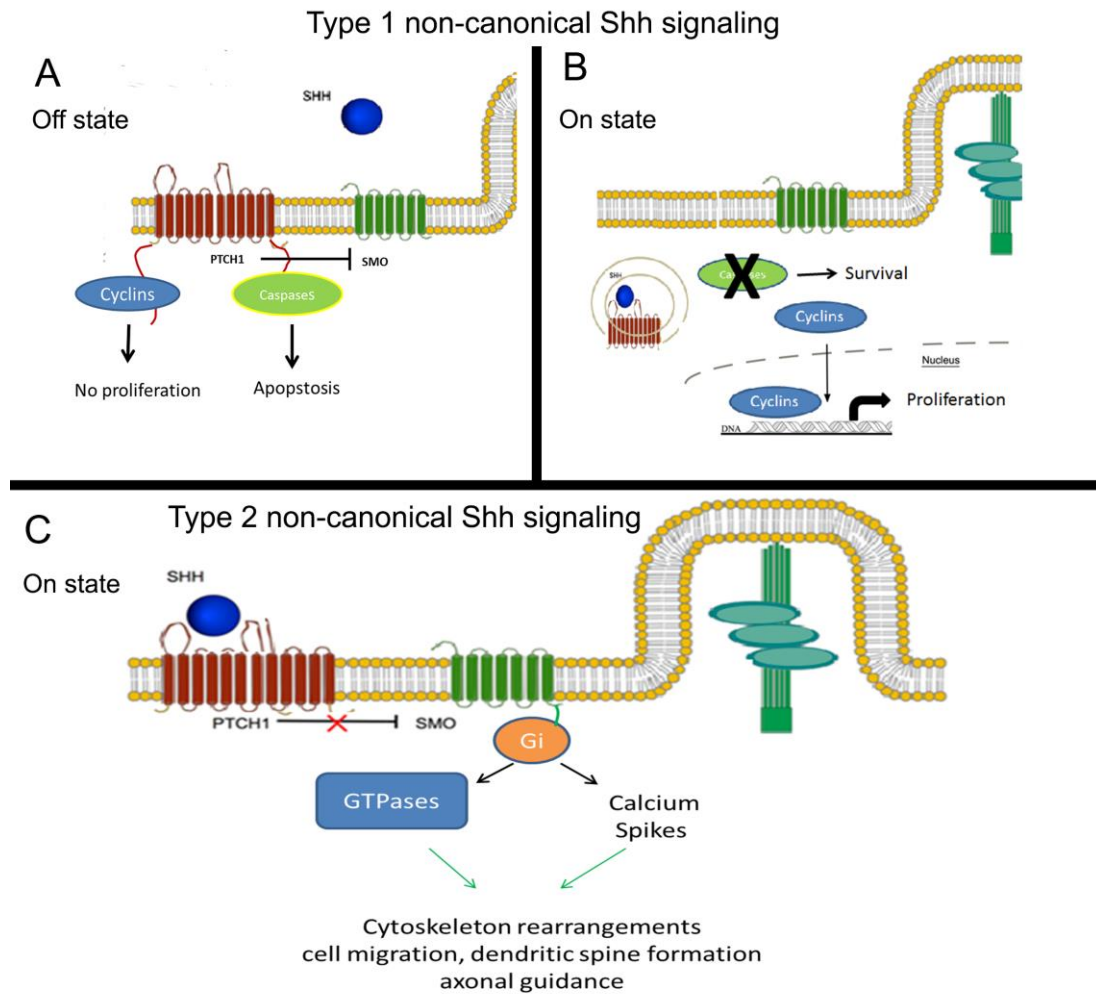


Figure 3: Simplified non-canonical Sonic hedgehog (Shh) signaling pathways. Non-canonical Shh signaling allows modulation of different cells functions without activation of Gli transcription factors. Type 1 non-canonical Shh signaling (A-B) is mediated only by receptor patched1 (PTCH1). In the absence of Shh ligand (Off state), receptor PTCH1 binds to caspases and cyclin proteins to induce apoptosis and prevent cell proliferation respectively (A). Upon Shh binding to PTCH1 (On State), PTCH1 is removed from cell membrane and releases cyclin proteins which can then enter the nucleus to promote cell proliferation. Removal of PTCH1 from the cell membrane also inactivates caspase which promotes cell survival (B). Type 2 non-canonical Shh signaling is mediated by receptors PTCH1 and Smoothened (SMO) and does not require activation of Gli transcription factors. Type 2 non-canonical Shh signaling is mediated by calcium spikes and GTPases that interact with SMO to promote cytoskeleton rearrangements, cell migration and axonal guidance among other cell functions (C). Modified from Moura et al., 2016 (16; 80).

IN VIVO DETECTION OF SHH SIGNALING

Genetic inducible fate mapping is an extremely valuable tool that enables in vivo labeling and tracking of cells expressing a specified gene. This technique utilizes two genetically engineered alleles; one allele expresses *Cre recombinase* under a promoter of interest. The other is a reporter allele designed to heritably label cells after recombinase mediated recombination (55). The generation of *Cre recombinase* fused with tamoxifen inducible estrogen receptor allows temporal control of genetic fate labeling. Transgenic mouse lines for genetic inducible fate mapping of Shh and Shh related genes have been developed and validated (2; 10; 47; 53). These transgenic mice have helped us understand how Shh signaling is activated and regulated in different tissues at different times (53; 89).

The *Gli1*^{CreERT2} transgenic mouse line expresses a *Cre recombinase* fused with taxomifen inducible estrogen receptor under the *Gli1* promoter. *Gli1* is a target gene of the canonical Shh signaling pathway and is known to enhance Shh signaling. Therefore, *Gli1* expression can be used as an effective readout of canonical Shh signaling (2). *Gli1*^{CreERT2} mice have been used to detect canonical Shh signaling in the CNS during postnatal development (106), in normal adults (3; 41; 53), and after injury (76; 94). These studies showed that canonical Shh signaling is highly expressed throughout the ventricular-subventricular zone (V-SVZ) during postnatal development and downregulated in adults. Interestingly canonical Shh signaling in the V-SVZ is increased in response to traumatic brain injury affecting the cerebral cortex but is decreased in traumatic brain injury when white matter damage is the major pathological feature (76). These studies demonstrate that genetic inducible fate mapping can accurately detect

changes in canonical Shh signaling in vivo. Therefore, *Gli1*^{CreERT2} and *Shh*^{CreERT2} mice can detect changes in canonical Shh signaling during different stages of myelination and remyelination.

REMYELINATION

In MS patients and in pre-clinical models of demyelination, OP cells in the parenchyma can proliferate and effectively remyelinate acute demyelinating lesions (7; 57; 59; 92). The amplification of the OP cell population is a common factor in demyelinating models that can spontaneously remyelinate. Interestingly the increase in OP cells correlates with upregulation of signaling molecules involved in OP amplification during developmental myelination including FGF2 and Shh (8; 35).

PDGF signaling can also mediate OP amplification in response to demyelination from cuprizone (bis-cyclohexanone-oxaldihydrazone) ingestion. Transgenic mice over expressing the human *PDGF-A* gene showed an increased number of OP cell after acute demyelination and enhanced remyelination after chronic demyelination (111). Consistent with the amplification role of PDGF signaling in remyelination, mice heterozygous for *PDGF α R* showed impaired remyelination after acute demyelination (82). Similarly, mice over expressing the human *EGFR* gene showed increased levels of OP cell proliferation and enhanced remyelination after acute demyelination (1). In contrast to the beneficial effects observed with increased PDGF and EGF signaling, increased FGF2 signaling after acute and chronic demyelination inhibits OP cell differentiation and limits remyelination. Genetic removal of FGF2 or associated receptor *FGFR1* enhanced remyelination and functional recovery after acute and chronic demyelination (8; 77; 105).

The role of the Shh signaling in the remyelination process is not yet clear since different models of demyelination have reported inconsistent results. In acute demyelinating lesions with lysolecithin, Shh and Shh related proteins (Smo and Gli1) were upregulated in the demyelinated corpus callosum. The upregulation of Shh contributed to spontaneous remyelination since overexpression of Shh in the V-SVZ adjacent to the demyelinated corpus callosum enhanced remyelination and inhibition of Shh signaling impaired remyelination (35). Contrary to these findings, acute demyelination with cuprizone (CPZ) did not change levels of Shh protein or *Gli1* expression in the demyelinated corpus callosum. Furthermore, genetic and pharmacological inhibition of Gli1, a transcription factor known to amplify Shh signaling, enhanced the migration of Shh responsive cells in the V-SVZ to the demyelinated corpus callosum (94). These apparent inconsistencies can potentially be explained by understanding how Shh and related proteins interact with each other to modulate different cell functions under different pathological cues.

CUPRIZONE MODEL OF DEMYELINATION AND REMYELINATION

The use of pre-clinical models of demyelination is essential to understand the mechanisms mediating myelination and remyelination. CPZ is a copper chelator that can be fed to mice to induce demyelination. The exact mechanisms by which CPZ causes demyelination are unknown. However, the available evidence suggests that CPZ disrupts mitochondrial function in the CNS which leads to the death of myelinating oligodendrocytes due to their inability to maintain their high metabolic demand (69).

In C57BL/6 mice, CPZ causes reproducible demyelinating lesions in the corpus callosum (51). CPZ can be fed to mice for different lengths of time to induce acute or

chronic demyelinating lesion. Additionally, the ability to easily remove CPZ from the diet facilitates analysis of the remyelination process after acute and chronic lesions. Acute demyelinating lesions can be induced by feeding mice chow containing CPZ for 3-6 weeks (28; 51). Acute demyelinating lesions with CPZ have several pathological characteristics found in RRMS, including high levels of neuroinflammation, acute axonal damage, increased proliferation of OP cells and spontaneous remyelination (7; 92; 109; 114). Chronic demyelinating lesions can be induced by feeding mice CPZ for 12 weeks (7; 69). Prolonged CPZ ingestion in mice causes depletion of OP cells, and inefficient/limited remyelination when CPZ is removed from the diet (7). These pathological characteristics are also found in progressive stages of MS (59). Therefore, different protocols for CPZ induced demyelination can model different stages of MS.

CHAPTER 2:

Postnatal Sonic hedgehog (Shh) responsive cells give rise to oligodendrocyte lineage cells during myelination and in adulthood contribute to remyelination

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ABSTRACT

Sonic hedgehog (Shh) regulates a wave of oligodendrocyte production for extensive myelination during postnatal development. During this postnatal period of oligodendrogenesis, we fate-labeled cells exhibiting active Shh signaling to examine their contribution to the regenerative response during remyelination. Bitransgenic mouse lines were generated for induced genetic fate-labeling of cells actively transcribing *Shh* or *Gli1*. *Gli1* transcription is an effective readout for canonical Shh signaling. *Shh*^{CreERT2} mice and *Gli1*^{CreERT2} mice were crossed to either *R26*^{tdTomato} mice to label cells with red fluorescence, or, *R26*^{LAP} mice to label membranes with alkaline phosphatase. When tamoxifen (TMX) was given on postnatal days 6-9 (P6-9), Shh ligand synthesis was prevalent in neurons of *Shh*^{CreERT2};*R26*^{tdTomato} mice and *Shh*^{CreERT2};*R26*^{LAP} mice. In *Gli1*^{CreERT2} crosses, TMX from P6-9 detected *Gli1* transcription in cells that populated the corpus callosum (CC) during postnatal myelination. Delaying TMX to P14-17, after the peak of oligodendrogenesis, significantly reduced labeling of Shh synthesizing neurons and *Gli1* expressing cells in the CC. Importantly, *Gli1*^{CreERT2};*R26*^{tdTomato} mice given TMX from P6-9 showed *Gli1* fate-labeled cells in the adult (P56) CC, including cycling progenitor cells identified by EdU incorporation and NG2 immunolabeling. Furthermore, after CPZ demyelination of the adult CC, *Gli1* fate-labeled cells incorporated EdU and were immunolabeled by NG2 early during remyelination while forming myelin-like membranes after longer periods for remyelination to progress. These studies reveal a postnatal cell population with transient Shh signaling that contributes to oligodendrogenesis during CC myelination, and gives rise to cells that continue to proliferate in adulthood and contribute to CC remyelination.

INTRODUCTION

Myelination of axons is tightly regulated during postnatal mammalian development to achieve efficient neurotransmission in concert with the maturation of neuronal circuits (13; 98). This pattern of myelination is modulated by the generation of oligodendrocyte progenitor (OP) cells, which then differentiate into myelinating oligodendrocytes. Signaling molecules that regulate progressive stages from OP generation through myelination are critical to understand in order to develop strategies to mitigate developmental disabilities associated with myelination. These cellular processes and signaling molecules can also provide insights into the design of therapeutics to improve the capacity of OP cells to repair myelin in the adult CNS after an episode of demyelination (20; 37).

Indeed, the developmental origin of OP cells has recently been shown to influence their regenerative properties during remyelination in adulthood (23). The corpus callosum (CC) is populated by OP cells derived from both ventral (Gsh2) and dorsal (Emx1) regions of the ventricular zone during late embryonic and early postnatal development, respectively (58; 110). The dorsal (Emx1) derived cells account for the majority of the oligodendrocytes in the postnatal and adult CC (23; 110). After an episode of demyelination, OP cells derived from the dorsal (Emx1) domain repopulated the CC and dominated the remyelination response (23).

We initiated studies to decipher the signaling molecules that are active during the postnatal amplification of the dorsally derived OP cells which later in life contribute to the remyelination response in the CC. We hypothesized that Sonic hedgehog (Shh) signaling may be an important candidate pathway. In the postnatal and adult ventricular-subventricular zone (V-SVZ), Shh regulates proliferation of activated neural stem cells

and is required to maintain progenitor populations in the telencephalon (27; 66; 88). Relative to oligodendrogenesis, Shh signaling regulates the postnatal wave of oligodendrocyte production from the dorsal V-SVZ to populate the CC prior to myelination (106). This postnatal Shh signaling for OP cell generation activates *Gli1* transcription, which is an effective readout of the canonical Shh pathway (2; 106). However, given the complex and dynamic regulatory interactions of the developing V-SVZ, it is important to determine whether the postnatal Shh responsive cells are part of the dorsally derived population that generates progenitors which contribute to remyelination. Therefore, the current studies focus on the postnatal period of OP amplification and oligodendrogenesis to determine whether Shh responsive cells generate oligodendrocyte lineage cells that contribute to remyelination.

Our studies used induced genetic fate labeling for *in vivo* cell tracking based on postnatal Shh signaling. *Gli1*^{CreERT2} mice were crossed to *R26*^{tdTomato} or *R26*^{LAP} reporter lines to evaluate the distribution of Shh responsive cells in the CC. This heritable approach enabled the Shh responsive population to have expression of the reporter gene, either tdTomato or alkaline phosphatase, induced by tamoxifen (TMX) administration during early postnatal development. The fate and distribution of these genetically labeled cells was then analyzed in the CC after short intervals to examine postnatal myelination, and at much longer intervals to examine the contribution to adult CC populations. Complementary analysis of Shh responsive cells using the *Gli1*^{CreERT2} line and Shh synthesizing cells using the *Shh*^{CreERT2} line examined the postnatal period of active Shh signaling during the progression of myelination in the CC. Healthy adult *Gli1*^{CreERT2} mice were analyzed to identify the CC cell types that were generated from the postnatal *Gli1*

fate-labeled population under homeostatic conditions. Cuprizone (CPZ) mediated demyelination in adult *Gli1*^{CreERT2} mice was used to test whether the postnatal *Gli1* fate-labeled population continued to generate oligodendrocyte lineage cells that contributed to remyelination of the CC. Together, these experiments identify a transient postnatal Shh responsive cohort that gives rise to cells which contribute to CC myelination during development and in adulthood continue to generate oligodendrocyte lineage cells for homeostasis and for myelin repair.

METHODS

Mice for Genetic Inducible Fate Labeling

Mice were housed and cared for in accordance with the guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences. *Shh*^{CreERT2} (B6.129S6-*Shh*^{tm2(cre/ERT2)Cjt}/J; Jackson Laboratories, 005623, Bar Harbor, ME) or *Gli1*^{CreERT2} (*Gli1*^{tm3(cre/ESR1)Alj}/J; Jackson Laboratories, 007913, Bar Harbor, ME; provided by Dr. Sohyun Ahn, National Institutes of Health) mice express Cre recombinase fused to a mutated estrogen receptor, which requires TMX binding for nuclear translocation (2; 3; 47). *Gli1*^{CreERT2} mice were backcrossed to C57BL/6J mice (Jackson Laboratories) for at least six generations. *Shh*^{CreERT2} or *Gli1*^{CreERT2} mice were crossed to reporter mouse lines, either *R26*^{tdTomato} (B6.Cg-Gt(*ROSA*)26^{Sortm14(CAG-tdTomato)Hze}/J; Jackson Laboratories, 007914, Bar Harbor, ME) or *R26*^{IAP} (B6;129-Gt(*ROSA*)26Sortm2Nat/J; Jackson Laboratories, 009253, Bar Harbor, ME; provided by Dr. Tudor Badea, National Institutes of Health). The *R26*^{tdTomato} mouse line carries a floxed stop signal preceding the *tdTomato* gene (67). The *R26*^{IAP} reporter mouse line expresses the human alkaline

phosphatase (AP) gene, with a floxed inverted exon 2 (9). TMX (4-hydroxytamoxifen, H7904, Sigma, St. Louis, MO) was dissolved in sunflower seed oil (S5007, Sigma) to a concentration of 4 mg/ml and delivered via intraperitoneal injection (0.033mg/g) on each of four consecutive days, either postnatal day 6-9 (P6-9) or P14-17.

Analysis included both male and female mice, as noted in the figure legends. Male mice were used for demyelination since CPZ results in similar demyelination in male and female mice but causes toxicity resulting in estrus cycle disruption in female C57BL/6 mice, so that gender differences cannot be appropriately examined (103). Female mice were used for the analysis of the normal conditions during postnatal myelination through to adulthood.

Demyelination of the Corpus Callosum

Acute demyelination in the CC was induced in 8 week old *Gli1^{CreERT2}; R26^{IAP}* male mice by adding 0.3% CPZ to the chow pellets for 6 weeks. After acute demyelination, mice used for analysis of remyelination were given normal chow during a 3 week recovery period after removal of CPZ from the diet.

Chronic demyelination was induced in *Gli1^{CreERT2}; R26^{tdTomato}* and *Gli1^{CreERT2}; R26^{IAP}* mice by adding 0.3% CPZ to the chow pellets for 12 weeks. *Gli1^{CreERT2}; R26^{tdTomato}* mice were perfused 6 days after removal of CPZ from the diet. *Gli1^{CreERT2}; R26^{IAP}* were perfused either at the end of the 12 week CPZ exposure or after a subsequent 6 weeks on normal chow for analysis of remyelination.

Immunohistochemistry and Reporter Detection

Mice were perfused with 3% paraformaldehyde at time points noted in the results for analysis of postnatal development, or as adults with or without CPZ treatment. Brains

were further post-fixed by immersion overnight in 3% paraformaldehyde. Female mice were used for analysis of *Gli1* or *Shh* fate labeling at P11, P13, P14, P28 and P56. Male mice were used for CPZ studies including age matched control mice perfused at P119, P146 and P182. Coronal sections (14 μ m) were used for immunohistochemistry or reporter detection, except for *Shh*^{CreERT2}; *R26*^{IAP} tissue which was better visualized in 20 μ m sections. Antibody information is provided in Table 1. Myelin was immunostained for myelin oligodendrocyte glycoprotein (MOG) (15; 62). Oligodendrocyte lineage cells were identified by Olig2 expression. OP cells were identified as being small, elongated process-bearing cells immunolabeled for NG2. CC1 was used as a marker for mature oligodendrocyte. GFAP was used as a marker for astrocytes in the corpus callosum and neural stem cells in the V-SVZ. Neurons were identified by detection of NeuN. TdTomato reporter signal was visible without additional processing. AP activity was detected as previously described (76). Briefly, endogenous AP activity was deactivated by incubating mounted brain section in heated phosphate buffer saline (69 °C for 90 minutes). Sections were then incubated in nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indoly-phosphate toluidine salt (BCIP) substrate (DAKO, Cat. #: K0598) overnight followed by nuclear staining with methyl green (Vector Laboratories, Cat #: SP5000).

Cell Proliferation In Vivo

Ki67 antigen detection (Table 1) was used to identify cells in an active stage of cell division at the time of perfusion. An ABC kit (Vector Laboratories) was used to detect biotinylated Ki67 antibody followed by brown diaminobenzidine enzymatic reaction (Vector Laboratories) in *Gli1*^{CreERT2}; *R26*^{IAP} mice and combined with the

NBT/BCIP reaction to detect AP (see above). To label cycling cells based on DNA synthesis, *Gli1*^{CreERT2}; *R26*^{tdTomato} mice were injected i.p. with 5-ethynyl-2'-deoxyuridine (EdU) (100 mg/kg). Healthy adult mice were given two injections of EdU at 12 hours apart and perfused 12 hours after the second EdU injection on P56. Chronically demyelinated and age-matched control *Gli1*^{CreERT2}; *R26*^{tdTomato} mice received EdU over five days at 12 hours intervals (10 injections in total) starting two hours after the end of CPZ treatment. Mice were perfused 12 hours after the last EdU injection. EdU was detected in tissue sections using a Click-iT® Plus EdU kit (C10637; Life Technologies).

Image Acquisition, Quantification and Statistical Analysis

Fluorescent images were acquired using a SPOT RT3 camera (Diagnostic Instruments, Sterling Heights, MI) or a Zeiss LSM 700 (Carl Zeiss Microscopy, Thornwood, NY). AP and Ki67 reactions were digitized using a NanoZoomer 2.0-RS (Hamamatsu Instruments, Hamamatsu, Japan). Quantification of each region-of-interest (Fig. S17) included 3 sections per animals with at least 3 animals per group, unless otherwise specified. The CC was subdivided into medial and lateral regions in coronal sections to enable quantification based on the developmental progression of myelination in postnatal mice (112). The medial CC region was defined as the region from the midline to point under the peak of the cingulum. The lateral CC region extended from the point under the peak of the cingulum laterally to edge of the lateral ventricle. Rostral through caudal regions of the CC were examined qualitatively. Quantitative analysis focused on the caudal region of the CC over the hippocampus between approximately -1.06 to -2.30 mm from bregma (36), where CPZ demyelination is prominent (114). Spot Advanced Software (<http://www.spotimaging.com>; RRID: SCR_014313; Spot imaging

solutions, Sterling Heights, MI) was used to measure the area of each region-of-interest. The areas of MOG immunolabeling and AP reaction in the CC were estimated based on the number of pixels with intensities above the background intensity level using the thresholding function in MetaMorph software (<https://www.moleculardevices.com>; RRID: SCR_002368; Molecular Devices, Downingtown, PA). Cells labeled with tdTomato, NG2 or EdU were quantified in the CC as cells/mm². Figure panels were prepared using Adobe Photoshop version 7.0 or above (<http://www.adobe.com>; RRID: SCR_014199; Mountain View, CA). Prism 7.01 (<http://www.graphpad.com>; RRID: SCR_002798; Graphpad; San Diego, CA) was used to prepare graphs and perform statistical analysis.

Both quantitative and qualitative analyses were performed with blinding and the number of mice included in each analysis is listed in each figure legend. Myelination progression was analyzed using one-way analysis of variance followed by Dunnett's post hoc comparison to P56. Two-way analysis of variance was used to analyze cells in the CC as well as AP labeling in the CC. Sidak's multiple comparisons test was used for posthoc analysis. Statistical significance was set as $p < 0.05$.

RESULTS

Shh responsive cells populate mainly lateral CC regions during early postnatal myelination

Gli1^{CreERT2}; R26^{tdTomato} mice were used for induced genetic fate labeling of cells responding to canonical Shh signaling during postnatal myelination of the CC (Fig. 4). Mice were given TMX on postnatal days 6-9 (P6-9) to examine Shh signaling during the period when oligodendrocytes populate the CC (112). When mice were perfused on P10,

Gli1 fate-labeled cells expressing tdTomato reporter (Gli1-Tom) were prominent in the V-SVZ (Fig. 4A-D). In addition, *Gli1*-Tom cells were detected in the lateral CC in coronal sections from both rostral and caudal levels of the brain (Fig. 4A-D). Extending the time after TMX until P14 resulted in higher levels of reporter expression, which facilitated double labeling with Olig2 to identify oligodendrocyte lineage cells among the Gli1-Tom cells in the CC (Fig. 4E).

To examine the distribution of *Gli1* fate-labeled cells relative to the progression of CC myelination, *Gli1*^{CreERT2}; *R26*^{tdTomato} mice were given TMX from P6-9 and analyzed at P14, P21, P28 and P56 (Fig. 5A). Across these time points, immunolabeling of myelin for myelin oligodendrocyte glycoprotein (MOG) demonstrated a lateral to medial progression of myelination in the CC that was evident at the early time points, i.e. P14 (Fig. 5B-D) and P21 (Fig. 5E-G), and continued through full myelination of the CC by P56 (Fig. 5H-J). Quantification shows this progression in detail (Fig. 5K). This myelination pattern in *Gli1*^{CreERT2}; *R26*^{tdTomato} mice is consistent with prior studies of the progression of postnatal myelination in mice (112).

The vast majority of Gli1-Tom cells were localized in the lateral CC, as compared to the medial CC (Fig. 5L). These Gli1-Tom cells could be the initial cells that underwent recombination, or the progeny that inherited the recombined transgene for reporter expression. The density of Gli1-Tom cells did not redistribute in step with the progression of myelination in the medial CC. Gli1-Tom cells were maintained in the lateral CC at similar levels out to adulthood, i.e. P56. Gli1-Tom cells were also distributed within the cerebral cortex (Fig. 5C, F, I). Prior studies from our lab and others

have identified similar *Gli1* fate-labeled cells as a subpopulation of cortical astrocytes in adult mice (41; 76).

Transient postnatal Shh responsive population elaborates myelin-like membranes in postnatal and adult CC

Gli1^{CreERT2} mice were crossed to *R26*^{LAP} mice to visualize membranes formed by *Gli1* fate-labeled (Gli1-AP) cells during postnatal myelination (Fig. 6). When TMX was administered at P6-9, Gli1-AP cells accumulated in the lateral CC at P21, P28, and into adulthood (P56) (Fig. 6A-D). Moreover, the AP reaction revealed that Gli1-AP cells elaborated membranes that extended from P21 (Fig. 6B, E) to P28 (Fig. 6C, F) in the plane of CC axons and appeared in clusters consistent with a myelin morphology in P56 adult mice (Fig. 6D, G). The distribution of Gli1-IAP cells was also examined at rostral levels in adults and again Gli1-IAP cells were mainly found in the lateral CC regions adjacent to the V-SVZ (Fig. S18)

In contrast to these findings with TMX on P6-9, studies using the *Gli1*^{CreERT2} line with TMX administered in adult mice have shown only rare labeling of cells in the CC (41; 53; 76). It is not clear at what point during postnatal development *Gli1* expression is down-regulated among cells that go on to populate the CC. Therefore, we next delayed TMX administration until after the peak of oligodendrogenesis for analysis at a later stage in the progression of CC myelination (96). The delay of TMX to P14-17 significantly reduced the density of *Gli1* fate-labeled cells in the CC (Fig. 6H-L).

Differential Shh signaling may occur among cortical regions in comparison with the CC. Analysis of the cerebral cortex in caudal regions showed a lateral distribution of Gli1-AP cells in mice given TMX from P6-9 in caudal regions (Fig. S18). However, in rostral regions, cortical Gli1-AP cells were localized in medial regions (Fig. S18).

Cortical Gli1-AP cells were also present at much lower levels with TMX administered from P14-17, in comparison with P6-9 (Fig. S18).

