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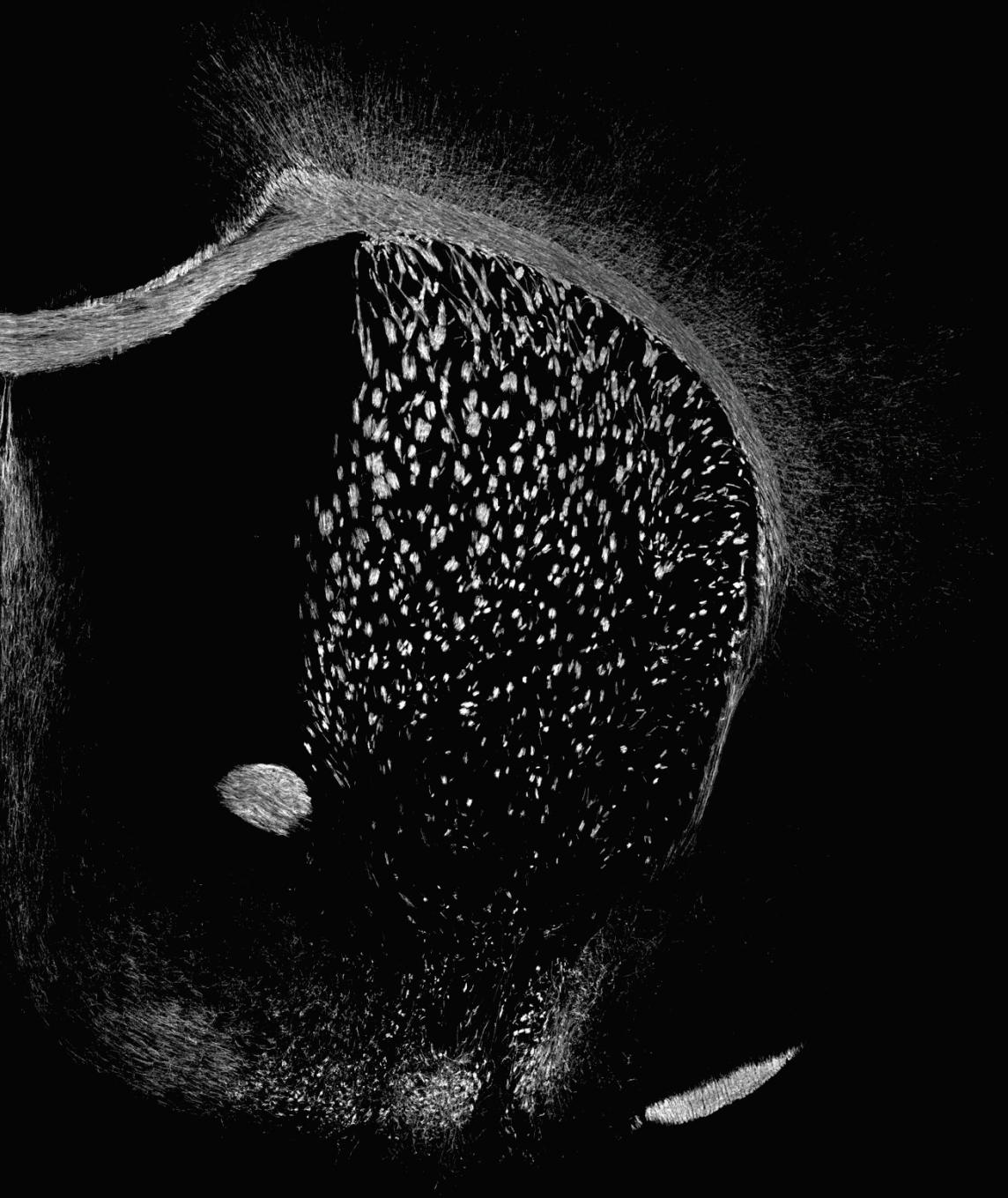
Doctoral Thesis

Genetic approach to study the role of Sonic Hedgehog in physiological CNS myelination and remyelination



Sonia Nocera

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Doctoral Thesis

Genetic approach to Study the role of Sonic Hedgehog in physiological CNS myelination and remyelination

Universidad de Castilla la Mancha

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Alla mia famiglia, la mia vita.
A mio padre perchè farei di tutto per farti stare bene.

"Per un solo dolcissimo umore del sangue,
per la stessa ragione del viaggio viaggiare"

(Fabrizio De André)

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ABBREVIATION

AOBS: Acousto-Optical Beam Splitter
BrdU: 5-Bromo-2-deoxyuridine
CC: Corpus Callosum
CDK: Cyclin Dependent Kinase
CGE: caudal ganglionic eminence
CNS: central nervous system
CPZ: Cuprizone
CreER: Cre-Estrogen Receptor
DG: dentate gyrus
Dhh: Desert hedgehog
EAE: Experimental Autoimmune Encephalomyelitis
EdU: 5-ethynyl-2'-deoxyuridine
FGF2: Fibroblast Growth Factor type 2
GFAP: Glial Fibrillary Acid Protein
GFP: Green Fluorescent Protein
GF-Smo: Gain of Function of Smoothened
HBSS: Hank's Balanced Salt Solution
ICH: Immunohistochemistry
IFN-γ: Interferon-γ
IL: Interleukin
Ihh: Indian Hedgehog
Hh: Hedgehog
LGE: lateral ganglionic eminence
LPC: Lysolecithin
MACS: Magnetic-Activated Cell Sorting
MAPK: Phosphorylated Mitogen-Activated Protein Kinases
MBP: Myelin Basic Protein
MGE: medial ganglionic eminence
MyRF: myelin regulator factor
MRI: Magnetic Resonance Imaging
MS: Multiple Sclerosis
MWB: Miltenyi Wash Buffer
NAWM: Normal-Appearing White Matter
NDS: Normal Donkey Serum
NFH: Nerufilament Heavy
NSC: Neural Stem Cells
OLs: Oligodendrocytes
OPC: Oligodendrocyte Precursor Cells
P7: Postanatal day 7
P14: Postanatal day 14
PB: Phosphate Buffer
PBS: Phosphate Buffered Saline
PDGF-AA: Platelet-Derived Growth Factor
PDGFR: Platelet-Derived Growth Factor receptor
PFA: Paraformaldehyde
PNS: Peripheral Nervous System
Ptch: Patched
RA: Retinoic Acid
ROI: Region of Interest
RT: Room Temperature
SEM: Standard Error of the Mean
SAG: Smoothened Agonist
Shh: Sonic Hedgehog
SGZ: Subgranular Zone
Smo: Smoothened
SVZ: Subventricular Zone
tdT: tandem dimer Tomato
TNF-α: Tumor Necrosis Factor- α

RESUMEN

La vía de señalización de Hedgehog es determinante en la regulación de la organogénesis en los vertebrados y juega un papel clave en la organización del SNC. De todos los miembros de la familia, Sonic Hedgehog (Shh) es el ligando mejor estudiado. Durante el desarrollo, Shh actúa como morfógeno secretable, dando lugar a gradientes de concentración. En el cerebro adulto, Shh continúa activo, modulando la autorrenovación y la especificación de las células madre neurales (NSC). Además, la señalización de Hedgehog se sobre-expresa después de una lesión cerebral aguda. Estudios recientes han demostrado que los progenitores de la zona subventricular ventral (V-SVZ) postnatal del prosencéfalo dorsal requieren señalización Shh para dar luegar a nuevos oligodendrocitos (OL) en el cuerpo calloso (CC). Los OL son células gliales que mielinizan los axones y desempeñan un papel fundamental en el desarrollo y correcta transmisión de los impulsos nervioso. Existe una población sustancial de células precursoras de oligodendrocitos (OPC) que permanece quiescente pero activable durante en el SNC adulto: estos OPCs son necesarias para mantener la plasticidad y el mantenimiento de la mielina, así como después episodios de desmielinización como los que ocurren durante la esclerosis múltiple (EM). Una de las moléculas que se sobre-expresan en las lesiones desmielinizantes que caracterizan a esta enfermedad es Shh lo que, potencialmente, regula las respuestas de los OPC y el proceso de remielinización espontánea postlesión. Sin embargo, el efecto directo de la señalización de Shh sobre los OPCs durante el desarrollo posnatal y en respuesta a la desmielinización siguen siendo importantes cuestiones abiertas. La presente tesis doctoral estudia cómo la sobre-expresion y la inhibición parcial de la vía de Shh afecta la diferenciación de los OPC tanto durante la mielinización postnatal, como durante los fenómenos de desmielinización y remielinización del CC del cerebro adulto. Al trasplantar OPCs en zonas del SNC que no presentan mielina en condiciones fisiológicas, como la retina, se demuestra que el efecto de la activación/inhibición de esta cascada es dependiente de factores ambientales que difieren en función de el estadio de desarrollo. Finalmente, estudiamos por primera vez el efecto directo del ligando Shh sobre OPCs aislados de corteza cerebral de humano adulto.

ABSTRACT

The Hedgehog signaling pathway is decisive in the regulation of vertebrate organogenesis and plays a key role in the organization of the CNS. Sonic Hedgehog (Shh) is the best-studied ligand of the Hedgehog signaling pathway. During development, Shh acts as a secreted morphogen, forming a concentration gradient that has different effects on the cells. Shh gradient continues to act in the adult brain modulating self-renewal and specification of neural stem cells (NSC). Furthermore, hedgehog signaling is often upregulated after acute brain injury. Recent studies showed that progenitors in the postnatal ventral subventricilar zone (V-SVZ) of the dorsal forebrain require Shh signaling to generate oligodendrocytes in the corpus callosum. Oligodendrocyte are glial cells that myelinate axons and play a fundamental role in the development and correct transmission of nerve impulse. There is a substantial population of oligodendrocyte precursor cells (OPCs) that remains quiescent during the adult CNS: These OPCs are required to support myelin plasticity and maintenance, as well as after episodes of demyelination such as those that occurs during Multiple Sclerosis. Shh has been shown over-expressed in Multiple Sclerosis lesions, and this pathway has the potential to regulate OPC responses that impact remyelination. One of the molecules that are over-expressed in the demyelinating lesions that characterize this disease is Shh, which potentially regulate OPC responses and the post-injury spontaneous remyelination. However, the direct effect of Shh pathway on OPCs during postnatal development and in pathological conditions is still an open question. The current PhD Thesis studies how the up or down-regulation of Shh pathway affects OPCs differentiation both during postnatal and adult (re)myelination. By transplanting OPCs in areas of the CNS that do not present myelin under physiological condition, it is shown that the effect of the activation/partial inhibition of this cascade is dependent on environmental factors that differ depending on the stage of developing. Finally, we study for the first time the direct effect of Shh ligand on OPCs isolated from adult human cerebral cortex.

Prologue

Close your eyes and wave your arms around: you can tell where they are at every moment. How is it possible? Like those revealing muscles position, some kinds of signals travel on super-fast nerve impulses at speeds of up to 120 meters/second. Other types of messages, as the pain signals, travel much slower. If you stub your toe, you will feel the pressure right away because the touch signals travel around 75 meters/second. However, you will not feel the pain for another two or three seconds because the pain signals generally travel only up to 2 meters/second (Siegel e Sapru, 2006).

The brain, the nervous system driver, is an extraordinary "machine" to plan and execute actions. These actions are designed and implemented in response to the conditions of the external environment. For this reason, above its planning capacity, the brain is also an incredible machine for managing sensory information, which informs the individuals about the state of the environment and encourages them to react correctly.

Where the extraordinary effectiveness in the genesis of our actions, our feelings, our thoughts of this "gray matter" come from? The merits are generally attributed to the "noble brain cells": the neurons. The role of these neurons is indeed essential, although, as equally important, there are other categories of cells in the nervous system: the "glial cells." Glial cells make up most of the brain cells. In the last few years, glia has been demonstrated essential for the correct development and organization of the neural circuity, playing a critical role in every function performed by the brain. Although there are several glial cell types including, the main ones are: astrocytes, oligodendrocytes, and microglia; each one is showing characteristic morphology, localization and function (Zuchero e Barres, 2015). Oligodendroglial cells are the only ones involved in the myelin formation, the "white matter" of the central nervous system (CNS), consisting of the parts of the nervous tissue particularly rich in bundles of nerve fibers -or "axons"- sheathed with myelin.

The emergence of myelin is probably dated 425 million years ago (Zalc, Goujet, e Colman, 2008). A lipid mixture (70%) and proteins (30%), myelin represents an opportunity for more evolved vertebrates: it accelerates, up to 100 times, the nerve

impulses conduction speed. Without this insulating sheath, the movements, the thoughts would have overwhelming delays. Its richness in lipids gives it a light color, hence the term "white substance," which defines myelinated nerve fibers bundles.

This present work focuses on the biology of oligodendroglial cells and how they are involved in myelin formation. This study aims to understand the cellular and molecular mechanism involved in the proliferation and differentiation of oligodendrocyte precursor cells during development and demyelinating pathologie

FROM MYELIN TO OLIGODENDROCYTE PRECURSOR CELLS (OR VICE VERSA)

Historical Sketch:

In 1838, the physiologist and neurologist Robert Remak observed for the first time the coexistence of two types of nerve fibers (now called axons) in the peripheral nervous system (PNS). He noted that certain fibers are covered with a thick sheath, while others, finer, appear to be deprived of it (Grzybowski e Pietrzak, 2013). In 1854 the biologist and pathologist Rudolf Virchöw proposed the term "myelin." The histologist Louis Ranvier was the first to show that the myelin sheath is not continuous along the axons: it is interrupted at regular intervals, later known as "Ranvier nodes"(Boullerne, 2016).

The identification of oligodendrocytes is dated back to 1919 and was completed along the following decade by the Spanish neurohistologist and neuropathologist Pío del Río-Hortega, a disciple of the famous Santiago Ramón y Cajal, who is considered the founder of modern Neuroscience. Indeed, Río-Hortega discovered the right composition of the “third element” of the CNS described by Cajal (1913): microglia and oligodendroglia, which are the only myelin-forming cells of the CNS and would be equivalent to Schwann cells in the PNS ad scribing all its classic functions (Río Hortega, 1919; Río Hortega, 1928; for a review of the historical achievement see: del Rio-Hortega, 2012; Pérez-Cerdá et al., 2015). He named these cells “oligodendroglia” because they present just a few branches (“oligo,” in classic Greek) in their tree of cell extensions (“dendro”).

A better understanding of myelin constitution was achieved in the mid-twentieth century with the development of electron microscopy. The works of Sjòstrand and Fernández-Morán, in 1949-1950, on peripheral nerves, as well as that of Fernández-Morán and Finean in 1957 on the CNS, allowed to establish that the myelin is formed by the spiral wrapping of membrane prolongations of the myelinating cells (Schwann cells in the PNS, or Oligodendrocytes in the CNS). Each of these cells has a cellular body, which can produce the myelin up to a hundred times its weight (Boullerne, 2016). So, myelin is a differentiation of cellular membrane: it is formed of the extension of the oligodendrocyte cells.

Nowadays, the presence of myelin is associate with three essential functions:

1- Axon insulation and protection: The myelin sheath, consisting mostly of fats (lipids), is a good insulator of the nerve fibers that envelops, thus avoiding electrical interference between neighboring cells and increasing resistance to the axonal membrane. It is essential for the rapid and reliable conduction of the action potentials along neuronal axons.

2-Speed up the nerve impulses: as said above, the myelin sheath is interrupted by short segments in which the bare axon is exposed to the interstitial space. These segments, the nodes of Ranvier, are the location of clusters of sodium channels. The nerve impulse jumps from node to node: this saltatory conduction increases the impulse speed from 50 to 100 times (Huxley e Stampfli, 1949).

3- A "nurse" for the axon. To guarantee enough energy supply along the whole axon, the oligodendrocytes (the cells forming myelin) collaborate with the astrocytes. Astrocytes capture glucose through the blood capillaries and transform it into lactate through anaerobic glycolysis. But the astrocytes can distribute lactate only to the soma of neurons. Instead, the oligodendrocytes can carry the lactate recovered from the astrocytes, in addition to the one produced by themselves, and distribute it throughout the axon (Fünfschilling et al., 2012; Lee et al., 2012). Oligodendrocytes can also provide other energy substrates, i.e., glucose, to axons, indicating different metabolic support among different white matter microdomain (Meyer et al., 2018).

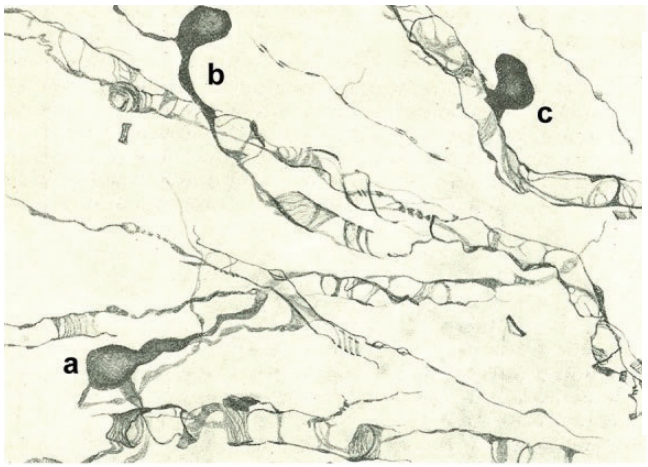

Figure 1 Drawing of the subcortical white matter after staining with the Golgi-Hortega method.

Oligodendrocytes of the third type with different kind of processes around axons are represented: one has two clear and long processes (a), while others are endowed with a single process divided into an acute angle for two axons (b) or in T that ensheathes a nerve fiber. Modified from Del Río Hortega (1928). Pérez-Cerdá et al., 2015

Oligodendrogenesis & Myelination

Part of macroglia, oligodendrocytes (OLs) are generated during embryonic development from oligodendrocyte precursor cells (OPCs). All OLs are considered to be a progeny of neural stem cells (NSCs). NSCs are self-renewing, multipotent cells that generate neurons and various glial cell types (astrocytes, OLs, ependymal cells) in the CNS during embryonic development. Murine NSCs proliferate during the early embryonic stages, produce neurons during the middle embryonic stages, then generating OLs and astrocytes from the late embryonic to the postnatal stages (Naruse et al., 2017, Fig.2).

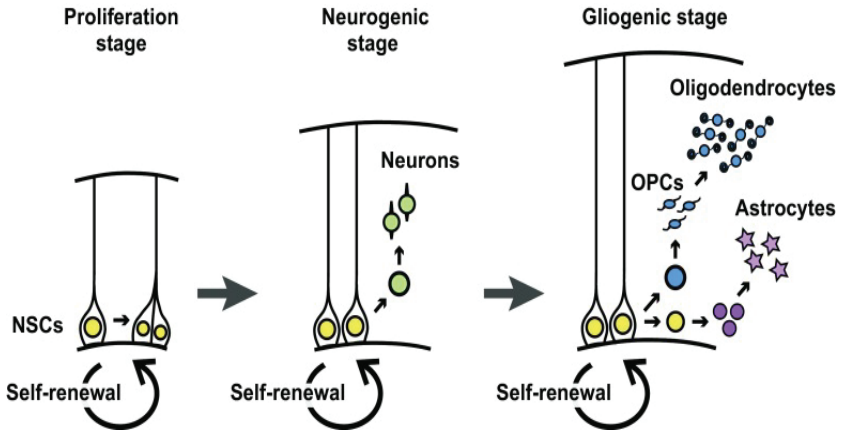

Figure 2 Proliferation and differentiation of NSC in the embryonic mouse brain. *NSC (yellow), or radial glial cells, produce neurons (green) in the middle embryonic stage, and then oligodendrocytes (blue) and astrocytes (purple) in the late embryonic stage. Naruse et al., 2017*

NSCs are also found in the adult brain, arising in specific neurogenic regions of the mature brain, subventricular zone (SVZ) and subgranular zone (SGZ), capable of both neurogenesis and gliogenesis (Yamaguchi et al., 2016; Rusznák et al., 2016). OPCs represent an intermediate stage in oligodendrogenesis, and they are capable of self-renewal and maintenance of OLs lineage in the adult CNS (Crawford et al., 2014).

There is a temporal gradient of OPCs production, in the mammalian telencephalon, from ventral to dorsal (Kessaris et al., 2006). The first wave of OPCs (which are $Nkx2.1^{+}$) arises from the medial ganglionic eminence (MGE) at E11.5, arrive at the cortex about E16, and are immigrants from ventral territories (Fig. 3). A second wave ($Gsh2^{+}$) originates from the lateral-to-caudal ganglionic eminence (LGE/CGE) around E15.5. Therefore, at E18, all OL lineage cells in the cortex are ventral-derived cells. After E18, ventral cells contribution starts to decrease, and a third wave ($Emx1^{+}$) joins this scenario. This third OPC wave comes from the cortical ventricular zone and debuts

around birth (Richardson et al., 2006; Fig. 3). Although there is a replacement of most of the OPCs generated during the first waves, some studies demonstrated the existence of a reservoir of OPCs from the first wave, which remains able to survive, differentiate and myelinate the telencephalon in case the next waves were impeded, as in the case of preterm infants (Kessaris et al., 2006).

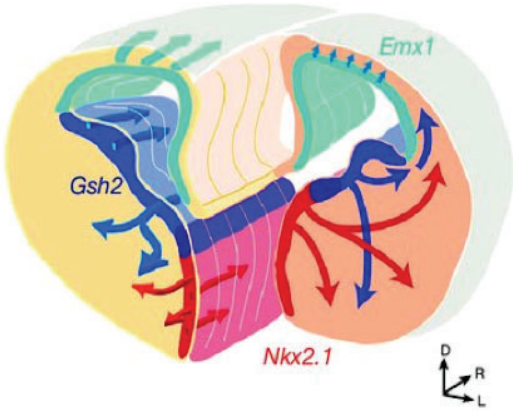

Figure 3 temporal gradient of OPCs production. *In the telencephalic subventricular zone (SVZ), three oligodendrogenic domains can be identified: Nkx2.1+ Gsh+ and Emx1+. During embryonic development, Nkx2.1+ OPCs are the first to appear, although the cells originated in this ventromedial domain die later in development, while the OPCs generated in the first two domains colonize the basal and lateral telencephalon, those generated in the Emx1+ domain will myelinate the entire neocortex (together with a minor contingent entring from the upper GsH2+-domain) (de Castro e Zalc, 2013)*

OPCs can maintain a tight balance between survival, proliferation, and differentiation. This balance is crucial to keep the OPC pool during adult life, when myelin is fundamental for brain physiological plasticity, as well as to remyelinate in diseases, including primary demyelinating disorders like multiple sclerosis (MS; Marie et al., 2018). Although we know that numerous extrinsic signals and transcription factors are involved in this process, the complete explanation of how these signals are integrated into the nucleus to regulate the OPC behavior is still unknown (Emery e Lu, 2015; Bergles e Richardson, 2015).

OPCs exhibit a unipolar morphology in the early stages of their maturation. They proliferate in response to the Platelet-Derived Growth Factor (PDGF-AA), Fibroblast Growth Factor type 2 (FGF-2), and Sonic Hedgehog (Shh), which are known for their mitogenic role (for a specific review, see: Rowitch e Kriegstein, 2010; de Castro e Zalc, 2013). During migration, OPCs acquire a bipolar morphology and become respondent to chemoattractant and chemorepellent signals, such as secreted semaphorins, PDGF, FGF-2, VEGF or HGF, extracellular matrix components such as laminin, fibronectin, vitronectin, tenascin-C, and anosmin-1, as well as molecules associated to axonal

guidance such as semaphorins, netrin-1 and CXCL1. In addition to that, endothelial cell signals seem to be essential for OPC migration through the interaction between the CXCR4 from the OPC surface and CXCL12 of the endothelial cells (D. H. Miller et al., 2002; Spassky et al., 2002; Tsai et al., 2002; H. Zhang et al., 2004; Bribián et al., 2006; Okada et al., 2007; Bribián et al., 2008; Hayakawa et al., 2011; Murcia-Belmonte et al., 2016; Tsai et al., 2016; for specific reviews on this subject, see: de Castro e Bribián, 2005).

Upon arrival at their destination, the OPC population expands to reach the homeostatic equilibrium. The OPC expansion is mediated by the combination of proliferation activating signals and the differentiation inhibiting signals. For example, the process involves the stimulation of the growth factor PDGF and the inhibition of the Wnt and Notch signaling pathways, as well the activation of the transcription factors Olig1/Olig2 and Sox10 through the differentiation inhibitors ID2, ID4, and Hess5 (Fancy et al., 2011; Kremer et al., 2011).

The inhibition of proliferating factors allows the activation of an intrinsic complex network of transcription factors including Nkx2.2, Olig1/2, Sox10, and MyRF (myelin regulator factor, H. Li et al., 2009; Emery, 2010). Those factors are involved in the maturation of OPCs into differentiated oligodendrocytes. Mature oligodendrocyte acquires a ramified morphology and, with their plasma membrane, the capacity to ensheath neuron axons, thus reaching a final size proportional to the myelinated axon (Fig. 4) (D. H. Miller et al., 2002; de Castro e Bribián, 2005). The helix-loop-helix transcriptional factor Olig2, for example, is expressed throughout the OL lineage (Fig. 4). It is shown that the induction of Olig2 expression leads to increased OPC production, while the loss of Olig2 during development suppresses their generation (Ligon et al., 2006; Maire et al., 2010). Nevertheless, in Olig2 null mutant mice, the OPC production is not entirely abolished, maybe due to the compensation achieved by Olig1, other members of the same family of transcription factors (Lu et al., 2002).

Sox10 is another marker for the OL lineage required for the final differentiation of these cells (Fig. 4; Stolt et al., 2004). Sox10 can cooperate either with Olig1 or MyRF to drive the transcription of myelin genes and myelin formation.

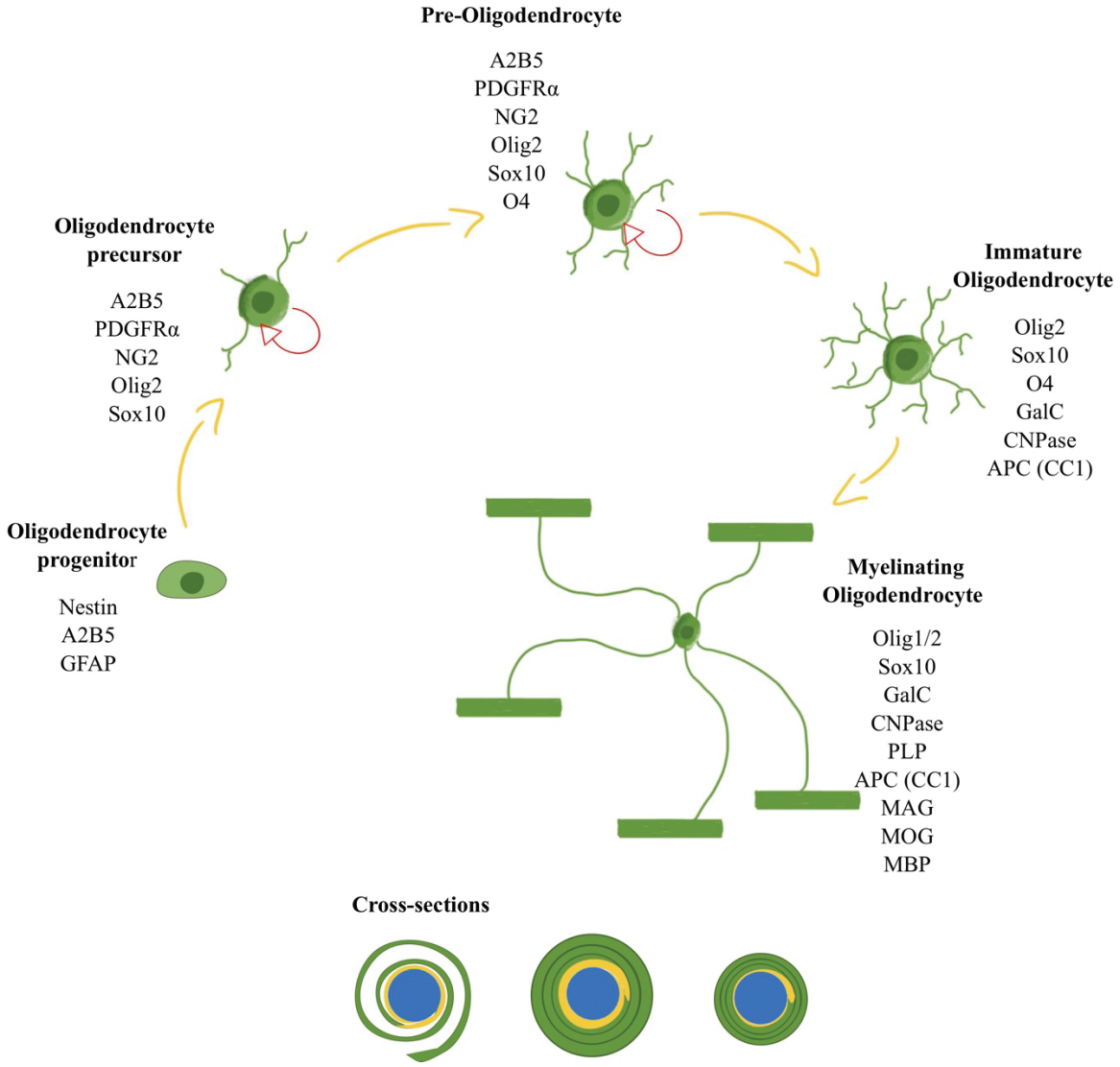

Figure 4 Oligodendroglial markers along maturation. *During the maturation process, oligodendrocyte progenitors undergo progressive differentiation until they become mature oligodendrocyte, with the ability to myelinate. These cells produce large membranous extensions that wrap the axons in successive layers that are then compacted by the exclusion of cytoplasm to form the myelin sheath. It should be noted that only OPCs and pre-oligodendrocyte possess a certain capacity for self-renewal during development and in adult*

Myelination starts with the extension of many cellular processes that contact the axon and form a specialized membrane that allows the communication between the oligodendroglial process and axon. The glial process then expands laterally along the axon and wraps the entire internodal length until forming a dense layer of highly compacted cell membrane. The lateral cytoplasmatic domain at the edge of each myelin layer remains uncompacted and in contact with the axonal membrane, forming the paranodal loops that will flank the future node of Ranvier. Most OLs generate about 30

myelin sheaths, and some may even extend up to 100 membrane turns (Young et al., 2013; Fields, 2014). In this way, it has been estimated that one oligodendrocyte may sum up to 5,000-50,000 μm^2 of myelin membrane, a surface that any other cell in the body reaches (Pfeiffer et al., 1993).

As mentioned before, not all the OPCs generated during embryonic development mature and differentiate: many of them persist in the adult CNS white and gray matters. These OPCs are capable of proliferating and differentiating into myelin-producing oligodendrocytes in vivo and in vitro. In the adult mouse, these OPCs represent about 8-9 % of the white matter total cells and 2-3% of the grey matter (Dimou et al., 2008; Mangin e Gallo, 2011; de Castro e Zalc, 2013). The primary function of OPCs is to replace the oligodendrocytes that die and, in this way, maintain the myelin sheath of the axons and the proper functioning of the CNS. They also have an essential role in cell repair since they can replace OLs destroyed by pathologies affecting white matter. The reestablishment of myelin sheaths involves several steps: 1) factors produced by astrocytes and microglia after demyelination are able to activate OPCs, switching from a resting state to a regenerative phenotype; 2) once activated, OPCs respond to different mitogens, mitogenic and chemotropic factors and are able to populate demyelinated areas and 3) these already recruited OPCs begin to differentiate into mature myelin-producing oligodendrocytes, which repair the damage caused (Franklin e Ffrench-Constant, 2008; Bradl e Lassmann, 2010; de Castro e Zalc, 2013). Therefore, the existence of OPCs in the adult CNS is a crucial fact to keep in mind as they are cells that, if the environment is favorable, could migrate to areas of demyelination, representing great potential for lesion repair. This is why OPCs are under the focus of many studies in demyelinating diseases as multiple sclerosis (MS). Among this research, it is essential the study the molecules that control their survival, proliferation, migration, and differentiation, both during normal development and in pathological scenarios, like this present research.

Myelination in humans

As in other mammals, myelination in humans begins before birth in the caudal brain stem and progresses rostrally to the forebrain. During the first year, myelin spreads through the entire brain, proceeding from the central to superficial, and from the occipital and parietal lobes to the frontal and temporal lobes (Barkovich e Kjos, 1988; Aubert-Broche et al., 2008). Myelination is a continuous process that pursues beyond adolescence and into early adulthood (D. J. Miller et al., 2012). Even more surprising is the myelination of the corpus callosum (CC; one of the associative areas of the cortex that allows the communication between the two hemispheres), where myelination begins around the third month of life, but it does not end until around the age of twenty-five, or even more. Magnetic Resonance Imaging (MRI) studies showed that although the CC is not myelinated at birth, there are 70% of myelinated axons in the human adult, while the other 30% remain unmyelinated (Fields, 2008).

Several studies highlight how practicing a skill can increase the volume of white matter employed in executing the task. The one led by the Swedish Ullém's team demonstrated how extensive piano practicing has a regionally specific effect on white matter development (Bengtsson et al., 2005). In 2012, it was observed in a group of adults learning a foreign language, a link between the learning progress and the myelination of some brain bundles associated with the language area at the left hemisphere, but also of the frontal lobe that crosses the CC´s knee (Schlegel et al., 2012). Conversely, social isolation, with reduced external stimuli, leads to hypomyelination and impaired cognitive functions. Thus, once myelination of all brain regions is completed, new myelin production is still possible, suggesting that brain activity can impact new myelin production even in adolescence and adulthood. However, in contrast to observations in mice, myelination in adult humans seems to be carried out exclusively by mature OLs generated during development.

Studies of the human OPC development remain limited at the cellular level due to the material that encompasses a wide age range. OPCs are known to appear in the anterior brain around the 10th gestational week. Around the 15th gestational week, they can be observed in the forebrain ganglionar eminences and subventricular zone. Leong and colleagues, in 2014, described different human populations of OPCs, based on the

expression level of the markers A2B5 and O4 in nervous tissue of embryos (15-20 gestation week) and adult (26-39 years old). Using Fluorescence-Activated Cell Sorting (FACS), they showed that most OPCs are positive for A2B5 in neonates, while in adults, they are mainly positive for O4.

Recently, an interesting *ex-vivo* study analyzed the gene expression of oligodendroglial cells isolated from surgical biopsies of fetal, pediatric, adolescent, and adult specimens by single whole-cell RNA sequencing. The analysis of 5,613 genes identified three distinct cellular subpopulations: the early-OPC, the late-OPC, and the mature OL. They found age-specific differences in the distribution of these subpopulations. While early-OPCs were detected only in the fetal group and a minimal percentage in the pediatric group, the late-OPCs were the main population represented at the peak of myelination (pediatric) and the only present in adulthood. Moreover, they found that top biological pathways enriched each subpopulation in the oligodendroglial lineage. For example, in early-OPCs, the biological processes implicated in the cell cycle, microtubule cytoskeletal organization, and chromatin conformation are upregulated. In late-OPCs, the extracellular matrix, lipids, and involved in myelination are upregulated. Finally, mature OLs upregulate pathways involved in myelination, cell transport regulation, organelle organization, and intracellular iron sequestration (Perlman et al., 2020). Overall, those studies provide insights into the identity of cell subpopulations within the oligodendroglial lineage that differ with respect to maturity and functional properties.

Oligodendroglia and myelin in pathological conditions

One of the unique qualities of OPCs is their ability to respond to several types of CNS injury that may provoke the death of the OLs. Several studies have been reported the increase of the levels of OPCs such as traumatic brain injury, ischemia, vascular brain disease, spinal cord injury, and reactive OPCs are also found in neurodegenerative disease or schizophrenia (McTigue et al., 2001; Kang et al., 2010; Goldman et al., 2012; Zhang et al., 2013).

Destruction or loss of myelin results in impairment of the saltatory conduction that perturbs the neural circuitry and could lead to neurodegeneration if sustained. Among the primary demyelinating diseases, the more frequent is MS. MS is defined as a chronic, autoimmune, and neurodegenerative disease characterized by partial loss of central myelin, axonal damage, and CNS parenchymatous inflammation/immune cells infiltration. Myelin destruction leads to the subsequent formation of sclerotic plaques found in any location in the CNS, white and gray matter (Lassmann, 2018). Three kinds of lesions have been distinguished: active, chronic-active, chronic-inactive, depending on the ability to spontaneously remyelinate and on the presence and distribution of infiltrated immune cells (for a specific review, see Lassmann, 2018; Absinta et al., 2019)

The neurological symptomatology in MS is variable and can be total or presented as episodes of partial and reversible affectation, lasting days or weeks. The main clinical signs, depending on the affected CNS area, include vision loss, sensorial and motor dysfunction, fatigue, neurocognitive changes, depression, or urinary, digestive and sexual dysfunctions (Compston e Coles, 2008). Those symptoms can be diverse and confusing, often coming and going without any clinical patterns, making it difficult to diagnose. In MS, OPCs spontaneously migrate to the area of injury and differentiate into mature oligodendrocytes that can partially replenish the myelin sheath lost, forming the so-called "shadow plaques" that determine the clinical evolution of symptoms (Frohman et al., 2006; Clemente et al., 2013; Frischer et al., 2015).

Even if remyelination can be efficient in the early stages of MS, its effect seems to be limited in the long term, mainly because of the reduced capacity of adult OPCs to

proliferate and differentiate into myelin-forming oligodendrocytes (Franklin et al. 2012). The reason for this reduced differentiation remains unknown but points to the predominance of inhibitory signals present in the extracellular environment (Zhou et al., 2012; Franklin e Goldman, 2015; Baxi et al., 2017).

Most of the currently available therapies of MS are immuno-modulatory, immunosuppressants, and anti-inflammatory drugs, and they focus on the reduction of the inflammation in the CNS, and therefore on the intensity and frequency of the clinical relapses, but they do not face the issue of the lesion remyelination (Steinman et al., 2005). The need to target adult OPCs to promote remyelination in demyelinating diseases such as MS is an important therapeutic goal (Fernandez-Castaneda e Gaultier, 2016). Current clinical trials evaluating neuroprotective and/or remyelinating drugs search to induce regeneration or repair damaged myelin (Green et al. 2017).

Animal model of MS

MS is characterized by several clinical patterns that involve immune-mediated inflammation and primary oligodendrogliopathy. Nowadays, more and more researchers are focused on understanding why OL fails to remyelinate to develop new treatments to restore myelin.

Due to the great difficulty of accessing to the brain to study the mechanisms underlying CNS disorders, experimental animal models are a helpful tool to enhance the understanding of the molecular pathogenesis of these disorders. As no such model is comparable to MS, multiple models are used to reproduce the several features of this disease; each one mimics only a part of MS pathology. Thus, the choice of the model depends on which feature of the disease has to be elucidated. Table 1 (adapted from Gudi et al., 2014) summarizes the common experimental approaches to induce demyelination in rodent CNS.

Table 1. Advantages and disadvantages of different MS animal model

Animal model	Advantages	Limitation
Experimental Autoimmune Encephalomyelitis (EAE). Adoptive transfer of myelin reactive T cells induced by myelin peptides (MBP, PLP, MOG). (Most frequently used model).	-Mimic Relapsing-Remitting MS symptoms and pathology. -The interaction between various immunopathological and neuropathological mechanisms leads to an approximation of the key pathological features of MS: inflammation, demyelination, axonal, and gliosis. -Display a relatively sensitive readout system. -Used to test putative immunosuppressive and neuroprotective drugs, to study the response of the immune cells' types and their interplay with the resident glial cells, mechanisms of neuronal damage and loss, to study BBB dysfunction.	-Required adjuvant to activate the innate immune system -High complexity due to involvement of different cell type -High variety in susceptibility to EAE dependent on strain, gender, or even different animal colonies -Does not reflect the aspect of progressive MS. -Unpredictable localization of lesions that classically are analyzed in the spinal cord. -Not useful for remyelination studies since both de- and remyelination can proceed simultaneously.
Viral murine encephalitis (TMEV, MHV, SFV)	-Supports the environmental compound (early infections) of MS etiology -Useful to study mechanisms of viral infection of neuronal, glial cells and viral persistence. -Useful to study immune-mediated and virus-triggered demyelination and test different regenerative, neuroprotective, and immunosuppressive therapeutics.	-The assessment of remyelination is at least problematic since both de-and remyelination can proceed simultaneously -Some protocols require surgery -Difficult to determine to what extent disease is caused by virus activity or by the inflammatory response. -C57Bl/6 mice clear TMEV → difficulties in using genetically modified strains
Cuprizone (systemic oral application)	-Easy reproducible -Predictable kinetics of de- and remyelination -Clear detection/evaluation system. -Simple induction protocol -Can be induced in different rodents and strains	-Irreversible damage of mature oligodendrocytes via a toxin of an unknown mode of action -T, B cells independent → not useful for the development of immunomodulating drugs.

	-Available as acute and chronic demyelination model. -Useful to study remyelination and cellular behavior in the absence of peripheral immune cells. -Localised lesion.	-No useful clinical readout. -Not useful to study spinal cord demyelination since demyelination occurs only in the brain. -Time-consuming (acute model: 5–6 weeks; chronic: 12 weeks).
Lysolecithin and Ethidium bromide (focal injection of a toxin)	-Well reproducible -Predictable kinetics of de- and remyelination -Localised lesions can be induced in the brain or spinal cord. -Useful to study remyelination and cellular behavior -Useful to test putative immunosuppressive, neuroprotective, and especially regenerative agents	-Artificial way of demyelination induction due to irreversible selective damage of myelin-producing cells in the lysolecithin model. Ethidium bromide damages all nucleolus containing cells. -T, B cells independent → only of limited relevance for MS - Injection procedure damage tissue -High complexity and difficulties in assessing remyelination in the spinal cord since different myelinating cells are involved in the remyelination.

Considering the pros and contras of animal models for MS, for the present study, we chose the cuprizone model (see below). Cuprizone model allows us to study demyelination and remyelination in a defined white matter tract without immune system involvement.

THE MORPHOGEN SONIC HEDGEHOG

In mammals, the Hedgehog (Hh) pathway is composed of three family members named: Indian Hedgehog (Ihh), Desert hedgehog (Dhh), and Sonic hedgehog (Shh), due to their somewhat spiky appearance. Sonic Hedgehog is the most studied (and yes, his name comes from the comic book that then became a videogame!). Shh is classified as a morphogen and acts as a signaling molecule that elicits several cellular responses depending on its concentration, driving the organization of regional group of cells into patterns. The absolute concentration of the morphogen acting in the cell determines its developmental fate (Gurdon e Bourillot, 2001).

Once produced, Shh undergoes post-translational modifications. The precursor protein is first cleaved and then becomes biologically active by adding cholesterol at the C-terminus and palmitate at the N-terminus (ShhNp), forming a high molecular weight complex (Dessaud et al., 2008). Shh binds the Patched1 (Ptch1) receptor of the responding cells, relieving the inhibition on the seven-pass transmembrane G protein-coupled receptor Smoothened (Smo). Thus, the activated Smo initiates an intracellular signaling cascade that involves the transcription factors of the Gli-A family. Eventually, Gli is transported toward the nucleus and leads to the transcription of target genes (Fig.5 A). The hedgehog-GLI pathway plays a critical role in controlling cell specification, cell-cell interaction, and tissue patterning during embryonic development (Briscoe e Thérond, 2013).

Besides the classical canonical Shh-Ptch1-Smo route, additional non-canonical pathways involving Shh exist. In the last few years, it was proved the existence of at least two types of non-canonical Hh signaling, both of them occurring through Gli-independent mechanisms:

- Type I signaling involves the activation, by Smo, of couple Gi proteins (G), a small GTPases RhoA and Rac, ending with calcium (Ca^{2+}) release (Fig.5 B).
- Type II signaling is not related to Smo. The binding of Shh with Ptch disrupts the interaction between Ptch and Cyclin B1, leading to increased cell proliferation and survival (Fig.5 B; Carballo et al., 2018).

Cyclins act as regulatory subunits for cyclin-dependent kinases and are responsible for regulating the cell cycle progression (Murray, 2004). In particular, the cyclin B1-Cdk1 complex is involved in the G2/M checkpoint (essential for mitotic progression) (Takizawa e Morgan, 2000).