Primary cilia required for Shh signaling are present in the CC of adult mice

Activation of canonical Shh signaling and subsequent expression of *Gli1* is dependent on the translocation of Smoothened (Smo) to the primary cilium (84). Previous studies have shown that primary cilia are found on immature oligodendrocyte lineage cells and lost during differentiation into myelinating cells (32; 100). To investigate if loss of primary cilia could explain the reduction in *Gli1* expression after the second postnatal week, tissue sections were immunolabeled to detect Arl13b, which is a marker of cilia and regulates Smo entry into the cilium (60) (Figure S19). Cilia were examined in either C57BL/6J mice at P7 or in *Gli1*^{CreERT2}; *R26*^{tdTomato} mice given TMX at P6-9 that were perfused at P14, P21 and P56. Arl13b labeled small rod shaped structures in cells of the choroid plexus and ependyma, which served as internal positive controls for immunolabeling of cilia that are known to regulate the circulation of cerebral spinal fluid (107). Arl13b labeling consistent with the morphology of primary cilia was also found in the V-SVZ, the CC, and the cerebral cortex. Compared to P7, lower densities of Arl13b labeling continued to be detected in the cerebral cortex and in the CC at P14, P21, and P56. Therefore, loss of primary cilia is not likely to fully account for the lack of *Gli1* fate-labeling in the CC with TMX administered at P14-17.

Transient *Shh* ligand fate-labeling of neuronal populations during postnatal myelination

Shh^{CreERT2} mice were used to investigate Shh ligand synthesis relative to the temporal and spatial patterns of *Gli1* fate-labeling observed with TMX administered from

P6-9 (Fig. 7A-F) or delayed until P14-17 (Fig. 7G-L). *Shh*^{CreERT2}; *R26*^{tdTomato} mice given TMX on P6-9 showed that neurons in the cerebral cortex and in multiple ventral regions are the major source of Shh ligand, i.e. tdTomato labeled based on transcription from the *Shh* promoter (Fig. 7C-E). Shh-Tom cortical neurons were localized mainly in the somatosensory and motor regions at rostral levels (Fig. 7B) as well as in the retrosplenial region at caudal levels (Fig. 7C). Shh-Tom neurons were markedly reduced in the cerebral cortex when TMX was delayed until after the second postnatal week (Fig. 7G-I). Shh-Tom cells in regions adjacent to the V-SVZ were also identified by cell type-specific markers (Fig. 8 for P6-9 TMX; unpublished data for P14-17 TMX). Shh-Tom cells only rarely exhibited an astrocytic morphology or GFAP immunoreactivity. In contrast, the majority of Shh-Tom cells had a neuronal morphology which was confirmed with NeuN immunolabeling (Fig. 8).

Shh^{CreERT2}; *R26*^{LAP} mice were used so that the AP reaction and a longer survival time after TMX administration could increase the detection of the neuronal morphology and distribution of *Shh* transcribing cells across brain regions (Fig. 4). Mice fate labeled at P6-9 and perfused at P21 showed Shh-AP labeling in neurons in somatosensory, motor, and retrosplenial regions of cerebral cortex (Fig. 7D-F), consistent with our findings with tdTomato reporter. At rostral levels, Shh-AP labeling also indicates Shh synthesis in cells of multiple ventral regions and particularly high expression in the claustrum (Fig. 7E). Delaying TMX to P14-17 reduced Shh-AP labeling in the cortical neurons, including somatosensory neurons at both rostral and caudal levels (Fig. 7J-L). Shh-AP labeling in the claustrum was strong with TMX at both P6-9 and P14-17 and served to confirm adequate AP reaction across tissue sections (Fig. 7E, K).

Postnatal Shh responsive cells proliferate in germinal niches and CC into adulthood

We next asked whether the transient postnatal Shh responsive cell population may contribute to the adult regenerative potential, i.e. capacity to generate new cells.

Immunohistochemistry for Ki67 was used to identify cycling cells in sections from *Gli1^{CreERT2}; R26^{LAP}* mice given TMX on P6-9 (Fig. 9). Gli1-AP cells exhibited Ki67 nuclear immunoreactivity in each of the major germinal zones of the adult brain - the V-SVZ (Fig. 9B), subgranular zone (Fig. 9C), and subcallosal zone (Fig. 9D). In the CC, cycling cells were abundant at P28 (Fig. 9E), as expected during late postnatal myelination, whereas fewer cycling cells were observed in adult P56 mice (Fig. 9F). Interestingly, Gli1-AP cells were double labeled for Ki67 in the CC at P28 (Fig. 9E inset) and at P56 (Fig. 9F inset).

Postnatal Shh responsive cells generate cycling oligodendrocyte lineage cells in adult CC

The regenerative potential of the Gli1-Tom fate labeled population in adult mice was further examined based on incorporation of EdU during DNA synthesis and immunolabeling to identify actively proliferating Shh responsive cells that can give rise to oligodendrocyte lineage cells (Figs. 10, 11). *Gli1^{CreERT2}; R26^{tdTomato}* mice given TMX at P6-P9 were administered EdU on P56 (Fig. 10A, 11A). We examined the V-SVZ to determine whether postnatal *Gli1* fate-labeled parent cells or their progeny continued to be present in the adults (Fig. 10). Gli1-Tom cells persisted in dorsal and ventral regions of the V-SVZ cells, and included cycling cells that incorporated EdU (Fig. 10B). These observations indicate that TMX on P6-9 heritably labels self-renewing neural stem cells that continue to give rise to transient amplifying cells in adults. Consistent with this interpretation, immunolabeling for GFAP demonstrated Gli1-Tom labeling of neural stem

cells in the V-SVZ at P56 (Fig. 10C-E). In addition, NG2 was expressed by Gli1-Tom cells in the region that extends from the V-SVZ under the CC (Fig. 10B).

More specific analysis of the oligodendrocyte lineage focused on the CC in adult *Gli1^{CreERT2}; R26^{tdTomato}* mice to examine the fate of the Gli1-Tom population that was heritably labeled with TMX from P6-9 (Fig. 11). Olig2 nuclear immunoreactivity identifies oligodendrocyte lineage cells from OP cells through mature oligodendrocytes. Olig2 immunolabeling demonstrated that Gli1-Tom cells in the CC included oligodendrocyte lineage cells (Fig. 11B). Many of the Gli1-Tom cells with nuclear Olig2 were aligned in rows that are characteristic of interfascicular oligodendrocytes (Fig. 8B). OP cells can continue to proliferate in the CC and give rise to myelinating oligodendrocytes into adulthood (7; 26; 82). Indeed, Gli1-Tom cells in the CC included NG2 immunolabeled cells with small cell bodies and processes characteristic of OP cells (Fig. 11C, D). EdU incorporation enabled detection of cells in the CC that had undergone mitosis during the day prior to perfusion (Fig. 11D). Gli1-Tom cells were identified as cycling OP cells by triple labeling for tdTomato, NG2, and EdU and often appeared as doublets, consistent with recent cell division (Fig. 12D, inset). Gli1-Tom fate-labeling from P6-9 was not specific to the oligodendrocyte lineage as Gli1-Tom astrocyte populations were also present in the adult CC and cerebral cortex (Fig. 12).

Postnatal Shh responsive cells continue to generate oligodendrocyte lineage cells after acute and chronic demyelination with CPZ

The regenerative potential of postnatal Shh responsive cells was investigated during the course of CC demyelination and remyelination using the CPZ model. We first examined an acute demyelination protocol which undergoes effective remyelination after the removal of CPZ from the diet (25). *Gli1^{CreERT2}; R26^{LAP}* mice given TMX on P6-9 were

fed CPZ starting from P56 (8 weeks of age) and continuing for 6 weeks, with or without a subsequent 3 week period on normal chow to allow for remyelination (Fig. 13A).

Gli1^{CreERT2}; R26^{LAP} mice given TMX on P6-9 and perfused at P56 were used as controls without demyelination (No CPZ). Control mice illustrate normal myelination of the CC with MOG immunolabeling of myelin (Fig. 13B). Gli1-AP cells extend clusters of thin membranes parallel to axons consistent with myelin formation in the CC (Fig. 13C-D). After 6 weeks of CPZ, CC demyelination is evident from loss of MOG immunoreactivity (Fig. 13E). Gli1-AP cells in the CC have thickened processes (Fig. 13F-G). After a 3 week period for recovery, MOG immunolabeling is again detected across the CC (Fig. 13H). Consistent with remyelination, Gli1-AP cells in the CC exhibit long, thin membranes with myelin-like morphology (Fig. 13I-J). These findings indicate that postnatal Shh responsive cells contribute to remyelination after acute demyelination.

We next tested the potential of postnatal Shh responsive cells to contribute to remyelination after chronic demyelination in the CPZ model (Figs. 14, 15). Chronic CPZ administration results in continued death of oligodendrocytes, and the OP population that is amplified during acute CPZ demyelination is reduced during chronic CPZ demyelination (7). Therefore, the regenerative potential of the postnatal *Gli1* fate-labeled cells is important to examine in this chronic lesion context. *Gli1^{CreERT2}; R26^{tdTomato}* mice given TMX on P6-9 were fed CPZ for 12 weeks to induced chronic demyelination in the CC. At the end of CPZ, *Gli1^{CreERT2}; R26^{tdTomato}* mice were returned to normal chow and administered EdU every 12 hours for 5 days. Mice were perfused 12 hours after the last dose of EdU (Figs. 14A). Gli1-Tom cells in the CC continued to localize to lateral CC more frequently than to medial CC in both chronically demyelinated mice and age

matched control mice (No CPZ)(Fig. 14B). Therefore, Gli1-Tom cells did not redistribute into the medial CC after CPZ demyelination. However, chronic demyelination significantly reduced the density of Gli1-Tom cells in the lateral CC, as compared to control mice (Fig. 14B). Chronic demyelination also caused an obvious change in the morphology of Gli1-Tom cells in the CC. This change in Gli1-Tom cell morphology is consistent with the morphology previously characterized for reactive astrocytes in the CC after chronic CPZ (49). Immunohistochemistry with GFAP identified Gli1-Tom cells with normal white matter astrocyte morphology in the CC of control mice (Fig. 14C) and reactive astrocytes in the chronic CPZ mice (Fig. 14D). To examine the contribution of Gli1-Tom cells to oligodendrocytes relative to remyelination, immunolabeling with CC1 was used to identify oligodendrocytes and EdU incorporation distinguished oligodendrocytes generated after chronic demyelination (Fig. 11E-P). In control mice, Gli1-Tom cells in the CC were often immunolabeled for CC1 but did not incorporate EdU (Fig. 14E-H). After chronic demyelination, Gli1-Tom cells contributed to newly generated oligodendrocytes based on labeling for CC1 and EdU incorporation (Fig. 14I-P). Gli1-Tom cells with the morphology of reactive astrocytes also proliferated after chronic demyelination (Fig. 14M-P).

The generation of new oligodendrocytes from Gli1-Tom cells during the first 6 days after ending chronic CPZ was further examined by quantitative analysis of cell proliferation and OP cells that give rise to oligodendrocytes during remyelination (Fig. 15A). After chronic demyelination, cell proliferation in the CC was significantly increased overall and within the Gli1-Tom population (Fig. 15B-E). Small, elongated process bearing OP cells immunolabeled for NG2 were also increased in the CC after

chronic demyelination (Fig. 15F-H). Posthoc analysis showed the total OP cell increase to be significant due to the increase in the medial CC (Fig. 15H). In contrast, the Gli1-Tom cells expressing NG2 were most prevalent in the lateral CC in control mice and did not increase after chronic demyelination (Fig. 15I). Analysis of only NG2 cells that incorporated EdU identified OP cells that continued to proliferate after demyelination, both with and without Gli1-Tom labeling (Fig. 15J-M).

This contribution of *Gli1* fate-labeled cells to the generation of new oligodendrocytes after chronic demyelination was then examined in *Gli1^{CreERT2}; R26^{IAP}* mice with a longer recovery period to allow visualization of the membranes elaborated during remyelination (Fig. 16). *Gli1^{CreERT2}; R26^{IAP}* mice were given TMX on P6-9 and fed CPZ for 12 weeks to induce chronic demyelination followed by a 6 week period on normal chow for spontaneous remyelination (Fig. 16A). CPZ mice were compared to age matched control mice (No CPZ) perfused at 182 days of age (26 weeks). Control mice illustrate normal myelination of the CC with MOG immunolabeling of myelin (Fig. 16B). Gli1-AP cells elaborate large clusters of thin membranes parallel to axons consistent with myelin formation in the CC (Fig. 16C-D). After 12 weeks of CPZ, CC demyelination is evident from loss of MOG immunoreactivity (Fig. 16E). Gli1-AP cells in the CC have thickened processes and reduced overall AP labeling within the CC (Fig. 16F-G). After the 6 week period for recovery, MOG immunolabeling is again detected across the CC (Fig. 16H). Consistent with remyelination, Gli1-AP cells in the CC exhibit long, thin membranes with myelin-like morphology (Fig. 16I-J). Gli1-AP membranes appear to also extend along axons in the cingulum after chronic demyelination (Fig. 16I-J). These

findings indicate that postnatal Shh responsive cells contribute to CC remyelination after chronic demyelination.

DISCUSSION

Understanding the regulatory signals that generate postnatal OP cells is important for designing strategies to optimize the capacity for myelin maintenance and repair in adults. The current study identified cells with active Shh signaling during postnatal development that generate CC oligodendrocytes during postnatal myelination and during remyelination in adults. Crossing of *Gli1*^{CreERT2} mice and *Shh*^{CreERT2} mice to reporter lines demonstrated transient canonical Shh signaling associated with cells populating the CC. *Gli1* fate-labeling was evident in the postnatal CC, particularly in lateral regions adjacent to the V-SVZ, when TMX was given from P6-9. *Gli1* fate-labeling of cells in the CC was significantly reduced when TMX was delayed until P14-17. Our findings demonstrate active Shh signaling, i.e. *Gli1* fate-labeling, during the postnatal wave of oligodendrogenesis. We go on to show that cells which are *Gli1* fate-labeled from P6-9 are present in the CC not only during postnatal myelination but continue to be found in the CC in adulthood. Furthermore, this cohort of postnatal *Gli1* fate-labeled cells gives rise to oligodendrocyte lineage cells in adults during remyelination in response to either acute or chronic demyelination.

We used induced genetic labeling to demonstrate active Shh signaling *in vivo* during the specific time period of high oligodendrogenesis for postnatal myelination. Importantly, this heritable labeling approach enabled long term *in vivo* tracking of the fate of this Shh responsive postnatal cohort and subsequent progeny. Heritable labeling at P6-9 was followed out to as long as 26 weeks to examine *Gli1* fate-labeled cells in adult

mice during homeostasis and in response to chronic demyelination. The *Gli1*^{CreERT2} mouse conditional driver line employed for induced genetic labeling of cells responding to Shh has been characterized to show that reporter labeling corresponds with Gli1 mRNA transcript expression patterns during embryonic development (2). In addition, *Gli1*^{CreERT2} mice exhibited reporter labeling consistent with *Gli1* expression detected by in situ hybridization for mRNA transcripts in adult mice (53). *Gli1*^{CreERT2} mice have enabled analysis of the lineage and/or fate of cells with active canonical Shh signaling *in vivo* in postnatal and adult mice (3; 106). Interestingly, *Gli1*^{CreERT2} mice that exhibit extensive labeling of cells populating the CC after TMX from P6-9 have shown extremely rare labeled cells in the CC after TMX administration to healthy adult mice (41; 53; 76).

Shh signaling and transcriptional activation targeting *Gli1* varies with age and specific localization within germinal zones (4; 17; 106). Gli1 expression is highest in ventral regions of the V-SVZ in adult mice (53; 89). In contrast, during early postnatal development Gli1 is also expressed dorsally in the V-SVZ adjacent to the lateral CC. Gli1 mRNA transcripts are enriched in the dorsal V-SVZ at P0 and P7 but diminished by P14 and almost absent by P21 (106). Our experiments in *Gli1*^{CreERT2} mice confirmed that *Gli1* expression, as detected by reporter fate-labeling, was high in the V-SVZ between P6-9. Furthermore, with TMX from P6-9 the lateral CC was populated by cells that were Gli1 fate-labeled, which was dramatically reduced with TMX given from P14-17.

Shh signaling regulates postnatal oligodendrocyte production from the dorsal V-SVZ. Genetic activation or inactivation of a critical Shh pathway signaling component, *Smoothed*, in the dorsal V-SVZ increased or decreased oligodendrocyte production,

respectively, in early postnatal mice (106). Our experiments provide initial evidence regarding temporal regulation of Shh signaling during this postnatal wave of oligodendrocyte production. We used *Shh*^{CreERT2} mice to examine cells exhibiting active *Shh* ligand transcription during the time period when the *Gli1*^{CreERT2} mice were used to heritably label cells with active Shh signaling. *Shh*^{CreERT2} mice have been characterized previously as exhibiting reporter labeling consistent with endogenous Shh expression levels and have appropriately reduced *Shh* fate-labeling after homozygous or hemizygous deletion of floxed *Shh* alleles (52; 53). In regions surrounding the V-SVZ and CC in postnatal *Shh*^{CreERT2} mice we identified neurons as the major Shh synthesizing cell type, based on morphology and immunolabeling with NeuN. Comparison of TMX on P6-9 versus P14-17 showed fewer neurons with active *Shh* ligand transcription after the second postnatal week. The labeling in *Shh*^{CreERT2} mice with TMX on P14-17 is consistent with the labeling in cortical neurons with TMX administered in adult *Shh*^{CreERT2} mice and in overall distribution in Shh expressing neuronal populations in adult brain using multiple techniques (4; 41). Therefore, transcriptional control of Shh ligand synthesis corresponds temporally with the transient wave of Shh responsive cells that generate oligodendrocytes in the lateral CC during postnatal myelination. However, *Shh* transcribing neurons are not closely co-localized with *Gli1* fate-labeled cells. Neurons have extensive processes and Shh peptides can have direct short or long-range actions, which may involve secretion through multiple forms of extracellular vesicles (44; 46; 113). Shh peptides have been shown to bind to astrocytes which then present Shh to OP cells to influence proliferation and migration in developing optic nerve (73; 87). Given this complexity, the spatial

distribution of Shh ligand synthesized from *Shh* transcribing neurons cannot be fully appreciated from the current results.

The dorsal V-SVZ developmental site of origin markedly influences the OP population response to demyelination and contribution to remyelination in the adult mouse spinal cord and CC (23). Specifically, OP cells that are derived from the dorsal V-SVZ populate the forebrain and CC during development, become the majority OP population in the adult CC, and then dominate oligodendrocyte production for remyelination. These studies exploited *Emx1-Cre* mice crossed to dual fluorescent reporter lines to fate-label cells derived from the dorsal V-SVZ. Our experiments in *Gli1^{CreERT2}* mice extend these findings to show that Shh signaling regulates the postnatal generation of this dorsally derived OP population. Analysis of two time points, P6-9 and P14-17, with an inducible Cre construct delineated the postnatal OP generation period as mainly prior to the third postnatal week. With TMX from P6-9, *Gli1* fate-labeled cells in the CC were identified as generating oligodendrocyte lineage cells based on immunolabeling with NG2, Olig2, and CC1 along with formation of myelin-like membranes. Few *Gli1* fate-labeled cells populated the postnatal CC when TMX was administered from P14-17. Additional preliminary studies confirmed only rare *Gli1* fate-labeling of CC cells with TMX from P14-17 after chronic demyelination (Fig. S20). Analysis of *Shh^{CreERT2}* mice with TMX also from P6-9 and P14-17 illustrated a parallel temporal pattern of labeling to that observed in the *Gli1^{CreERT2}* mice. These concordant results for the Shh ligand synthesis and Shh responsive cells support a model in which Shh signaling regulates the postnatal generation of this dorsally derived OP population that contributes to remyelination after acute or chronic demyelination (Fig. 21).

The current studies are the first to demonstrate a connection from Shh responsive cells during postnatal myelination to the CC populations that respond to demyelination in the adult CC. This dorsal population of Shh responsive cells does not appear to overlap completely with the dorsal Emx1 domain of the V-SVZ. Emx1 derived cells distribute throughout the medial and lateral regions of the CC during postnatal development (58; 110). In contrast, our results show that the postnatal Shh responsive cells populated more lateral regions of the CC during myelination and in adulthood. This lateral distribution of *Gli1* fate-labeled cells was found into adulthood in both male and female mice, and continued during remyelination. More detailed studies would be needed to fully explore the relationship of this transient dorsal Shh population relative to the population derived from the Emx1 domain.

Further studies will be of interest to test the implications of this *Gli1* fate-labeled postnatal cohort for developmental myelination, adult myelin maintenance, and remyelination. Experimentally induced apoptosis of oligodendrocytes during early postnatal myelination impairs remyelination in adult mice (19). Therefore, disruption of normal Shh signaling in oligodendrocyte lineage cells during this postnatal period may have long term implications, and potential vulnerabilities, related to the available pool of OP cells and oligodendrocytes in the adult CC. Through adulthood, new oligodendrocytes are generated for myelin maintenance and activity dependent myelin plasticity (43; 70). In the case of injury or disease, OP cells are required to generate new oligodendrocytes for myelin repair (57; 82; 91). Since we now show that progeny from this postnatal cohort of Shh responsive cells from the dorsal V-SVZ contribute to

remyelination in the adult, future studies may also be able to determine whether these cells exhibit different molecular controls that can be targeted to enhance remyelination.

The current results based on postnatal Shh signaling raise questions of whether similar Shh signaling is active during demyelination and remyelination. Prior studies have indicated conflicting roles of Shh signaling, either reporting Shh positively regulates oligodendrocytes for remyelination, or, that *Gli1* negatively regulates recruitment of remyelinating cells from the V-SVZ (35; 94). Therefore, our ongoing work is analyzing *Shh*^{CreERT2} mice and *Gli1*^{CreERT2} mice given TMX at multiple time points during the CPZ time course to examine Shh signaling in reactive microglia and astrocytes and in oligodendrocyte lineage cells during acute demyelination or after chronic demyelination. Although beyond the scope of the current study, these ongoing complementary studies using the same reporter lines will determine whether the Shh signaling that is active during postnatal population of the CC may be reactivated during repopulation of the CC after acute or chronic demyelination.

Table 1: List of antibodies 1

Primary antibodies	Secondary antibodies
CC1 (mouse mAb) Anti-APC (Ab-7)1:20 Millipore, Cat # OP80 RRID: (AB_2057371)	donkey anti-mouse F(ab') ₂ IgG (Alexa Flour® 647) 1:100 Jackson ImmunoResearch, Cat # 715-606-151 RRID: AB_2340866 donkey anti-mouse (H+L) IgG (Alexa Flour® 488) 1:50 Jackson immunoResearch, Cat # 715-545-150 RRID: AB_2340866
GFAP (rabbit polyclonal) 1:500 DAKO Cat # Z0334 RRID: (AB_10013382)	goat anti-rabbit (H+L) IgG (Alexa Flour® 488) 1:200 Thermo Fisher Scientific, Cat # A11034 RRID: AB_2576217
Ki67 (rabbit monoclonal) 1:100 Abcam, Cat # AB16667 RRID: (AB_302459)	goat anti-rabbit (H+L) IgG (biotinylated) 1:50 Vector, Cat # BA-1000 RRID: AB_2313606
MOG (mouse monoclonal 8-18C5) 1:100 Millipore, Cat # MAB5680 RRID: (AB_1587278)	donkey anti-mouse F(ab') ₂ IgG (Cy TM 3) 1:100 Jackson immunoResearch, Cat # 715-166-150 RRID: AB_2340816
MOG (mouse monoclonal 8-18C5) 1:20 Dr Minettia Gardinier, Department of Pharmacology, University of Iowa, Iowa City, IA U.S.A. RRID: (AB_2631445)	donkey anti-mouse (H+L) IgG (Alexa Flour® 488) 1:50 Jackson immunoResearch, Cat # 715-545-150 RRID: AB_2340846
NeuN (mouse monoclonal, clone A60 conjugated with Alexa Flour® 488) 1:100 Millipore, Cat # MAB377 RRID: (AB_2298772)	
NG2 (rabbit polyclonal) 1:500 gift from Dr. William Stallcup RRID: (AB_2572306)	donkey anti-rabbit F(ab') ₂ IgG (Alexa Flour® 647) 1:100 Jackson ImmunoResearch, Cat #: 711-606-152 RRID: AB_2340625
Olig2 (rabbit polyclonal) 1:100 Millipore, Cat # AB9610 RRID: (AB_570666)	goat anti-rabbit (H+L) IgG (Alexa Flour® 488) 1:200 Thermo Fisher Scientific, Cat # A11034 RRID: AB_2576217

FIGURES

Figure 4: Tamoxifen (TMX) from P6-9 fate labels Shh responsive cells in the ventricular-subventricular zone (V-SVZ) and lateral corpus callosum (CC).

Gli1CreERT2; R26tdTomato mice were administered TMX from postnatal day 6-9 (P6-9) to induce genetic fate-labeling of cells expressing Gli1, which is an effective readout of Shh signaling (A). In mice perfused on P10, the tdTomato (Tom) reporter expression in Gli1 fate-labeled (Gli1-Tom) cells was strong in the dorsal and ventral regions of the V-SVZ, respectively labeled dV-SVZ and vV-SVZ, along the lateral wall of the lateral ventricle (LV) (B). The yellow line divides the CC under the peak of the cingulum (Cg) designating regions as medial (M_CC) and lateral (L_CC) (B). Higher magnification shows small process-bearing Gli1-Tom cells in the white matter tracts of the CC and Cg (C). Gli1-Tom cells are also found along the lateral wall of the lateral ventricle, in the subcallosal zone (SCZ), and distributed within the L_CC in sections from more caudal brain regions (D). In mice with perfusion delayed until P14, immunohistochemistry for Olig2 identifies oligodendrocyte lineage cells among the Gli1-Tom cells in the CC (E; arrows show example of Gli1-Tom cell with nuclear Olig2; enlarged as inset). Images shown are representative from n = 4 (P10) and n = 4 (P14) female mice. Scale bar in B = 400 μ m, C, D = 200 μ m, E = 100 μ m.

Figure 4

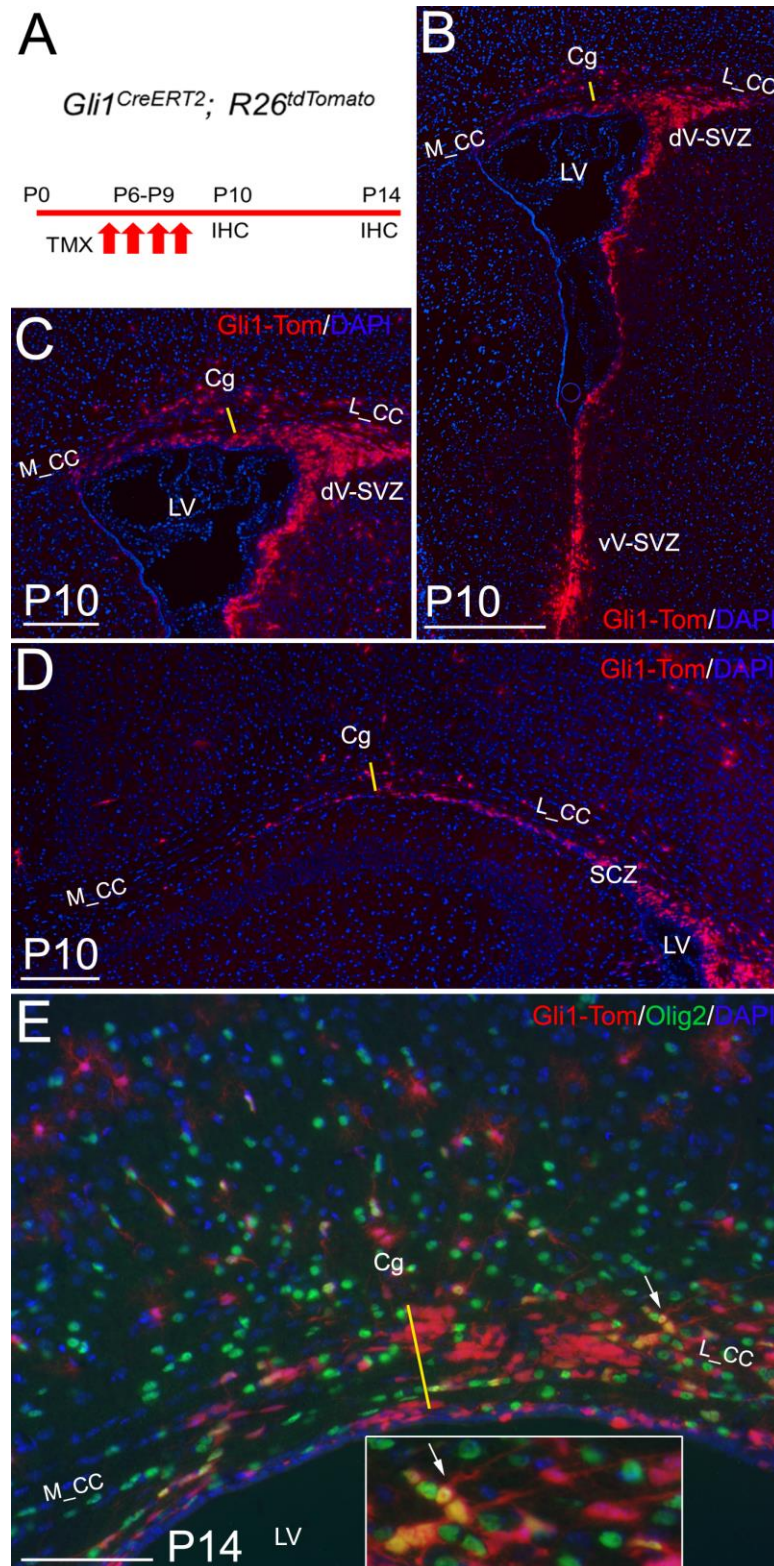


Figure 5: Tamoxifen (TMX) from P6-9 fate labels Shh responsive cells that localize in the lateral corpus callosum (CC) during postnatal myelination and into adulthood.

Gli1^{CreERT2}; *R26*^{tdTomato} mice were administered TMX from postnatal day 6-9 (P6-9) to induce genetic fate-labeling of cells expressing *Gli1* (**A**). The CC was analyzed for myelination and tdTomato (Tom) reporter expression in *Gli1* fate-labeled (Gli1-Tom) cells at P14 (**B-D**), P21 (**E-G**), P28 (not shown) and P56 (**H-J**). The yellow line across the CC under the peak of the cingulum (cg) divides the CC regions into medial (M_CC) and lateral (L_CC) (**B-J**). Immunohistochemistry (IHC) to detect myelin oligodendrocyte glycoprotein (MOG) demonstrates the lateral to medial progression of myelination in the CC between P14-P56 (**B, E, H**). Myelination in the M_CC is delayed compared to myelination in the L_CC (**K**). Gli1-Tom cells are localized mainly in the lateral CC during postnatal myelination (**C, F**) and this pattern is maintained in adult mice (**I**). The low density of Gli1-Tom cells in the medial CC (**L**) does not increase in parallel with postnatal myelination (**K**). One-way ANOVA, Dunnett's multiple comparisons to P56 (**K**). Two-way ANOVA (**L**). Female mice used in quantification: n = 4 for P14, P21 and P56; n = 3 for P28. Scale bar = 400 μ m.

Figure 5

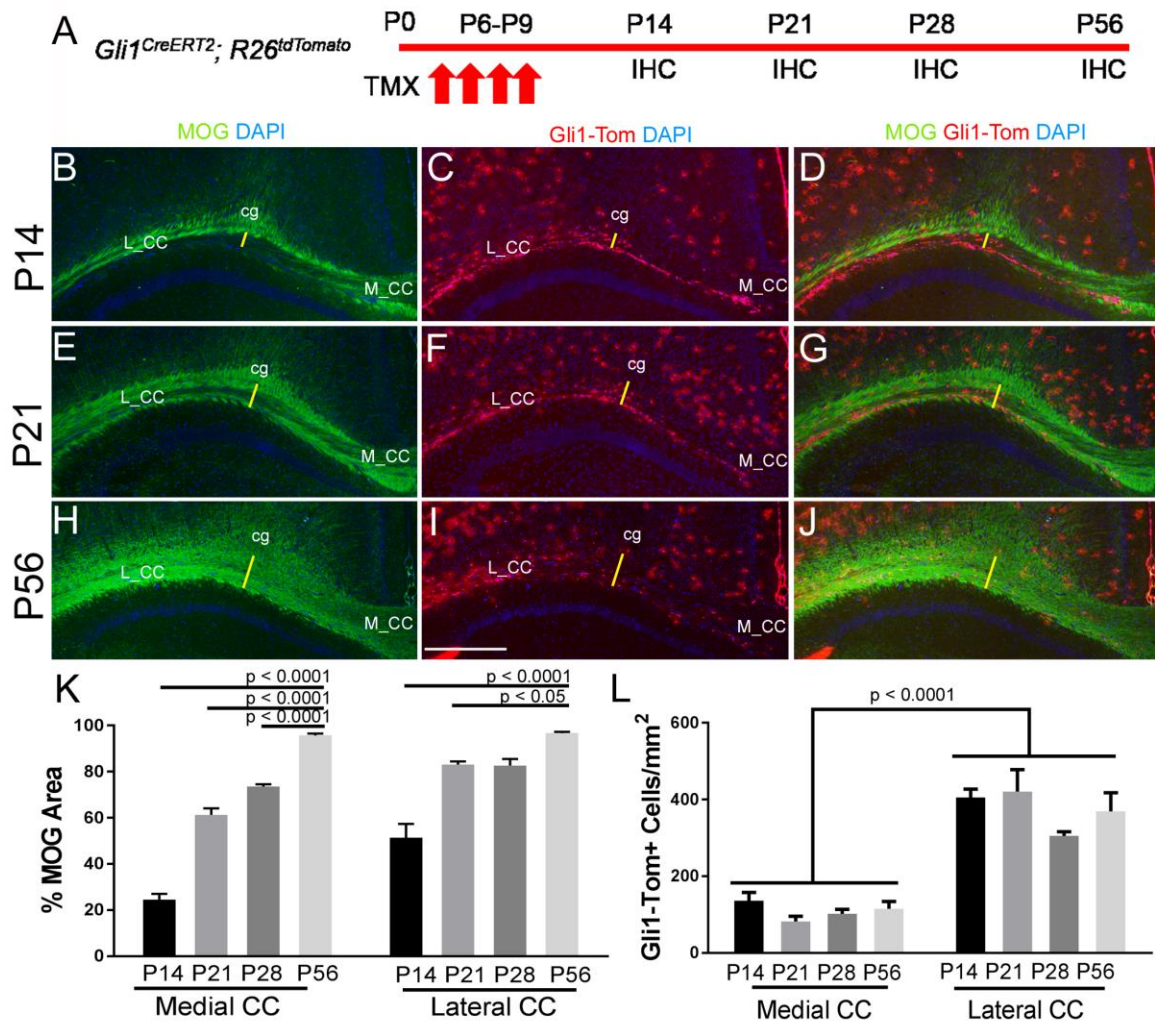


Figure 6: *Gli1*^{CreERT2}; *R26*^{IAP} cross demonstrates membrane elaboration from transient Shh responsive population.

Gli1^{CreERT2}; *R26*^{IAP} mice were given tamoxifen (TMX) from postnatal days 6-9 (P6-9) (**A-G**) or from P14-17 (**H-K**). Representative images show alkaline phosphatase (AP) reaction in *Gli1* fate-labeled (Gli1-AP) cells distributed mainly in the lateral corpus callosum (L_CC) with few in the medial region (M_CC) when analyzed at P21 (**B**), P28 (**C**), or P56 (**D**). Gli1-AP cells initially have short processes at P21 (**E**) that become more extensive and aligned along the plane of CC axons by P28 (**F**), and exhibit a myelin-like morphology by P56 (**G**). When TMX administration was delayed until P14-17 (**H**), Gli1-AP labeling was dramatically reduced across all time points examined (**I-K**). Quantification shows the more lateral distribution of Gli1-AP cells in the CC of mice given TMX at P6-9 and the significant loss of Gli1-AP labeling with TMX given at P14-17 (**L**). Female mice used in quantification: n = 4 with TMX at P6-9 for P21, P28 and P56; n = 3 with TMX at P14-17 for P21 and P56; n = 5 with TMX at P14-17 for P28. Scale bar in K = 400 μ m for B-D and I-K; in E-G = 50 μ m.

Figure 6

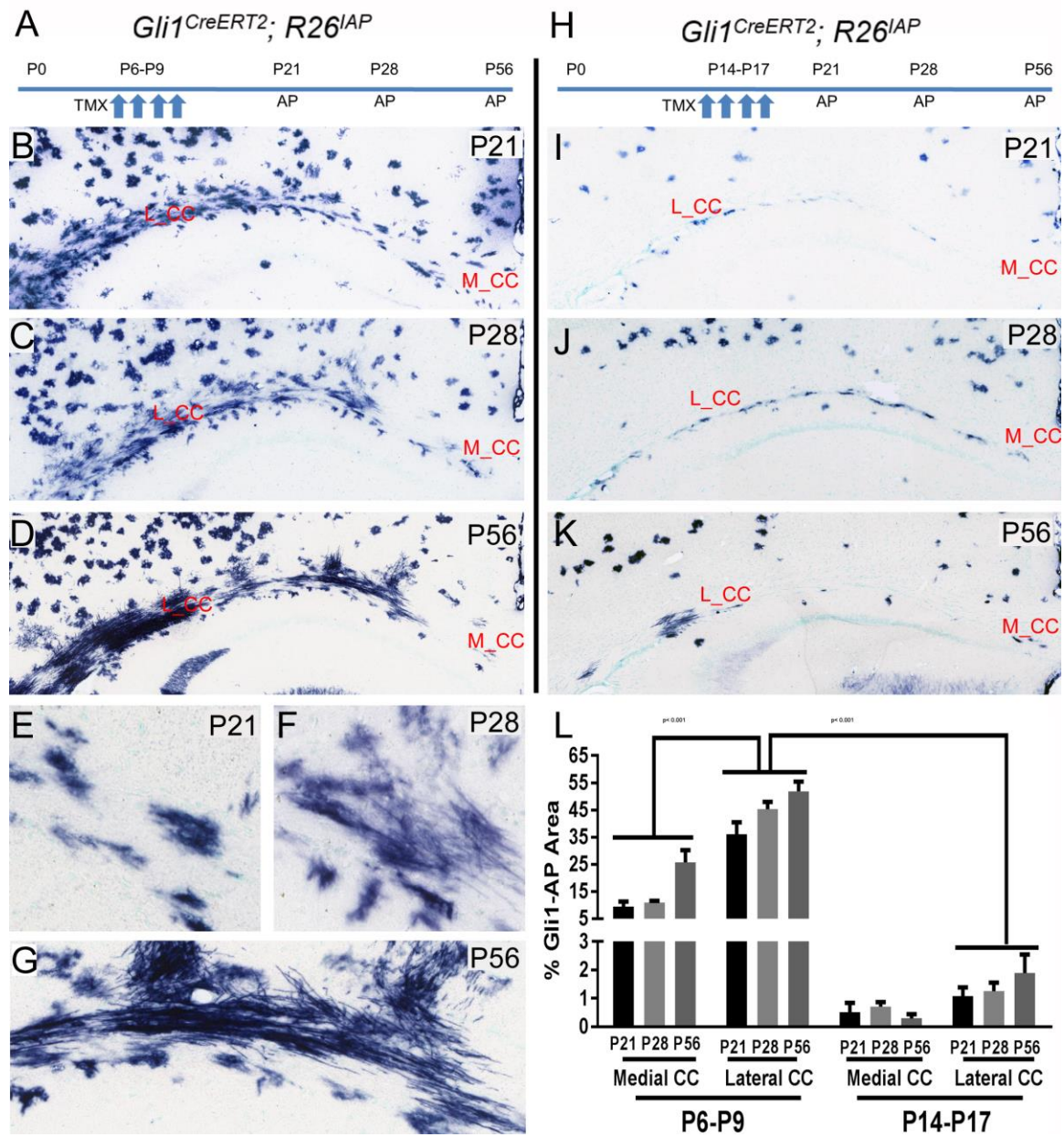


Figure 7: *Shh*^{CreERT2} fate-labeling identifies active *Shh* transcription in neuronal populations during postnatal development.

Shh^{CreERT2} mice were given tamoxifen (TMX) on postnatal days (P6-9) (A-F) or P14-17 (G-L). With TMX from P6-9 in *Shh*^{CreERT2}; *R26*^{tdTomato} mice, the tdTomato (Shh-Tom) reporter labeling shows that cortical neurons (A-C) are the main source of Shh ligand in the dorsal forebrain. Cortical neurons were found in the somatosensory (S) and motor (M) regions of the cerebral cortex at rostral levels (B) along with the retrosplenial (RS) region at caudal levels (H). Similar analysis in *Shh*^{CreERT2}; *R26*^{IAP} mice shows more detail of the neuronal processes labeled with alkaline phosphatase (AP) with TMX given from P6-9 (D-F). Delaying TMX until P14-17 in *Shh*^{CreERT2}; *R26*^{tdTomato} mice identified relatively few Shh synthesizing neurons in the somatosensory cortex, with almost no labeled cells in the motor or retrosplenial regions (G-I). Similarly, TMX from P14-17 in *Shh*^{CreERT2}; *R26*^{IAP} mice shows much less Shh fate labeling (J-L). The claustrum (cl) is strongly labeled in rostral sections with TMX at either P6-9 or P14-17 and so serves as an internal control demonstrating appropriate AP reaction across time points (E, K). Shh synthesis was not detected in the cingulate cortex (CgC) at either time point (B, H). Immunohistochemistry (IHC) for cell type detection in *Shh*^{CreERT2}; *R26*^{tdTomato} mice is shown in supplemental materials. Images shown are from representative female mice: n = 5 for *Shh*^{CreERT2}; *R26*^{tdTomato} mice with TMX at P6-9 and analyzed at P13; n = 4 for *Shh*^{CreERT2}; *R26*^{tdTomato} mice with TMX P14-17 and analyzed at P21; n = 3 for *Shh*^{CreERT2}; *R26*^{IAP} mice with TMX at P6-9 or P14-17 and analyzed at P21 or P28. Scale bars in B = 100 μ m for B-C and H-I; in E, F, K, L = 500 μ m.

Figure 7

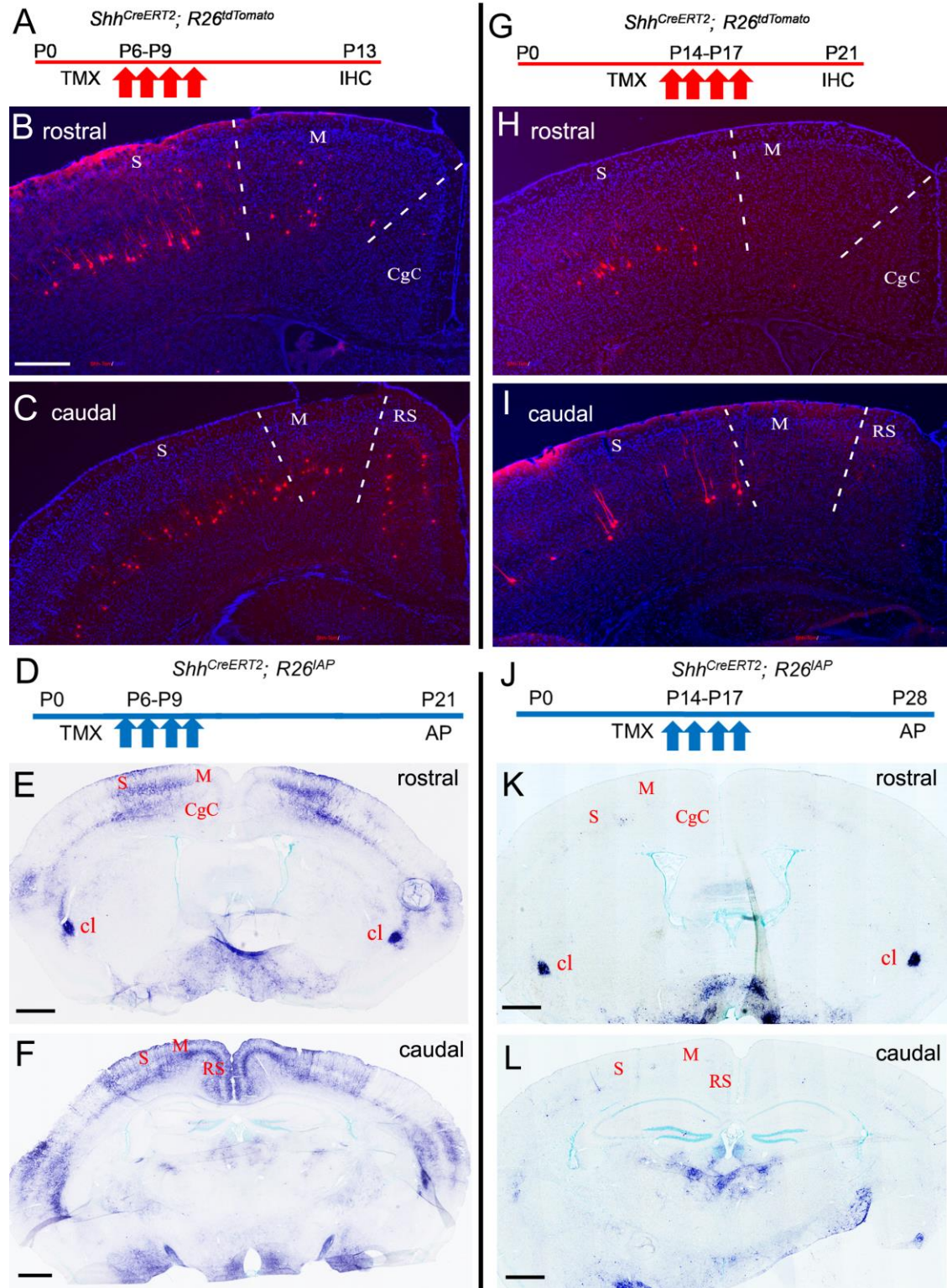


Figure 8

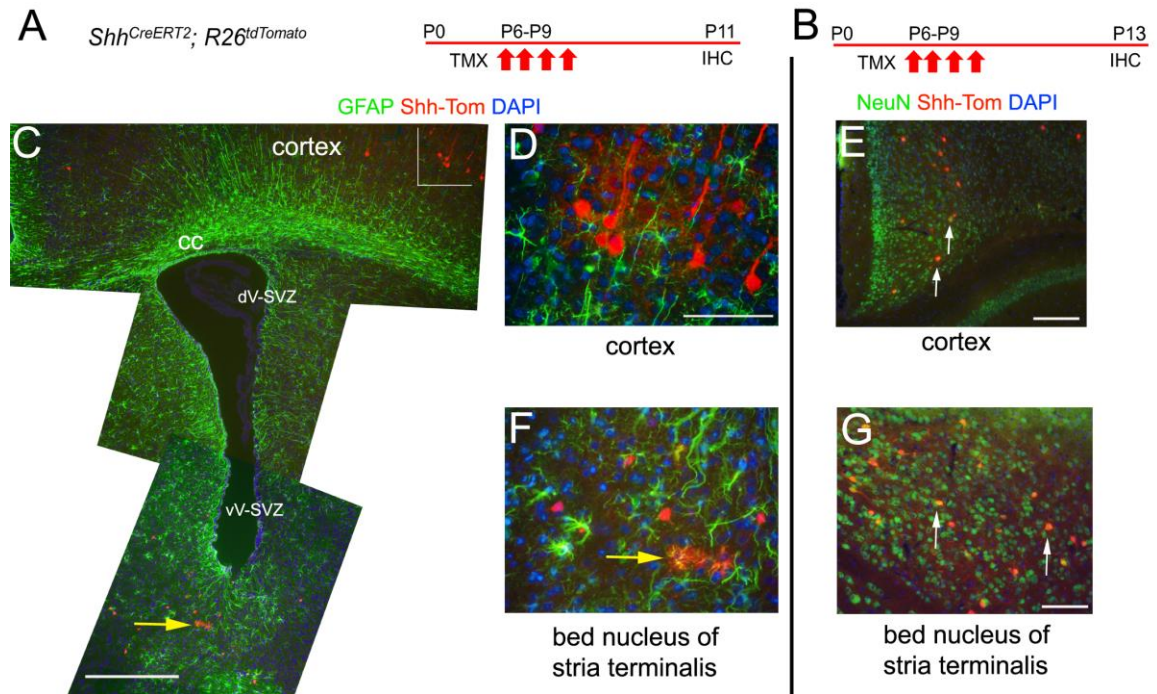


Figure 8: Neurons are the main source of Shh ligand in postnatal development.

Shh^{CreERT2}; R26^{tdTomato} mice were given tamoxifen (TMX) at postnatal days 6-9 (P6-9) and perfused at P11 (A) or P13 (B). *Shh* fate-labeled (*Shh*-Tom) cells were found in the cerebral cortex (C-E) and in ventral regions (F, G). Most *Shh*-Tom cells had neuronal morphology (area marked off at top corner in C is enlarged in D) and were immunolabeled with neural marker NeuN in the cerebral cortex (E, arrows) and in ventral regions (G, arrows). Rare *Shh*-Tom cells were astrocytes based on immunolabeling for the astrocytic marker GFAP (arrow in C and F). Images shown are representative from $n = 2$ (P11) and $n = 5$ (P13) female mice. Scale bars C = 400 μm ; D = 100 μm for D, F; E = 200 μm ; G = 100 μm .

Figure 9

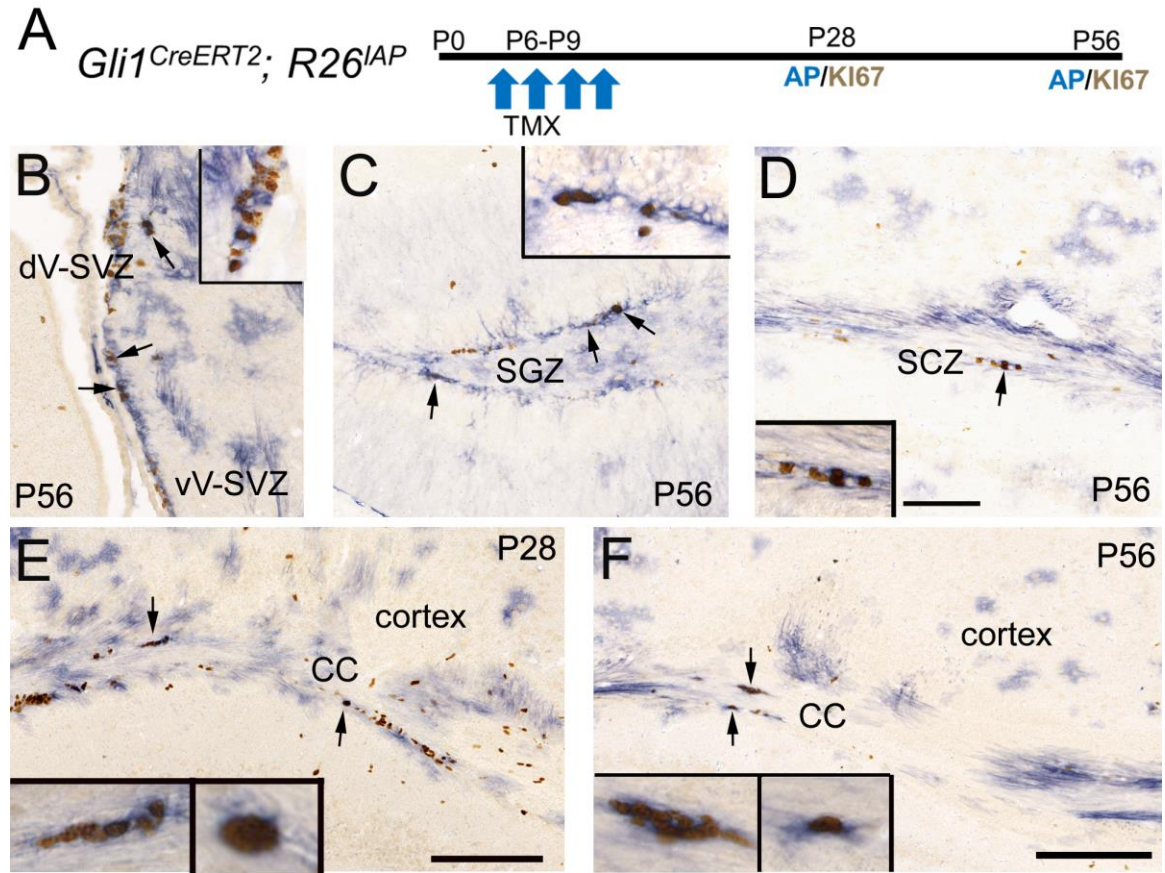


Figure 9: Tamoxifen (TMX) from postnatal days 6-9 (P6-9) fate labels Shh responsive cells that include cycling cells in adult mice.

Gli1^{CreERT2}; R26^{lAP} reporter mice were analyzed with immunolabeling to detect Ki67, a marker of actively proliferating cells, late in postnatal myelination (P28) and in adult mice (P56) to compare the contribution to cycling cell populations (A). Localization of Ki67 in nuclei of cells fate labeled with alkaline phosphatase (AP) showed that *Gli1* fate-labeled (*Gli1*-AP) cells continue to proliferate in each of the major germinal zones (B-D). In the ventricular-subventricular zone (V-SVZ) (B), *Gli1*-AP cells with nuclear Ki67 (arrows) were observed in both the dorsal (dV-SVZ; inset) and ventral (vV-SVZ) regions. *Gli1*-AP cells with nuclear Ki67 (arrows) were also located in the subgranular zone (SGZ) within the hippocampus (C; inset) and the subcallosal zone (SCZ) overlying the hippocampus (D; inset). *Gli1*-AP cells with nuclear Ki67 were detected in the corpus callosum (CC) and were more prevalent at P28 (E) than at P56 (F). Examples shown in insets are rows of *Gli1*-AP cells double labeled for nuclear Ki67 (E and F, downward arrow; inset) along with double labeled cells in the adjacent SCZ (E and F, upward arrow; inset). Images shown are representative from $n = 4$ female mice for each time point, P28 and P56. Scale bar in D = 100 μm for B-D, in E, F = 200 μm .

Figure 10

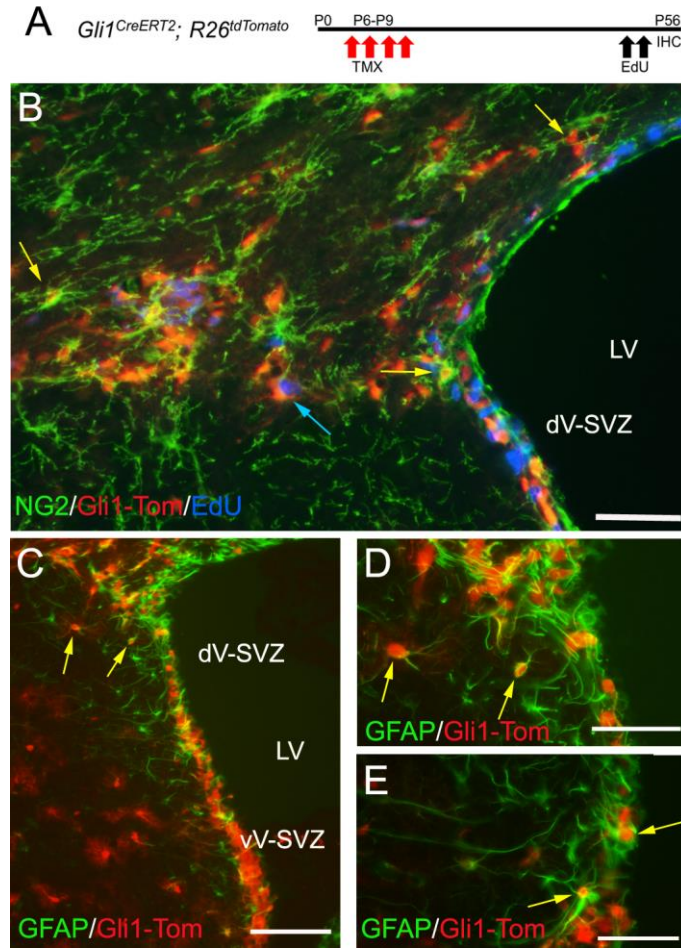


Figure 10: Tamoxifen (TMX) from P6-9 fate labels Shh responsive neural stem cells and their progeny in the adult ventricular-subventricular zone (V-SVZ).