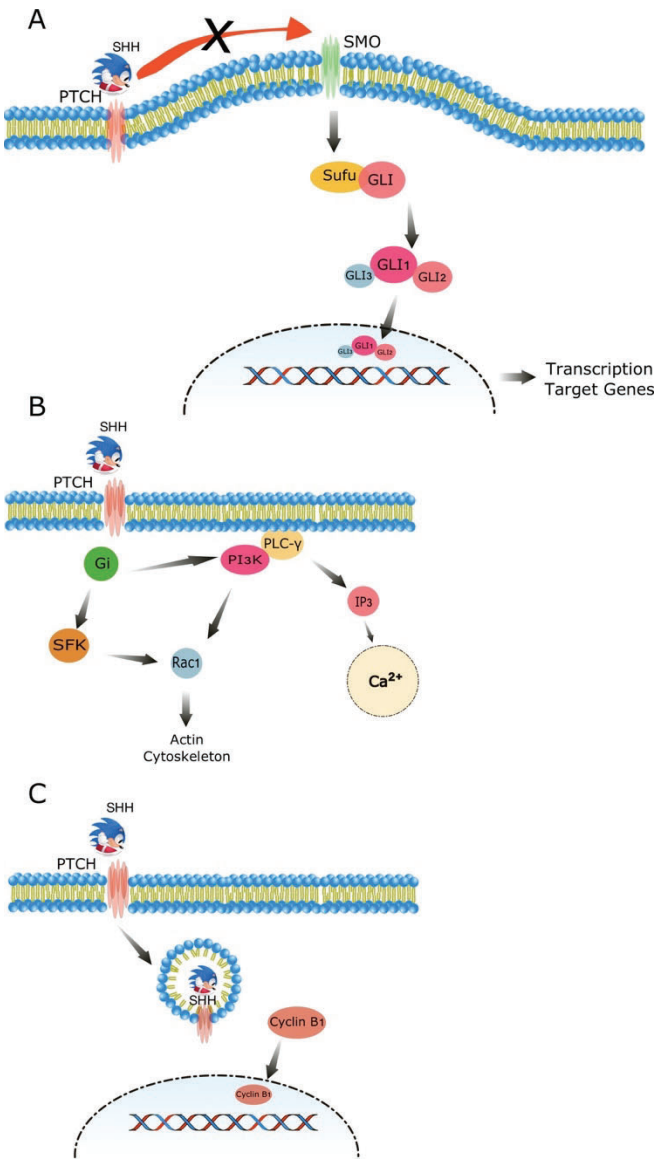

Figure 5 Shh signaling cascade

A. The canonical activation of Shh pathway in vertebrates. The activation occurs by ligand-dependent interaction when Shh binds to Ptch at the cell membrane. In response to this binding, Ptch no longer inhibits Smo, which accumulates at the primary cilium (PC) initiates the downstream signaling pathway cascade. When Gli is activated, it translocates to the nucleus, where it activates target genes.

B-C. The non-canonical activation of Shh pathway. *The non–canonical activation occurs through Gli-independent mechanisms and it can be of two types:*

- Type I which modulates Ca^{2+} and actin cytoskeleton (center). When Shh binds the receptor Ptch, Smo is no longer inhibited and couple Gi proteins (G) and small GTPases RhoA and Rac1 activated. In addition, Smo stimulates calcium (Ca^{2+}) release from the endoplasmic reticulum (ER) and PLC-γ-catalyzed the opening of IP3-dependent channels by the generation of IP3. C)

-Type II which is independent of Smo. When Shh binds Ptch, the interaction of Ptch with cyclin B1 is disrupted, leading to an increase in cell proliferation and survival (right). (Adapted from Carballo et al., 2018).

However, unraveling the exact function of Shh pathways has turned out to be complicated, not only because Shh is implicated in different processes at different developmental times but also because of the discordant findings published by several research groups.

Sonic Hedgehog signaling involvement in oligodendrogenesis.

The involvement of Shh in oligodendrogenesis has been specially studied in the spinal cord. During embryonic development within the spinal cord, functionally distinct neurons are generated in a spatially segregated manner in response to the crucial release of morphogen and extracellular signals emanating from the neural tube and the surrounding tissue. Some of the most studied counteracting gradients of long-range signals include Shh. It is secreted by the notochord and floor plate that induces ventral identities, while Wnt and BMP, produced dorsally, promote dorsal identities (Ulloa e Briscoe, 2007). The spread of Shh from ventral to dorsal establishes a gradient of activity within the ventral neural tube necessary to determine the neural progenitors pattern of specification (Fig. 6A). BMPs are also involved in establishing this activity gradient (Dessaud et al., 2008; Balaskas et al., 2012). The time at which neuronal progenitors exit the cell cycle and differentiate also influences cell fate. In this way, Shh can generate five distinct progenitor domains, each one characterized by a molecular code for the specification of the various ventral neuronal subtype (Fig. 6B). (J. Briscoe et al., 2000; Balaskas et al., 2012). In particular, the three most ventral progenitor domains, pMN, p3 and FP, are identified by the expression of Olig2, Nkx2.2, and Foxa2 transcription factors (from dorsal to ventral, respectively). The expression domain of Olig2, pMN, expands dorsally, and it is downregulated in the cells expressing Nkx2.2. Nkx2.2 and Olig2 give rise to motoneurons and oligodendrocytes (Dessaud et al., 2008; Balaskas et al., 2012). Independent groups evaluated the time-window during which Shh is required for the specification of OPCs by using floorplate ablation and/or blocking Shh antibody. The different results reported suggest that Shh is likely active at that time. The progenitors lose their competence to generate MNs or floorplate cells in response to Shh, and then, Ptc (Shh receptor) is expressed by the neuroepithelial cells of the ventricular domain producing OPCs (Fig.6 C; Orentas et al. 1999; Soula et al., 2001).

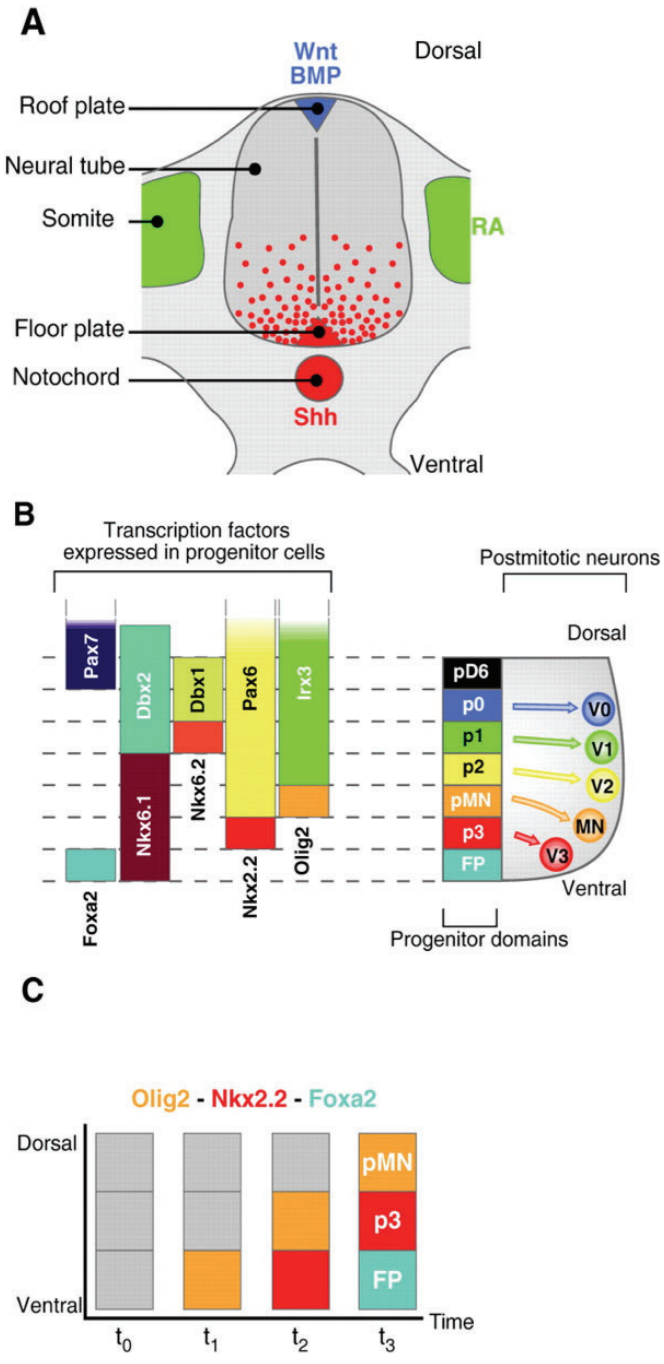

Figure 6 Secreted signals establish the dorsal-ventral pattern of progenitor domains in the neural tube by regulating the spatial expression of transcription factors.

A*Schematic of a transverse section of an amniote embryo. The key signals Shh (red), secreted by the notochord and floor plate; retinoic acid (RA, green), produced by the somites that flank the neural tube; and BMP and Wnt family members (blue), which are produced dorsally. The spread of Shh from ventral to dorsal establishes a gradient of activity within the ventral neural tube (red dots).*

***B** Schematic of the ventral half of the neural tube, where the ventral gradient of Shh activity controls position identity by regulating the expression, in neural progenitors, of a set of transcription factors. These include Pax7, Pax6 and Irx3 (repressed by Shh signaling), Dbx1, Dbx2, Nkx6.1, Olig2, Nkx2.2 and Foxa2 (require Shh signaling). The differential response of these genes to graded Shh signaling establishes distinct dorsal and ventral boundaries of expression for each factor. The combinatorial expression of the transcription factors defines domains of progenitors (p). Each progenitor domain is identified by its transcription factor code, and this code determines the neuronal subtype progeny the progenitors produce.*

***C** The three ventral-most progenitor domains of the neural tube, FP, p3 and pMN, can be identified by the expression of the transcription factors, Foxa2, Nkx2.2 and Olig2, respectively. Their expression follows a dorsal-to-ventral progression, resulting in the temporally distinct establishment of each progenitor domain.*

Initially, ventrally located progenitors express Olig2 prior to the initiation of Nkx2.2 and Foxa2As the expression domain of Olig2 expands dorsally, Nkx2.2 and Foxa2 are induced ventrally. Olig2 is then downregulated in cells expressing Nkx2.2. Hence, Nkx2.2 expression defines the ventral limit of the Olig2-expressing, pMN domain. Subsequently, in cells of the ventral midline, Nkx2.2 expression is downregulated by an as yet undefined mechanism. This generates a Nkx2.2+ Foxa2- p3 domain, and a Foxa2+ Nkx2.2- FP. One consequence of the progressive induction and modification of ventral progenitor identity is that Nkx2.2- and Olig2-expressing cells share a lineage (Adapted from Dessaud et al., 2008).

Shh signaling also regulates cell proliferation and fate in the developing forebrain and hindbrain (Machold et al., 2003; Blaess et al., 2006; Balordi e Fishell, 2007a;2007b; Xu et al., 2010). Also, it is important in the initial generation and proliferation of postnatal NSCs in the SVZ and DG, but whether it has a role in the specification of regional identity in the adult is not yet known (Lai et al., 2003; Balordi e Fishell, 2007b; Han et al., 2008). Álvarez-Buylla and his collaborators found a neuronal source of secreted Shh ligand close to the ventral SVZ. They show how this pathway is sufficient to determine neuronal cell fate also in adult SVZ NSCs(Ihrie et al., 2011). However, Sánchez and Amstrong (2018) used transgenic mice to show how Shh ligand synthesis is prevalent in neurons, while Gli1 transcription is detected in cells that populate the CC during postnatal myelination. They also found Gli1 fate-labeled cells in the adult. $Gli1^{+}$ cells included cycling progenitor cells revealing that Shh signaling contributes to the oligodendrogenesis during CC myelination and adulthood.

Shh signaling is also required to regulate the postnatal wave of OLs production that populates the CC prior to myelination (Kessaris et al. 2006; Ihrie et al. 2011; Sánchez e Armstrong 2018). Moreover, in the embryonic mouse, Shh cascade cooperates with FGFRs to generate OPCs in the ventral forebrain (Furusho et al. 2011). Consistent with this, precursors isolated from the medial ganglion eminence E14 mice responded to PDGF, FGF2 and Shh by increasing the proliferation and/or survival of these cells. The same effect was found on the neonatal purified cortical OPC cultures (Lelievre et al., 2006), while it was found reduced when inhibiting the Shh pathway with the Shh inhibitor cyclopamine (Chojnacki e Weiss, 2004). Similarly, Kessaris et al. (2004) and Furusho et al. (2011) showed that the generation of Shh-induced OPCs required a basal level of the phosphorylated mitogen-activated protein kinases (MAPKs). However, only FGF2 increased MAPK activity, suggesting that Shh effect depends on FGF activity (Kessaris et al., 2004; Furusho et al., 2011). All these evidences suggest an intracellular cross-talk between Hh and FGF signaling pathways. Furthermore, several groups reported cyclins being an essential player in mediating OPC proliferation via Shh signaling by regulating the cell cycle progression (Casaccia-Bonnefil e Liu, 2003; Murray, 2004; Xu et al., 2020)

Despite the bulk of studies that outline the relevance of Hh signaling in the generation

of OPCs, there are controversial reports on its role in the regulating of OLs differentiation and maturation.

Shh role in demyelinating disease

After a demyelinating insult, there is an upregulation of several genes involved during oligodendroglial development, like the transcription factor Olig2 and NKX2.2 (Watanabe et al., 2004; Fancy et al., 2004). Furthermore, local adult OPCs or CNS stem cells undergo a switch from a quiescent state to a regenerative phenotype. They are induced to proliferate and migrate by the grow factors PDGF and FGF2, both of which are upregulated during remyelination (Hinks e Franklin, 1999; Levine e Reynolds, 1999; Messersmith et al., 2000; Reynolds et al., 2002; Dawson et al., 2003; Sim et al., 2006). After reactivation and proliferation, the adult OPCs must differentiate into remyelinating OLs following three steps: 1) establish contact with the axon to be remyelinated, 2) express myelin genes, and 3) generate a myelin membrane that finally wraps and compact the membrane to form the sheath.

During demyelinating diseases, such as MS, remyelination failure may be associated with either insufficient recruitment of OPCs to the lesion site or, more commonly, failed OPCs or OLs differentiation. At present, it is not possible to establish which of the two modus operandi is required for successful remyelination because there are no biomarkers or imaging strategies able to predict if pro-recruitment or pro-differentiation therapies would be appropriate (Franklin e Ffrench-Constant, 2008). Moreover, even if there have been many similarities between development and regenerative processes governing the phases of OPCs proliferation, maturation, and myelination, differences in this regulation do occur. For example, a clear beneficial effect of PDGF on remyelination in chronically demyelinated lesions induced by cuprizone was proved, showing that this factor has not only a mitogenic effect but may work differently in specific lesion types. Other factors as Olig1, which is not essential for developmental myelination, instead are required during remyelination (Arnett et al., 2004; Vana et al., 2007; Aguirre et al., 2007).

In addition to participating in myelination processes during the development of the CNS, Shh relationship with demyelinating pathologies has been described both in

humans and in various demyelination models, showing its implication in tissue repair. Studies in samples of patients with MS show an increase in immunoreactivity for Shh in hypertrophic astrocytes of chronic-active lesions, located mainly in their periplaque and in the adjacent normal-appearing white matter (NAWM) (Wang et al., 2008). They also observed a lower expression of the transcription factor Gli1 both in chronic-active and chronic-inactive lesions but not in the active lesion. These results seem to suggest a positive involvement of Shh cascade in injury areas as response to the beginning of the inflammation. The chronic inflammatory environment becomes unfavorable and blocks the repair process by inhibiting Shh signaling. In the same work, the authors have shown similar results in murine EAE. They noted upregulation of Shh, Smo and Gli1 during early inflammation before the onset of clinical signs. Despite an initial increase, Gli1 expression markedly decreased after the onset and throughout the disease. They finally concluded that cell response to Shh is attenuated by inflammation, causing the Gli1 down-regulation (Yue Wang et al. 2008).

In 2013, the studies carried out by the scientist Ferent confirmed, in part, the results obtained by Wang et al. (2008). Using a model of focal demyelination induced by lysolecithin (LPC) in the CC of adult mice, Ferent et al. (2013) reported a time-dependent broad reactivation of Shh pathway. Firstly, OPCs trigger Shh cascade in the area overlapping the LPC-induced demyelination at the earliest step of repair, as well as Smo and Gli1. Shh started to decrease and stayed at similar level at late phases of repair while Smo and Gli1 continued to increase. Shh signal was visible in cells expressing the OPC marker PDGFRα and OL lineage marker Olig2. To study Shh involvement, they used, on the one hand, an adenoviral vector to transfer Shh at the level of the LPC-induced demyelination, and, on the other hand, they transferred a Shh antagonist. In the first case, Shh promoted OPCs proliferation, survival and differentiation, attenuating the lesion. In the second case, the antagonist had the opposite effect preventing tissue repair (Ferent et al., 2013).

In the last few decades, conditional transgenic animals have helped research to map the fate of cell populations. It has also given the possibility to study the effect of a gain or loss of function in specific cells in a determined period by controlling, in this way, the expression of target proteins in space and time. Samanta et al. (2015) used Gli1

transgenic mice to study remyelination in cuprizone, another CC demyelination model. They could map the destiny of cells expressing this transcriptional factor and also downregulate its expression. The results obtained confirmed the activation of Shh pathway (through Gli-1), and they found that $Gli1^{+}$ cells were recruited to the demyelinating areas. They claimed that in a healthy adult brain, the mapped cells correspond to NSCs in the SVZ and a subset of astrocytes, but not OPCs. After the cuprizone model, $Gli1^{+}$ NSCs were recruited in the CC and continued to generate glial cells for a prolonged period after demyelination, most of which were oligodendroglia. In contrast with previous results, they found that genetic downregulation of Gli1 ($Gli1^{null}$ -mice), or its pharmacological inhibition, amplified the recruitment of NSCs into the CC and increased their differentiation into mature OL and remyelination (Samanta et al., 2015).

Although Shh signaling involvement is a shared feature in the works mentioned above, the authors disagree about the source of Shh and the target cells. Sánchez and Armstrong (2018) answered this question using two transgenic mice lines. One transgenic line was used to map the cells expressing Shh, while the other one to map the cells expressing Gli1. The authors discovered that the fate of cells labeled at postnatal day P6-9 showed Shh ligand synthesis mainly in the neurons. At the same time, Gli1 was detected in cells that populate the CC during postnatal myelination and in adults (P56)(Sánchez e Armstrong, 2018). Moreover, the cells labeled at P6-9 were also found in the CC after the demyelination induced by cuprizone. These cells corresponded to OPCs and were able to proliferate and to contribute to CC remyelination, suggesting the involvement of Shh pathway during myelin repair. However, when the authors used the same transgenic lines to label Shh^{+} and $Gli1^{+}$ cells during or after the acute or chronic cuprizone-induced demyelination, no Shh or Gli1 signal was found in CC. Finally, they microinjected SAG, an agonist of both canonical and non-canonical Hedgehog signaling pathway, after chronic demyelination and noted an increase in proliferation and remyelination. SAG did not increase Gli fate-labeled cells in CC, indicating signaling through the non-canonical pathway (Samanta et al., 2015).

Up to date, it is unclear how to reconcile the results concerning the functions of Shh signaling activity during remyelination. Since Shh pathway seems to regulate proliferation, cell cycle, survival and differentiation, an obvious question is how a single

signaling pathway can control these distinct cell behaviors.

HYPOTHESIS

The myelination process is vitally important for a healthy Central Nervous System physiology. Since its discovery, more and more functions have been attributed to myelin. Its absence, leads to the emergence of pathologies that reduce the quality and lifespan. However, many questions remain regarding the complexity of this signaling activity during myelin regeneration. Therefore, one of the major therapeutic challenges is to design new treatments enhancing sequentially effective neuroprotection, OPCs proliferation, OL differentiation and axonal support.

The data accumulated over the years on the role of the morphogen Sonic Hedgehog signaling in the genesis of oligodendrocyte lineage demonstrates that Hedgehog is one of the essential pathways in this process. Shh has been shown to promote proliferation and migration on OPC during physiological development (Machold et al., 2003; Merchán et al., 2007; Ortega et al., 2012). Shh has been shown over-expressed in MS lesions (Wang et al., 2008; de Castro et al., 2013), and his pathway has the potential to regulate OPC responses that impact remyelination (Ahn and Joyner, 2005; Ferent et al., 2013; Samanta et al., 2015; Sánchez et al., 2018).

Although many works have shown the relevance of Shh pathway on the OPC physiology both during development and in pathological conditions, none of them explain if its effect is due to a direct or indirect mechanism. To date, no information has been reported about what happens if these signaling pathways are over or downregulated directly on the committed-OPCs.

Shh signaling is a good candidate mechanism for controlling OPCs proliferation during development since it is known to directly regulate proliferation in other contexts. However, it has been an open question whether and how it works. The work presented here aimed to investigate the Shh potential as (re)myelinating pathway in development and adult rodents. For this reason, we choose an *in vivo* approach using transgenic mice in which up or downregulate Shh signaling directly on committed-OPC. Moreover, through *in vitro* assay we studied the effects of this pathway on humans adult OPC.

Pharmacological approaches to remyelination represent a promising new frontier in the therapy of demyelinating pathologies, like MS, and might provide novel tools to

improve adaptive myelination in aged individuals. A deeper understanding of the implication of the components that regulate oligodendrogenesis and myelination should beneficially influence the therapeutic strategies in the field of myelin diseases.

AIM

1) Study the direct role of Shh pathway on committed-OPCs when major morphogenetic and proliferative processes are no longer in operation. Does Shh continue to contribute to the proliferation and differentiation of OPCs during late development?

2) Study the role of Shh pathway on the OPCs during the repair of demyelinated lesions. Is remyelination a recapitulation of the mechanism involved in the development, or is it different?

3) Study the Shh pathway role in relation to the surrounding environment.

MATERIAL & METHOD

ANIMALS

Mice were housed at Cajal Institute (Madrid) on a 12 h light/dark cycle with ad libitum access to food and water. All procedures were approved by a regional ethics committee CSIC (440/2016, Madrid, Spain) and Spain Authorization (RD 53/2013 and 178/2004, Ley 32/2007 y 9/2003, Decree 320/2010) in accordance with the European Communities Council Directive (2010/63/EU, 90/219/EEC).

Conditional mice

In the last few decades, conditional gene targets allowed the spatial and temporal control of genetic modifications. The most widely used approach to generate conditional mice is the Cre-loxP system that uses a site-specific recombinase, whereby a region of interest is flanked by two unique 34-bp sequences, identified as loxP sites. Recombination between loxP sites is catalyzed by Cre recombinase allowing deletion, inversion, insertion, or translocation of the gene of interest (Heffner et al., 2012). By using cell-specific drivers for Cre is possible to generate conditional mice that express the Cre-recombinase only in a target cell-type.

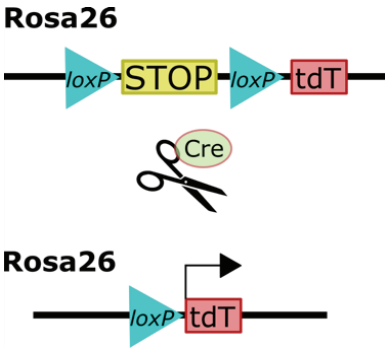

Figure 7 Cre catalize recombination between loxP sites.

To confirm that Cre-recombinase is active only in specific tissues/cells, reporters' strains have been developed. Those mice express a visible marker, such as green fluorescent protein (GFP), tandem dimer Tomato (tdTomato, tdT), lacZ, etc., only after Cre recombinase excises a lox-flanked stop sequence. For a good visualization of the reporters, it is also crucial to choose an appropriate promoter. The most commonly used promoter for ubiquitous expression of a transgene is the *ROSA26* promoter (Fig. 7). Rosa26 gene is located in chromosome 6, the loss of this gene is not lethal, and no affect the animal viability or fertility. Therefore, *ROSA26* locus is widely used as a permissive site for targeted placement of transgenes in mice. Finally, to add inducibility to the Cre/lox system, have been developed ligand-dependent chimeric Cre recombinases, CreER (Cre-Estrogen Receptor). The CreER recombinase is inactive, but it is highly sensitive to the synthetic estrogen antagonist tamoxifen. In this way, it is

possible the external temporal control of the Cre activity (see: Feil et al., 2009) for a more detailed description of the inducible Cre mice generation).

Although the Cre-lox system provides control over gene expression, when both the Cre recombinase and lox sites are present in the experimental mouse, it is possible to have unexpected transient expression of Cre recombinase in the germline or during early development. To avoid unwanted recombination events, it is important to use only heterozygous Cre recombinase mice.

NG2-CreERT2 Knock-In Mice:

Homozygous Wild-type (NG2-TdTomato/TdTomato) and heterozygous (NG2 CreERT2- TdTomato/TdTomato) were gently donated by Kirchhoff lab (University of Saarland, Homburg, Germany), bred in the animal facility of the Cajal Institute, and PCR determined their genotype at weaning. The original targeting strategy used for NG2-Cre TdT creation is described by (Huang et al., 2014) and summarized in Fig.8.

This mouse line has the new generation of the open reading frame of the tamoxifen-inducible form of the Cre DNA recombinase (CreERT2) inserted into the NG2 locus by homologous recombination (Fig.8 A). CreERT2 is released from cytoplasmatic sequestration and translocated to the nucleus (activated) in the presence of tamoxifen (R. Feil et al. 1996).

To visualize the Cre activity and to fate-map NG2 glia in vivo, NG2- CreERT2 mice were crossbred to the reporter mouse lines TgH(Rosa26-CAG-floxed-stop-tdTomato) (Rosa26-tdTomato) (Madisen et al., 2010). The reporter allele contains a floxed stop sequence upstream of tdTomato used as a readout of recombinase activity. tdTomato exhibits a short maturation time and excellent brightness (Fig. 8B; S. Li et al., 2018).

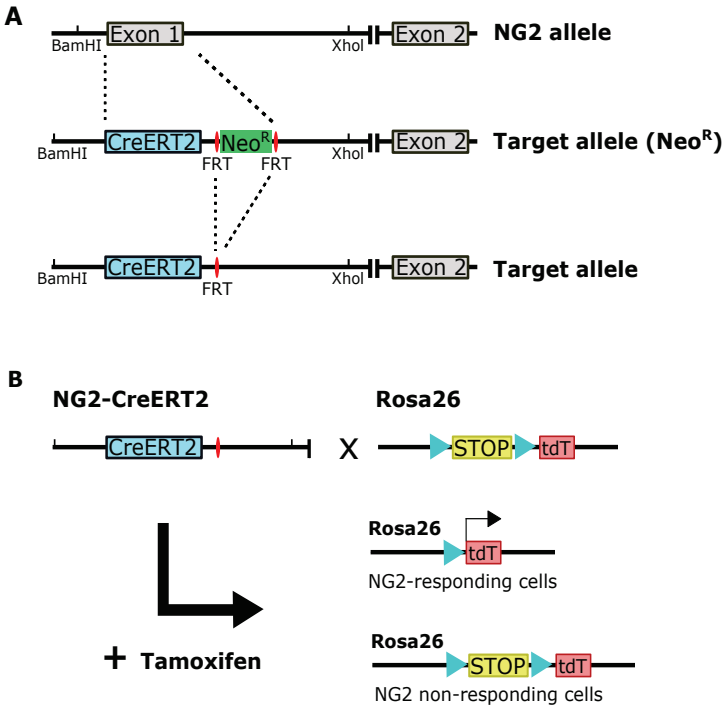

Figure 8 Generation of NG2-CreERT2 allele. ***A*** *Schemeatic representation of the targeting strategy. The first coding exon of the NG2 locus was replaced by the open frame of CreERT2 followed by a flanked Neo resistence cassette (FRT-Neo^R-FRT). The remotion of the Neo^R generate the final NG2-CreERT Knock-in mice. (See Huang at al., 2014).* ***B*** *NG2-CreERT2 were crossbred to a reporter mouse line: Rosa26-tdTomato (R26-tdT). In the absence of tamoxifen, CreERT2 is retained in the cytoplasm. Binding of tamoxifen results in the translocation of the recombinase into the nucleus where it causes recombination between its loxP sites (cyan triangle) in the R26-tdT allele, excision of stop sequence, and expression of the tdTomato. Permanently marked cells are detected by tdT activity (red fluorescence).*

Two cell populations are associated with the NG2 glycoprotein expression: OPCs and the vasculature pericytes (Stallcup, 2002; Horner et al., 2002). Those latter are easily distinguished by their elongated morphology along the brain vasculature, and they are not labeled with any other OL lineage marker (Huang et al., 2014). Compared with other transgenic NG2-Cre lines created with non-homologous recombination strategy (Zhu et al. 2011), the NG2-CreERT2-tdT mouse facilitates reliable gene targeting in NG2-expressing cells and their oligodendroglial progeny at different developmental stage, displaying higher recombination efficiency. For the subsequent experiments, only heterozygous NG2 CreERT2 and Rosa26-tdT were used (NG-2-Cre+/- and Rosa26-tdT +/-). The progeny was not reporting any alteration after birth.

SmoC mice:

The Smoothened (Smo) conditional allele Smo^C (*Smo$^{flox/flox}$*) mice were purchased from The Jackson Laboratory (JAX stock #004526, Bar Harbor, ME). These floxed mice possess loxP sites on either side of exon 1 of the Smo (smoothened) gene. In this way, exon 1 is successful excise when bred to a strain expressing Cre recombinase (Fig.9A). The mice did not report any gross physical or behavioral abnormalities, and they result viable, fertile, and normal in size.

R26-SmoM2 *mice:*

The R26-SmoM2 mice were purchased from The Jackson Laboratory (JAX stock #005130, Bar Harbor, ME). Mice contain a copy of the Smoothened homolog (Drosophila) gene inserted into the Gt(ROSA)26Sor locus. The mutant allele consists of a point mutation W539L, which constitutively active the mouse Smoothened homolog gene (SmoM2). The SmoM2 gene expression is blocked by a loxP-flanked STOP fragment placed between the Gt(ROSA)26Sor promoter and the SmoM2 sequence. When used in conjunction with a Cre recombinase-expressing strain, successful Cre-mediated excision results in the constitutive expression of mouse smoothened homolog and unrestrained Hedgehog signaling in Cre-expressing tissues, leading to unrestrained Hedgehog signaling (Fig.9A). The mice did not report any gross physical or behavioral abnormalities.

Generation of the conditional transgenic mice

To temporally and spatially control the modulation of Shh pathway in the OPCs, the conditional *NG2-Cre^{ERT2};Rosa26tdTomato* mice were crossbred with *Smo* floxed and *SmoM2* lines (Fig.9 A-B). The derivate offspring *NG2-Cre;SmoM2/tdT* mice was used to induce a gain of function (to make the manuscript more fluent, we will refer them from now on as *GF-Smo*) while *NG2-Cre;tdT-Smo$^{fl/+}$* to induce a partial loss of function of the Smo receptor (to make the manuscript more fluent, we will refer them from now on as *Smo$^{fl/+}$). NG2-Cre^{ERT2};Rosa26tdTomato* were used as Control for the entire experimental series (called from now on just as *Control*). The progeny was not reporting any alteration after birth.

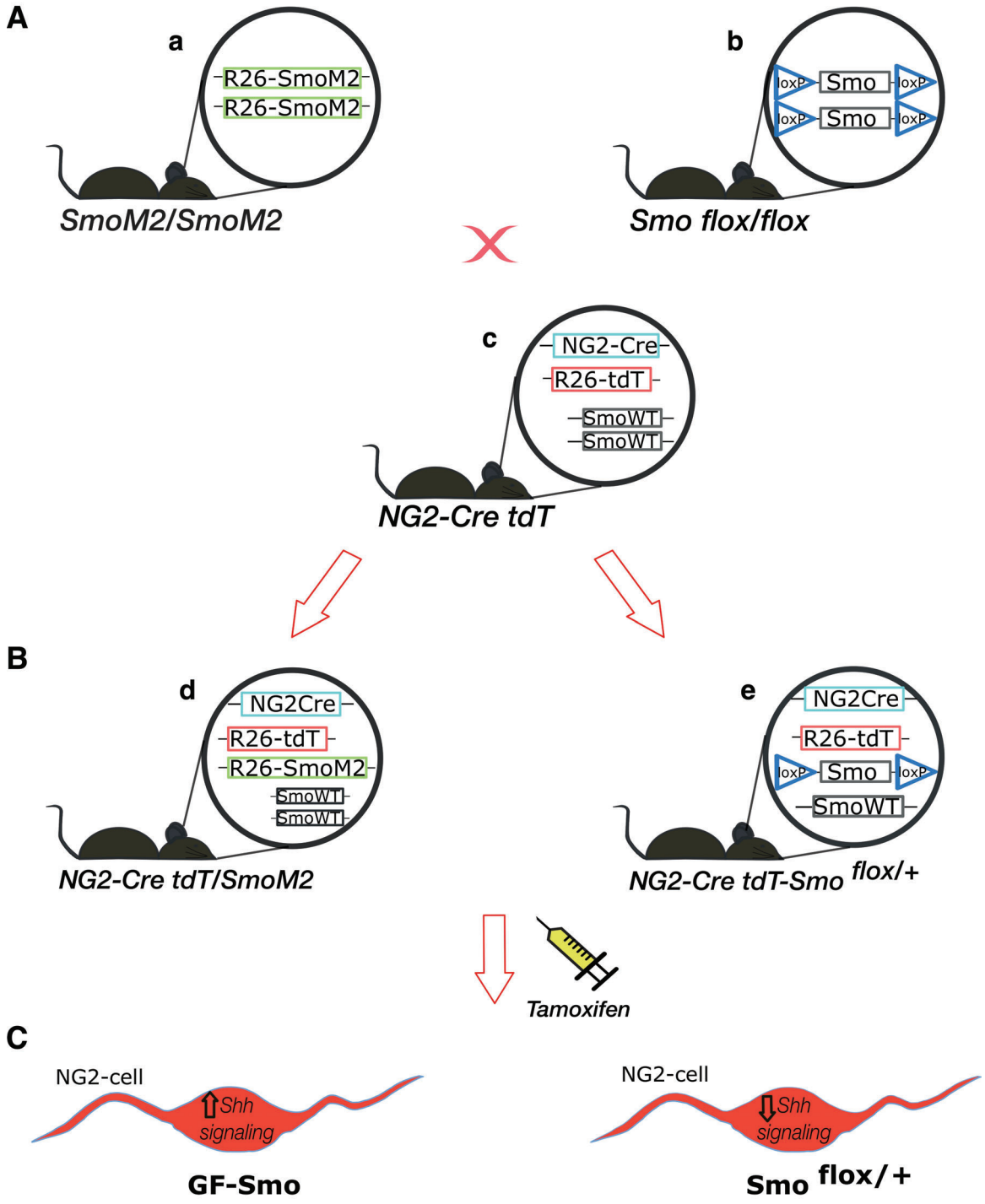

Figure 9. Generation of the conditional mice to explore the effect of Shh signaling on the OPCs in vivo.

A *R26-SmoM2 mice line (a, for the gain of function) and Smo floxed mice line (b, for the partial loss of function) were crossed with the NG2-Cre; R26-tdTomato (c).*

B *PCR was used to genotype the offspring in order to select the mice expressing both the Cre recombinase and the tdTomato. The gain of function line exhibited also a copy of the R26-SmoM2 allele, (d) while the partial loss of function line exhibited one floxed Smo allele (Smo $^{fl/+}$) (e).*

C *The administration of tamoxifen results in the transient activation of CreERT2 in NG2-Cre;R26tdT/SmoM2 or NG2-Cre;R26tdT;Sm $^{fl/+}$ transgenic mice. Therefore, increasing or decreasing the level of Smo expression in the OPCs.*

Tamoxifen preparation and administration

Tamoxifen is a potent CreER^{T2} system inducer, and it is used to activate the Cre-recombinase to generate time- and tissue-specific mouse mutants. Tamoxifen (T5648, Sigma-Aldrich, St. Louis, MO) was dissolved in corn oil (Sigma, St. Louis, MO) to achieve a concentration of 20 mg/ml.

To study how Shh signaling could affect the proliferation and differentiation of OPCs in vivo during the postnatal development, transgenic mice were administrated with tamoxifen at P1-2 by the lactating mothers (one intraperitoneal injection of 100 mg/kg body weight), and the brains were perfused at P14. Thus, Shh modulation was examined during the period when OPCs populate the CC (Kessaris et al., 2006). A cohort of 11 mice was used for this study, 3 *Smo$^{fl/+}$*, 5 *GF-Smo* and 3 control.

Tamoxifen (100 mg/kg body weight) was administered intraperitoneally once per day for five consecutive days for young adult mice.

Cuprizone murine model of demyelination

Although EAE is probably the most commonly used animal model of MS, it has an important immunopathogenic component (Procaccini et al., 2015). To avoid this, we decide to use a cuprizone model, which may be more appropriate to study remyelination itself (Gudi et al., 2014; Torre-Fuentes et al., 2020). Cuprizone targets oligodendroglia lineage directly, enabling predicting the timing of demyelination and remyelination and not involving the peripheric immune system (Gudi et al., 2014).
Males were used for demyelination since cuprizone could cause toxicity, resulting in estrus cycle disruption in females (Taylor et al., 2010). 8-9-week-old mice fed a diet of chow mixed with 0.25% cuprizone [w/w] [bis(cyclohexanone)oxaldihydrazone, C9012, Sigma-Aldrich] ad libitum integrated into a powdered standard rodent chow. Considering a food intake of about 5g/30g body weight/day for an average mouse, each mouse would ingest approximately 3.33 g cuprizone/kg body weight/day. Toxic demyelination was induced with five weeks Cuprizone diet (from week 0 to 5, 42 days in total) in 3 groups of mice for each line studied (at least three males for group/line). After five weeks of diet, mice were back to the standard chow to test if the modulation

of Smo protein affects remyelination. In addition, tamoxifen was given in different strategical period for five consecutive days (timeline is described in Fig. 10).

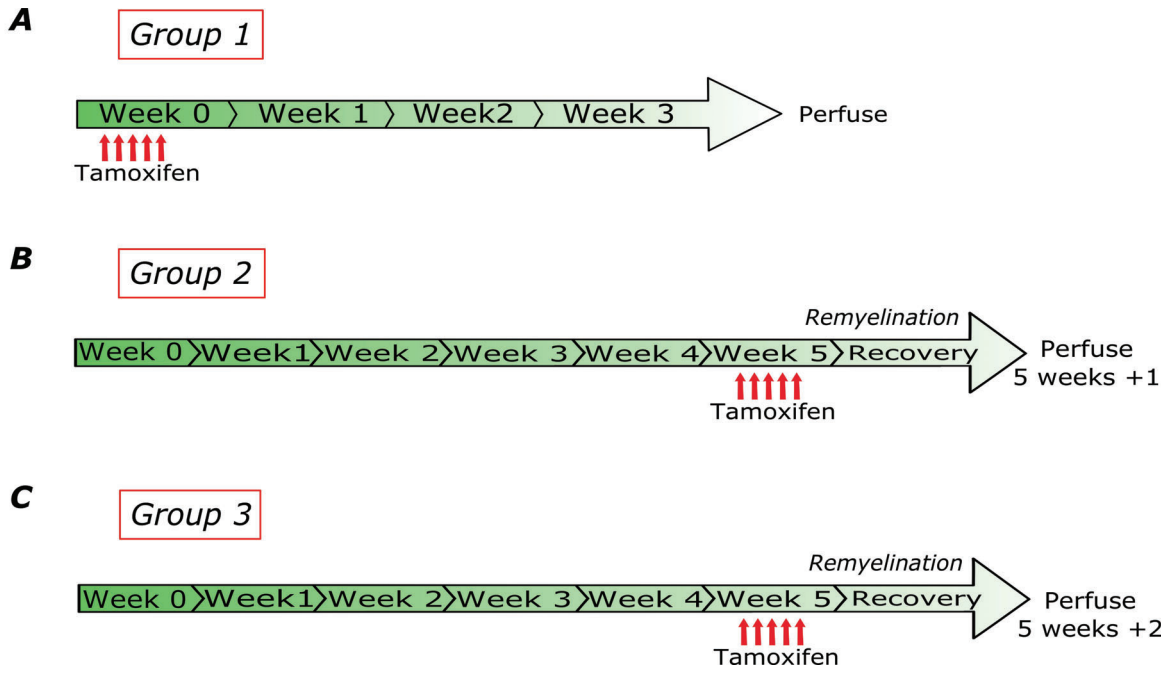

Figure 10 Cuprizone and tamoxifen timeline.

A. *Tamoxifen was given at the beginning of the cuprizone diet. Mice were perfused at the end of week 3 to test OPCs proliferation and microglia activation.* ***B.*** *Tamoxifen was given at week 5 and perfused after one week of recovery to test OL differentiation remyelination.* ***C.*** *Tamoxifen was given at week 5 and perfused after one week of recovery to test OL differentiation and remyelination.*

Additionally, aged-matched control mice with normal diet were taken from each group for immunohistochemical analysis during and after cuprizone-feeding to assess tissue structural changes and demyelination. In total, a cohort of 43 cuprizone-fed mice and 15 control mice were used. Cuprizone-fed and control animals were euthanized at 4, 7, and 8 weeks.

EdU labeling and detection

To detect proliferating cells, it was used the 5-ethynyl-2'-deoxyuridine (EdU), a thymine analog which is efficiently incorporated into cellular DNA during DNA replication. EdU (Invitrogen) was dissolved in the drinking water at 0.2 mg/ml, determined to be nontoxic. The water was exchanged every 48h to 72h (ad libitum), and mice were perfused after seven days of EdU exposure days.

EdU was visualized through its terminal alkyne group that reacts with organic azides in the double helix's major groove, without steric hindrance (Salic and Mitchson, 2008). Briefly, immediately after immunohistochemistry sections were rinsed with 100mM Tris HCl pH 8.5 and incubated with a Click-iT EdU reaction solution composed by 100mM Tris HCl pH 8.5 (10%), 100mM CuO4 (2%), Alexa488 azide 2nM (0.5%), dissolved in mqH2O, finally, ascorbic acid (10%) was added before adding the solution to the sample. After 30 minutes, at RT section were washed and mounted with a coverslip in Fluoromount-G (Southern Biotech).

Tissue sampling and processing for histology

Animals were euthanized with a letal dosis of Dolethal (Vetoquinol) and intra-cardially perfused using freshly prepared 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer pH 7.4 (PB). After perfusion, the brains were dissected and postfixed in the same fixative for 4h at RT. Brains were washed for 12h in PB at 4°C and then cryoprotected through increasing concentration (w/v) of sucrose from 10 to 20 and 30% in PB 0,1M, waiting each time that the brains were completely sunk. Subsequently, each brain was frozen embedded in OCT® (WVR Chemicals), carrying out the whole process in dry ice, and kept at -80°C until its use. OCT is a synthetic medium that stores brain sections at -20°C until its subsequent immunohistochemical analysis.

Brains obtained from postnatal day 14 (P14) mice were cut out with a sliding microtome (HM 450, Thermo Scientific). Coronals sections of 30 μm thickness were collected off the microtome knife with a pencil, placed in PB solution, and stored at 4ºC for subsequent histochemical processing.

Brains obtained from adult mice were cut out with a cryostat (CM1950, Leica), and coronal sections of 20 μm thickness were thaw-mounted on Superfrost® Plus slides (Thermo Scientific) and kept at -20ºC until use.

Immunofluorescence

The detection of the target cells was performed by immunofluorescence, using the antibodies listed in Table 2. Sections were rinsed with PB 0.1 M and pre-treated for 15 minutes with 10% methanol in PB, then washed with phosphate-buffered saline 1X (PBS) and pre-incubated for 1 hour at RT with blocking solution containing 5% normal donkey serum (DNS, Vector), 0.2% Triton X-100 (Sigma) dissolved in PBS 1X. Primary antibodies (listed in Table 2) were then diluted in the blocking solution, and sections were incubated overnight in a wet chamber at 4°C. Next, sections were washed with PBS and treated with fluorescent secondary antibodies (1:1000, Invitrogen) diluted in blocking solution for 2 hours in the dark at RT. After three washes and Hoechst incubation (10 μg/ml, Sigma) for nuclei staining, sections were mounted with a coverslip in Fluoromount-G (Southern Biotechm).

Table 2 List of primary antibodies

Target	Primary Antibody	Species	Dilution	Reference
OPC	PDGFR	Goat	1/120	#AF1062 R&D systems
Immature OL	O4	Mouse	1:5	DSHB, Developmental studies Hybridoma Bank
Mature OL	CC1	Mouse	1/100	#OP80 Millipore
Myelin	MBP	Rat	1500	#MCA409S BIO-RAD
Myelin	MOG	Mouse	1/100	#MAB5680 Millipore
Oligodendroglial Lineage	Olig2	Rabbit	1/200	#AB9610 Millipore
Heavy Neurofilament	NFH	Rabbit	1/800	#AB4680 Abcam

Microglia	Iba-1	Guinea Pig	1/500	#234004 Synaptic System
Paranode	CASPR	Rabbit	1/900	#AB34151 Abcam
Astrocyte	GFAP	Mouse	1/500	#MAB3202 Millipore
Astrocyte	S100	Rabbit	1/200	#AB868 Abcam
Neuron	NeuN	Mouse	1/500	#MAB377 Millipore
Neuroblast	Dcx	Rabbit	1/500	#4604 Cell Signaling

Intraretinal inyection

Mice were anesthetized with xylazine (20 mg / mL) + ketamine (100 mg / mL) in a cocktail with saline solution which is administered at 0.2 ml per 20g of mouse weight (87.5 mg / kg Ketamine and 12.5 mg / kg Xylazine) through intraperitoneal injection (IP). Cells were injected intravitreally, under direct visual observation, close to the RGC layer with a 33-gauge needle and 5 µL glass syringe (Hamilton). Positioning was as described in figure 11. OPCs were delivered together with the 1µL of cell suspension. Animals were perfused after five weeks from transplantation. Injected eyes were removed and placed in 4% PFA for additional 2 hours, then transferred to PBS. Retinae were dissected from the sclera for immunohistochemistry. Finally, after making 4-5 radial incisions, flat-mounted retinae were mounted on slides and examined under confocal microscopy (Fig. 11).