Gli1^{CreERT2}; *R26*^{tdTomato} mice given TMX on postnatal days 6-9 (P6-9) were raised to adulthood and given two doses of EdU to identify cells that had undergone DNA synthesis indicative of cell division within 24 hours prior to perfusion (A). EdU detection shows extensive proliferation in the dorsal V-SVZ (dV-SVZ) (B) including *Gli1* fate-labeled (Gli1-Tom) cells in lateral extension of the V-SVZ (B, blue arrow). Immunolabeling with NG2 shows Gli1-Tom progenitor cells in the V-SVZ and corpus callosum (B yellow arrows). Immunohistochemistry (IHC) with GFAP identified Gli1-Tom cells as neural stem cells in the dorsal (dV-SVZ) (C, D yellow arrows) and ventral (vV-SVZ) (C, E yellow arrows) V-SVZ. Images shown are representative from n = 4 female mice perfused at P56, with n = 2 given EdU. Scale bars in B, D-E = 50 μm, in C = 100 μm

Figure 11

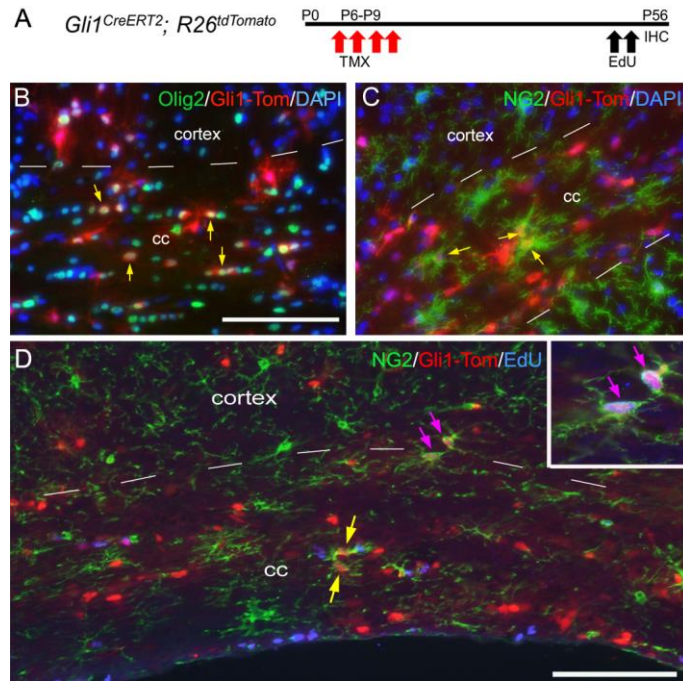


Figure 11: Tamoxifen (TMX) from P6-9 fate labels Shh responsive cells that generate oligodendrocyte lineage cells in the corpus callosum (CC) of adult mice.

Gli1^{CreERT2}; *R26*^{tdTomato} mice were administered TMX on postnatal days 6-9 (P6-P9) and perfused at P56 to identify oligodendrocyte lineage cells (A). Mice were given two injections of a thymidine analogue (EdU) to identify cells that had undergone cell division within 24 hours prior to perfusion. *Gli1* fate-labeled (Gli1-Tom) cells in the CC include oligodendrocyte lineage cells based on nuclear Olig2 immunoreactivity (B, yellow arrows). Gli1-Tom cells were aligned in rows characteristic of interfascicular oligodendrocytes (B). Immunohistochemistry (IHC) to detect NG2 identified small, elongated process-bearing Gli1-Tom cells as oligodendrocyte progenitors (yellow arrows in C, D). Proliferation of Gli1-Tom oligodendrocyte progenitor cells was evident from EdU incorporation in nuclei of Gli1-Tom cells immunolabeled for NG2 and appearing as doublets (D purple arrows). Scale bar for C shown in B. Images shown are representative from n = 4 female mice perfused at P56 with n = 2 given EdU. Scale bars B, D = 100 μ m.

Figure 12

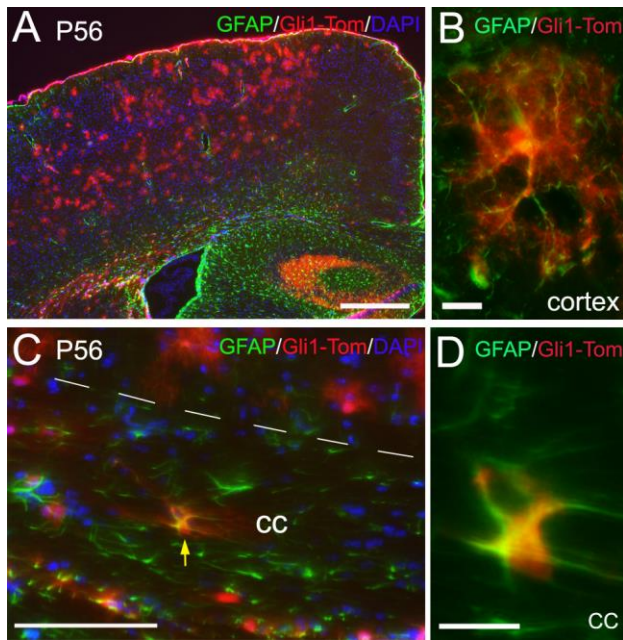


Figure 12: Tamoxifen (TMX) from postnatal days 6-9 (P6-9) fate labels Shh responsive cells that give rise to astrocytes in the corpus callosum (CC) and in the cerebral cortex.

Immunohistochemistry was used to detect expression of the astrocytic marker GFAP in coronal section from *Gli1*^{CreERT2}; *R26*^{tdtomato} mice given TMX at P6-9 and perfused at P56. Gli1-Tom cells include cells with an astrocytic morphology and co-labeling for GFAP in the cerebral cortex (A-B) and in the CC (A, C-D). Images shown are representative from n = 4 female mice. Scale bars in A = 500 μ m in B and D = 15 μ m in C = 100 μ m

Figure 13

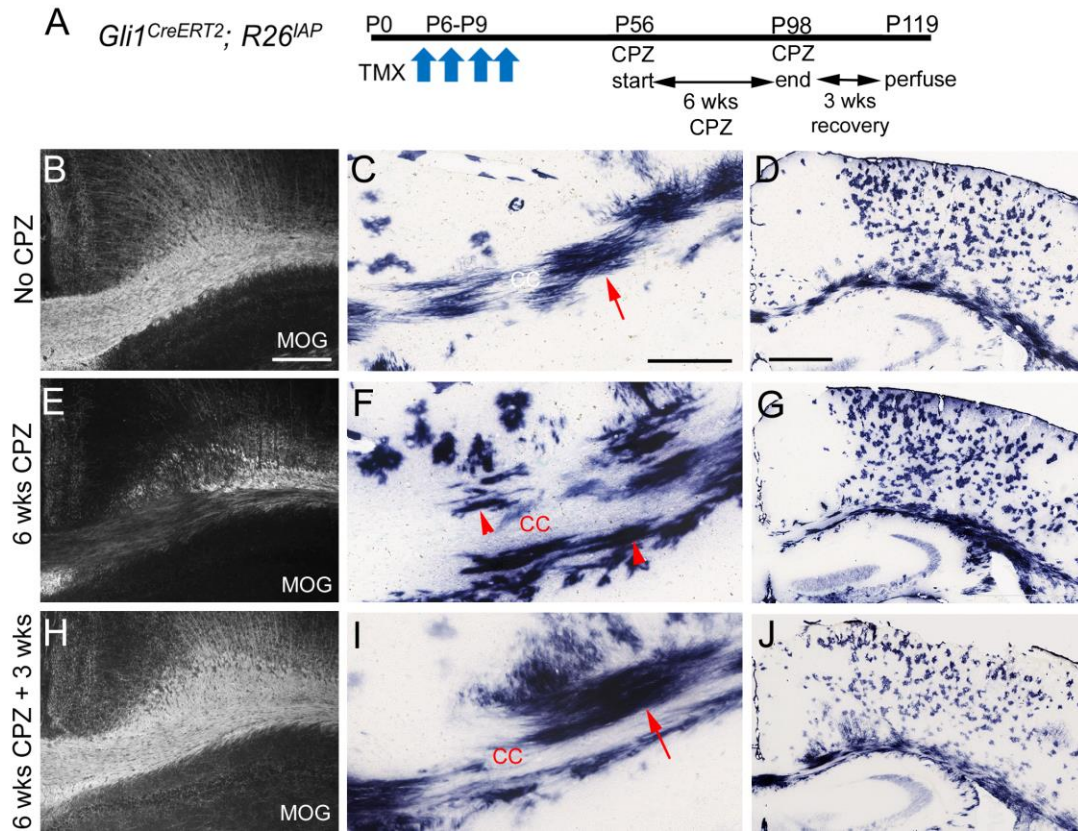


Figure 13: Response of Gli1 fate-labeled cells to acute cuprizone (CPZ) demyelination.

Gli1^{CreERT2};R26^{lAP} mice given tamoxifen (TMX) at postnatal days 6-9 (P6-9) were fed 0.3% CPZ starting at P56 (8 weeks of age) and continuing for 6 weeks to induce acute demyelination in the corpus callosum (CC). Mice were perfused at the end of CPZ, or after a recovery period of three weeks with normal chow to allow for remyelination (A). In adult (P56), immunostaining for myelin oligodendrocyte glycoprotein (MOG) shows that the CC is fully myelinated in control mice (No CPZ) (B). Prior to demyelination, the alkaline phosphatase (AP) reporter shows that *Gli1* fate-labeled (*Gli1*-AP) cells extend clusters of membranes along axons consistent with myelin formation in the CC (C, D arrows). After 6 weeks of CPZ feeding, MOG immunolabeling shows extensive demyelination in the CC (E) and many *Gli1*-AP cells in the CC have long, thickened processes (F, G, arrowheads). In mice given a three week recovery period after ending CPZ, MOG immunolabeling shows extensive remyelination (H) and *Gli1*-AP cells again have elaborated thin myelin-like membranes in the CC (I, J, arrows). Images shown are representative examples from female mice perfused at P56 No CPZ controls (n = 6) and male mice with 6 weeks CPZ (n = 3), and 6 weeks CPZ + 3 weeks recovery (n = 3). Scale bars in B = 250 μ m for B, E, H; in C = 150 μ m for C, F, I; in D = 500 μ m for D, G, J.

Figure 14: Chronic demyelination reduces Gli1 fate-labeled cells in the lateral corpus callosum (CC).

Experimental time line (A) for *Gli1*^{CreERT2}; *R26*^{tdTomato} mice administered tamoxifen (TMX) on postnatal days 6-9 (P6-9) and fed 0.3% cuprizone (CPZ) for 12 weeks to induce chronic demyelination of the corpus callosum. At the end of the 12 week period, mice received twice daily injections of EdU, a thymidine analogue, for five days to label cells that undergo cell division during the recovery period on normal chow. Mice were perfused on day 6, which was timed to be 12 hours after the last EdU injection. The density of *Gli1* fate-labeled (Gli1-Tom) cells was higher in the lateral (L_CC) than medial (M_CC) regions of the CC (B). Gli1-Tom cells in the lateral CC were significantly reduced by chronic demyelination (CPZ) as compared to age matched control mice (No CPZ) (B). Representative images from the lateral CC show the overall distribution of Gli1-Tom cells in the CC in control mice (C) and reduced levels after chronic demyelination (D). Gli1-Tom cells in the CC included astrocytes identified by GFAP (C, D; yellow arrows) that have fine processes in control mice (C) or long, thickened processes of reactive astrocytes after chronic demyelination (D). In control mice (E-H), Gli1-Tom cells were often immunolabeled with CC1, a marker of mature oligodendrocytes (E-G; yellow arrows) and EdU labeled proliferative cells were rare (E, H; arrowheads). After chronic demyelination (I-P), although overall Gli1-Tom cell density is reduced, examples of Gli1-Tom cells labeled by CC1 with EdU incorporation identify new oligodendrocytes generated from Gli1-Tom cells during the recovery period on normal chow (I-P; white arrows). Gli1-Tom cells with the morphology of reactive astrocytes also incorporated EdU after demyelination (M-P, purple arrows). Male mice used in quantification: No CPZ control mice (n = 4), CPZ mice (n = 5). Scale bars in C-D = 100 μ m in E, I and M = 50 μ m

Figure 14

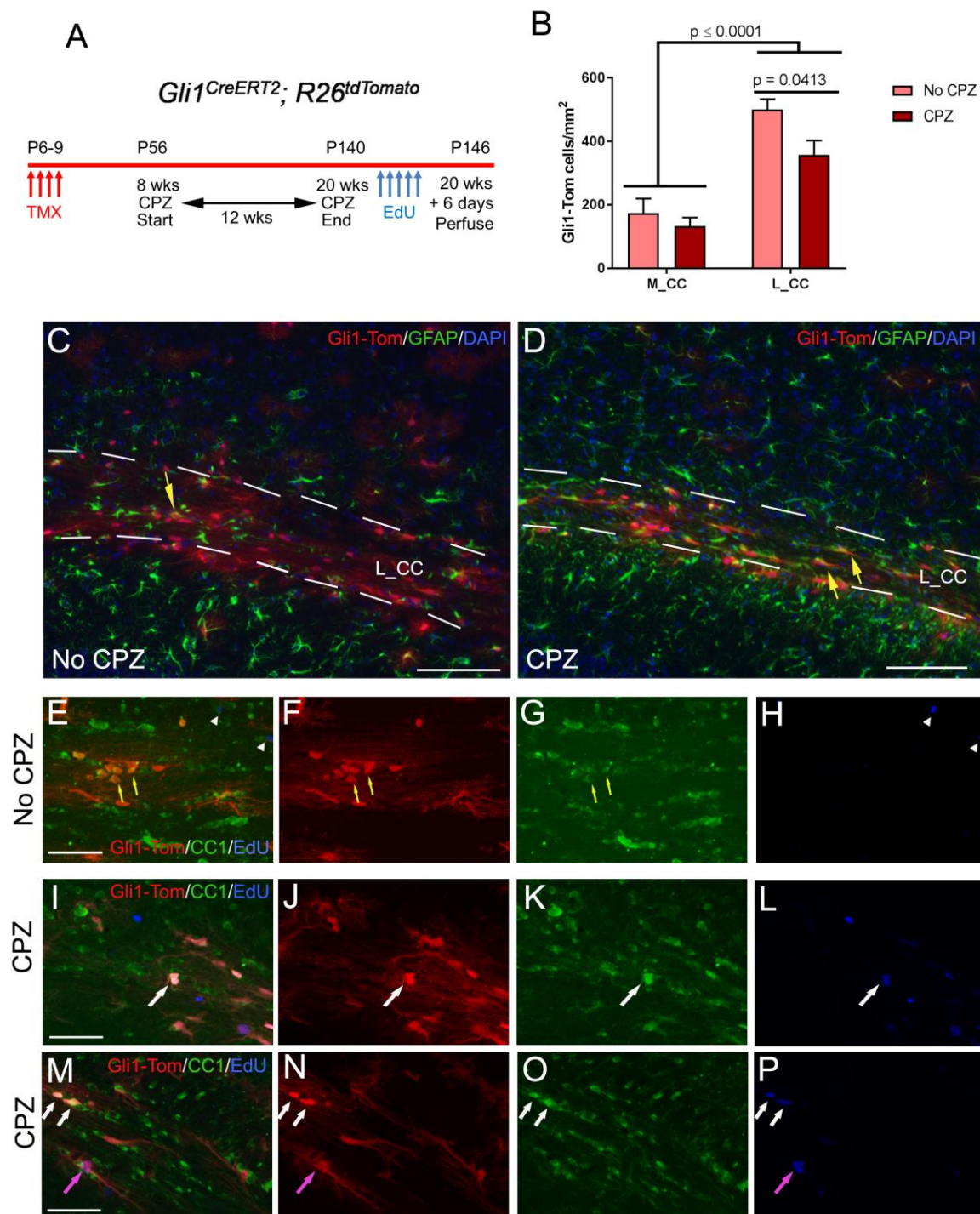


Figure 15: Gli1 fate-labeled cells contribute to cycling and oligodendrocyte progenitor (OP) populations in response to chronic demyelination.

Experimental time line (A) for *Gli1*^{CreERT2}; *R26*^{tdTomato} mice given tamoxifen (TMX) on postnatal days 6-9 (P6-9) and 0.3% cuprizone (CPZ) for 12 weeks to induce chronic demyelination in the corpus callosum (CC). At the end of CPZ, mice were administered EdU twice daily for five days to identify proliferating cells and perfused 12 hours after last EdU dose. Control mice without CPZ (No CPZ) were age matched. EdU labeled cells were rare in control mice (B; white arrow) and significantly increased after demyelination (C; white arrows) based on quantification (D). Double labeling with EdU (C; yellow arrow; inset) and the tdTomato (Tom) reporter shows that *Gli1* fate-labeled (Gli1-Tom) cells contribute to the overall increase of proliferation in response to demyelination (E). Immunohistochemistry for NG2 (F, G) shows an increase of small, elongated process-bearing OP cells after chronic demyelination (H), which was significant only in the medial CC following posthoc analysis (***p = 0.0002). NG2 expressing OP cells with Gli1-Tom fate-labeling (F, G; yellow arrows, insets) were significantly higher in the lateral CC (L_CC) but did not have a significant difference in density after demyelination (I). In both control and demyelinated mice, proliferation of NG2 expressing OP cells was evident from EdU labeling (J, K; white arrows) and included Gli1-Tom cells (K, purple arrows). Proliferation of OP cells with or without Gli1-Tom labeling was not significantly elevated after demyelination (L, M). Male mice used in quantification: No CPZ control mice (n = 4), CPZ mice (n = 5). Scale bars in B-C = 100 μ m in F-G and J-K = 50 μ m.

Figure 15

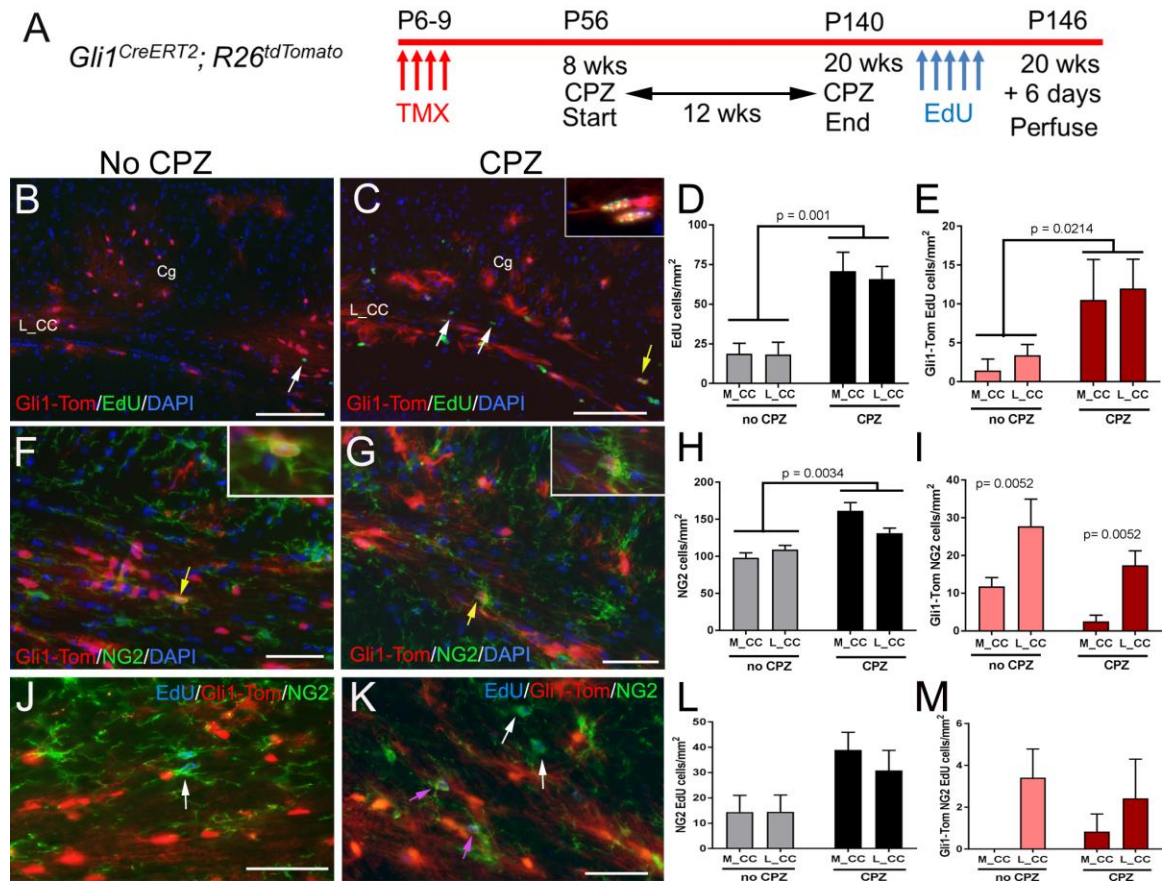


Figure 16

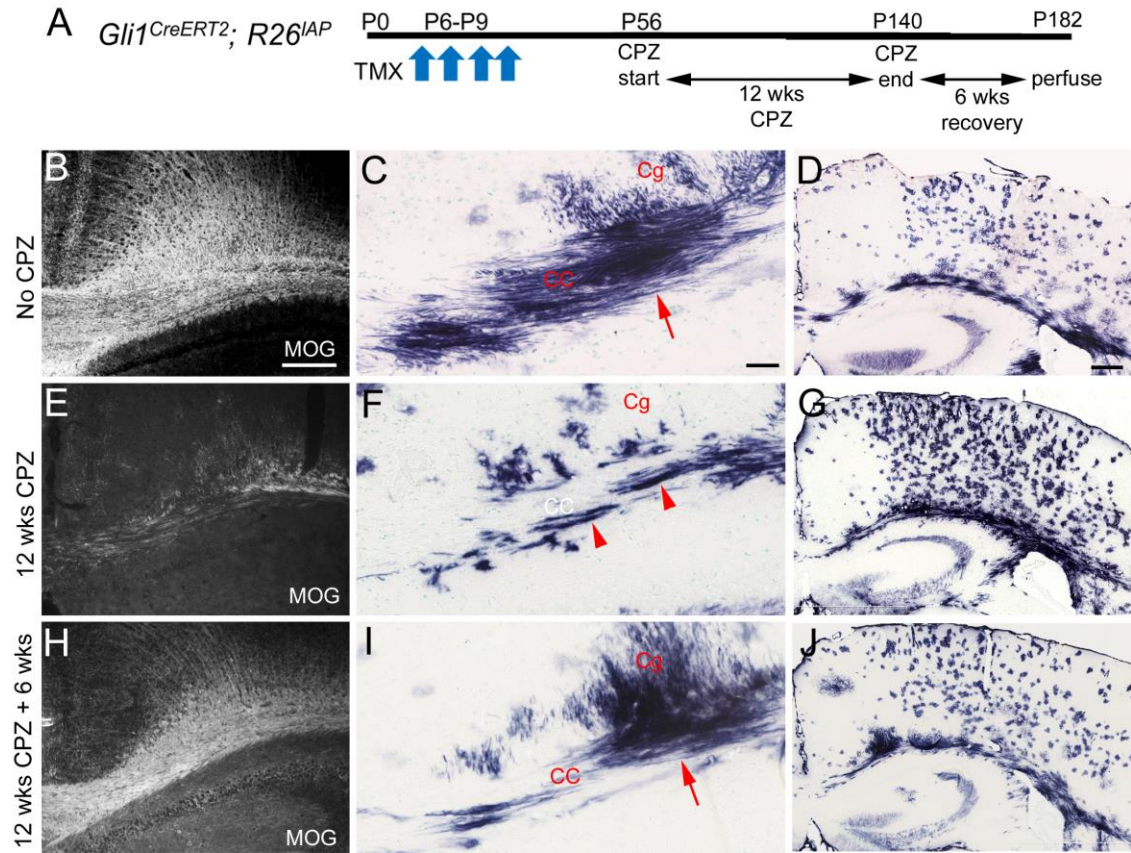


Figure 16: Response of Gli1 fate-labeled cells during recovery after chronic cuprizone (CPZ) demyelination.

Experimental time line (A) for *Gli1*^{CreERT2}; *R26*^{lAP} mice given tamoxifen (TMX) on postnatal days 6-9 (P6-9) and fed 0.3% CPZ for 12 weeks to induce chronic corpus callosum (CC) demyelination. Mice were perfused at the end of CPZ or after a further six week recovery period on normal chow for remyelination to progress. Control mice (No CPZ) were age matched to the end of the recovery period, i.e. 182 days of age. In control mice, immunostaining for myelin oligodendrocyte glycoprotein (MOG) shows that the CC is fully myelinated (B) and the alkaline phosphatase (AP) reporter shows that *Gli1* fate-labeled (*Gli1*-AP) cells extend clusters of membranes along axons consistent with myelin formation in the CC (C, D arrows). After 12 weeks of CPZ feeding, MOG immunolabeling shows extensive demyelination in the CC (E) and many *Gli1*-AP cells in the CC have long, thickened processes (F, G, arrowheads). In mice given a 6 week recovery period after ending CPZ, MOG immunolabeling shows extensive remyelination (H) and *Gli1*-AP cells have elaborated thin myelin-like membranes in the CC (I, J, arrows). Scale bars in B 200 μ m for B, E, H; in C = 50 μ m for C, F, I; in D = 300 μ m for D, G, J. Images shown are representative examples from male mice perfused at P182 for No CPZ control (n = 3), 12 weeks CPZ (n = 4), and 12 weeks CPZ + 6 weeks recovery (n = 4).

Figure S17

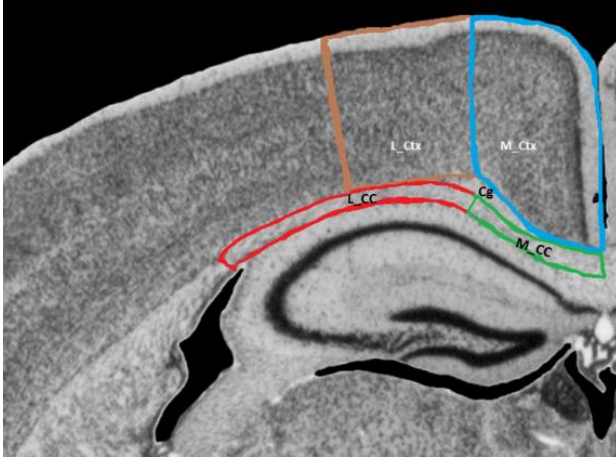


Figure S17: Image of a coronal section of the mouse brain showing regions-of-interests.

The corpus callosum (CC) was subdivided into medial (M_CC) and lateral (L_CC) regions. The M_CC was identified as the region between the midline and the peak of the cingulum (Cg). The L_CC was identified as the region from the peak of the Cg to the medial wall of the lateral ventricle. The adjacent cortical areas were similarly divided into medial (M_Ctx) and lateral (L_Ctx) regions for comparison with CC regions. The M_Ctx was identified as the area between the midline and the peak of the cingulum. The L_Ctx was identified by the area obtained from one picture frame starting at the peak of the cingulum.