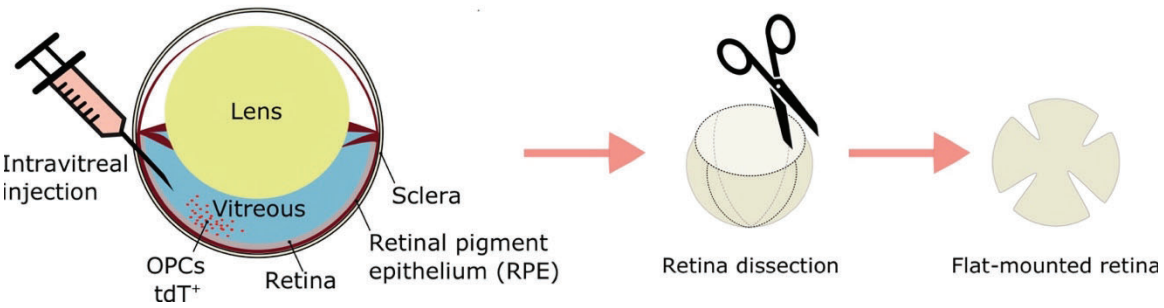

Figure 11 Scheme of intravitreal retinal injection

HUMAN BIOPSIES

Human biopsies were obtained from the adult brain cortex (25-35 years old) proceeding from epilepsy surgeries of patients who did not respond to drug treatment or brain trauma. The sample was obtained from the Neurosurgery Services of Hospital Universitario La Princesa (Madrid) and Hospital 12 de Octubre (Madrid). All samples were transported in Hibernate-A (Gibco) at 4°C as quickly as possible to reduce cell damage. The studies were conducted in accordance with the guidelines, and the Research Ethics Committee approved protocol of the Instituto Cajal-CSIC (440/2016 and 2016/049/CEI3/20160411). All subjects gave their informed consent for inclusion before they participated in the study.

Culture primary cell

For the study of the Shh signaling effect on the OPCs, primary cultures were made from transgenic mice at 7 days (P7) or from adult human samples. For this, the accepted method for the purification of OPCs was magnetic-activated cell sorting (MACS; Fig. 11; Dincman et al., 2012).

Tamoxifen induction was made to lactating mothers at P1-P2, and five to seven P7 mice were used for each primary culture. First, the P7 mice were decapitated and brain dissected by carefully removing the skull. The brain was extracted with blunt forceps and placed in cooled $HBSS^{+/+}$ (Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+}; Gibco) to slow the metabolism. After removing meninges and choroidal plexuses, the cortex was cleared to eliminate the brain tissue rest (including the hippocampus). Clean cortices were cut with the help of a scalpel in a glass Petri dish and transferred to a 15 mL conical bottom falcon tube. According to the manufacturer's instruction, after two rinses with $HBSS^{-/-}$ (Hank's Balanced Salt Solution without Ca^{2+} and Mg^{2+}; Gibco) to recovery and cell debris removal, the tissue was enzymatically digested using a commercial neural tissue dissociation kit (Miltenyi). Briefly, the tissue was resuspended in a previously tempered enzymatic digestion solution containing papain (mix 1), then incubate in closed tubes at 37 °C under slow, continuous rotation. After 25 minutes, a second enzymatic solution (mix 2) was added, and we proceed with the

crushing using a 5 ml pipette to dissociate the tissue mechanically. After another incubation at 37°C for 10 minutes in continuous rotation, another crushing step was repeated. The cell suspension was filtered through a 70 µm pore size mesh (BD Falcon) to a 50 mL falcon tube, and 10 mL of HBSS was applied through the strainer. The cell suspension was centrifuged at 300×g for 10 minutes at RT. Cells were, then, labeled with the primary antibody from hybridoma cells, O4 (DSHB, Developmental Studies Hybridoma Bank, Iowa) diluted 1:5 in a Miltenyi wash buffer solution (MWB, containing 1X PBS, 2mM sodium pyruvate, 0.5% BSA and 2mM EDTA, adjusted to a pH of 7.3) for 30 minutes at 4°C to prevent capping of antibodies on the cell surface and non-specific cell labeling (Fig. 11). Cells were then washed with 5 mL of MWB, followed by the addition of 20 µL/ten million cells of secondary antibody rat anti-mouse IgM (anti-mouse IgM microbeads; Miltenyi) for 15 minutes. After incubation, cells were washed and pass through a previously MWB-coated medium-size column (MS, Miltenyi) coupled to the magnetic field of MACS Separator (Fig.12). The column was charged with the cell suspension, rinsed twice with MWB, and flush out with commercial culture Neuromedium (miltenyi) supplemented with 2% of Neurobrew-21 (Miltenyi), 20 mM of Glutamine, and 10 ng / mL of the growth factors PDGF-AA and FGF2 (proliferation medium) (Fig. 12). Isolated O4+ cells were counted and seeded or kept in suspension depending on the desired paradigm described in the next epigraphs. For the isolation of OPCs in human samples, the same protocol was used with few modifications. After filtration through the 70 µm mesh, cells were washed and the pellet treated in a 20% percoll (GE Healthcare) gradient. The suspension was centrifuged at 800 g for 20 minutes without braking to avoid distortion of the gradient and facilitate cell myelin removal. Then, a solution of Red Blood Cell Removal Solution (miltenyi)

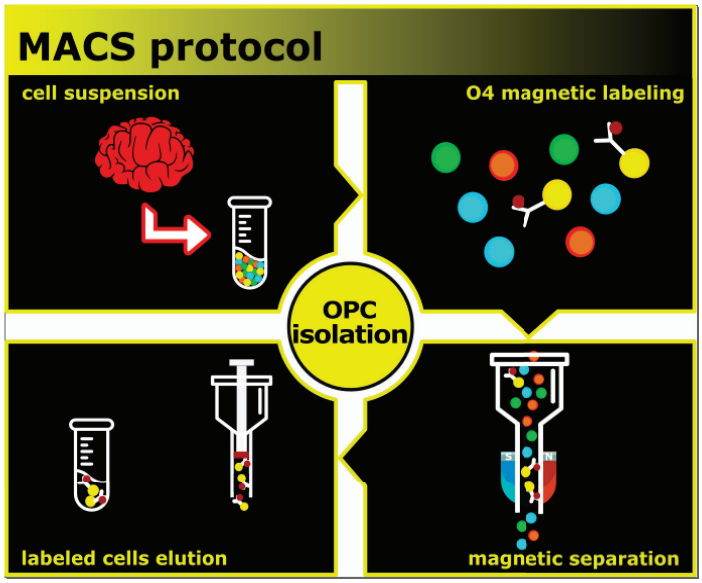

Figure 12 Schematic rappresentation of MACS protocol to isolate O4+ OPC from cerebral cortex of P7 mice and adult human brain.

dissolved in ddH_20 was added and incubated for 10 minutes in the refrigerator (2–8 °C). After washing with MWB, the pellet was resuspended in 97.5 µL of MWB per up to 10^7 total cells. 2.5 µL of Anti-O4 MicroBeads antibody (Miltenyi) was added and incubated for 15 minutes in the dark in the refrigerator (2–8 °C) in slow agitation. In presence of higher cell numbers, we scaled up all reagent volumes and total volumes accordingly.

Proliferation assay

In this paradigm, $O4^+$ cells were seeded in a 24-well plate previously coated overnight with Poly-L-Lysine (Sigma, 0.1 mg/ml in borate buffer, pH 8.5) and laminin (Sigma, 10 µg / ml in PBS1X). According to our group's protocol (E. M. Medina-Rodríguez et al., 2013), a concentration of 50.000 cells was seeded in each well in a final volume of 500 µL of proliferation medium. After 24h, half of the medium was replaced by fresh medium to allow cells to recover. After three days *in vitro* (DIV 3), Shh ligand was added to a final concentration of 200 nM with the corresponding control condition. The pulse of 50 µM of BrdU (5-Bromo-2-deoxyuridine; Sigma-Aldrich) was also performed at the same time for 24 hours, after which the medium was totally refreshed (in the same condition) for additional 24 hours, following the protocol of our group. Cells were fixated for 10 minutes with 4% PFA and processed for immunolabelling with anti-Olig2 and anti-BrdU (refer to the antibody table).

Differentiation assay

In this case, $O4^+$ cells were seeded at a density of 50.000cells/well in a 24-well plated previously coated with Poly-L-Lysine and laminin. The culture was maintained by replacing 50% of the prepared medium to allow recovery with a proliferation medium every other day. After six days *in vitro*, the volume was replaced by new medium without growth factors but with the addition of Shh (200nM), and the medium was changed every other day for a total of 10, 16, 17, 18 and 20DIV. Cells were fixed and immunolabelled for CC1 and MBP.

Immunocytochemistry

The detection of the different OPC and OLs markers was performed by immunocytochemistry, using the antibodies listed in Table 1. For that purpose, after three washing steps with 1X PBS, cells were incubated for one hour in a blocking solution containing 5 % normal donkey serum (NDS, Vector) and 0.02% Triton X 100 (Sigma), and later in the corresponding primary antibodies O/N in a wet chamber, at 4ºC and dark. After three washing steps, plates/coverslips were incubated with the secondary antibodies (at a 1:1000 dilution in blocking solution) for 1 hour in the dark at RT. After washes and Hoechst incubation for nuclei staining, coverslips were mounted in Fluoromont® on slides.

The BrdU staining has a particular procedure. After Olig2 staining steps, the coverslips were fixed in 4% PFA for 10 min at RT followed by denaturation in 2N HCl at 37°C for 45 min, followed by three washing steps with borate buffer and the blocking step (with a solution containing 5 %FBS, 0.03% Triton, 0.2% gelatin and 0,2M glycine in 1X PBS), for 3 hours at RT. The coverslips were then washed in PBS 0.03% Triton and treated with anti-BrdU antibody overnight. Sequentially after the washes, the secondary antibody was added to the blocking solution for 6 hours, cells were processed as explained before.

CONFOCAL MICROSCOPY

Images were acquired with an inverted Leica SP5 confocal microscopy from the Microscopy and Image Analysis Service at the National Hospital for Paraplegics (Toledo). This microscopy has an additional resonant scanner for obtaining quick images and a special acousto-optical beam splitter (AOBS) to simultaneously obtain several colors. Microphotographs were captured at a magnification of 20X with a z-plane depth of 3 μm or at 40X with a z-plane depth of 1 μm. For CASPR-MBP staining, a 63X objective was used, and microphotographs were taken in a single z-stack with the major MBP intensity. All the images were taken with a 1024 x 1024 pixels resolution.

Confocal images were processed using the freeware software Image J, processing package FIJI (Schindelin et al., 2012). Cell count was performed manually. The rostral CC was analyzed between the Bregma 1.10 and 0.14 μm. For P14 experiments, the region

of interest (ROI) was defined from the midline to the point under the peak of the cingulum, while for the cuprizone demyelinating model, ROI included the middle of the CC, whereas area measurement

ELECTRON MICROSCOPY

For electron microscopy, animals were transcardially perfused first with 0,9% NaCl for one min, followed by a fixative solution containing 1% glutaraldehyde and 4%paraformaldehude in 0.1 M PB (pH 7.4). Brains were removed from the skull and postfixed overnight at 4°C. Next, brains were washed with PB solution for two hours and then 60 µm-thick coronal sections were obtained using a vibrating microtome (VT1000 S, Leica). Tissue was rinsed, postfixed with 1% OsO_4 in PB for 30 min, counterstained with 1% uranyl acetate in dH_2O for 30 min, dehydrated with a series of graded ethanol until reach 100%, infiltrated with propylene oxide, and embedded in epoxy resin Durcupan (Fluka). Ultrathin section of the CC (70 nm-thick sections) were cut using an ultramicrotome (Reichert Ultracut E, Leica), collected in single slot copper grids at room temperature, and analised using a transmission electron microscope Jeol-1010 at 1500X magnification. ImageJ/Fiji was also used to obtain axon diameter measurements (Feret diameter), as the longest distance between any two points along the selection boundary (https://imagej.nih.gov/ij/docs/menus/analyze.html. For G-ratio, the inner myelin sheath diameter was divided by the outer myelin sheath diameter. Analyses took at least 500 axons measured for each animal, 3 animal/genotype in the cuprizone group, 1 animal/genotype in the no treated group.

STATISTICAL ANALYSIS

All the statistics were performed with the GraphPad Prism version 7 (GraphPad Software, La Jolla California). Data are expressed as the mean ± the standard error of the mean (SEM). The Student's *t*-test was used to compare two populations/groups and One-Way-ANOVA to compare more than two groups. Their corresponding non-parametric tests (on ranks) were used when normality tests failed.

Statistical signification was established at $p < 0.05$. The results of the different statistical analysis are represented in figures follows: *$p < 0.05$; **$p < 0.01$; y ***$p < 0.001$.

RESULTS

Shh modulation in OPCs affects their differentiation during postnatal myelination

Shh was established as a mitogen, however, it is unclear whether this factor expands multipotent cells or those already committed to a specific lineage (Loulier et al., 2006; Merchán et al., 2007; Ortega et al., 2012). To investigate whether OPCs respond to Shh during postnatal myelination, the current studies focus on the postnatal period of OPCs differentiation in the corpus callosum (CC). Previously studies indicate that Shh null mutant mice generate OLs, implying that other Hh proteins (Dhh or Ihh) may compensate the lack of Shh (Carballo et al., 2018). Furthermore, Ptch1 is expressed at basal levels in the absence of any direct Hh signaling input (Bai et al., 2002), while Gli1, an accurate readout of Shh activation, is implicated only in the canonical-pathway. Thus, to rule out the possibility of compensatory Hh signaling and activate both canonical and non-canonical pathways, we have examined the phenotype generated by the modulation of the Smoothened receptor in committed-OPC.

To temporally and spatially control the modulation of Shh pathway in the OPCs, we used the conditional *NG2-Cre^{ERT2};Rosa26tdTomato* line. NG2 expression in this line was demonstrated occurs mainly in OPCs, serving to produce OLs throughout life (Huang et al., 2014). *NG2-Cre^{ERT2};Rosa26tdTomato* mice were crossbred with *SmoM2* and *Smo* floxed lines. The derivate offspring was used to induce a gain of function, *GF-Smo* mice, and partial loss of function, *Smo$^{fl/+}$* mice, of the Smo receptor (Fig. 9; for more details, see methods). *NG2-Cre^{ERT2};Rosa26tdTomato* was used as Control, and in the purpose of clarity, they will be designed as “control” group along the present work.

Recombination was induced with tamoxifen at P1-P2 (see methods), and the effect of Shh modulation was examined at P14 when OPCs and OLs populate the CC during postnatal myelination (Kessaris et al., 2006; Sánchez e Armstrong, 2018). The differentiation of recombined OPCs was identified through the tdTomato reporter.

According to the markers used for characterization, much tdT^{+} glia appeared in the region analyzed, and most of them were restricted to the OL lineage (Fig. 13 A-I). The differentiation status was determined by PDGFRα (OPCs) and CC1 (mature oligodendrocyte) immunostaining (Fig. 13 A-I). Although P14 time point is considered the peak

of OPCs differentiation in the CC (Sánchez e Armstrong, 2018), however, OPCs still expressed PDGFRα (Fig. 13 A-F)). The total OPCs or OLs density (cells/mm^2) did not show significant differences between the groups (Fig. 13 J). However, the proportion of OPCs or mature OLs (CC1$^+$) respects the total number of cells (PDGFRα + CC1) showed that *GF-Smo* mice display a higher percentage of OPCs (and, as consequents, a lower number of mature OLs) to both respect *Smo$^{fl/+}$* and Control mice (Fig. 13 K). Moreover, analyzing the recombined cell population (tdT$^+$), those differences between the groups appear clearer: the *GF-Smo* line has a significantly higher number of OPCs (tdT-PDGFRα$^+$) with respect to the Control and Smo$^{fl/+}$ groups (Fig. 13 L). The significantly larger OPC population in the *SF-Smo* line results in a significantly lower number of mature OLs when compared to Control and *Smo$^{fl/+}$* (Fig. 13 M). There were no differences between CT and *Smo$^{fl/+}$* groups when were directly compared (Student's *t*-test), which suggests that Smo haploinsufficiency does not affect the differentiation of OPCs, at least at this stage of the OLs differentiation. All together, these results indicate that during the first 14 postnatal days, OPCs develop into mature OLs in both CT and *Smo$^{fl/+}$* groups, but cell differentiation was delayed when they over-express Smo (*GF-Smo*).

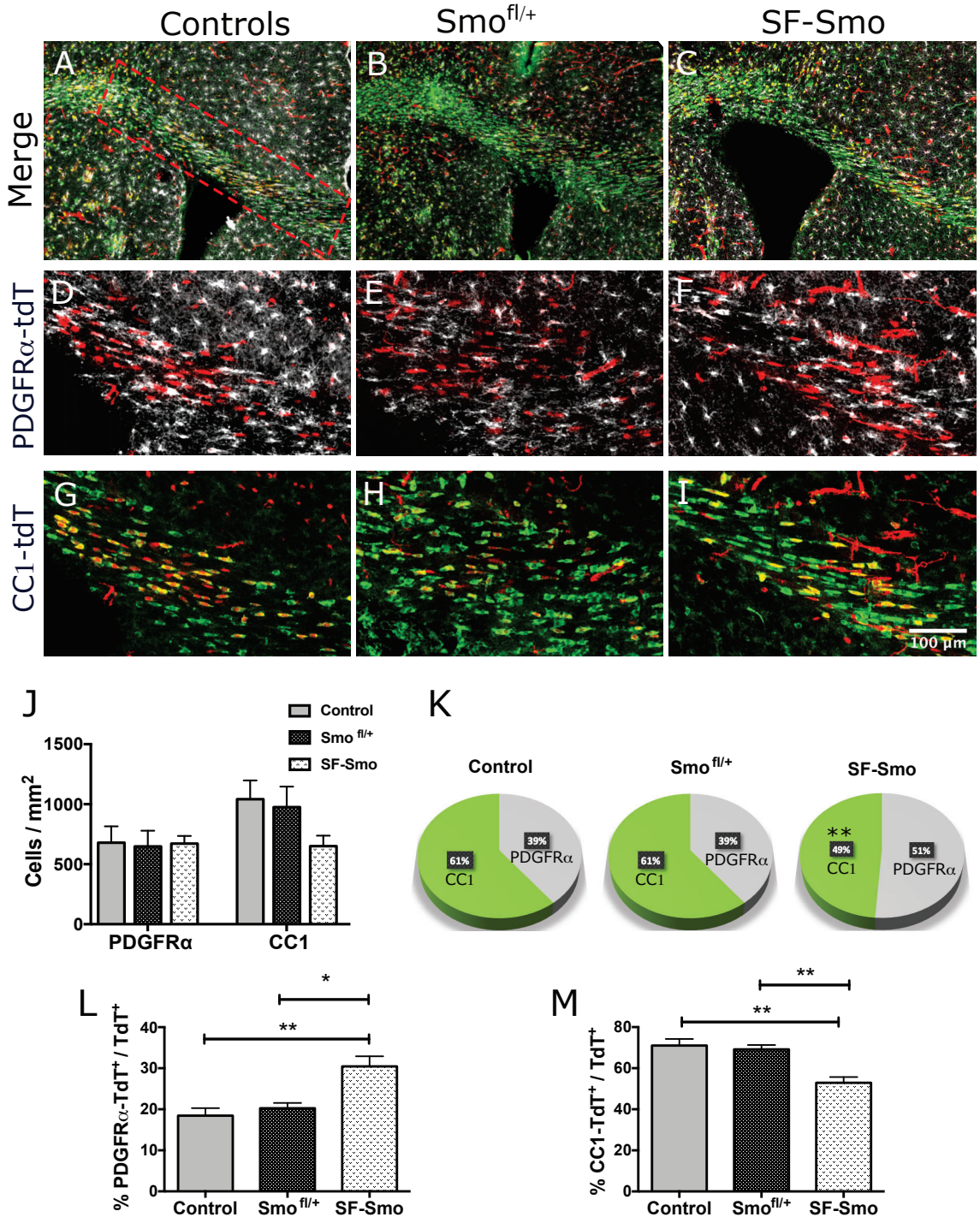

Figure 13 Smo gain of function increases the number of OPCs and impedes their differentiation into mature OLs*. Panoramic* ***(A-C)*** *and detailed* ***(D-I)*** *views of the CC in P14 mice injected with tamoxifen at P1-P2. Red outlines (A) highlight the region used for the quantification. Immunostaining for PDGFRα OPCs (white), CC1 mature OLs (green), and tdTomato (red). NG2 mice line is used as Control (A, D, G), Smo$^{fl/+}$ partial loss of function (B, E, H) and GF-Smo gain of function (C, F, I). Objective 20X.* ***(J)*** *Quantification of the percentage of PDGFRα-tdT$^+$cells shows a higher number of OPCs in the GF-Smo mice.* ***(K)*** *Quantification of the rate of CC1-tdT$^+$cells shows a lower number of mature OLs in the GF-Smo mice.* ***(L)*** *No differences are found in the total number of OPC (PDGFRα) and mature OL (CC1). Scale bar100µm (Scale Bar is equal to 300µm in A-C). The statistical analysis was carried out using the one-way ANOVA test and the post-hoc Tukey's multiple comparisons test, where *p< 0.05; **p< 0.01.*

The specific potentiation of Shh signaling pathway in OPCs disrupts normal myelination during the development

There is a peak of OL differentiation and CNS myelination by P14 in the mouse (Parnavelas, 1999; Sánchez e Armstrong, 2018). Having demonstrated a delay in the OLs differentiation in the *GF-Smo* mice, we wanted to study how this delay could affect myelination. There were no gross differences in the organization of axonal tracts and the distribution of myelin between the three groups (Fig. 14 A-K). But the area occupied by MBP in the CC was significantly lower in the *GF-Smo* than in CT animals (Fig. 14 A-B, G-H), and MBP expression was decreased by 24.71% compared to the Control (Fig. 14 K). This decrease in MBP expression ties in with the observed -1.34-fold decrease in the number of mature OLs (Fig 13 M). As in the differentiation assay, there were no differences between CT and $Smo^{fl/+}$ groups, which suggests that Smo haploinsufficiency does not affect neither myelination at this stage of the development.