Figure S18

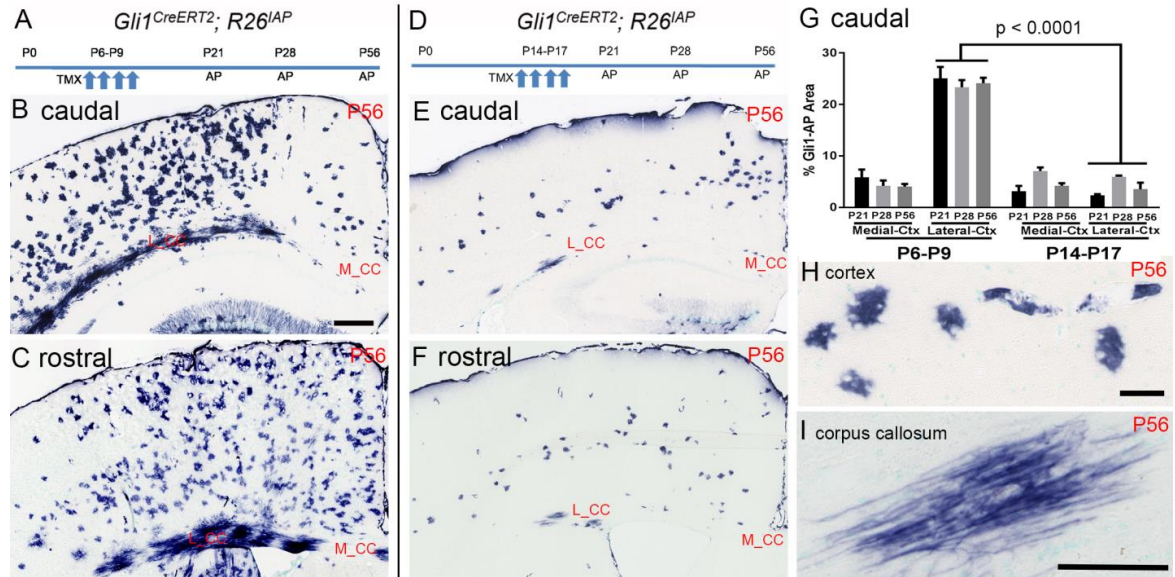


Figure S18: Distribution of Shh responsive cells in cerebral cortex relative to adjacent corpus callosum (CC).

Gli1^{CreERT2}; R26^{lAP} mice were given tamoxifen (TMX) on postnatal days 6-9 (P6-9) (A-C) or P14-P17 (D-F). In mice given TMX P6-9, a lateral distribution of alkaline phosphatase (AP) reaction in *Gli1* fate-labeled (Gli1-AP) cells was observed in the CC and cerebral cortex at caudal levels (B). At rostral levels, Gli1-AP cells continued to be localized to lateral region in the CC but appeared in medial regions of the cerebral cortex (C). Delaying TMX to P14-17 drastically reduced the Gli1-AP cells in both the CC and cerebral cortex at both rostral (F) and caudal (E) levels. Quantification of the AP reaction in membranes from Gli1-AP cells in caudal sections shows a drastic reduction of Gli1-AP cells in the lateral cortex when TMX is given at P14-17 compared to P6-9 (G). The cortical Gli1-AP cells typically exhibited an astrocytic morphology (H) while Gli1-AP cells in the CC extended myelin-like membranes (I). Female mice used in quantification: n = 4 with TMX at P6-9 for P21, P28 and P56; n = 3 with TMX at P14-17 for P21 and P56; n = 5 with TMX at P14-17 for P28. Scale bars B-C and E-F, shown in B = 300 μ m; H-I = 50 μ m

Figure S19: Gli1 fate-labeled cells exhibit cilia into adulthood.

Gli1^{CreERT2}; *R26*^{tdtomato} mice were given tamoxifen (TMX) from postnatal days 6-9 (P6-9) to fate-label Shh responsive cells and perfused at P14, P21, P28, or P56 (A). Immunohistochemistry (IHC) for Arl13b was used to detect cilia that are associated with Shh signaling (B-L). Arl13b labeled small rod shaped structures in ependymal cells along the wall of the lateral ventricle (LV) (B, double arrows) and in choroid plexus cells (B, arrowhead). Boxed area in B shown enlarged in C. The small rod shaped structures are consistent with cilia morphology. Cells of the choroid plexus and ependymal cells are known to use cilia to regulate the circulation of cerebral spinal fluid. Arl13b also labeled cilia in cells in the ventricular-subventricular zone (V-SVZ; B-C, arrows pointing upward). Arl13b immunolabeled cilia of *Gli1* fate-labeled (Gli1-Tom) cells in the corpus callosum (CC; D, arrows). Boxed area in D is shown enlarged in E. **F-I:** Arl13b showed a relatively high density of cilia in the cerebral cortex at P7 (F, arrows) with lower cilia densities noted in the cerebral cortex at P14 (G, arrows), P21 (H, arrows) and P56 (I, arrows). Primary cilia associate with cells with and without *Gli1* fate labeling from TMX at P6-9 (G-I, arrows). **J-L:** Primary cilia were also detected in cells of the CC at P14 (J), P21 (K) and P56 (L). Cilia of cells in the choroid plexus (J, arrowhead) and ependymal cells (J, double arrows) are shown for comparison. Scale bars B-E, G, I, K, L = 20 μ m; F, H = 10 μ m; J = 50 μ m

Figure S19

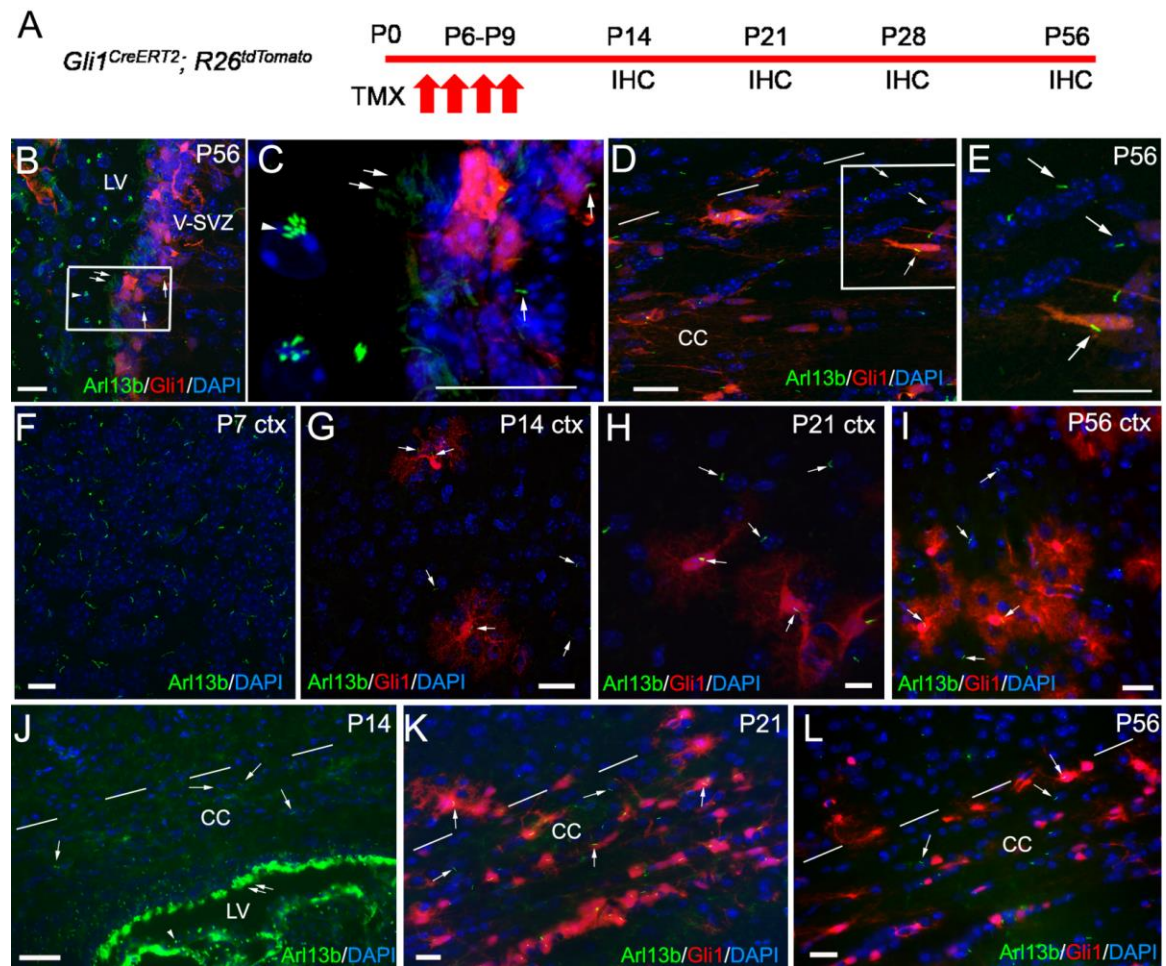


Figure S20: Tamoxifen (TMX) from postnatal days 14-17 (P14-17) results in a low level of Gli1 fate-labeled cells in the corpus callosum (CC) after chronic demyelination.

Gli1^{CreERT2}; R26^{tdtomato} mice were given TMX at one of two time points, either P6-9 or P14-17, and then grown to adults and given cuprizone (CPZ) for 12 weeks to induce chronic demyelination in the CC (A). *Gli1^{CreERT2}; R26^{tdtomato}* mice given TMX from P6-9 that were not fed cuprizone were used as age-matched, non-demyelinated control mice (No CPZ). Analysis of tissue sections in caudal regions from mice given TMX on P6-9 showed *Gli1* fate-labeled (Gli1-Tom) cells in the CC, mainly in lateral (L_CC) rather than medial (M_CC) areas, in both control (No CPZ; B) and demyelinated mice (CPZ; C). In mice given TMX at P14-17, Gli1-Tom cells were rarely observed in the CC after chronic demyelination (CPZ; D). Higher magnification of the lateral CC (L_CC) in caudal levels shows the distribution and morphology of Gli1-Tom cells after demyelination in mice given TMX from P6-9 (E, inset) or from P14-17 (F, inset). Gli1-Tom cells were also observed in the subcallosal zone (SCZ) in sections from caudal levels (E-F). Analysis of tissue sections from rostral levels (G-I) allowed visualization of Gli1-Tom cells in CC relative to the dorsal ventricular-subventricular zone (dV-SVZ). Gli1-Tom cells were observed in the dV-SVZ in control (No CPZ; G) and in chronically demyelinated mice given TMX at P6-9 (CPZ; H) or at P14-17 (CPZ; I). Mice given TMX from P6-9 showed Gli1-Tom cells in the lateral CC (L_CC) regions adjacent to the dV-SVZ (G, H). However in the CC at rostral levels, Gli1-Tom cells were rarely observed in mice given TMX from P14-17 (I). Mice given TMX from P6-9 were given the thymidine analogue EdU for detection of cell proliferation for analysis shown in the main text. To match, the additional mice in this figure that had TMX from P14-17 were also administered EdU although not further analyzed for EdU detection given the low number of Gli1-Tom cells found in the CC. Images shown are representative from n = 2 female mice given TMX at P14-17 and male mice given TMX at P6-9; n = 4 (no CPZ) and n = 5 (CPZ). Scale bars in B = 500 μ m for B-D; in E = 200 μ m for E-F; in G = 200 μ m for G-I. LV = lateral ventricle; Cg = cingulum

Figure S20

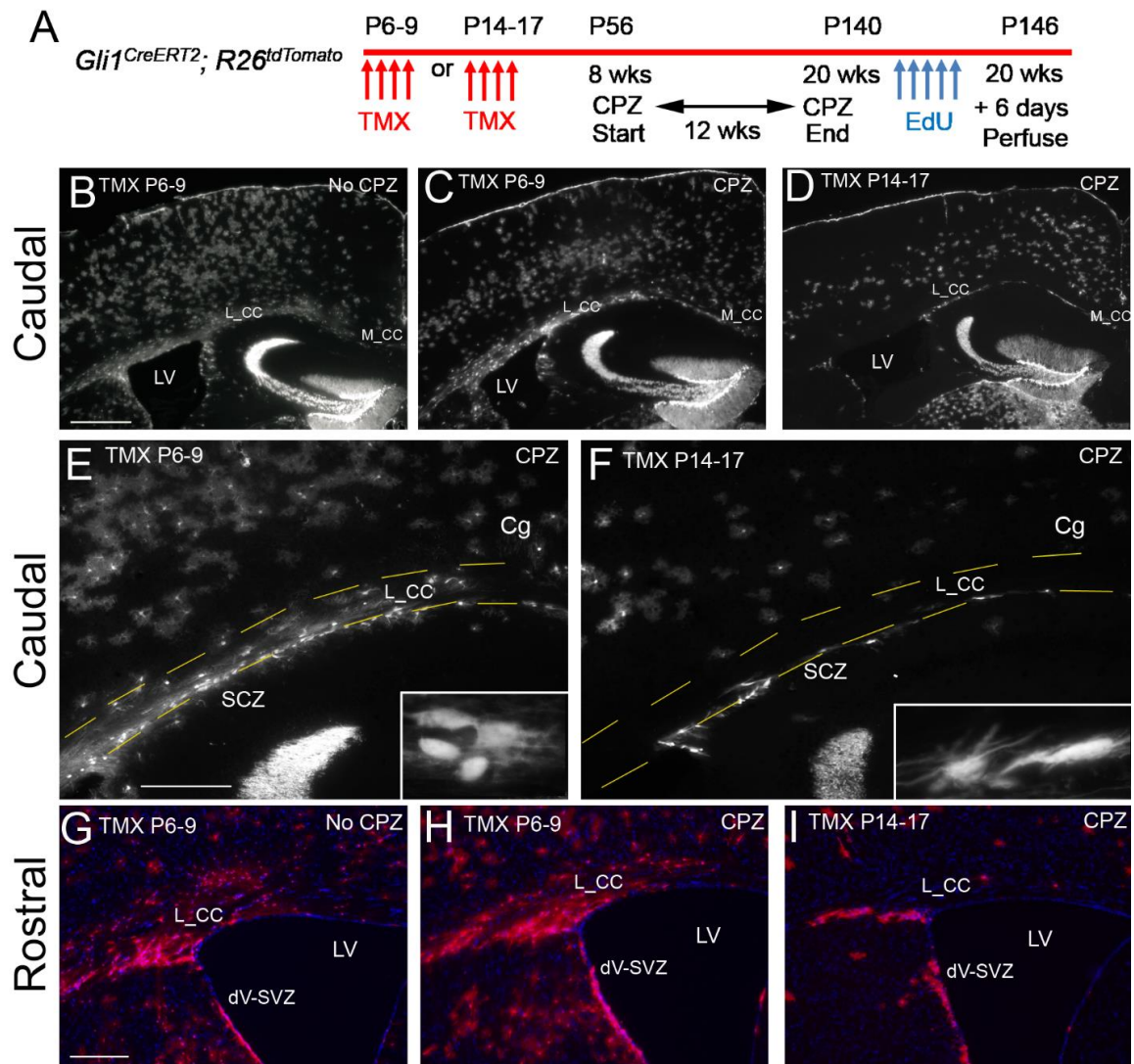


Figure 21

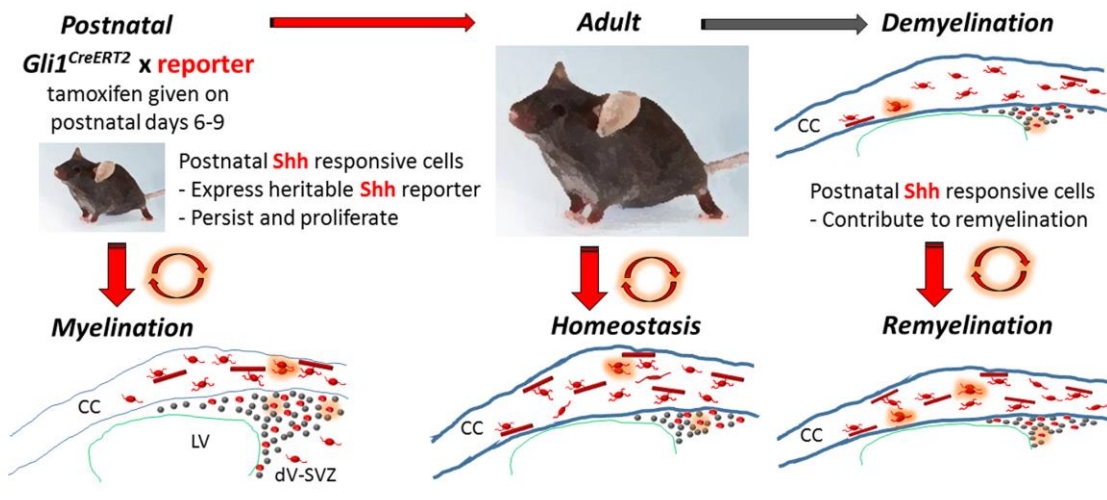


Figure 21: Sonic hedgehog responding cells fate-labeled from postnatal days 6-9 (P6-9) contribute to remyelination after chronic demyelination.

Gli1^{CreERT2}; *R26*^{tdtomato} mice were given tamoxifen (TMX) at P6-9 to fate label cells responding to canonical Shh. Genetic fate mapping of *Gli1* expressing cells from P6-9 showed that Shh responding cells from P6-9 give rise to myelinating cells in the adult corpus callosum (CC). Demyelination with cuprizone induced proliferation of *Gli1* fate-labeled cells and these cells contributed to remyelination.

CHAPTER 3

Genetic detection of Sonic hedgehog (Shh) expression and cellular response in the progression of acute through chronic demyelination and remyelination

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ABSTRACT

Multiple sclerosis is a demyelinating disease in which neurological deficits result from damage to myelin, axons, and neuron cell bodies. Prolonged or repeated episodes of demyelination impair remyelination. We hypothesized that augmenting Sonic hedgehog (Shh) signaling in chronically demyelinated lesions could enhance oligodendrogenesis and remyelination. Shh regulates oligodendrocyte development during postnatal myelination, and maintains adult neural stem cells. We used genetic approaches to detect Shh expression and Shh responding cells *in vivo*. *Shh*^{CreERT2} or *Gli1*^{CreERT2} mice were crossed to reporter mice for genetic fate-labeling of cells actively transcribing *Shh* or *Gli1*, an effective readout of canonical Shh signaling. Tamoxifen induction enabled temporal control of recombination at distinct stages of acute and chronic cuprizone demyelination of the corpus callosum (CC). *Gli1* fate-labeled cells were rarely found in the CC with tamoxifen given during acute demyelination stages to examine activated microglia, reactive astrocytes, or remyelinating cells. *Gli1* fate-labeled cells, mainly reactive astrocytes, were observed in the CC with tamoxifen given after chronic demyelination. However, *Shh* expressing cells were not detected in the CC, even during chronic demyelination, indicating that *Gli1* fate-labeled cells may be recruited into chronic lesions. Finally, SAG, an agonist of both canonical and type II non-canonical Shh pathways, was microinjected into the CC after chronic demyelination. SAG delivery increased proliferation and enhanced remyelination, but did not increase *Gli1* fate-labeled cells in the CC. Therefore, while cells within CC lesions did not exhibit Shh transcription or canonical signaling, interventions to increase non-canonical Shh signaling may promote remyelination after chronic demyelination.

INTRODUCTION

Multiple sclerosis (MS) is a demyelinating autoimmune disease that results in long term disability due to limited remyelination capacity and cumulative damage to axons (30). Effective remyelination in MS cases has been demonstrated after early stage, or acute, demyelination and may correspond with symptom remission (92). Relatively limited remyelination is observed in chronic MS, which is attributed to multiple factors including prolonged immune attack, axon damage, an insufficient oligodendrocyte regenerative response, and inhibitory conditions within the sclerotic lesion environment (29; 59). To complement immunosuppressive therapies, interventional strategies are sought to accelerate and/or promote the extent of remyelination to protect vulnerable demyelinated axons from further damage and to recover function of intact axons.

Pre-clinical studies in mice have shown that the cellular processes responsible for generating oligodendrocytes depend on the biological context, therefore developmental myelination and acute demyelination models may provide insights for optimization of oligodendrogenesis in chronic disease. Neural stem cells (NSCs) of the ventricular-subventricular zone (V-SVZ) contribute to postnatal myelination (102; 106). NSCs generate oligodendrocyte progenitor (OP) cells that migrate into white matter regions, such as the corpus callosum, and continue to proliferate as well as differentiate into myelinating oligodendrocytes (23; 95; 110). In adulthood, much lower homeostatic levels of oligodendrogenesis are required to support myelin plasticity and maintenance (31; 72). In response to acute demyelination, OP cells within the adult white matter proliferate to generate sufficient oligodendrocytes for efficient remyelination (7; 8; 23). OP cells continue to be present in chronically demyelinated lesions but exhibit reduced capacity

for proliferation and differentiation, possibly due to the presence of inhibitory signals (7; 33; 116).

The current studies examine the cellular response of the Sonic hedgehog (Shh) signaling pathway in the context of chronic demyelination. The Shh pathway has both the potential to serve as a marker of NSCs from the V-SVZ in normal adult mice, and the potential to regulate NSC and/or OP responses that impact remyelination (3; 35; 53; 94). Canonical Shh signaling is required for a postnatal wave of oligodendrogenesis from the V-SVZ (106). Postnatal Shh responsive NSCs in the V-SVZ generate OP cells that populate the corpus callosum, persist into adulthood, and contribute to remyelination (95). In healthy adult mice, NSCs in the V-SVZ exhibit active Shh signaling (3). However, Shh signaling is down regulated with differentiation along the oligodendrocyte lineage (53; 76; 94). Indeed, genetic reporter mice demonstrate a stark absence of canonical Shh signaling among all cell types in the adult corpus callosum (53; 76; 94). Furthermore, canonical Shh signaling through Gli1 appears to inhibit NSC mobilization from the V-SVZ into the corpus callosum after acute demyelination (94). However, NSC recruitment from the V-SVZ may not be required in this acute demyelination condition; remyelination of acute lesions is mainly carried out by amplification of OP cells that populated the corpus callosum during postnatal myelination (57).

To discriminate potential beneficial or detrimental effects of Shh signaling relative to repair of chronic demyelination, we examined the Shh pathway during adult homeostasis and during progressive stages of cuprizone (CPZ) demyelination. *In vivo* Shh signaling at specific time points was detected by inducing heritable genetic labeling of cells actively transcribing *Shh* or *Gli1*. *Gli1* transcription is an effective readout for

canonical Shh signaling (3). *Shh*^{CreERT2} mice or *Gli1*^{CreERT2} mice were crossed either to *R26*^{tdTomato} mice to label cells with red fluorescence, or, to *R26*^{LAP} mice to label membranes with alkaline phosphatase. Time points throughout the progression of acute through chronic demyelination were analyzed, as well as after remyelination. Finally, we delivered a Shh agonist into the chronically demyelinated region of the corpus callosum to evaluate *Gli1* activation, the regenerative response, and remyelination capacity. This combination of approaches revealed novel insights of Shh signaling *in vivo* during the progression of acute through chronic demyelination, and the potential for non-canonical Shh signaling to enhance remyelination of chronic lesions.

METHODS

Mice for Genetic Inducible Fate Labeling

Mice were housed and cared for in accordance with the guidelines of the National Institutes of Health and the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences. *Shh*^{CreERT2/+} (B6.129S6-*Shh*^{tm2(cre/ERT2)Cjt}/J; 005623; Jackson Laboratories; Bar Harbor, ME) or *Gli1*^{CreERT2/+} (*Gli1*^{tm3(cre/ESR1)Alj}/J; 007913; Jackson Laboratories; provided by Dr. Sohyun Ahn, National Institutes of Health) mice express *Cre* recombinase fused to a mutated estrogen receptor, which requires tamoxifen (TMX) binding for nuclear translocation (2; 3; 47). *Gli1*^{CreERT2} mice were backcrossed to C57BL/6J mice (000664, Jackson Laboratories) for at least six generations. *Shh*^{CreERT2/+} or *Gli1*^{CreERT2/+} mice were crossed to reporter mouse lines, either *R26*^{tdTomato} homozygous mice (B6.Cg-Gt(*ROSA*)26^{Sortm14(CAG-tdTomato)Hze}/J; 007914; Jackson Laboratories) or *R26*^{LAP} homozygous mice (B6;129-Gt(*ROSA*)26Sortm2Nat/J; 009253; Jackson Laboratories; provided by Dr. Tudor Badea,

National Institutes of Health). The $R26^{tdTomato}$ mouse line carries a floxed stop signal preceding the *tdTomato* gene (67). The $R26^{IAP}$ reporter mouse line expresses the human *alkaline phosphatase* (AP) gene, with a floxed inverted exon 2 (9). TMX (T5648; Sigma; St. Louis, MO) was dissolved in corn oil (C8267; Sigma) to a concentration of 20 mg/ml and was administered via oral gavage (10mg/day) on two consecutive days.

Demyelination with Cuprizone

First generation crosses were used as heterozygotes for the promoter and reporter alleles for $Gli1^{CreERT2}; R26^{tdTomato}$, $Gli1^{CreERT2}; R26^{IAP}$, $Shh^{CreERT2}; R26^{tdTomato}$ or $Shh^{CreERT2}; R26^{IAP}$ mice. At 8 weeks of age, male mice were given chow containing 0.3% CPZ for 3 or 6 weeks to induce acute demyelination in the corpus callosum or for 12 weeks to induce chronic demyelination. Some cohorts were returned to normal chow to follow fate-labeled cells without CPZ present. The specific experimental design for each cohort is shown with the results. Only male mice were used for demyelination due to toxicity effects of CPZ in C57BL/6J female mice that results in the disruption of the estrus cycle and prevents analysis of gender differences (103).

Immunohistochemistry and Reporter Detection

Mice were perfused with 3% paraformaldehyde and brains were further post fixed in 3% paraformaldehyde overnight. Coronal brain sections, 14 μ m thick, were used for immunohistochemistry and/or reporter detection for all studies. Thicker 20 μ m sections were used to improve detection of fine processes in $Shh^{CreERT2}; R26^{IAP}$ tissue. Antibodies used for immunolabeling are detailed in Table 2. Myelin was immunostained for myelin oligodendrocyte glycoprotein (MOG) (15; 62). Cell type-specific immunolabeling used Iba1 for microglia, glial fibrillary acidic protein (GFAP) for white matter astrocytes, NG2

for OP cells, and GSTpi for oligodendrocytes. The tdTomato reporter signal was visible without additional processing. AP activity was detected as previously described (76). Briefly, endogenous AP activity was deactivated by incubating mounted brain sections in heated phosphate buffer saline (69 °C for 90 minutes). Sections were then incubated in nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indoly-phosphate toluidine salt (BCIP) substrate (K0598; DAKO/Agilent; Santa Clara, CA) overnight and then incubated in methyl green for nuclear staining (SP5000; Vector Laboratories; Burlingame, CA).

Cell Proliferation *In Vivo*

Ki67 antigen detection (Table 1) was used to identify cells in an active stage of cell division at the time of perfusion. Biotinylated secondary antibody was detected using an ABC kit (PK-6101; Vector Laboratories) followed by brown diaminobenzidine chromogen reaction (SK-4100; Vector Laboratories) in *Gli1^{CreERT2};R26^{IAP}* mice and combined with the NBT/BCIP reaction to detect AP (see above). To label cycling cells based on DNA synthesis, *Gli1^{CreERT2};R26^{tdTomato}* mice were injected i.p. with 5-ethynyl-2'-deoxyuridine (EdU) (E10187; Thermo Fisher Scientific; Waltham, MA). Chronically demyelinated *Gli1^{CreERT2}; R26^{tdTomato}* mice received EdU (100 mg/kg) over five days with twice daily injections given 2 hours apart (10 injections in total) starting approximately two hours after SAG or vehicle delivery (see below). EdU was detected in tissue sections using a Click-iT ® Plus EdU kit (C10637; Thermo Fisher Scientific).