The function of myelinated axons is also dependent on the structure of the nodes of Ranvier. Their density and organization, together with the distribution and concentration of Na^+ and K^+ channel, has a strong influence on axonal excitability and axonal conduction velocity (Fields, 2008). To further analyze myelination in our three experimental groups, the number of Ranvier nodes was calculated by identifying doublets of CASPR staining per mm^2, associated with the Na^+ channel clustering in the paranodes (Murcia-Belmonte et al., 2016). Consistent with the previous findings, Smo gain of function revealed the disorganization of the nodes with significantly reduced expression and number of doublets compared with Control and $Smo^{fl/+}$ groups (Fig.15 A-G), indicating, as indirect measurement, longer internodal length. The ROI in this analysis was smaller with respect to the MBP-NFH study due to the increased magnification. To analyze the myelin relating to the number of nodes of Ranvier, we quantified the MBP area also in the MBP-CASPR study. Then, we normalized the percentage of MBP expression and the number of CASPR doublet with respect to the control group (Fig.15 H). This analysis reinforces the MBP-NF study results and shows how a lower rate of myelination in the *GF-Smo* line is associated with a lower percentage of node density. Again, $Smo^{fl/+}$ mice did not show any difference with respect to the Control. Altogether, these results suggest that the increase of Shh signaling selectively regulates OL

maturation of OLs, negatively impacting myelination with an aberrant distribution of ion channels at the node of Ranvier in the CC.

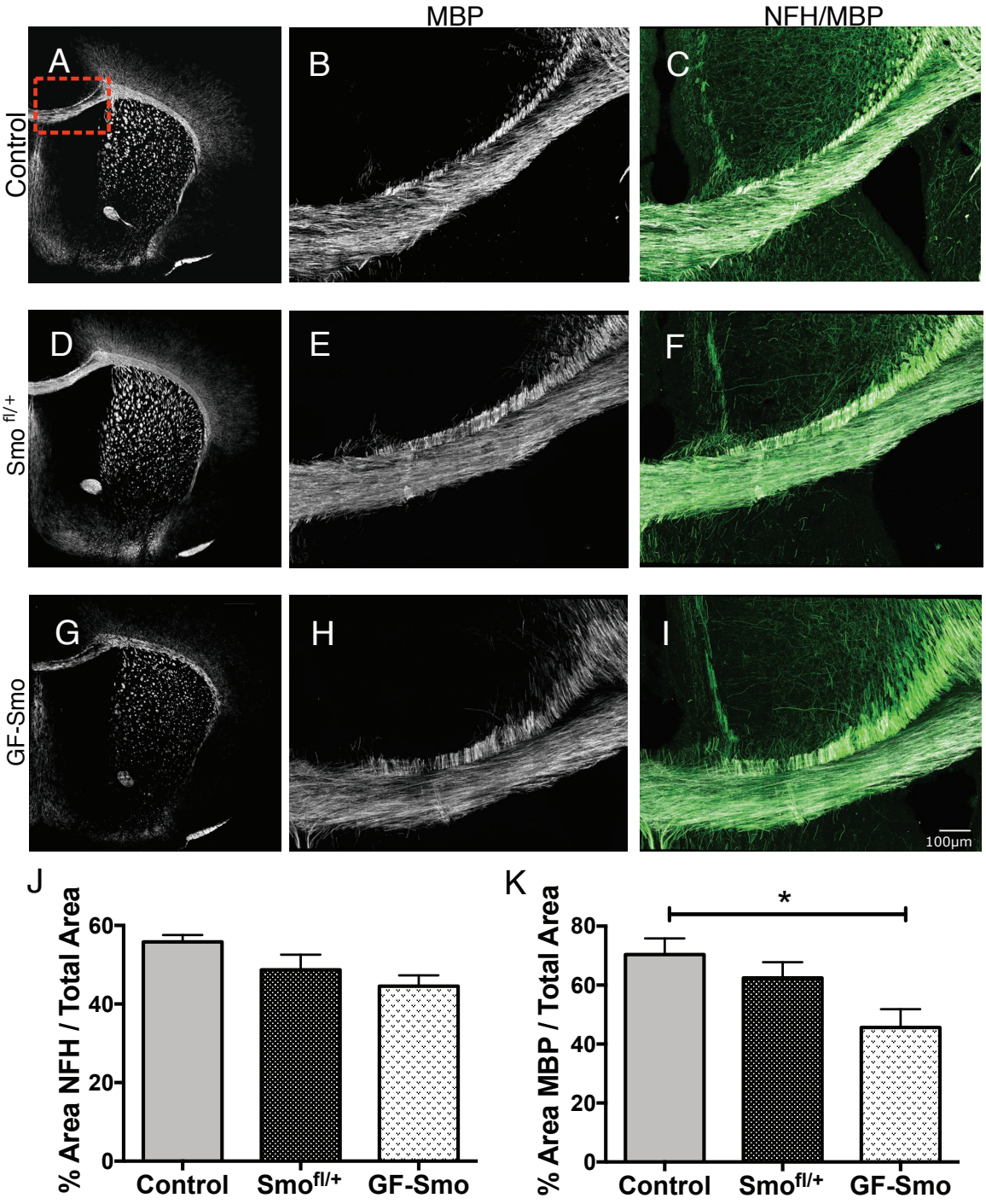

Figure 14. Increasing the Smo receptor expression leads to a deficit in CNS myelination* in vivo.** ***A, D, G. *Overview of the rostral CC, myelin is stained with the MBP antibody. P14 mice were injected with tamoxifen at P1-P2.* ***B, E, J*** *MBP and heavy chain of neurofilament (NFH) stain* ***C, F, K*** *of the CC.* ***L.*** *Quantification of the percentage of axons (NF) at CC level.* ***M*** *Quantification of myelin (MBP) percentage found at the CC level of each mice line. Scale Bar 100µm (Scale bar is equal to 500µm in A, D, G). The statistical analysis was carried out using the one-way ANOVA test and the post-hoc Tukey's multiple comparisons test, where *p< 0.05.*

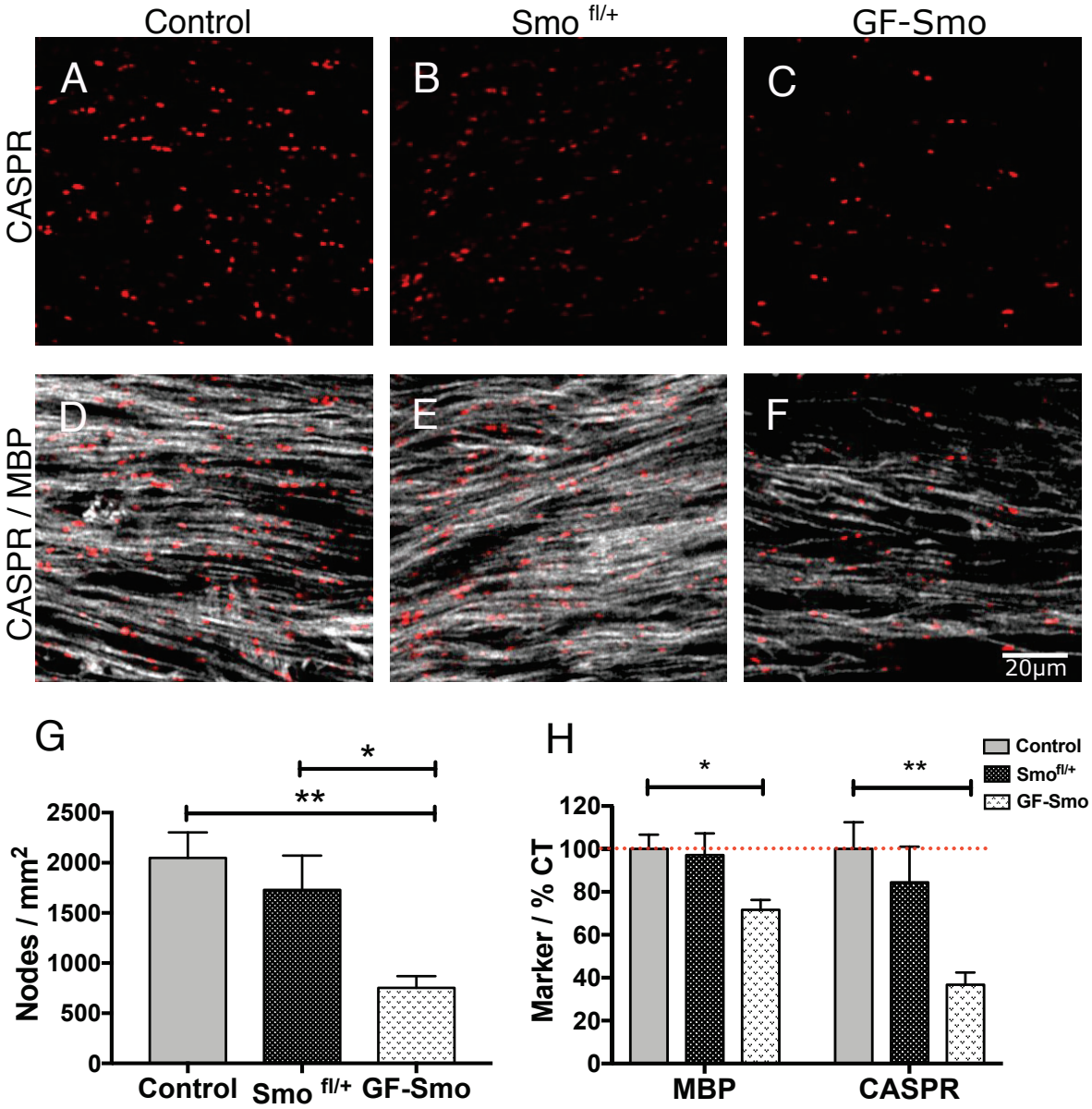

Figure 15 Deficit of myelination in the GF-Smo animals. A-F *Paranodal stain with CASPR and myelin stain with MBP antibody in the CC of the P14 mice. GF-Smo mice appear less myelinated, with a disorganized and lower number of CASPR pair versus Control and Smo $^{fl/+}$ mice.* **G** *Quantification of CASPR doublet counted per mm² shows a decrease of paranode in the GF-Smo line.* ***H*** *Normalization of the percentage MBP area and doublet CASPR number of Smo $^{fl/+}$ and GF-Smo line over the Control confirms a significative difference in myelination and node of Ranvier distribution in the GF-Smo line. Scale Bar 20µm. The statistical analysis was carried out using the one-way ANOVA test and the post-hoc Tukey's multiple comparisons test, where *p< 0.05; **p< 0.01.*

The murine cuprizone-induced demyelination model triggers the glial reaction.

To investigate the function of Shh signaling during OL regeneration and CNS remyelination, the transgenic lines *Smo*$^{fl/+}$, *GF-Smo* and Control, were subjected to the demyelination model Cuprizone. Although cuprizone-fed mice did not show significant neurological symptomatology, its toxic effect was observed in weight loss (Fig. 16). Mice lost between 7 and 8% of their original weight during the first week of cuprizone induction and remained stable during the treatment duration. These observations were congruent with previous works, including our group (Hiremath et al., 1998; Medina-Rodríguez et al., 2017). Weight increased in all groups once the diet was back to the standard chow (Fig. 16).

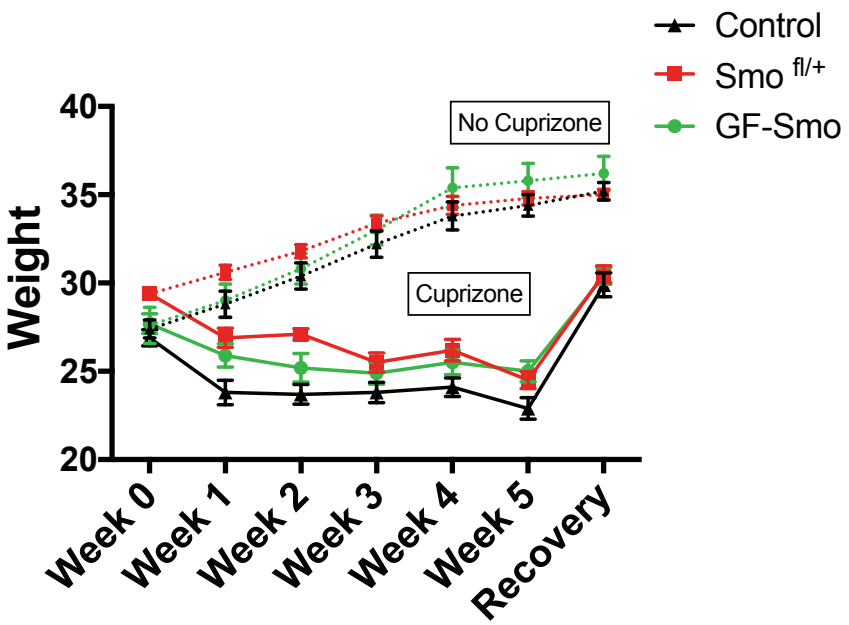

Figure 16 Animal weight is an important variable for reliable cuprizone-induced demyelination. *The three lines show weight loss starting from the first week for the entire duration of the treatment.*

Microglial activation and OPC proliferation during the demyelinating phase of cuprizone model

Cuprizone diet induces, directly or indirectly, oligodendrocyte cell death after a few days of exposure. This triggers the activation of microglia and the spontaneous response of OPCs to remyelinate (Gudi et al., 2014). Both were studied in our model (Fig. 17). Microglia are involved in the inflammatory and anti-inflammatory processes; it is

responsible for the phagocytosis and clearing of myelin debris. The removal of myelin debris is a prerequisite for efficient myelination repair (Gudi et al., 2014; Robinson e Miller, 1999; Syed et al., 2008). The period following week 3 is characterized by a significant increase of highly activated microglia, numerously present in the demyelinating regions such as the CC (Fig. 18 E-H), cortex, and hippocampus. Moreover, after 3 weeks of diet, it is possible to detect the degradation of myelin proteins by IHC.

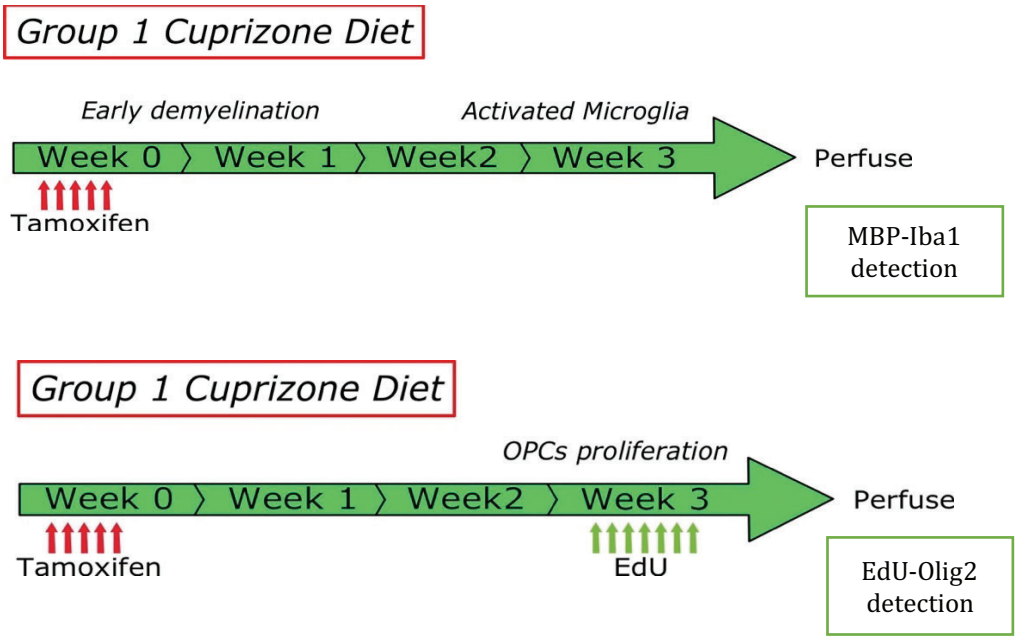

Figure 17 Timeline for the macroglia activation and proliferation study. A. *Simultaneously to the cuprizone induction, tamoxifen was injected. After week 3, microglia activation was studied together with the ongoing demyelination.* ***B.*** *Simultaneously to the cuprizone induction, tamoxifen was injected. At week 3, EdU was injected in drink water for a week to test OPCs proliferation during the demyelination phase.*

At 3 weeks post cuprizone diet, there was a significant reduction in the area occupied by MBP (Fig. 18 A-D) in the three experimental groups compared to the control with no cuprizone diet. Remarkably, there were no differences between the three experimental groups fed with the cuprizone diet (Fig. 18 B-D, M). Along with myelin loss, in the animals exposed to the cuprizone diet, we observed a significant increase of activated microglial cells accumulated in the medial CC. Upon CNS insult, activated microglia/macrophages change their shape from ramified to amoeboid (Gudi et al. 2014). There were no activated microglia in the control mice fed with standard chow: all the cells of this population showed their typical ramified morphology (Fig.18 E). In contrast, we observed a switch of the microglia/macrophage polarization in favor of the amoeboid phenotype in the cuprizone-fed mice (Fig. 18 E-H). Moreover, in the *Smo*$^{fl/+}$

group, we observed a significantly larger area occupied by microglia (Fig. 18 G, N), suggesting that the deletion of one Smo allele in the OPC population is sufficient to enhance microglial activation and proliferation at the lesion site during the demyelination phase. However, the activated microglia increase was not significative comparing to the *Smo$^{fl/+}$vs. GF-Smo* groups.

Parallel to the myelin clearance in the CC, a massive proliferation of OPCs is produced prior to remyelination, starting from week 1-2 of cuprizone treatment and peaking after week 3-4 (Gudi et al. 2014). We started studying the effect of Shh pathways during demyelination in the pick of the inflammatory contest. Mice were injected with tamoxifen simultaneously with the beginning of the cuprizone diet, and they were perfused at the end of week 3. To study OPC proliferation of the recombined cells, in a similar experimental series EdU was delivered through drink water during the seven days before the perfusion.

The adult brain analysis from all genotypes not treated with cuprizone showed a similar level of Olig2- tdT$^+$ cells (Fig 19 a-c). Moreover, 8 to 20% of the tdT$^+$cells were incorporating EdU (Fig 19 A-C), but no differences were found between the groups (data not shown). The cuprizone diet significantly increased the number of tdT$^+$ proliferating cells in all groups: 53-55% in the control and *Smo$^{fl/+}$* mice, respectively, and 63% in the *GF-Smo* mice (Fig. 19 D-F).

Then we analyzed the density of recombined-OPCs (Olig2-tdTomato$^+$) in the cuprizone-fed mice (Fig. 19 d-f). As hypothesized, the total number of oligodendroglial cells (OPCs or more differentiated cells) was significantly higher in *GF-Smo* than in the control group (1.84-fold change; Fig 19 d-f, G). Moreover, the total amount of proliferating OPCs (Olig2-EdU$^+$) was significantly higher in *GF-Smo* than in the other experimental groups (Fig. 19 H), and even more substantial differences were shown when analyzed just the proliferation of OPC induced with tamoxifen (2.25-fold change; Fig 19 D-F, I).

Unlike P14 analysis, the higher density of Olig2-EdU$^+$ in the *GF-Smo* line shown that the gain of function affects the total cell yield in this genotype with respect to the

Control (Fig. 20 H). As in the P14 study, *Smo*$^{fl/+}$ haploinsufficiency displays no effect in the total number of proliferating OPCs or recombined cells.