Sonic Hedgehog Agonist Administration

Smoothed agonist SAG (566661; InSolution™ Smoothed Agonist, EMD Millipore Sigma; Burlington, MA) was microinjected into the corpus callosum as

previously described (76). Briefly, chronically demyelinated *Gli1*^{CreERT2}; *R26*^{tdTomato} mice were anesthetized with isofluorane and placed in a stereotaxic frame to drill a small hole in the skull for microinjection. A 10- μ l Hamilton syringe (7653-01; Hamilton Company, Reno, NV) with removable needle (RN) compression fitting adapters (55750-01; Hamilton Company) was used to mount a pulled glass pipette (outer diameter 50 μ m) and microinject 1 μ l of SAG (SAG; 10 μ M) or vehicle (NDC 0409-4888-10; 0.9% sodium chloride INJ, USP, Hospira; Lake Forest, IL) into the posterior corpus callosum (1.0 mm posterior to bregma, 0.5 mm to the right of midline and 1.3 mm deep). Each microinjection was completed over a 5 minute period. The needle was left in place for 1 minute to reduce backflow along the needle track. The skin was closed using Tissumend II SC (3006178; Veterinary Products Laboratories, Phoenix, AZ).

Image Acquisition, Quantification and Statistical Analysis

Fluorescent images were acquired using a SPOT RT3 camera (Diagnostic Instruments, Sterling Heights, MI) or a Zeiss LSM 700 (Carl Zeiss Microscopy, Thornwood, NY). Images of AP and Ki67 chromogen reactions were acquired using a NanoZoomer 2.0-RS (Hamamatsu Instruments, Hamamatsu, Japan). Quantification included 3 coronal tissue sections per animal with at least 3 animals per group, unless otherwise specified. Quantitative analysis focused on the caudal region of the corpus callosum and overlying cortical regions between approximately -0.94 to -1.58 mm from bregma (36), where CPZ demyelination is prominent (114). For quantification purposes, the regions-of-interest in these caudal coronal sections were defined for the corpus callosum as extending from the midline to the inner wall of the lateral ventricle, and for the cerebral cortex as extending from the midline to the lateral end of cingulum.

Additional qualitative analysis included coronal tissue sections within this caudal region as well as a rostral region between 0.14 and -0.22 mm from bregma. Spot Advanced Software (<http://www.spotimaging.com>; RRID: SCR_014313; Spot imaging solutions, Sterling Heights, MI) was used to measure the area of each region-of-interest. The areas of MOG immunolabeling in the corpus callosum were estimated based on the number of pixels with intensities above the background intensity level using the thresholding function in MetaMorph software (<https://www.moleculardevices.com>; RRID: SCR_002368; Molecular Devices, Downingtown, PA). Cells labeled with tdTomato, NG2, GSTpi or EdU were quantified as cells/mm². Figures were prepared as panels using Adobe Photoshop version 7.0 or above (<http://www.adobe.com>; RRID: SCR_014199; Mountain View, CA).

Prism 7.01 (<http://www.graphpad.com>; RRID: SCR_002798; Graphpad; San Diego, CA) was used to prepare graphs and perform statistical analyses. Both quantitative and qualitative analyses were performed blinded and the number of mice included in each analysis is listed in each figure legend. Acute demyelination and astrocyte reactivity were analyzed using one-way analysis of variance followed by Tukey's post hoc test. Two-way analysis of variance was used for cell density values in the corpus callosum and in the cerebral cortex. Sidak's multiple comparisons test was used for post hoc analysis. Quantification is presented as mean $\pm$ standard error of the mean. Statistical significance was set as $p < 0.05$.

RESULTS

In acute demyelination, *Gli1* does not fate-label activated microglia, reactive astrocytes, or remyelinating cells in the corpus callosum.

Shh signaling was evaluated during acute demyelination as integral in the progression to chronic demyelination and to directly compare the potential contributions of Shh signaling to remyelination after acute versus chronic demyelination. *Gli1*^{CreERT2} mice and reporter lines were backcrossed to the C57BL/6J genetic background and showed similar timing and extent of pathology from CPZ for neuroinflammation and demyelination in the corpus callosum of C57BL/6 mice (49). Shh signaling in *Gli1*^{CreERT2} mice was examined at distinct stages of cellular responses during acute CPZ demyelination with subsequent remyelination. *Gli1*^{CreERT2}; *R26*^{tdTomato} mice exhibited robust microglial activation at 3 weeks of CPZ as assessed by staining with microglia activation marker Iba1 (Fig. 22A-D). In the subsequent 2 week period without CPZ, microglial activation persisted and demyelination progressed (Fig. 22E-F). *Gli1* fate-labeling induced by TMX given at 3 weeks of CPZ demonstrates clear tdTomato reporter protein in *Gli1* expressing (Gli1-Tom) cells in the cerebral cortex (Fig. 22G), consistent with Shh signaling in cortical astrocytes in healthy adult *Gli1*^{CreERT2} mice (41; 76). The absence of Gli1-Tom cells in the adjacent corpus callosum demonstrates a lack of canonical Shh signaling in activated microglia during the initiation of acute CPZ demyelination (Fig. 22G).

Shh responsive cells were next examined toward the end of the acute demyelination period and the fate of *Gli1*-labeled cells was followed into the remyelination period (Fig. 23). *Gli1*^{CreERT2} mice were crossed to the *R26*^{IAP} reporter line to take advantage of the AP membrane localization, which can reveal myelin-like membrane formation during remyelination (95). *Gli1*^{CreERT2}; *R26*^{IAP} mice given TMX after 5 weeks of CPZ were perfused either on week 6 of CPZ, or after a subsequent 3

week period without CPZ, to allow for remyelination (Fig. 23A). MOG immunolabeling showed extensive demyelination in the corpus callosum after 6 weeks of CPZ and extensive remyelination after the 3 week recovery period (Fig. 23B-D, N).

Immunolabeling with astrocytic marker GFAP demonstrated astrogliosis in the corpus callosum after 6 weeks of CPZ that continued at a lower level after a 3 week recovery period (Fig. 23E-J, O). *Gli1* expressing AP labeled (Gli1-AP) astrocytes were prominent in the cerebral cortex of healthy adults and after CPZ (Fig. 23K-M). In contrast, the corpus callosum was absent of Gli1-AP cells in healthy adults (Fig. 23K), at the peak of demyelination (Fig. 23L), or during remyelination (Fig. 23M). This absence of Gli1-AP cells in the corpus callosum after TMX induction at 3 (Fig. 22) or 5 weeks (Fig. 23) of CPZ ingestion demonstrates that canonical Shh signaling is not detectable in activated microglia or reactive astrocytes in the corpus callosum during acute demyelination. In addition, the OP cell population in the corpus callosum expands several-fold at 5-6 weeks of CPZ (7; 24) yet OP cells were not *Gli1* fate-labeled prior to remyelination.

Shh signaling is known to regulate the NSC population in adult V-SVZ (3; 53). Therefore, NSCs of the V-SVZ were examined as a potential Shh responsive population that may be recruited into the adjacent corpus callosum in response to acute demyelination (Fig. 24). In *Gli1^{CreERT2}; R26^{tdTomato}* mice with TMX induction at 3 weeks of CPZ, Gli1-Tom cells were localized in the V-SVZ, particularly in ventral regions (Fig. 24A-C). Similarly, in *Gli1^{CreERT2}; R26^{LAP}* mice given TMX at 5 weeks of CPZ, Gli1-AP cells were observed in the V-SVZ (Fig. 24D-F). *Gli1* fate-labeled oligodendrocytes were very rarely observed in the corpus callosum (Fig. 24F).

***Gli1* expressing cells localize within the corpus callosum after chronic demyelination but do not contribute to remyelination.**

To investigate whether Shh signaling may change during chronic demyelination, *Gli1^{CreERT2}; R26^{tdTomato}* mice were fed chow containing CPZ for 12 weeks. At the end of CPZ, mice were administered TMX to fate-label Shh responding cells, and were then switched to normal chow for 2 weeks (Figure 25A). As in our prior studies with TMX given in adult mice (75; 101), control mice (No CPZ) did not have Gli1-Tom cells in the corpus callosum (Fig. 25B-C), indicating that normal aging does not increase Shh signaling through *Gli1* in the corpus callosum. However, in the CPZ mice, MOG immunohistochemistry confirmed demyelination and expression of tdTomato reporter showed *Gli1* expressing (Gli1-Tom) cells localized within the corpus callosum (Fig. 25D-E).

To investigate if *Gli1* expressing cells in the corpus callosum could directly contribute to remyelination, *Gli1^{CreERT2}; R26^{IAP}* mice were chronically demyelinated with CPZ for 12 weeks, administered TMX at the end of CPZ, and then analyzed after a 6 week recovery period without CPZ to allow remyelination to progress (Fig. 26). Consistent with our finding in *Gli1^{CreERT2}; R26^{tdTomato}* mice, AP reporter expression showed Gli1-AP cells in the corpus callosum (Fig 26B-C). This initial analysis focused on caudal regions of the corpus callosum, where demyelination is most prominent (114). The Gli1-AP cells in the corpus callosum had thick processes consistent with the morphology of reactive astrocytes (Fig. 26C and inset). Gli1-AP cells did not extend long, thin myelin-like membranes along axons. Therefore, we did not find evidence of *Gli1* expressing cells forming new myelin after chronic demyelination. Compared to caudal regions in chronic CPZ mice, Gli1-AP cells were less frequently observed in the corpus callosum at rostral levels adjacent to the V-SVZ, which is a site of *Gli1* fate-

labeled NSCs in adults (Fig. 26D-F). Proliferating cells immunolabeled by Ki67 were not *Gli1* fate-labeled in the corpus callosum (Fig. 26F). Throughout the V-SVZ, Ki67 immunolabeled actively proliferating cells with and without *Gli1* fate-labeling (Fig. 26G and inset).

Active Shh transcription is not detected in cells within the corpus callosum during acute or chronic demyelination.

Shh^{CreERT2} mice were then used to examine Shh ligand expression relative to acute and chronic demyelination. *Shh^{CreERT2}; R26^{IAP}* and *Shh^{CreERT2}; R26^{tdTomato}* mice were administered TMX to detect Shh synthesis in healthy adults, during active demyelination, and after chronic demyelination (Figs. 27, 28). *Shh* expression was not observed in cells within the corpus callosum in healthy adult mice (Fig. 27A-E), during acute demyelination (Fig. 23F-J), or after chronic demyelination (Fig. 27K-O). *Shh* fate-labeling identified neurons as the major source of Shh based on neuroanatomical distribution and morphology (Figs. 27-28). *Shh* fate-labeled cells with neuronal morphology were distributed within the cerebral cortex and in ventral brain regions. Shh-AP axons were consistently detected superficial to the medial corpus callosum, and rarely within the corpus callosum (Fig. 27). Overall, Shh-AP labeling appeared similar across the three time points (Fig. 27). These patterns of Shh-AP and Shh-Tom cells are consistent with our prior study with TMX given to adult healthy mice which also showed co-labeling for the neuronal marker NeuN (101).

Shh agonist delivery enhances remyelination in corpus callosum after chronic demyelination.

These experiments show a lack of local Shh expression in the corpus callosum during acute or chronic demyelination. However, this result leaves open the question of

whether Shh, if available, could regulate cellular responses in the chronic lesion environment. A small molecule agonist of the Shh signaling pathway (SAG) was used to test the effect of augmenting Shh signaling in the corpus callosum of chronically demyelinated mice. SAG is an agonist of the Smoothed receptor, which activates Shh signaling through both canonical and non-canonical pathways (17). SAG was microinjected into the corpus callosum of *Gli1*^{CreERT2}; *R26*^{tdTomato} mice after chronic demyelination with CPZ (Fig 29A). EdU, a thymidine analogue, was administered daily for five days to label cycling cells. TMX was administered on days 2 and 3 after CPZ to fate-label *Gli1* expressing cells. Mice were perfused for analysis after a 10 day recovery period without CPZ. As illustrated in Figure 25, chronic CPZ results in Gli1-Tom cells in the corpus callosum that are not found in mice age-matched to this time point. Both SAG and vehicle injections reduced Gli1-Tom cells in the ipsilateral corpus callosum, indicating an effect of the injection procedure (Fig. 29B). Rare Gli1-Tom cells with highly branched thin processes indicative of oligodendrocytes were detected adjacent to the V-SVZ (Fig. 29C). Gli1-Tom cells were mainly astrocytes, with the vast majority being cortical astrocytes, as are found in healthy adults (Fig. 29D)(41). Gli1-Tom cells were less frequent within the corpus callosum and had processes similar to reactive astrocytes (Fig. 29D).

Cell type-specific immunolabeling was used to examine the response to SAG injection among cells with and without *Gli1* fate-labeling (Fig. 29D-P). OP cells immunolabeled for NG2 were not *Gli1* fate-labeled in the cerebral cortex or in the corpus callosum (Fig 29D-E). EdU labeled cycling cells were observed within the cerebral cortex and corpus callosum, including NG2 cells, but these cells were not *Gli1* fate-

labeled (Fig. 29D-E). SAG increased EdU labeling in the cerebral cortex, which included cycling OP cells labeled with NG2 and EdU (Fig. 29F-G). In the corpus callosum, overall EdU incorporation and cycling OP cells with NG2 and EdU labeling were increased ipsilateral to the injection and the highest values were observed with SAG delivery (Fig. 29H-I). The total NG2 population was not changed with SAG or vehicle injection (Fig. 29J). However, NG2 cells may go on to differentiate into oligodendrocytes and so could be lost from the NG2 pool, resulting in normalized population levels. Immunolabeling for MOG showed a significant increase in the myelinated area of the corpus callosum, which demonstrated a positive effect of SAG on remyelination after chronic CPZ (Fig. 29K-M). Consistent with enhanced remyelination, SAG increased oligodendrocytes, which were identified by GSTpi, but this change was not statistically significant (Fig. 29N-O). The majority of Gli1-Tom cells in the corpus callosum were reactive astrocytes, based on morphology and GFAP immunolabeling (Fig. 29P). These results demonstrate that SAG enhanced remyelination even though active Shh signaling through Gli1 was not detected in NG2 and GSTpi cells, or in other cell types within the corpus callosum. Therefore, SAG enhanced remyelination through the non-canonical Shh pathway, either directly and/or indirectly through other cell types in the corpus callosum.

DISCUSSION

These studies demonstrate differences in the cellular response to acute versus chronic demyelination, and the potential for non-canonical Shh signaling to stimulate proliferation and increase remyelination after chronic demyelination. We show that acute CPZ demyelination did not induce canonical Shh signaling, i.e. *Gli1* fate-labeling, in activated microglia, reactive astrocytes, or OP cells that generate oligodendrocytes for

remyelination. Only chronic demyelination resulted in *Gli1* fate-labeled Shh responsive cells in the corpus callosum. Importantly, delivery of SAG, a Shh pathway agonist, into the corpus callosum increased proliferation and remyelination. SAG delivery did not induce *Gli1* fate-labeling, indicating beneficial SAG effects after chronic demyelination were due to non-canonical Shh signaling (Fig. 30).

The current results showing lack of *Gli1* fate-labeled cells in healthy adults or after acute demyelination are consistent with prior studies, and validate our interpretation that differences in *Gli1* fate-labeling are due to the chronic demyelination condition (41; 76). Canonical Shh signaling, detected by *Gli1* fate-labeling, acts mainly in NSCs of the ventral V-SVZ to generate olfactory bulb interneurons in healthy adult brain (53). *Gli1* fate-labeling was only rarely detected in the corpus callosum of healthy adults, consistent with the low level of oligodendrocytes generated from the V-SVZ (41; 53). *Gli1* fate-labeling was also rarely detected in the corpus callosum after acute demyelination (94).

Gli1 fate-labeled cells in chronically demyelinated corpus callosum appear to be derived from outside the corpus callosum, rather than from local white matter cells, based on several findings. First, *Gli1* fate-labeled cells in the corpus callosum were identified as mainly reactive astrocytes, while few were oligodendrocytes, and immunolabeling with OP markers was not observed. Second, *Shh* expression was not upregulated in cells within chronic lesions of the corpus callosum. Third, *Gli1* fate-labeled cells within the corpus callosum were not increased by local delivery of Shh agonist. Fourth, the V-SVZ continues to exhibit high *Gli1* expression after chronic demyelination and thus could be a source of *Gli1* fate-labeled cells. These findings indicate that chronic demyelination

increases recruitment of *Gli1* fate-labeled cells, which become reactive astrocytes in the corpus callosum. Further testing of this model may provide insights into the formation of sclerotic lesion conditions in chronic demyelinating disease.

In addition to exploiting *Gli1*^{CreERT2} mice to monitor Shh responsive cells, the current studies are the first to use complementary *Shh*^{CreERT2} mice to evaluate changes in Shh synthesis during the course of acute and chronic demyelination. Neuronal cell types synthesize Shh in the cerebral cortex, septal regions, and ventral forebrain in healthy adult mice (4). Upregulated Shh expression in glial cells has been reported using a variety of other techniques. Shh immunolabeling was upregulated in oligodendrocytes in acute demyelinated lesions of the corpus callosum resulting from lysolecithin injection (35). After cortical injury in adult mice, interleukin-1 β from activated microglia/macrophage cells was reported to induce Shh immunolabeling of reactive astrocytes (6). However, Shh immunolabeling may detect Shh binding to reactive astrocytes in lesion areas (94). In contrast, using quantitative array analysis, we did not find an increase of Shh mRNA transcript levels in the corpus callosum throughout stages of acute and chronic CPZ demyelination (49). Our analysis in *Shh*^{CreERT2} mice utilizes *Shh* promoter activity for gene-specific analysis of *Shh* expression in acute and chronic demyelination. An early demyelination stage was examined at 3 weeks of CPZ feeding based on our prior work showing prominent microglial activation along with an approximately 10-fold elevation of interleukin-1 β transcript levels in the corpus callosum (49). The current study confirmed robust microglial activation after 3 weeks of CPZ yet no *Shh* fate-labeling was observed in cells within the corpus callosum in *Shh*^{CreERT2} mice. Consistent with this result, Shh levels were not sufficient to activate *Gli1* fate-labeling of corpus callosum

cells after acute demyelination. Furthermore, Shh synthesis was not observed in cells within the corpus callosum after chronic CPZ demyelination, or in age-matched healthy control *Shh*^{CreERT2} mice.

Experimental modulation to activate or inhibit Shh signaling can identify Shh roles in the generation and regeneration of glial cells in adult mice. Genetic conditional activation of Shh signaling in NSCs of the V-SVZ expanded the NSC pool but depleted the generation of direct progeny, including oligodendrocyte lineage cells in the corpus callosum of healthy adult mice (34). Therefore, low levels of Shh activation may need to be maintained in the V-SVZ for oligodendrocyte and myelin homeostasis. Recent studies of Shh signaling in acute demyelination models have involved relatively small corpus callosum lesions, either from lysolecithin injection (35) or from 0.4% CPZ in mice of a non-C57BL/6 genetic background (94). In acute demyelinated lesions, mRNA transcripts for key Shh signaling components, *Gli1* and *Smoothed*, have been reported as upregulated in oligodendrocytes and microglia, respectively (35). In addition, adenoviral-mediated overexpression of Shh increased remyelination in the adjacent corpus callosum, while an opposite effect was observed with overexpression of a Shh pathway antagonist (35). These Shh effects may be acting through NSCs in the V-SVZ, since adenovirus was delivered into the lateral ventricle to drive expression in ependymal cells. However, broader genetic and pharmacological inhibition of Shh signaling, specifically through *Gli1*, enhanced NSC mobilization from the V-SVZ and promoted remyelination of acute lesions in the corpus callosum (94). These studies present an inconsistent interpretation of the role of Shh signaling in acute demyelination models.

As a simple, direct test of Shh effects on cellular responses after chronic demyelination, we microinjected an activator of Shh signaling, SAG, into the corpus callosum. SAG is an agonist of the Smoothed receptor, which acts through canonical and type II non-canonical Shh pathways (16; 17). In our prior study in healthy adult mice, SAG microinjection into the corpus callosum increased *Gli1* fate-labeled cells in the adjacent V-SVZ but not in cells of the corpus callosum (76). However, the current results show that after chronic demyelination the corpus callosum was more permissive for recruitment and/or local activation of *Gli1* expressing cells. Thus, SAG may have more potential to enhance Shh responses after chronic demyelination. Indeed, we observed that SAG injection increased cell proliferation and remyelination in the corpus callosum. Additionally, SAG injection had a positive, but non-significant effect, of increasing cycling OP cells and oligodendrocytes. Surprisingly, SAG enhanced remyelination without increasing *Gli1* expression in any cell types within the chronically demyelinated corpus callosum. Therefore, SAG appears to have acted through non-canonical Shh signaling to enhance remyelination after chronic demyelination. Non-canonical Shh signaling can regulate cell migration, cell proliferation and survival without activation of *Gli1* expression (16). In fact in acute demyelination with CPZ, administration of *Gli1* inhibitors increased recruitment OP cells from the V-SVZ to the demyelinated corpus callosum indicating that downregulation of canonical Shh signaling might promote remyelination from NSC derived OP cells (94).

A limitation of the current study is that cells responding to SAG were genetically fate-labeled for only canonical Shh signaling. Similar *in vivo* genetic approaches are now warranted for analysis of non-canonical Shh signaling, specifically alternatives within the

type II non-canonical pathway that involves Smoothened exclusive of *Gli*-dependent transcription (16). Additionally, Shh signaling is also known to modulate blood-brain barrier function and the immune response (5; 99) so further studies that utilize immune mediated models of chronic demyelination are also needed to better understand the potential benefits of augmenting Shh signaling in chronic multiple sclerosis lesions.

CONCLUSIONS

These results demonstrate the potential to enhance remyelination by endogenous cells through modulation of Shh signaling, and reveal differences in population dynamics in chronic lesions. Shh transcription does not appear to be upregulated in cells within demyelinated lesions. However, cells with active canonical Shh signaling begin to localize within areas of chronic demyelination, which is not observed during adult homeostasis or during acute demyelination. This canonical Shh response may initiate in NSCs and could indicate a source of potential cells to repopulate lesions. Gli1 fate-labeling may be a useful tool for further in vivo studies to redirect differentiation to inhibit reactive astrogliosis and favor remyelination of chronic lesions. Furthermore, our results demonstrate that Gli1 expression in oligodendrocyte lineage cells was not required for SAG to enhance remyelination. Therefore, SAG acts through type II non-canonical Shh signaling, either directly or indirectly, to promote remyelination after chronic demyelination.

Table 2: List of antibodies 2

Primary antibodies	Secondary antibodies
GFAP (rabbit polyclonal) 1:500 Z0334; DAKO/Agilent; Santa Clara, CA RRID: AB_10013382	goat anti-rabbit (H+L) IgG (Alexa Flour® 488) 1:200 A11034; Thermo Fisher Scientific; Waltham, MA; RRID: AB_2576217
GSTpi (rabbit polyclonal) 1:200 312; MBL; Woburn, MA RRID: AB_591792	donkey anti-rabbit F(ab') ₂ IgG (Alexa Flour® 647) 1:100 711-606-152; Jackson ImmunoResearch; West Grove, PA RRID: AB_2340625
Iba1 (rabbit polyclonal) 1:500 019-19741; WAKO; Richmond, VA RRID: AB_2665520	goat anti-rabbit (H+L) IgG (Alexa Flour® 488) 1:200 A11034; Thermo Fisher Scientific RRID: AB_2576217
Ki67 (rabbit monoclonal) 1:100 AB16667; Abcam; Cambridge, MA RRID: AB_302459	goat anti-rabbit (H+L) IgG (biotinylated) 1:50 BA-1000; Vector; Burlingame, CA RRID: AB_2313606
MOG (mouse monoclonal 8-18C5) 1:100 MAB5680; EMD Millipore Sigma; Burlington, MA RRID: AB_1587278	donkey anti-mouse F(ab') ₂ IgG (Cy TM 3) 1:100 715-166-150; Jackson ImmunoResearch RRID: AB_2340816
MOG (mouse monoclonal 8-18C5) 1:20 Hybridoma cells a gift of Dr Minettia Gardinier, Department of Pharmacology, University of Iowa, Iowa City, IA U.S.A. RRID: AB_2631445	donkey anti-mouse (H+L) IgG (Alexa Flour® 488) 1:50 715-545-150; Jackson ImmunoResearch RRID: AB_2340846
NG2 (rabbit polyclonal) 1:500 gift from Dr. William Stallcup, Burnham Institute, La Jolla, CA RRID: AB_2572306	donkey anti-rabbit F(ab') ₂ IgG (Alexa Flour® 647) 1:100 711-606-152; Jackson ImmunoResearch RRID: AB_2340625

FIGURES

Figure 22

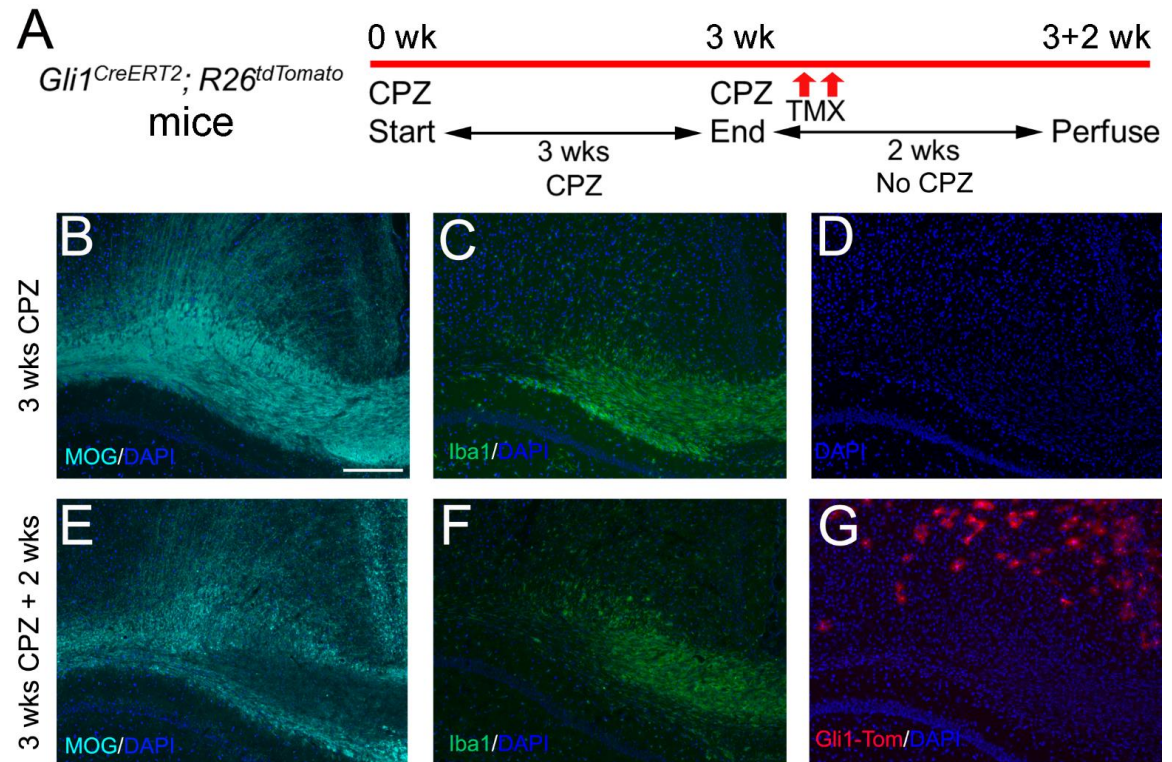


Figure 22: During early demyelination, *Gli1* does not fate-label activated microglia in the corpus callosum.

Gli1^{CreERT2}; *R26*^{tdTomato} mice were fed cuprizone (CPZ) for 3 weeks to initiate demyelination and then either perfused or given tamoxifen (TMX) to fate label cells expressing *Gli1* (A). After 3 weeks of CPZ, myelin pathology is not evident, based on immunolabeling to detect myelin oligodendrocyte glycoprotein (MOG) (B). Microglial activation, detected with immunolabeling for Iba1, is robust (C) and corresponds with high cell density in the corpus callosum (D). In mice given TMX after 3 weeks of CPZ and analyzed after a subsequent 2 week period on normal chow, extensive demyelination is observed (E) along with persistent microglial activation (F). Expression of td-Tomato shows *Gli1* fate-labeled cells in the cerebral cortex but not the adjacent corpus callosum (G). Representative images from 3 wks CPZ (n = 4) and 3 wks CPZ+ 2 wks (n = 3). Scale bar in B = 200 μ m.