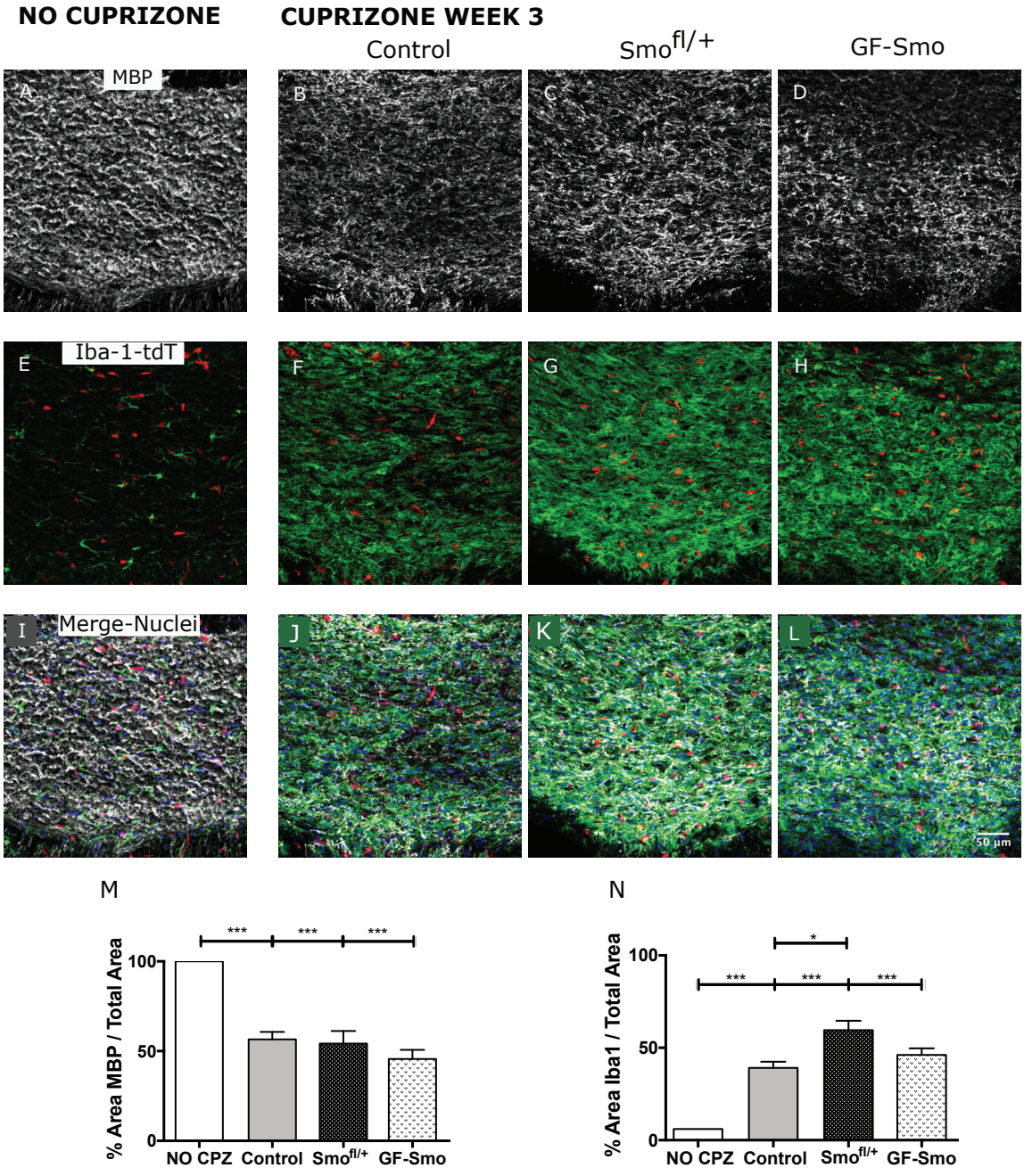

Figure 18 Demyelination and microglial increase were confirmed in the three lines. A-L *Triple labeling myelin (MBP, gray), microglia (Iba-1, green) and tdTomato (tdT) reporter show a decrease of the myelin during cuprizone diet* ***(A-D)****, and the increase of activated microglia* ***(E-H)*** *respect age-matched control mice without cuprizone (A, E, I).* ***M*** *Percentage of myelin in the middle of the CC over the total area analyzed showing myelin diminution with respect to the non-treated mice.* ***N*** *The rate of microglia in the middle of the CC over the whole area analyzed shows the increase of microglia with respect to the non-treated control. Scale Bar 50µm. The statistical analysis was carried out using the one-way ANOVA test and the post-hoc Tukey's multiple comparisons test, where *p< 0.05; **p< 0.01.*

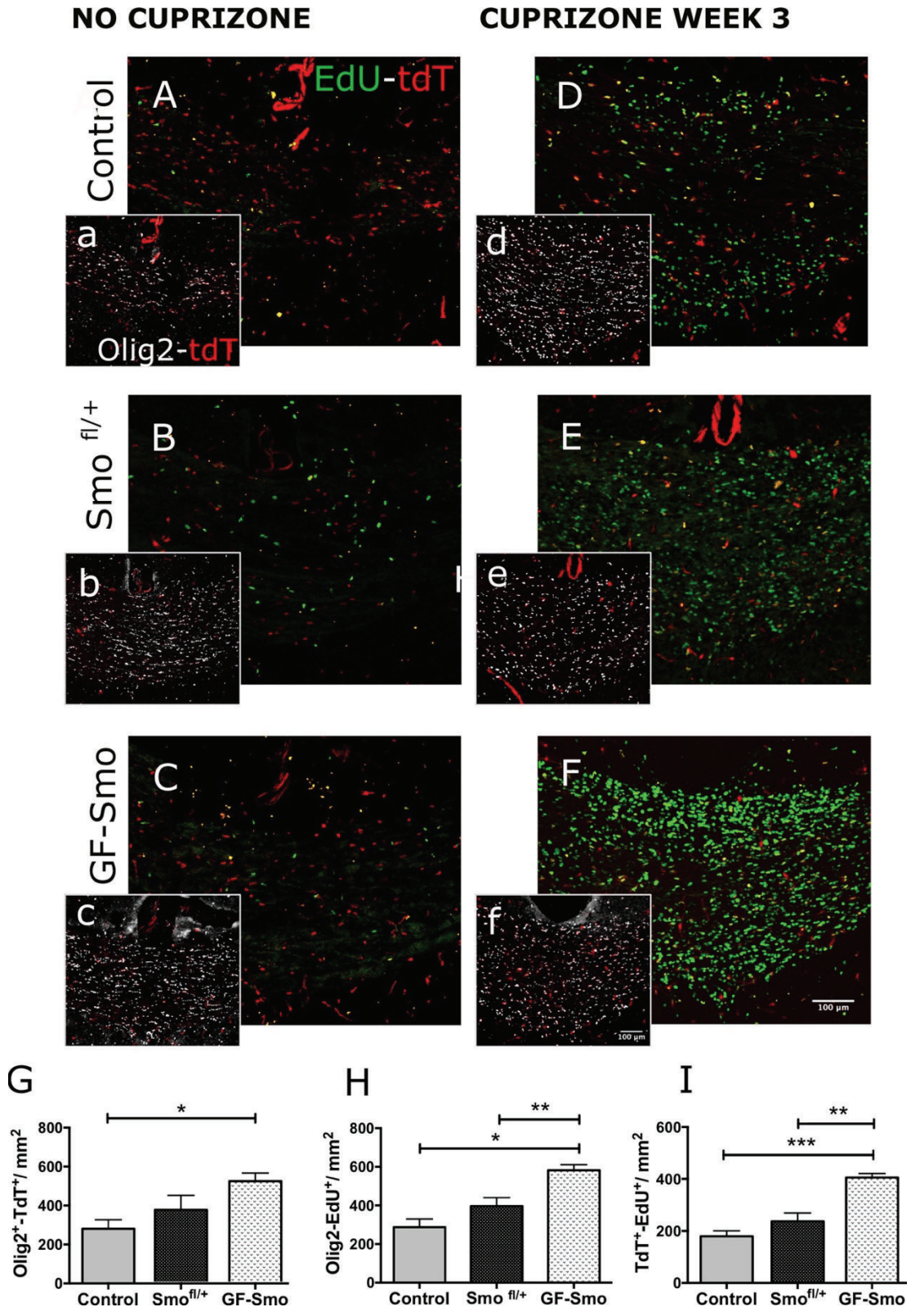

Figure 19 Proliferation at week 3 of the cuprizone diet shows an increase when Shh signaling is overactivated. *Double labeling with EdU (green) and tdT reporter* ***(A-F)*** *or Olig2 (gray) and tdT* ***(a-f)*** *shows that the activation of Shh pathway contributes to the overall increase of proliferation in response to demyelination* ***(G-I).*** *Control mice without cuprizone were age-matched. Scale bar 100 µm. The statistical analysis was carried out using the one-way ANOVA test and the post-hoc Tukey's multiple comparisons test, where *p< 0.05; **p< 0.01; **p< 0.001*

Remyelination study after Cuprizone demyelination

Remyelination occurs spontaneously after removing the cuprizone diet, and mice returned to standard chow (Gudi et al. 2014). To study if Shh signaling specifically intervenes in this spontaneous myelin repair, cell recombination was induced at 5 weeks, and brains were examined at 6 (5 weeks of cuprizone diet + 1 week of recovery) and 7 weeks (5 weeks of diet + 2 weeks of recovery; Fig. 20). In this way, we study the consequence of the Smo-modulation after the demyelinating insult.

***Figure 20 Timeline for the remyelination study**. Tamoxifen was injected during the last week of cuprizone, and remyelination was studied after one or two weeks of recovery.*

In this experimental design (delaying the tamoxifen injection at the last week of cuprizone exposure), the worst results were surprisingly obtained in the *Smo*$^{fl/+}$ group, while no differences were achieved between control and *Smo*$^{fl/+}$. The animals receiving standard chow diet did not show differences in the Olig2-TdT^{+} cells density (Table 3); however, in agreement with the results obtained during myelination in normal development (Fig. 13), *GF-Smo* animals showed less rate of mature OLs-tdT^{+} cells (Table 3, no statistical analysis was made due to the insufficient number of replications).

Table 3 OLs density and rate of mature cells in standard chow-fed mice.

NO Cuprizone	Control	*Smo*$^{fl/+}$	*GF-Smo*
Olig2-tdT density	519	485	417
% CC1tdT/tdT	73.4	70.5	43.4

After one week of cuprizone recovery, recombined-OLs (Olig2-tdT$^+$) density was significantly larger than in animals with a standard diet (Fig. 21A, D, G, J, M, Table 3). Moreover, the *Smo$^{fl/+}$* group showed significantly larger amounts of recombined-OPC than *GF-Smo* (Fig. 21D, G, J, M). Similar results were obtained for mature OLs population (CC- tdT$^+$; Fig. 22 F, I, L, N). Although these results just reflect the dynamics of the recombined-OPCs (tdT$^+$), the analysis of the global oligodendroglial population (Olig2$^+$) shown a significantly diminish in the *GF-Smo* (Fig. 21 O); no differences were detected in the amount of mature (CC1$^+$) OLs (Fig. 21 O). It should be noted that the percentage of recombined cells is approx. 40% of the total OLs cells and slightly less (approx. 35%) of them were CC1$^+$.

To study possible differences during the early phases of the remyelination, we also examined the component of central myelin. It has been reported, especially in adult mice, that some epitopes could result "hidden" within the tightly compacted myelin sheaths in the CNS (Gonsalvez et al. 2019). To explore this possibility, we used two different markers of mature central myelin, MBP and MOG. In this case, no differences between Control and any of the conditions modifying Shh signaling were found (Fig. 22).

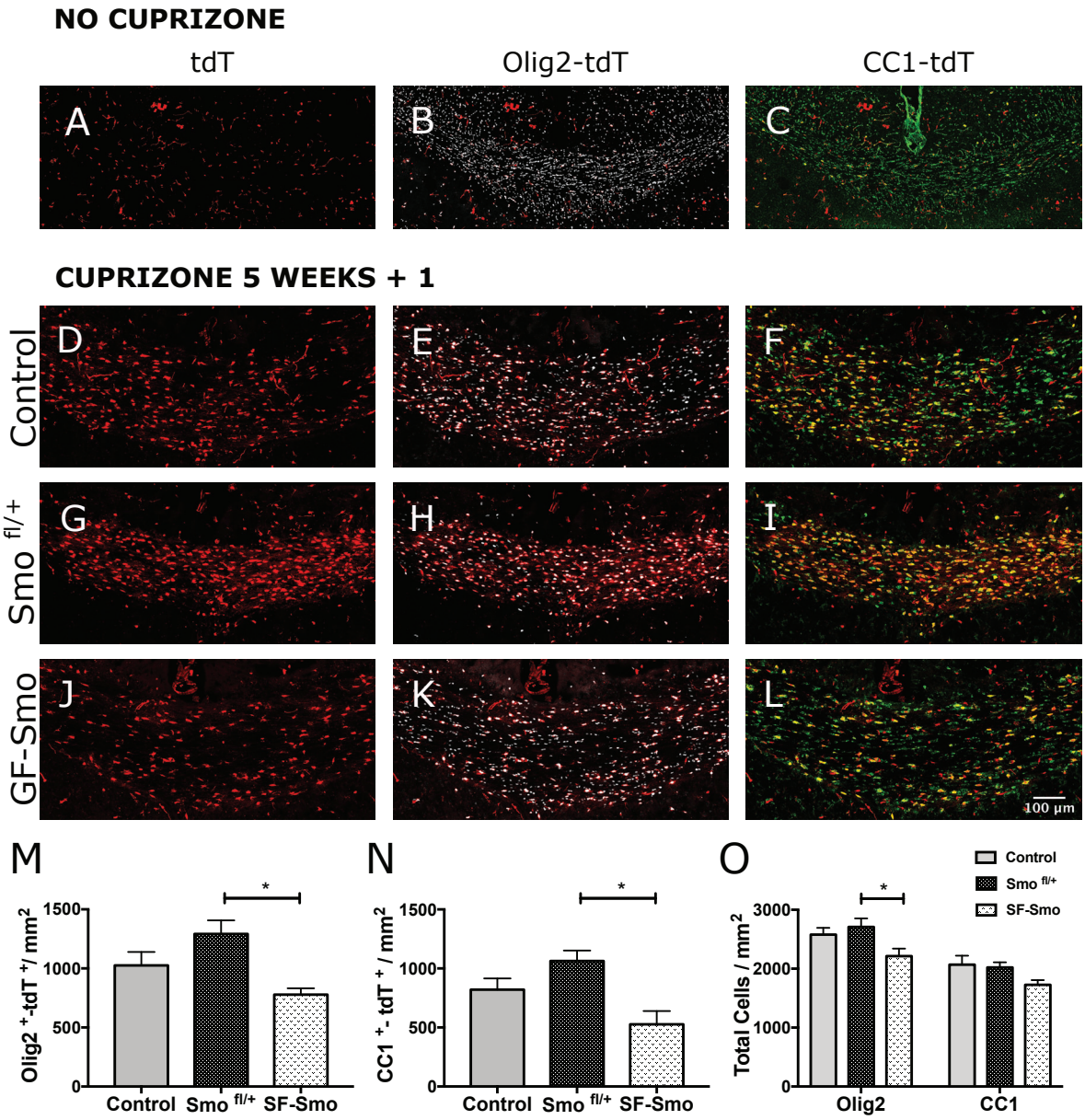

Figure 21 Differentiation of OL after one week of recovery from the cuprizone diet. A-L *Recombined OLs (tdT+) stained with Olig2 (gray) and CC1 (green) in the CC of naïve mice (A-C) and after one week of recovery from cuprizone treatment (D-L). Scale Bar 100 µm.* ***(M)*** *Recombined OLs density does not show differences between the three lines fed with cuprizone. However, the Smo$^{fl/+}$ group response is opposite to the GF-Smo group resulting in a higher number of the oligodendroglial lineage and mature OL* ***(N). (O)*** *The total density of OLs or mature OLs, fed with cuprizone, do not change between the groups; however, Smo$^{fl/+}$ and GF-Smo maintain the difference evidenced in the oligodendroglial lineage. Scale bar 100 µm. The statistical analysis was carried out using the one-way ANOVA test and the post-hoc Tukey's multiple comparisons test, where *p< 0.05.*

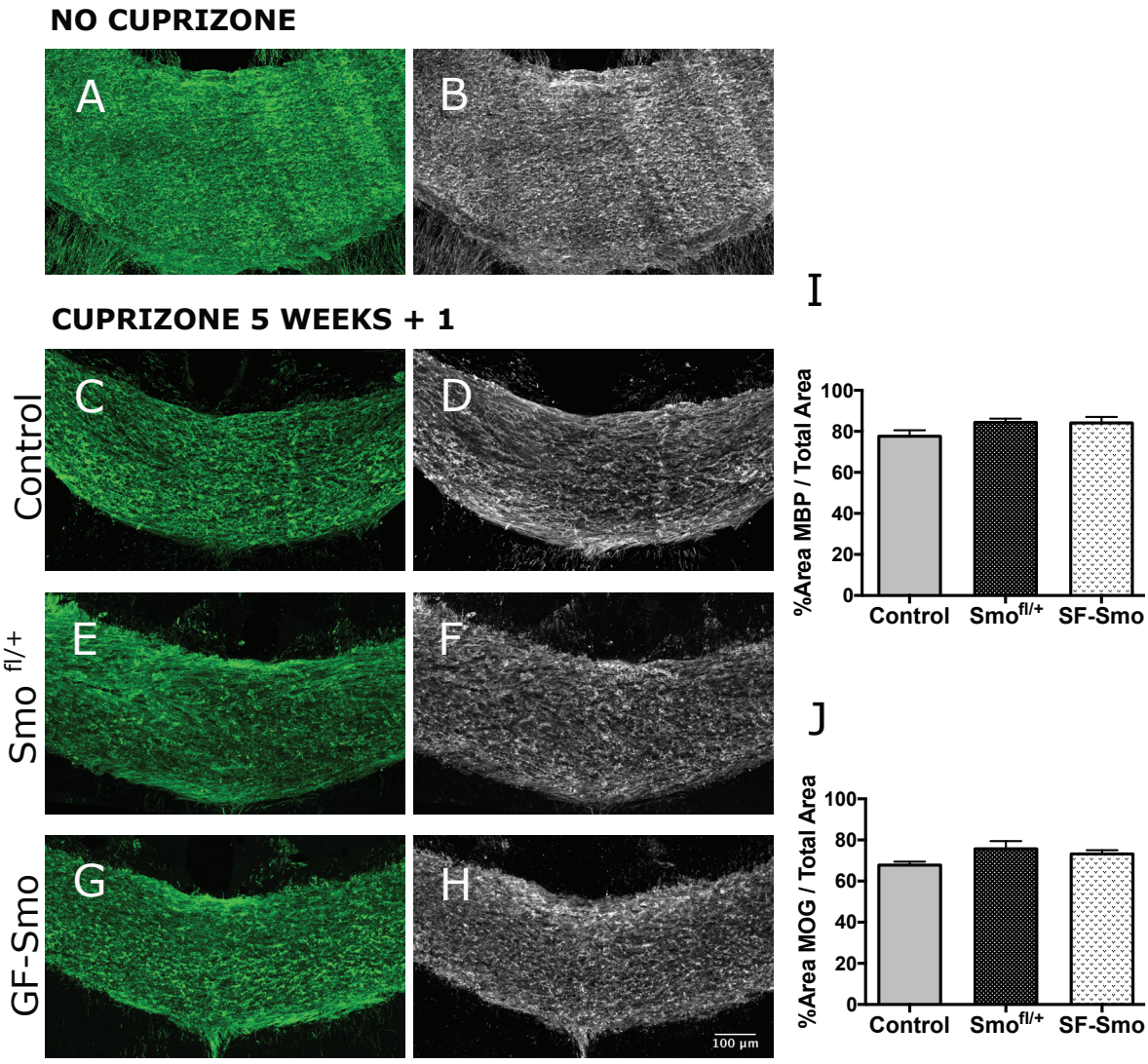

Figure 22 After one recovery week, the CC remyelination does not show differences between the treated groups. A-H *Double labeling with MBP (green) and MOG (gray) of the middle of CC in the control standard chow-fed (A, B) and cuprizone-fed (C-H) groups after one week of recovery.* ***I-J*** *Quantification of myelin percentage stained by MBP (I) or MOG (J) over the total area analyzed. Scale bar 100 µm. The statistical analysis was carried out using the one-way ANOVA test.*

At 2 weeks of recovery (5weeks+2; Fig. 23), the results obtained were slightly different: in this case, the better recovery was achieved by the *Smo*$^{fl/+}$ animals that showed significantly more recombined Olig2^{+} and CC1^{+} cells than the others groups (Fig. 23 A-K). Differences were more substantial when were quantified the entire oligodendroglial lineage (Fig. 23 J) than only the mature OLs (Fig. 23 K), suggesting that two weeks after cuprizone retrieval, spontaneous remyelination has not been completed and at least partially remain active.

When the entire population was quantified (both recombined or not recombined cells), no differences were observed between the three groups (Fig. 23 L). Those results suggest that Shh signaling act in different ways depending on the time window. During the initial phase of demyelination, Shh acts mainly as mitogen and could be useful for the early stage OPCs to promote their proliferation and subsequently enhance the number of remyelinating cells available. On the other hand, Shh does not help these OPCs to differentiate towards myelin-forming phenotypes (OLs): it seems that Shh may still be pushing the cell to undergo the cell cycle and proliferate. This dynamic behavior of Shh could explain the low number of mature OLs when the Shh pathway is overactivated, then resulting in worst remyelination/repair. On the contrary, the Shh pathway antagonization may promote remyelination, at least once the number of OPCs has already increased.

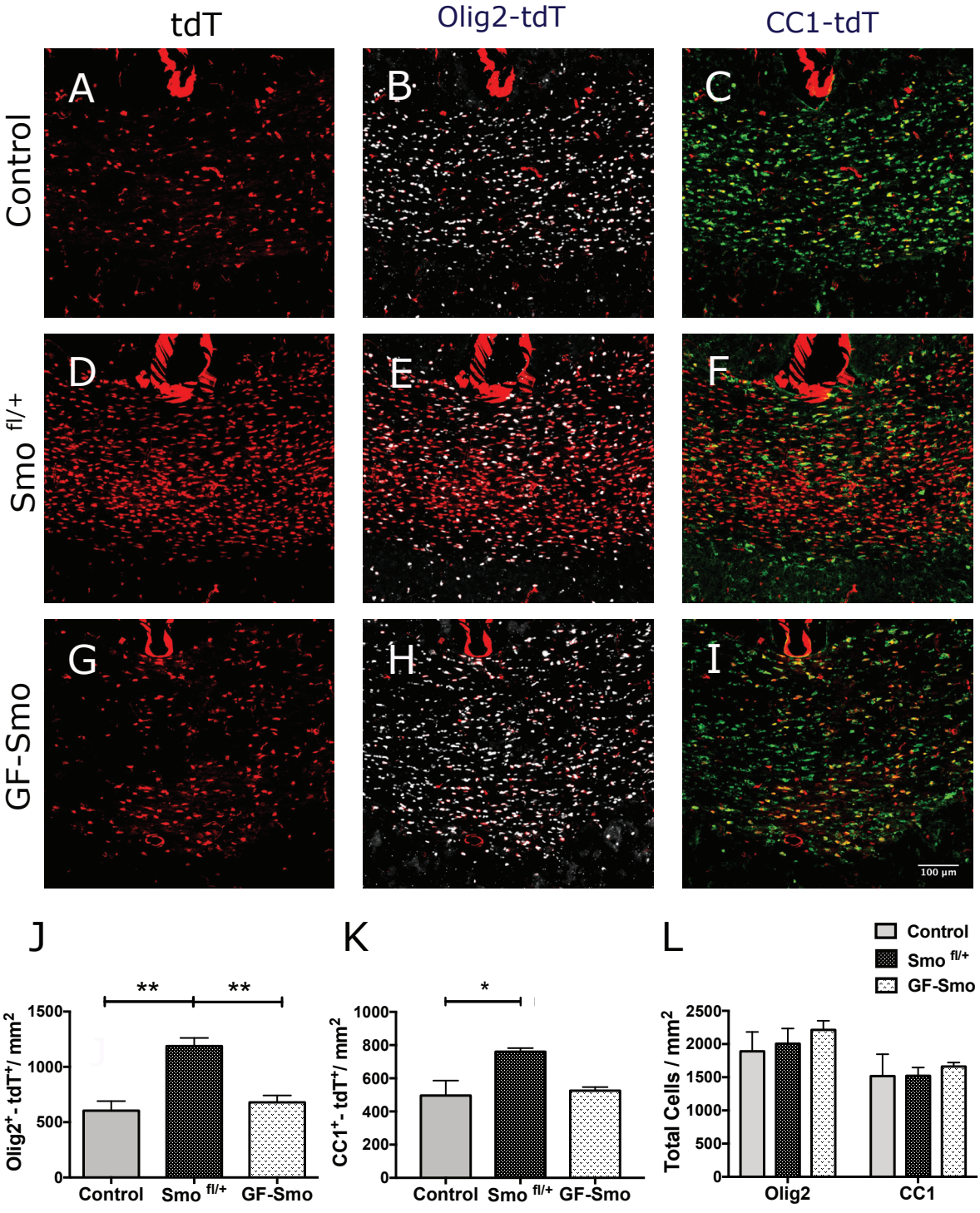

Figure 23 The partial loss of function affects the OLs differentiation after two weeks of recovery from the cuprizone diet. A-I *Triple labeling with Olig2 (gray), CC1 (green) and tdT reporter. Scale Bar 100µm.* ***J*** *Smo$^{fl/+}$ line shows that the partial inhibition of Shh pathway act on the increase of recombined OLs cells in response to demyelination, most of them are mature OLs* ***(K). L*** *The total density of OLs or mature OLs does not change between the groups. Scale Bar 100µm. The statistical analysis was carried out using the one-way ANOVA test and the post-hoc Tukey's multiple comparisons test, where *p< 0.05; **p< 0.01; **p< 0.001*

Electron microscopy study of remyelination

We analyzed the CC by electron microscopy after two weeks of recovery to enable a more detailed ultrastructural examination. The hallmark of remyelination is characterized by abnormally thin myelin sheaths (Franklin e Goldman 2015) measured by G-ratio (the ratio of axon diameter to myelinated axon diameter).