Figure 23: At the acute demyelination stage, *Gli1* does not fate-label reactive astrocytes or remyelinating cells in the corpus callosum.

Gli1^{CreERT2}; *R26*^{LAP} mice were given tamoxifen (TMX) after 5 weeks of cuprizone (CPZ) and then perfused at 6 weeks of CPZ, or after a subsequent 3 week recovery period (A). *Gli1*^{CreERT2}; *R26*^{LAP} control mice without CPZ (No CPZ) were age-matched to the longer time point. **B-D:** MOG immunohistochemistry was used to detect myelin, which is present throughout the corpus callosum in controls (B). The MOG immunolabeled area was reduced after 6 weeks of CPZ, due to extensive demyelination (C), and normalized after remyelination during the 3 week recovery period without CPZ (D). **E-G:** Normal GFAP immunolabeling of astrocytes in the corpus callosum (CC) of control mice (E), compared to increased GFAP immunolabeling after acute demyelination (F), which was only partially attenuated after a 3 week recovery period (G). **H-J:** Co-localization of demyelination with astrogliosis is illustrated in merged images of the MOG and GFAP immunolabeling. The same *Gli1*^{CreERT2}; *R26*^{LAP} mice analyzed by MOG and GFAP immunohistochemistry had proximate sections examined for alkaline phosphatase reporter expression (K-M). *Gli1* fate-labeled (Gli1-AP) cells were observed in cortical regions but not in the adjacent corpus callosum in healthy control mice (K), after acute demyelination (L), or following remyelination (M). Quantification of the immunolabeling for MOG (N) and GFAP (O) shows the progression of corpus callosum pathology and recovery. Quantification and representative images from no CPZ (n = 3), 6 wks CPZ (n = 3), and 6 wks CPZ + 3 wks (n = 4). Scale bar in B and K = 200 μ m.

Figure 23

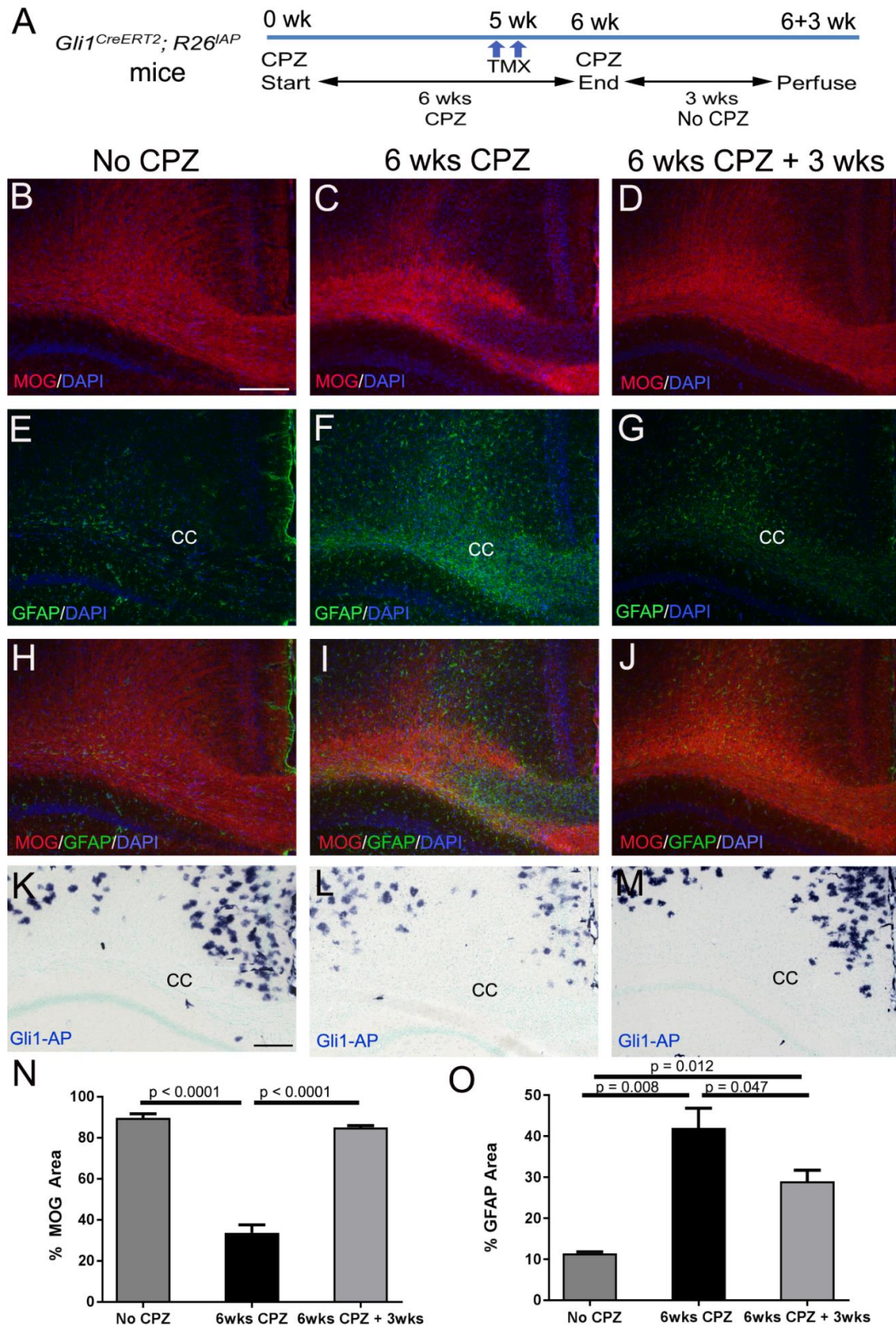


Figure 24

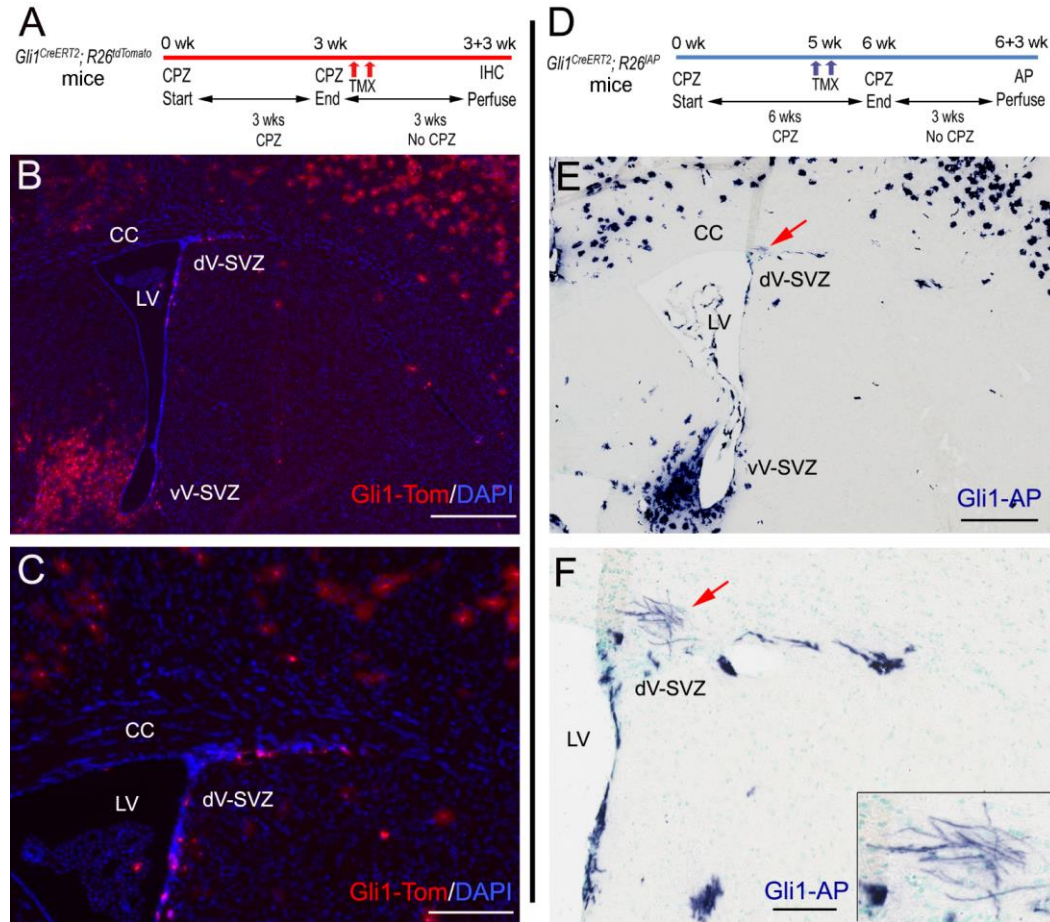


Figure 24: *Gli1* fate-labeled neural stem cells and their progeny are not recruited into the corpus callosum during acute demyelination or subsequent remyelination.

Gli1^{CreERT2}; *R26*^{tdTomato} mice were fed cuprizone (CPZ) for 3 weeks to initiate acute demyelination, administered tamoxifen (TMX) to fate-label *Gli1* expressing cells, then evaluated after a 3 week period on normal chow to allow for reporter expression (A). Coronal sections through the rostral corpus callosum (CC) show *Gli1* expressing (Gli1-Tom) cells in ventricular-subventricular germinal zone in both ventral (vV-SVZ) and dorsal (dV-SVZ) domains, including the dorsolateral extension under the corpus callosum (B, C). Gli1-Tom cells were not observed in the corpus callosum (C). *Gli1*^{CreERT2}; *R26*^{IAP} mice were given TMX after 5 weeks of CPZ and continued on CPZ through 6 weeks followed by a subsequent 3 week recovery period on normal chow without CPZ (D). Gli1-AP cells were prevalent in dV-SVZ and vV-SVZ domains (E). Gli1-AP cells were rarely found in the corpus callosum (F), with an example of an oligodendrocyte shown (F, inset). Representative images from *Gli1*^{CreERT2}; *R26*^{tdTomato} mice (n = 3) and *Gli1*^{CreERT2}; *R26*^{IAP} mice (n = 4). Scale bar in B = 500 μ m, C = 200 μ m, E = 500 μ m, F = 100 μ m.

Figure 25

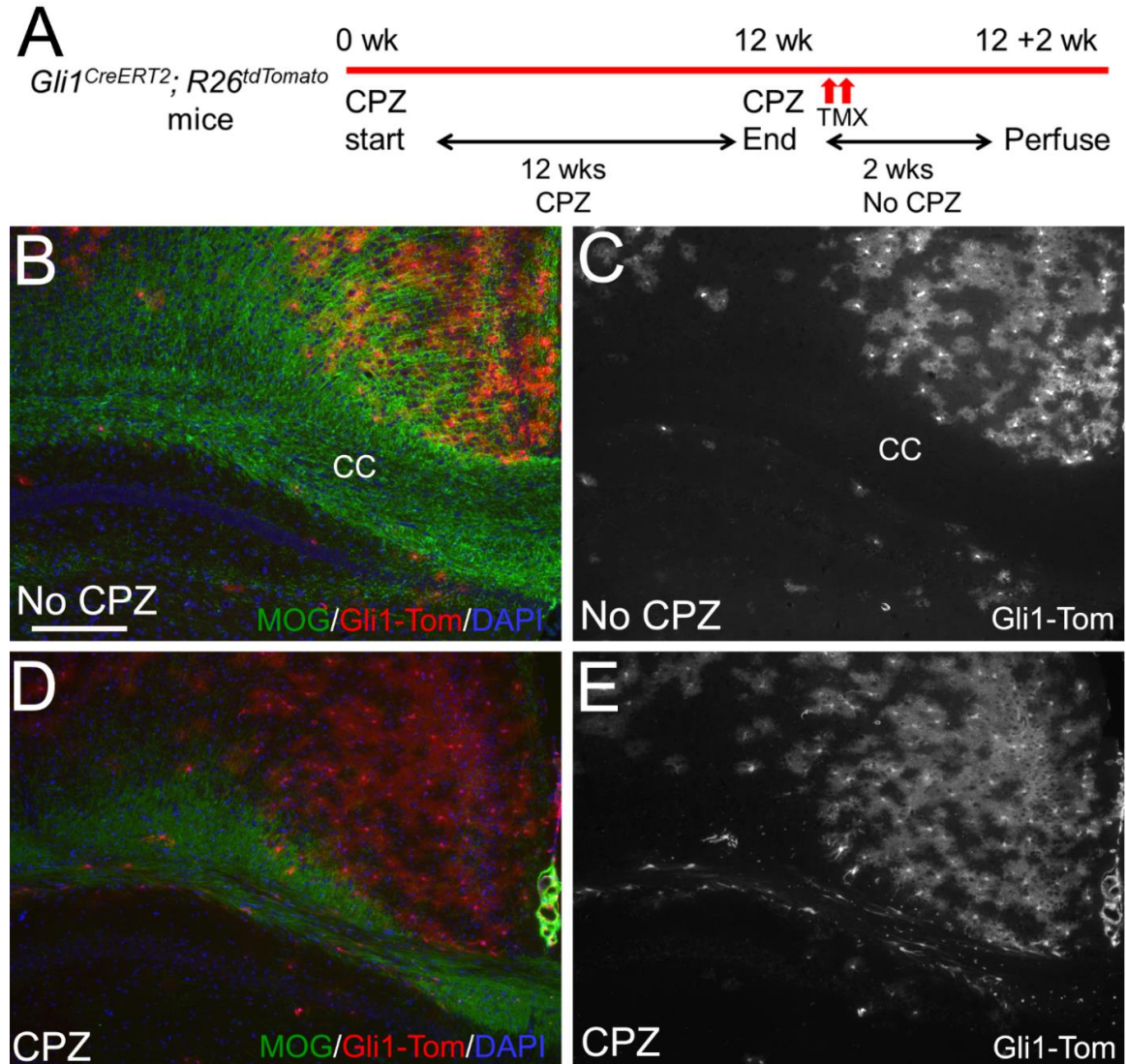


Figure 25: *Gli1* fate-labeled cells localize within the corpus callosum after chronic demyelination.

Gli1^{CreERT2}; *R26*^{tdTomato} mice were fed cuprizone (CPZ) for 12 weeks to induce chronic demyelination. Tamoxifen (TMX) was administered at the end of CPZ to fate-label *Gli1* expressing cells, which were analyzed 2 weeks later (A). *Gli1* fate-labeled (Gli1-Tom) cells were absent in the corpus callosum (CC) of age-matched control (No CPZ) mice (B-C). A low density of Gli1-Tom cells localized to the corpus callosum after chronic CPZ (D-E). Representative images from No CPZ (n = 4; with TMX and survival times age-matched to 10 (n = 2) or 14 (n = 2) days after ending CPZ) and CPZ (n = 2). Scale bar in B = 200 μm.

Figure 26

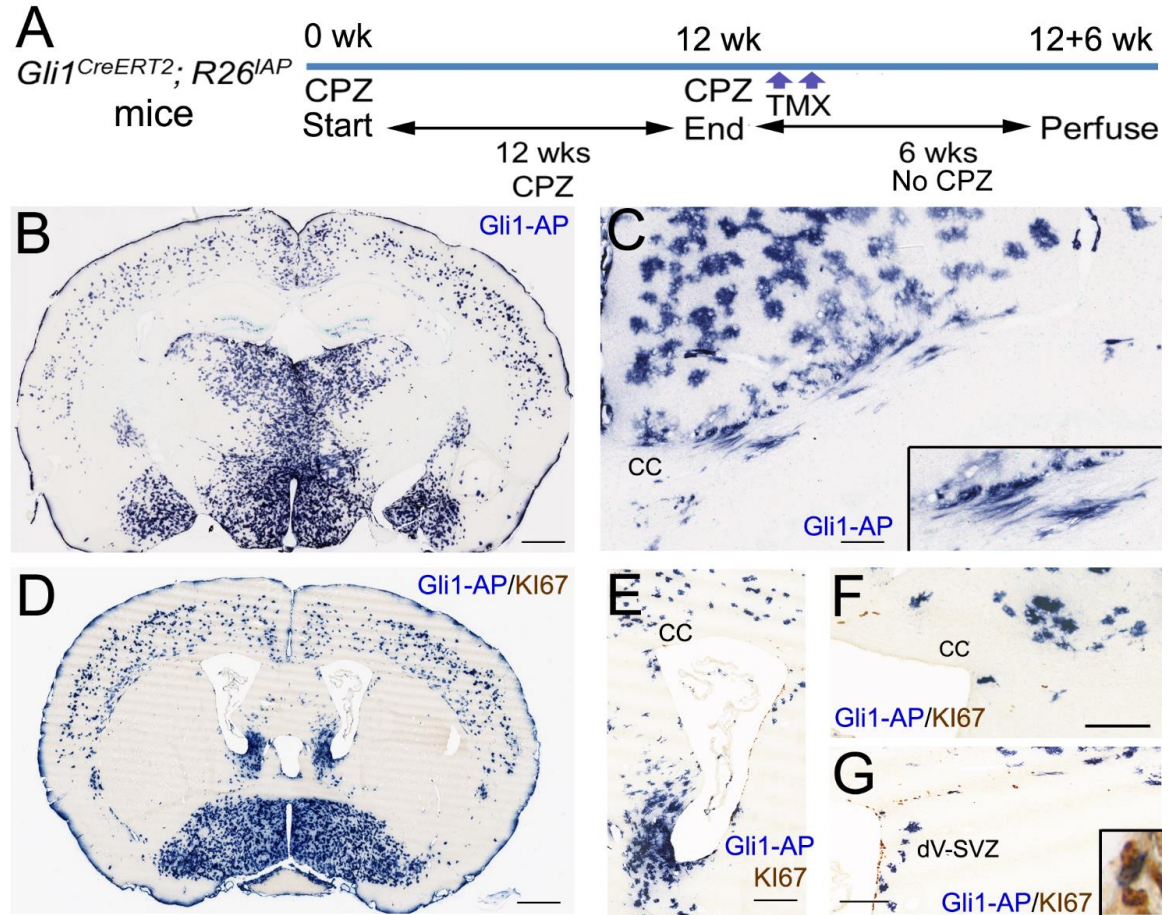


Figure 26: Cells fate-labeled for *Gli1* after chronic demyelination persist in the corpus callosum through remyelination.

Gli1^{CreERT2}; R26^{IAP} mice were given cuprizone (CPZ) for 12 weeks to induce chronic demyelination. Tamoxifen (TMX) was administered at the end of CPZ to fate-label *Gli1* expressing (Gli1-AP) cells, which were analyzed 6 weeks later (A). B-C: In caudal sections, Gli1-AP cells were found at high density in the cerebral cortex and in ventral regions while present at a low density in the corpus callosum (CC) (B-C). Gli1-AP cells in the corpus callosum extended thick processes with the morphology of reactive astrocytes (C, inset). D-G: In rostral sections containing the ventricular subventricular zone (V-SVZ), Gli1-AP cells localized within the corpus callosum less frequently than in caudal sections (D-F). Proliferating cells in the corpus callosum were labeled by Ki67 but did not express *Gli1* (F). In the V-SVZ, Gli1-AP cells were found in dorsal and ventral domains (E). Proliferating Ki67 labeled cells were also found throughout the V-SVZ, especially in the dorsal (dV-SVZ) region (G). Ki67 labeled cells with and without *Gli1* fate-labeling in the V-SVZ (G, inset). Representative images from *Gli1^{CreERT2}; R26^{IAP}* mice (n = 3). Scale bar for B = 800 μ m; C = 100 μ m; D = 800 μ m; E = 300 μ m; F = 150 μ m and G = 200 μ m.

Figure 27: Similar distribution of neuronal *Shh* expressing cells during acute and chronic demyelination, without detectable *Shh* transcription in cells within the corpus callosum.

Shh^{CreERT2}; *R26*^{LAP} mice were used to genetically label cells actively transcribing *Shh* in healthy adult mice (A-E), during acute demyelination (F-J), and after chronic demyelination (K-O). Alkaline phosphatase (AP) reporter was detected in *Shh* transcribing neurons in cerebral cortex and in ventral brain regions at both rostral (B, G, L) and caudal (D, I, N) levels. *Shh* expressing cells were rarely observed in the corpus callosum at rostral (C, H, M) or caudal levels (E, J, O). AP-labeled axons were only rarely visible within the corpus callosum, but AP-labeled axons were detected running superficial to the medial corpus callosum (C, E, H, J, M, O). Overall, the population of *Shh* expressing cells appeared similar across conditions. Representative images from No CPZ (n = 5), 6+ 3 (n = 2); 12+6 (n = 3). Scale bar for B, G and L, shown in B = 800 μ m; C, H, M shown in C = 300 μ m; D, I, N shown in D = 800 μ m; E, J, O shown in E = 300 μ m.

Figure 27

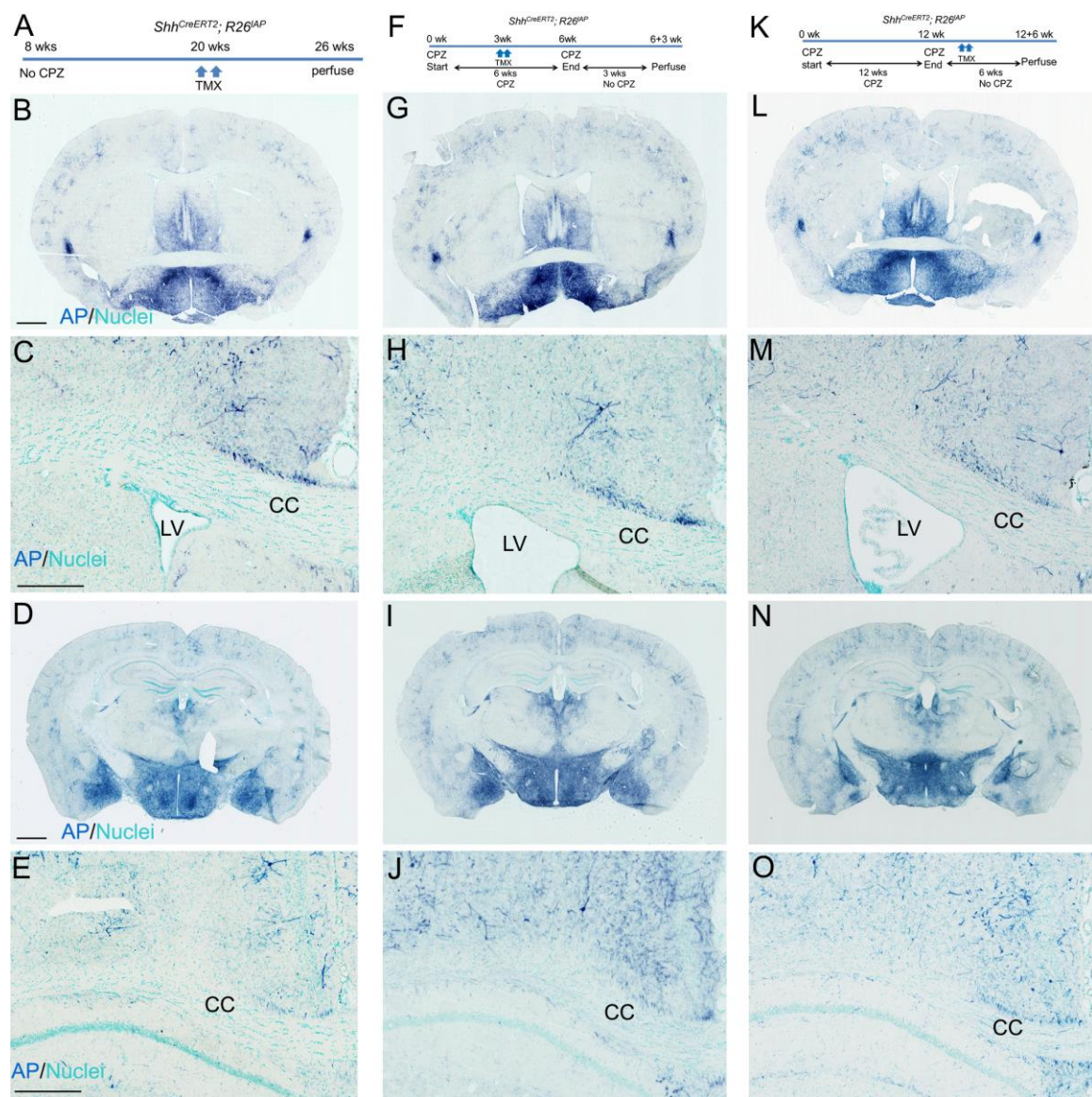


Figure 28

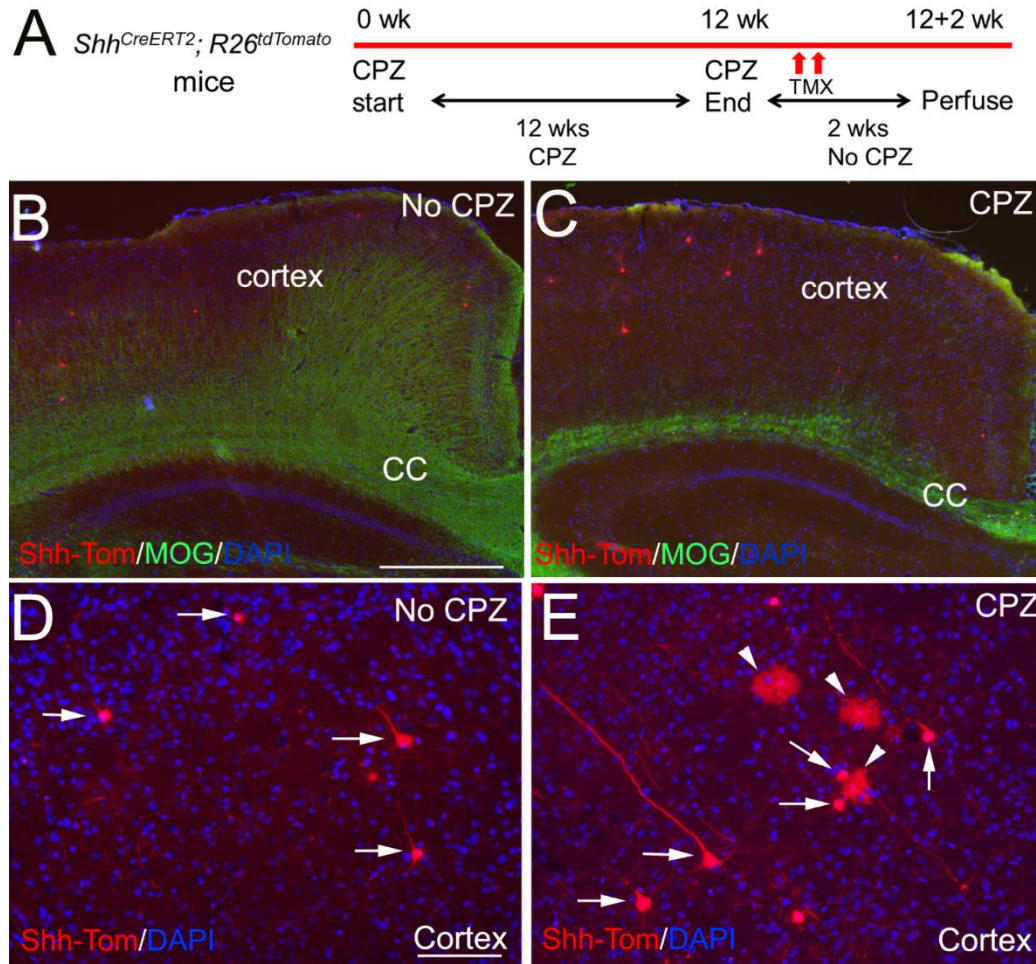


Figure 28: Chronic cuprizone demyelinates the cerebral cortex and corpus callosum without marked changes in *Shh* fate-labeled cells.

Shh^{CreERT2}; *R26*^{tdTomato} mice were given cuprizone (CPZ) for 12 weeks to induce chronic demyelination followed by tamoxifen (TMX) administration at the end of CPZ and a 2 week period without CPZ (A). This shorter 2 week recovery period after CPZ shows *Shh* expression at the same time point as the *Gli1* fate-labeling in Figure 4. Myelin was detected with immunohistochemistry for myelin oligodendrocyte glycoprotein (MOG). Age-matched healthy mice (No CPZ) show normal myelin throughout the corpus callosum (CC) (B). Following chronic CPZ, MOG immunolabeling is less extensive (C). *Shh* expressing (Shh-Tom) cells are not present in the corpus callosum but are prominent in the cerebral cortex (B, C). The majority of Shh-Tom cells in the cerebral cortex have neuronal morphology in both control (D, arrow) and CPZ (E, arrow) mice. Rare Shh-Tom cells with an astrocytic morphology were observed in the cerebral cortex of CPZ mice (E, arrowheads). Representative images from CPZ (n = 3); No CPZ (n = 3). Scale bar for B and C, shown in B = 500 μ m; D and E, shown in D = 100 μ m.

Figure 29: Shh agonist SAG enhances remyelination in the corpus callosum after chronic demyelination.

Shh agonist (SAG), or vehicle (VEH), was microinjected directly into the corpus callosum (CC) of *Gli1^{CreERT2}; R26^{tdTomato}* mice after 12 weeks of cuprizone (CPZ) to induce chronic demyelination (A). EdU was also administered at the end of CPZ for five consecutive days to label cycling cells. Tamoxifen (TMX) was administered on days 2 and 3 after the end of CPZ to fate-label cells actively transcribing *Gli1*; mice were perfused 10 days after the end of CPZ (A). Analysis of *Gli1* expressing (Gli1-Tom) cells in the corpus callosum showed a low density of Gli1-Tom cells in SAG and vehicle injected mice (B-E, red arrows). The number of Gli1-Tom cells was reduced in the area of the corpus callosum ipsilateral to the injection (B). Gli1-Tom cells in the corpus callosum included cells with mature oligodendrocyte morphology adjacent to the dorsal ventricular-subventricular zone (dV-SVZ) (C, white arrows). EdU labeling showed proliferating cells in the cerebral cortex and corpus callosum (D, blue arrows), some of which were oligodendrocyte progenitor (OP) cells identified by immunolabeling with NG2 (E, blue arrows). In the cerebral cortex, EdU labeling of proliferating cells in the cortical area ipsilateral to the injection was significantly higher in SAG than in vehicle injected mice (F). SAG significantly increased the number of proliferating OP cells (NG2 EdU double labeled cells) in the ipsilateral versus contralateral cortex (G). In the corpus callosum, EdU (H) and NG2 EdU doubled labeled cells (I) were significantly higher in the area of the corpus callosum ipsilateral to the injection, with the highest values observed in SAG mice. No difference was detected among total NG2 labeled cells in the corpus callosum across conditions (J). Immunohistochemistry to detect myelin oligodendrocyte glycoprotein (MOG) shows enhanced remyelination of the corpus callosum in mice given SAG vs vehicle (K-M). Remyelination was significantly higher in the corpus callosum ipsilateral to SAG injection (M). Oligodendrocytes, identified by GSTpi immunolabeling, were generally not among Gli1-Tom cells (N) or specifically an EdU labeled population (N, inset). Quantification of GSTpi cells shows an increase of oligodendrocytes with SAG injection, but this difference is not statistically significant (O). Gli1-Tom cells in the corpus callosum often had the morphology of reactive astrocytes (P) and were immunolabeled with the astrocytic marker GFAP (P, inset). Quantification and representative images from SAG (n = 7); Vehicle (n = 5). Scale bar in C = 100 μ m; D = 200 μ m; E = 50 μ m; K and L, shown in K = 200 μ m; N = 200 μ m; P = 100 μ m.

Figure 29

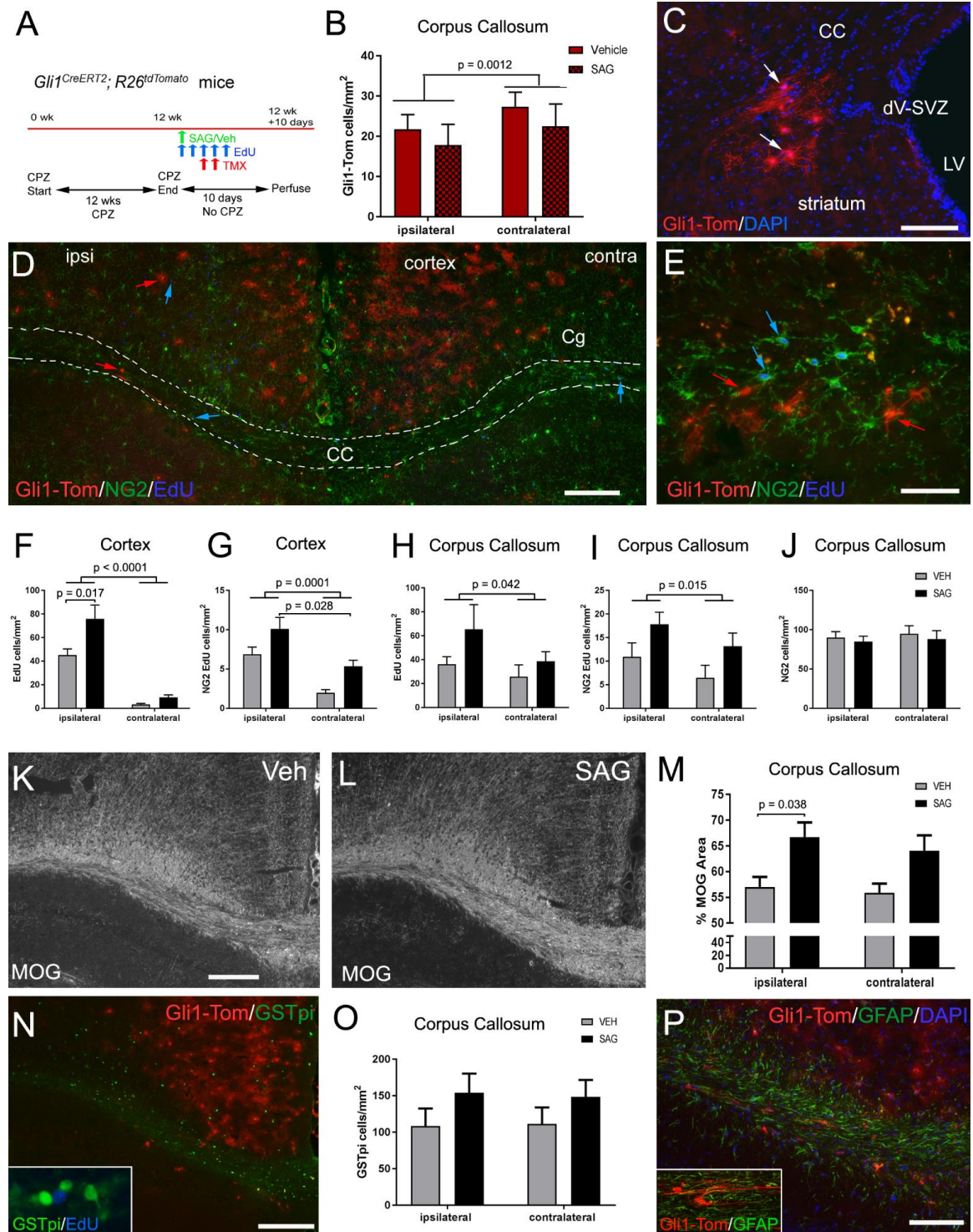


Figure 30

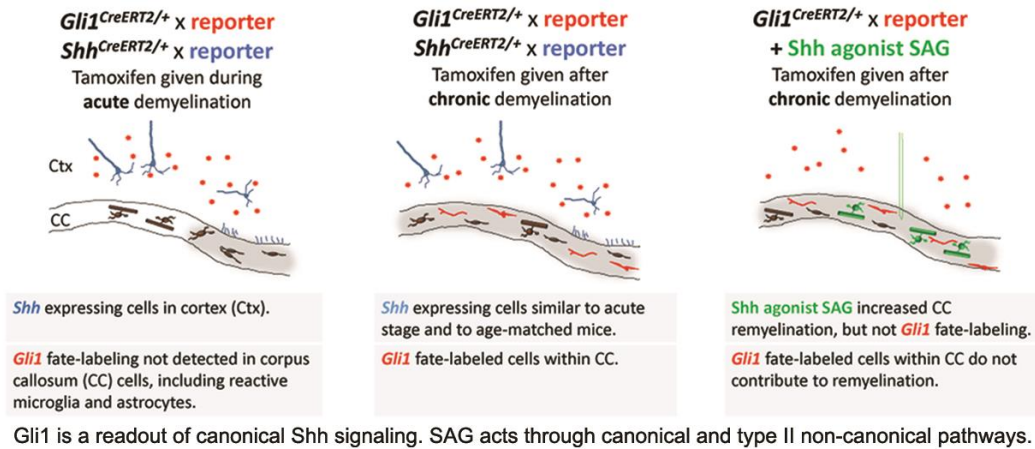


Figure 30: Canonical Sonic hedgehog signaling is not reactivated in response to acute or chronic demyelination, but Shh agonist (SAG) can enhance remyelination after chronic demyelination.

Gli1^{CreERT2} and *Shh*^{CreERT2} mice were used to detect in vivo activation of canonical Shh signaling during acute and after chronic demyelination with cuprizone (CPZ). Tamoxifen administration in *Gli1*^{CreERT2} mice showed rare labeling of Gli1 fate labeled cells in the corpus callosum (CC) after acute demyelination and low density of Gli1 fate-labeled astrocytes in the CC after chronic demyelination. Tamoxifen administration in healthy adult *Shh*^{CreERT2} mice and after acute or chronic demyelination showed similar distribution of *Shh* expressing cells in the cerebral cortex (Ctx). Microinjecting Shh agonist, SAG, into the chronically demyelinated CC showed increased proliferation and enhanced remyelination without increasing the Gli1 fate-labeled cell population in the CC.

CHAPTER 4: Discussion

The primary goals of our studies were to determine when Shh signaling is active during developmental myelination and remyelination, and to examine whether modulation of Shh signaling could be used as a therapeutic treatment to enhance remyelination after chronic demyelination. Shh signaling is involved in multiple steps of the myelination process during embryonic development and can contribute to repair of different CNS lesions (35; 76; 83). Therefore our studies used inducible genetic fate labeling to detect Shh signaling in vivo during postnatal myelination and different stages of demyelination and remyelination. Overall, our studies demonstrate that Shh signaling regulates oligodendrogenesis during normal postnatal myelination and can be used to enhance remyelination after chronic demyelinating lesions.

POSTNATAL SHH RESPONDING CELLS CONTRIBUTE TO REMYELINATION

Our studies in *Gli1*^{CreERT2} mice demonstrated that canonical Shh signaling is highly active during early postnatal myelination (postnatal days 6-9; P6-9) and reduced after the second postnatal week (P14-17). In situ hybridization detected downregulation of *Gli1* expression in the V-SVZ after the second postnatal week (106). Interestingly, fate labeling of *Shh* expressing cells in *Shh*^{CreERT2} mice showed that the downregulation of *Gli1* expression after the second postnatal week matches a reduction in *Shh* expressing cells in the cerebral cortex. Importantly, our studies showed that Shh responding (*Gli1* expressing) cells fate-labeled during P6-9 go on to populate and contribute to myelination of the corpus callosum in adulthood while those fate-labeled during P14-17 rarely do so.

The postnatal period in which high levels of *Gli1* expressing cells are observed, coincides with the amplification phase of OP cells necessary to myelinate the cerebral

cortex and corpus callosum in rodents (96; 106). During this early postnatal period, OP cells derived from cells expressing *Nkx2.1* and *Gsh2* in ventral regions of ventricular zone and from *Emx1* expressing cells in dorsal regions of the ventricular zone populate the CC, but not all of the generated cells survive to adulthood (58). Genetic fate mapping studies showed that most oligodendrocyte lineage cells in the adult corpus callosum are dorsally derived, and that these cells dominate the remyelination process after acute demyelinating lesions (23). During postnatal myelination, *Shh* responding radial glia in the dorsal V-SVZ also give rise to OP cells, but it is not clear if those radial glial cells are part of the *Emx1* cell population (106). OP cells derived from radial glial populate the corpus callosum until P28, but these studies did not show if this OP cell population survived to adulthood, or if this population could contribute to repair (106). In our studies, immunohistochemistry with Ki67 to detect cell proliferation demonstrated that *Gli1* fate-labeled cells from P6-9 (which likely includes the progeny of radial glial cells) that survive to adulthood continue to proliferate in germinal regions and in the corpus callosum. Additionally, immunohistochemistry with cell-type specific markers and administration of EdU, to detect cycling cells, showed that *Gli1* fate-labeled cells from P6-9 include cycling OP cells in the corpus callosum and neural stem cells in the V-SVZ in adult mice.

To further test the repair potential of *Gli1* fate-labeled cells from P6-9, we used CPZ to induce acute or chronic demyelination in the corpus callosum. Acute demyelinating lesions remyelinate spontaneously after CPZ is removed from the diet; chronic demyelinating lesions undergo limited remyelination (7). Interestingly, *Gli1* fate-labeled cells from P6-9 continued to localize to the corpus callosum and extend myelin-

like membranes after either acute or chronic demyelinating lesions. Furthermore, *Gli1* fate-labeled cells from P6-9 were able to incorporate EdU after chronic demyelination and go on to differentiate into mature oligodendrocytes. Therefore, our *Gli1* genetic fate labeling studies showed that high levels of Shh signaling during early postnatal development modulate a subset of cells that contribute to myelination of the healthy corpus callosum and to remyelination after acute or chronic demyelination.

SHH SIGNALING DURING DEMYELINATION

Our postnatal studies showed that *Gli1* expressing cells rarely localized to the corpus callosum between P14-17 after Shh signaling is downregulated. In adult mice, *Gli1* expressing cells are also very rarely observed in the healthy corpus callosum (41). To test if canonical Shh signaling could be reactivated in the corpus callosum during episodes of demyelination or remyelination, we fate labeled *Gli1* and *Shh* expressing cells after three weeks of CPZ ingestion. The three week time point was chosen because prior studies indicated that high levels of interleukin-1 β (IL-1 β) can induce Shh expression, and in the CPZ model, *IL-1 β* expression is increased 10-fold at this time point (6; 49). In *Gli1*^{CreERT2} mice, three weeks of CPZ ingestion resulted in extensive microgliosis in the corpus callosum. CPZ induced microgliosis did not induce *Gli1* expression in cells of the corpus callosum. In *Shh*^{CreERT2} mice, the distribution of *Shh* expressing cells was not significantly different between control mice and those fed CPZ for 3 weeks, suggesting that Shh signaling is not changed during initial stages of demyelination.

Canonical Shh signaling is increased in response to acute demyelination with lysolecithin; activation of Shh signaling contributes to remyelination in this model (35).

In the CPZ model of demyelination, demyelination and remyelination simultaneously take place after five weeks of CPZ (69). At this time point, a five-fold increase in OP cells has also been reported (7). To test if canonical Shh signaling could also contribute to remyelination after CPZ mediated demyelination, we also fate-labeled *Gli1* expressing cells after five weeks of CPZ. *Gli1* fate-labeled cells were then analyzed after 6 weeks of CPZ or after 6 weeks of CPZ followed by a 3 week period without CPZ to allow remyelination to occur. *Gli1* fate-labeled cells after five weeks of CPZ rarely localize to the corpus callosum even after mice were given time to remyelinate. Therefore canonical Shh signaling does not seem to be reactivated during acute demyelination with CPZ or during subsequent remyelination. The difference in canonical Shh signaling activation in acute demyelination with lysolecithin versus acute demyelination with CPZ might be explained by the breakage of BBB in demyelination with lysolecithin. Previous studies evaluating Shh levels after different pathological conditions reported that only invasive injuries with BBB disruption increase Shh signaling (99). The lack of BBB disruption in the CPZ model of demyelination could explain why canonical Shh signaling was not activated in this model.

To further test the repair potential of Shh signaling, we then fate labeled *Gli1* and *Shh* expressing cells after chronic demyelination from CPZ. *Gli1* fate-labeled cells after chronic demyelination were analyzed after a two or six week recovery period without CPZ. Surprisingly, *Gli1* fate-labeled cells now localized to the corpus callosum after chronic demyelination, although only at a low density. *Gli1* fate-labeled cells in the corpus callosum were not observed in age-matched control mice indicating that the localization of *Gli1* expressing cells to the corpus callosum was a result of chronic

demyelination rather than normal changes in Shh signaling associated with aging. Morphological analysis of *Gli1* fate-labeled cells in the corpus callosum indicated that these cells were reactive astrocytes rather than oligodendrocytes. Interestingly, analysis of *Shh* expressing cells after chronic demyelination did not show differences in *Shh* expression between chronically demyelinated mice and age-matched controls. These combined findings indicate that Shh signaling does not increase in response to chronic demyelination. Therefore, *Gli1* fate-labeled cells localized to the corpus callosum after chronic demyelination are more likely cells from other sites that migrated into lesion areas rather than cells within the corpus callosum. Our postnatal studies showed that *Gli1* expressing cells after the second postnatal week rarely localized to the healthy corpus callosum(95). *Gli1* expressing cells do not localize to the corpus callosum in healthy adults (41), after acute demyelinating lesions or in response to traumatic brain injuries (76); therefore, our results suggest that the pathology in chronic demyelinating lesions might make the corpus callosum more permissible for *Gli1* fate-labeled cells from other sites to migrate into the lesion areas.

Given that we found no indication of reactivation of endogenous Shh signaling in cells within the corpus callosum during acute or chronic demyelination, delivery of a Shh agonist was tested to increase Shh signaling in the chronically demyelinated corpus callosum. Increasing Shh signaling using Smo agonists (SAG or Purmorphamine) in pre-clinical models of stroke reduced cell death and lesion volume, increased survival of neural stem cells from the V-SVZ, reduced inflammation, and enhanced functional recovery (22; 54). In acute demyelinating lesions with lysolecithin, adenoviral-mediated overexpression of Shh in the V-SVZ reduced neuroinflammation and enhanced

remyelination (35). However, acute demyelinating lesions from lysolecithin injection can remyelinate spontaneously. Therefore, we tested whether Shh could have similar effects in chronic demyelinating lesions in which limited remyelination makes a more relevant target for testing enhanced repair. In our studies, Smo agonist, SAG, was microinjected directly into chronically demyelinated corpus callosum. SAG administration did not increase *Gli1* expression in the corpus callosum compared to mice administered vehicle. Importantly, SAG administration did increase cell proliferation in the cerebral cortex, as indicated by EdU incorporation. EdU labeled cells in the cerebral cortex and the corpus callosum included OP cells, but the OP cell population was not significantly different between SAG and vehicle treated mice. Importantly, analysis with MOG immunolabeling to detect myelin revealed enhanced remyelination in the corpus callosum of mice given SAG compared to those given vehicle. Together these findings indicate that increasing Shh signaling can increase remyelination through non-canonical Shh signaling.

LIMITATIONS AND FUTURE DIRECTIONS

In our studies, we identified a subpopulation of *Gli1* fate-labeled cells from P6-9 in the adult corpus callosum as OP cells and a subpopulation in the V-SVZ as neural stem cells. Our studies did not distinguish the repair potential between *Gli1* fate-labeled from P6-9 that migrate to the corpus callosum and those that remained in the V-SVZ. Therefore, *Gli1* fate-labeled cells that contributed to remyelination after acute or chronic demyelination could have derived from either source. Previous studies have shown that OP cells from the parenchyma and OP cells derived from neural stem cells in the V-SVZ remyelinate different regions of the corpus callosum after acute demyelination with CPZ (115). In demyelinating lesions with lysolecithin, OP cells from the parenchyma and the

V-SVZ are recruited to demyelinated regions but only OP cells from the parenchyma differentiate into myelinating cells (57). The difference in repair potential identified in these studies might be associated with the pathological conditions in each lesion. Potential differences in gene expression between OP cells from the parenchyma and V-SVZ might require different signaling molecules to engage each cell population in remyelination. Determining the contribution of each cell population to remyelination after chronic demyelinating lesions could provide insights on how to target each cell population to enhance remyelination.

Similarly, understanding the mechanism by which SAG mediated remyelination after chronic demyelination is important to determine under what circumstances Shh signaling could be beneficial. Our studies showed that delivery of Shh agonist, SAG, directly into the demyelinated corpus callosum was sufficient to enhance remyelination after chronic demyelination. SAG administration enhanced remyelination without inducing *Gli1* expression. However, the exact mechanisms by which SAG promoted remyelination were not examined in our studies. In acute demyelinating lesions with CPZ, downregulation of *Gli1* was necessary to promote migration of OP cells from the V-SVZ into the demyelinated corpus callosum while genetic removal of Smo did not have the same effects (94). In the chronic stage of MS, limited remyelination can be the result of depleted OP cells, inability to recruit OP cells to demyelinated regions, and/or inefficient OP cell differentiation (59). In pre-clinical models of stroke, administration of Shh agonists promoted functional recovery by decreasing neuroinflammation, increasing cell survival and reducing BBB permeability (22; 54). Further assessments of neuroinflammation and apoptosis are needed to determine how SAG modified the

chronically demyelinated corpus callosum or adjacent V-SVZ response to promote remyelination. SAG is an agonist of receptor Smo, therefore the first step to determine the direct effects of SAG would be to identify which cell population expresses Smo in the chronically demyelinated corpus callosum and/or adjacent V-SVZ. Activation of GTPases can then be measured using pull down assays from cell lysates of cells expressing Smo. Identifying the cell population that responds to SAG will identify if SAG enhances remyelination directly by modulating oligodendrocyte lineage cells or indirectly by modulating neuroinflammatory cells in the corpus callosum or NSCs in the V-SVZ.

Our studies detected enhanced remyelination associated with SAG delivery without detecting differences in OP cells between SAG and vehicle treated mice. However, the effects of SAG were assessed 10 days after SAG delivery, so it is possible that direct effects of SAG in OP cells were masked by their differentiation into myelinating cells. This lineage progression cannot be monitored in our *Gli1* reporter mice, which do not detect activation of the non-canonical Shh pathway. Further studies will need to examine different time points after SAG delivery to decipher the mechanism by which SAG enhanced remyelination and to assess its safety profile. Unfortunately, remyelination of axons does not always translate into functional recovery. Therefore, future studies should also test the functionality of SAG mediated remyelination. Future studies, should also consider using a combination of SAG and Gli1 inhibitors as a way to modify different aspects of the Shh signaling pathways and promote remyelination. Myelination of axons is a multistep process, so it is likely that a combination of drugs/treatments will be necessary to promote remyelination.

Microinjection delivery of drugs is an invasive way to administer a treatment and it is not ideal to use in patients. In our studies, we microinjected SAG directly into the demyelinated corpus callosum to assess the effects of augmenting Shh signaling directly within chronically demyelinated regions. Alternative routes of delivery are warranted to determine if less invasive methods of delivery can have the same beneficial effects. Systemic delivery of SAG and other Shh agonists showed beneficial effects in pre-clinical models of stroke in which disruption of the BBB is a pathological feature (22; 54). Interestingly, Shh signaling is known to modulate the BBB and Shh agonists have been shown to reduce BBB permeability (5; 22). BBB disruption is a pathological feature in MS that is not modeled in the CPZ model of demyelination (71). Therefore, systemic delivery of SAG to immune mediated models of chronic demyelination might reveal other potential benefits of augmenting Shh signaling in MS.

SUMMARY

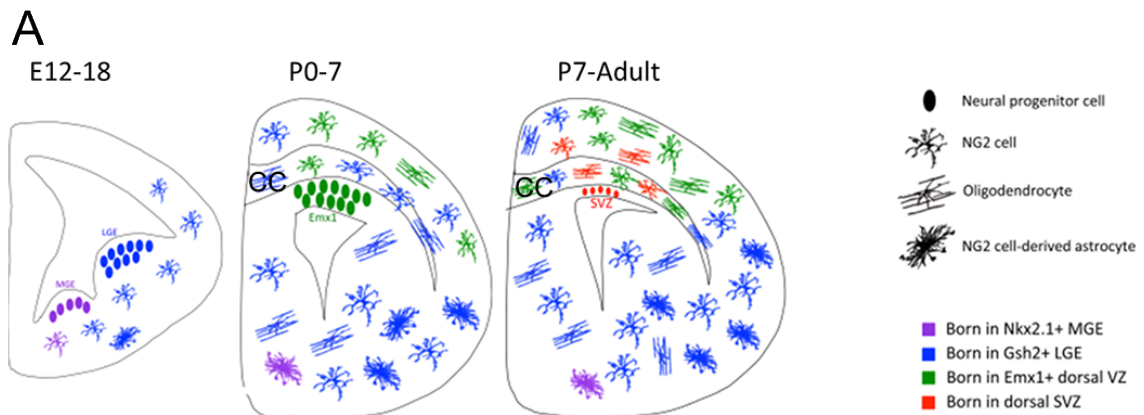
To our knowledge, our studies are the first to use inducible genetic fate labeling to detect in vivo activation of canonical Shh signaling during different stages of demyelination and remyelination. Our results indicate that canonical Shh signaling is not activated in cells of the corpus callosum in response to either acute or chronic injuries. Nevertheless, the chronically demyelinated corpus callosum is receptive to Shh signaling and augmenting Shh signaling enhances remyelination (Fig. 31). The mechanism by which Shh agonist mediated remyelination were not determined by our studies. However, our studies did identify two subpopulations of postnatal Shh responding cells that could be targeted for remyelination after chronic demyelinating lesions. Therefore, our

experiments laid out a path to further study the potential therapeutic effects of Shh signaling in chronic demyelinating lesions.

Figure 31: Thesis summary

Schematic shows origin of oligodendrocyte progenitor cells (OP) during embryonic development (E12-18), early postnatal myelination (P0-7) and through adulthood (P7-Adult). OP cells derived from LGE and Emx1 expressing cells contribute to myelination of the corpus callosum (CC), but OP cells derived from Emx1 expressing cells dominate the remyelination response after acute demyelination. Oligodendrogenesis from Emx1 expressing cells during P0-2 is dependent on Sonic hedgehog (Shh) signaling (A). Our studies used inducible genetic fate mapping to detect activation of canonical Shh signaling during different stages of postnatal myelination and after acute and chronic demyelination of the CC. The results of our studies are outlined in (B). Schematic modified from Hill and Nishiyama, 2014 (50).

Figure 31



B

**Postnatal days
0-7**

Tong et al., 2015 (Oligodendrogenesis from dorsal ventricular zone is Shh dependent).
Crawford et al., 2016 (OP cells derived from Emx1 expressing cells dominate remyelination).

**Postnatal days
6-9**

- High levels of Shh signaling
- *Gli1* fate-labeled cells populate and myelinate CC
- *Gli1* fate-labeled cells contribute to remyelination

**Postnatal days
14-17**

- Downregulation of Shh signaling
- *Gli1* fate-labeled cells do NOT populate CC
- *Gli1* fate-labeled cells do NOT contribute to repair

**Healthy
adult**

- Shh expressing cells in cortex and ventral regions
- *Gli1* fate-labeled cells do NOT populate CC
(Garcia et al., 2010 ; Mierzwa et al., 2014)

**Acute
demyelination**

Ferent et al., 2013 (Shh is increased and contributes to remyelination)
Samanta et al., 2015 (Gli1 inhibitors increase recruitment of NSC)
Kazanis et al., 2017 (NSC derived OP cells might not be needed to repair acute lesions)

- *Shh* expressing cells are not changed
- *Gli1* fate-labeled cells do NOT populate CC
- *Gli1* fate-labeled cells do NOT contribute to repair

**Chronic
demyelination**

- *Shh* expressing cells are not changed
- *Gli1* fate-labeled cells populate CC but do not contribute to remyelination

**Chronic
Demyelination
+ SAG**

- SAG increased proliferation and enhanced remyelination without activating *Gli1* expression in the CC.

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