The proportion of myelinated versus no myelinated axons was not significantly different in the Smo-mutants compared with Controls animals (data not shown), indicating that the modulation of the Smo gene does not result in abnormal myelination. The analysis of more than 1.500 axons from the three animals studied for each genotype showed that all three groups had a significant increase of the G-ratio with respect to the non-cuprizone treated mice, confirming that demyelination with cuprizone happened and remyelination is still ongoing (Fig. 24 A-G). When G-ratio was compared among remyelination groups, the $Smo^{fl/+}$ group shows significantly higher G-ratio with respect to the Control and *GF-Smo* mice, reflecting enhanced remyelination (Fig. 24 G). To reinforce this finding, we next sorted remyelinated axons (G-ratio ≥0.8) from pre-existing myelinated axons (<0.8). The result confirms a more significant number of axo ns undergoing remyelination in the $Smo^{fl/+}$ group (Fig. 24 H).

To determine how G-ratio changes with respect to the axon diameter, we plotted G-ratio against the diameter of individual myelinated axons (Fig. 24 I-J). G-ratio increases as axon get larger (1μm); however, as axonal diameter augments, the G-ratio increase slope becomes smaller. We found a widespread myelin thickness for axons of different diameters. As previously described, G-ratio is a valid measure of repair of large diameter myelinated axons (Franklin e Ffrench-Constant 2008; Michailov et al., 2004). However, it was not an accurate indicator of remyelination for small diameter axons (0.4-0.6μm) in the CC region analyzed in the present study, resulting in similar remyelinated and normally myelinated axons. All together, these findings confirm that Shh pathway antagonization could be a good target to promote remyelination.

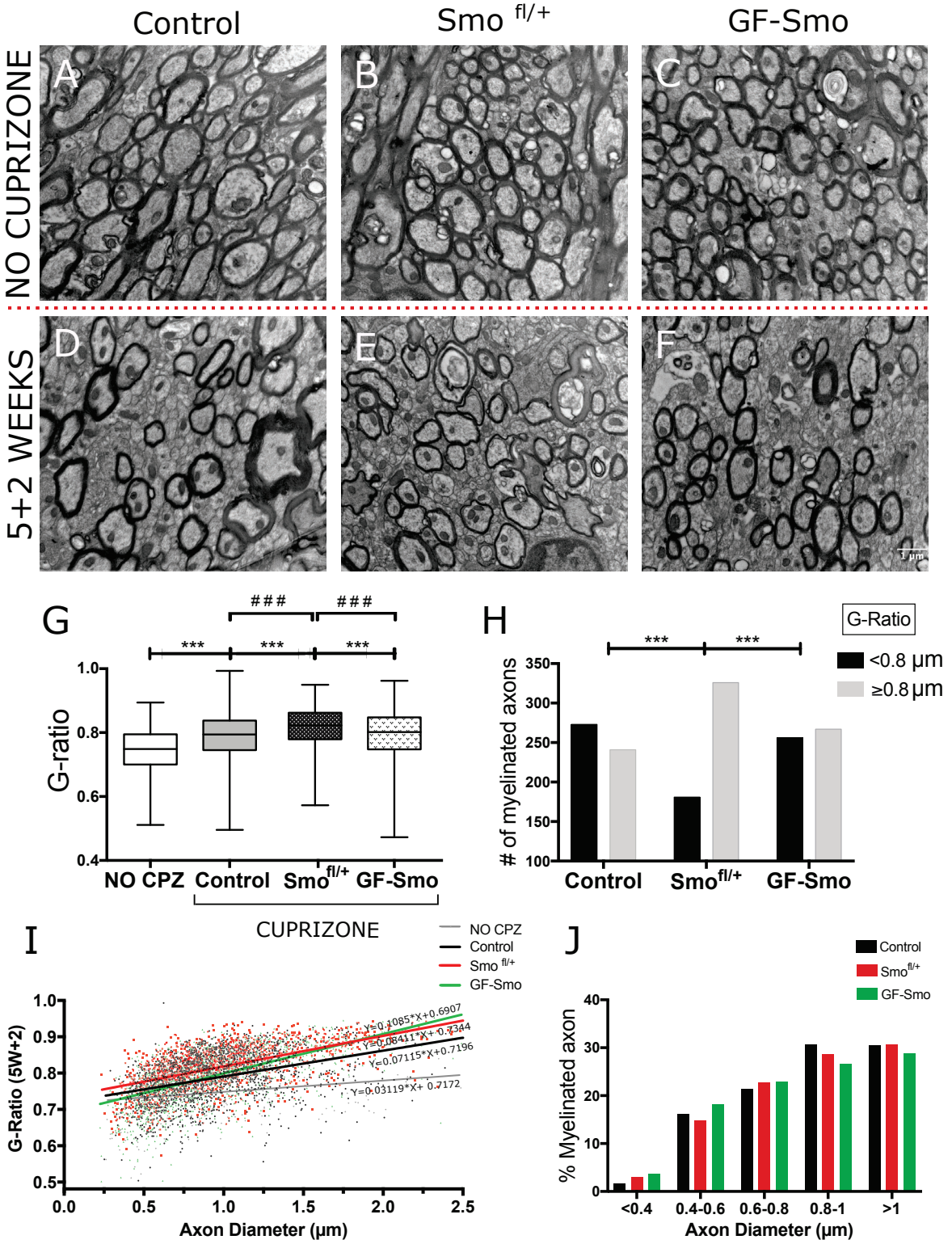

Figure 24 The partial loss of function increase remyelination. A-F *Electron microscopy of the CC from transgenic mice naive (A-C) and after 2 weeks of recovery from cuprizone (C-F). Scale Bar 1µm.* ***G*** *Box and whisker plot shows an increase of the G-ratio of myelinated axons in cuprizone-treated mice with respect to the NO-CPZ group. Smo $^{fl/+}$ group reveals a higher G-ratio with respect to the GF-Smo and Control lines.* ***H*** *Quantification of myelinated axons with G-ratio <0.8 or ≥0.8.* ***I*** *The scatterplot displays the G-ratio of individual axons as a function of axonal diameter.* ***J*** *Percentage of the myelinated axon as function of axonal diameter. Scale Bar 1µm. The statistical analysis was carried out using the chi-square and one-way ANOVA test with the post-hoc Tukey's multiple comparisons test, where ***p< 0.001*

Fate mapping analysis of Smo modulated OPCs after demyelinating insult

Depending on the combination of growth factor and culture condition, OPCs have been shown to exhibit lineage plasticity, and their differentiation can be directed towards protoplasmic astrocytes (Kondo e Raff 2000; Suzuki et al., 2017). Further, in response to traumatic injury, it has been shown that OPCs may adopt different fates depending on the environmental influences (Viganò et al., 2016). To study the potential fate of OPCs following cuprizone-induced demyelination in our experimental condition, we examined the CC for the identification of the tdT$^+$ cells that were positive for other neural markers like NeuN (neuron), Doublecortin (neuroblast), GFAP, or S100 (astrocyte). No tdT$^+$ cells exhibited immunoreactivity for NeuN or doublecortin in the CC of either Control or Smo-modulated mice at 5 +2 weeks (Fig 25). However, a variable percentage of astrocytes was found (between 6 and 17%, Fig. 26 A-I), although no statistical differences between them were obtained Fig. 27 J).

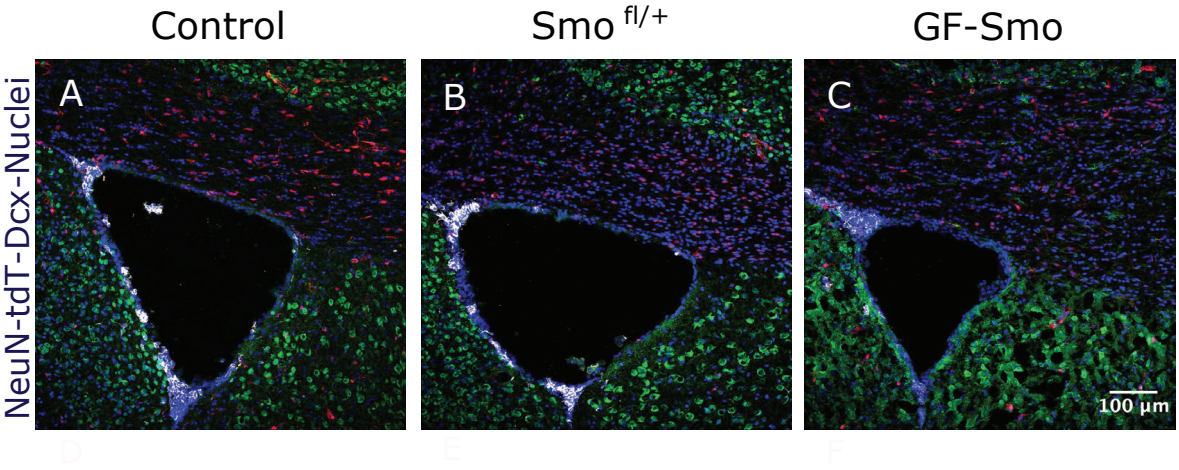

Figure 25 Fate mapping analysis shows no colocalization of tdT$^+$ cells with neuron or neuroblast markers. *After two weeks of recovery, tdT$^+$ cells do not produce fate neuron or neuroblast in response to acute demyelination, as shown by the double staining with NeuN (neuronal marker, green) or Doblecortin (neuroblast marker, white). Scale Bar 100µm*

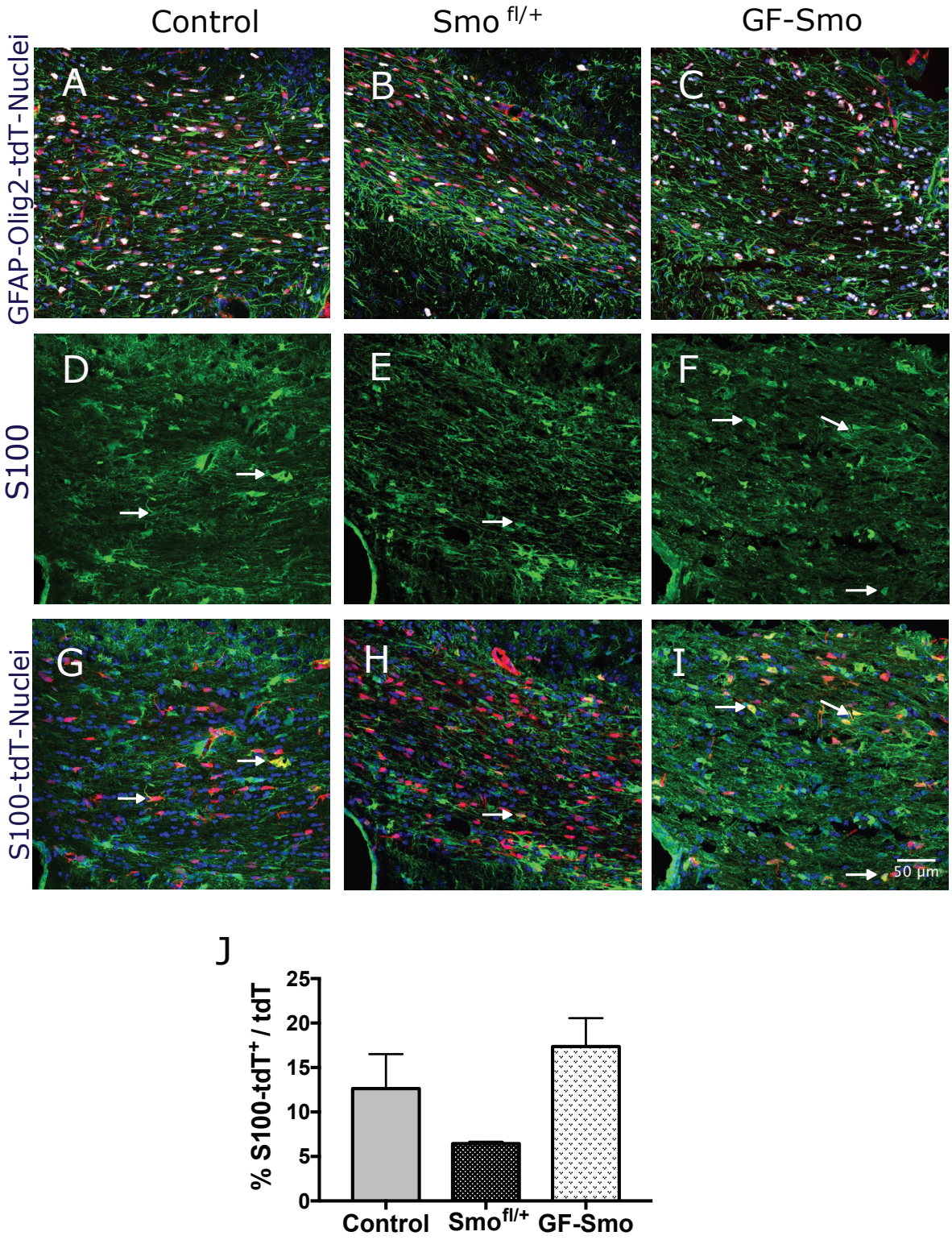

Figure 26 Fate mapping analysis shows colocalization of tdT+ cells with a small percentage of astrocyte marker. A-C *Double staining with GFAP (green) and Olig2 (white).* ***D-J*** *The astrocytes marker S100 shown a small percentage of tdT+ cells that differentiate into astrocytes. Arrows point to double labeled cells (S100+tdT+). Scale Bar 50µm. The statistical analysis was carried out using the one-way ANOVA.*

The downregulation of Smo increases myelination after OPCs transplantation into the retina.

The great difficulty of identifying the involvement of Shh pathway in the differentiation of OLs and (re)myelination is also due to the environmental factors (developmental or following demyelinating injury) that could affect the Shh pathway response.

It has been shown previously that transplantation of OPCs into the retina of young adult and aged rodents result in the formation of a myelinated patch of axons within the nerve fiber layer (Setzu, Ffrench-Constant, e Franklin 2004; Setzu et al., 2006). Axons in this layer remain unmyelinated throughout life due to the inability of OPCs to migrate out of the optic nerve and cross the lamina cribrosa (Ffrench-Constant et al., 1988; Perry e Lund, 1990; Spassky et al., 2002; for a review see: de Castro e Bribián, 2005). Thus, we took advantage of this method to test the hypothesis that the Smo receptor downregulation leads to an increase of OPC differentiation and myelination, while the upregulation delays it.

After the injection of tamoxifen at P1-P2 through lactating mothers, P7 brains were used to isolate OPCs from the cerebral cortex of the three transgenic lines. Isolated OPCs were then delivered to the retina of adult Wild Type mice via intravitreous injection. After five weeks, retinas were studied for the presence of tdT^+ cells and myelin. We found OPC transplants were associated with myelination of the retinal ganglion cell axons (Fig. 27 A-H). No labeling was observed from sham-injected or untreated retinas (data not shown). In all animals, myelin formed a distinct patch that was maximal at the optic nerve head, where the neurofilament layer is thickest and followed the fascicles radially toward the periphery (data not shown). The area of myelination achieved from OPC transplants isolated from the $Smo^{fl/+}$ and *GF-Smo* cortices was not significantly different from the control. However, significant differences were found comparing the *GF-Smo* and $Smo^{fl/+}$ myelin area. A possible explanation was that when OPC are injected into a relatively quiescent, non-inflammatory environment the extent of myelination is limited to a small area (Fig. 27 A-C; G-H). However, downregulation of Smo in this condition leads to an increase of OPC differentiation and myelination extension achieved by a similar number of transplanted OPCs (Fig. 27 D-F). This result indicates that the partial loss of the Smo has different effects depending on surrounded environment that could compensate the loss of one allele (as the ones that regulate postnatal

myelination or are upregulated during remyelination). On the other hand, the down-regulation of Smo in a "neutral" environment confirms its effect on promoting the maturation of OPCs and myelination.

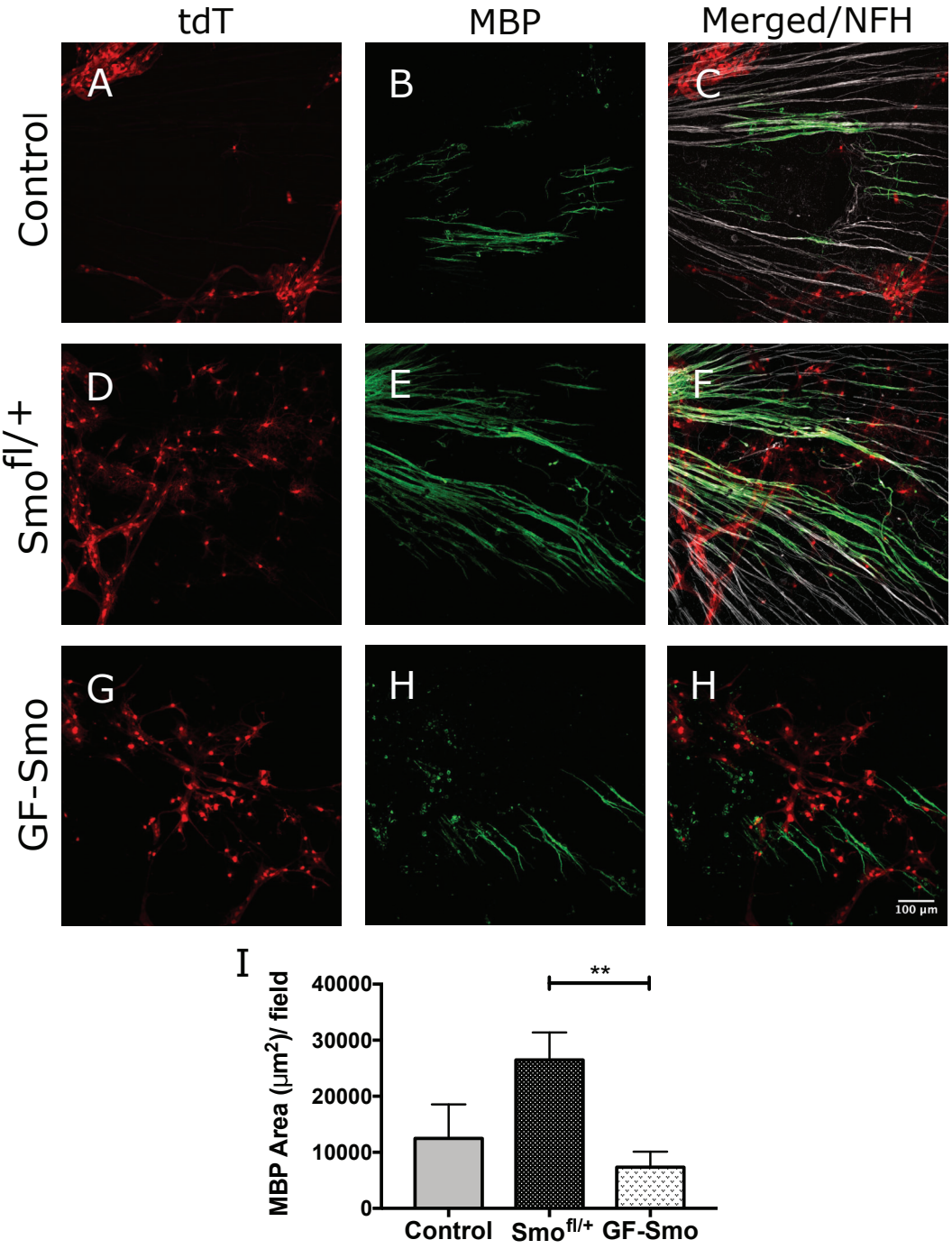

Figure 27 Intravitreous tdT+ OPCs integrate into the retina and differentiate into myelin-forming OLs. ***A, D, G*** *tdTomato cells differentiate into myelin* ***(B, E, H)*** *labeled with MBP antibody.* ***C, F, H*** *Merge showing extensive myelination of the ganglionar layer following 5 weeks after transplantation of primary OPCs. Scale Bar 100µm. The statistical analysis was carried out using the Student's t test, being the statistical level established as **p < 0.01.*

Shh ligand effect on adult human OPCs in vitro.

In view of what happened *in vivo* in the transgenic mice undergoing demyelination, we proceeded to study the effect of Shh on human adult OPCs (haOPCs) proliferation and differentiation. For this purpose, we used human cortex samples from non-tumoral biopsies (See methods). After isolation, we cultured the cells for six days to allow the recovery. As normally done in our group for such sensitive experiments (Bribián et al., 2020), first, we confirmed that the isolated haOPCs expressed the characteristic markers of OPC (Fig 28 A-D). We also analyzed the culture by EM, confirming that the isolated OPCs showed the typical undifferentiated aspect, an electron-dense nucleus, a distinctive feature of OLs, with chromatin in lumps associated with the internal nuclear membrane (Fig. 28 E). Furthermore, the presence of polyribosomes indicated that there was protein synthesis (Fig. 28 E).

After verifying that the OPCs were completely viable and functional for our study, we aimed to study the proliferation of human OPCs in the presence of the Shh ligand. For this purpose, after the 48h of recovery, a protocol of 24 hours of 5-Bromo-2'-deoxyuridine (BrdU) incorporation was applied to estimate the proliferation rate. Although the percentage of proliferating OPCs was low in both control and Shh treated condition, this latter showed a significant increase with respect to the control (Fig. 28 F-N). This finding suggests that, as viewed in mice, Shh stimulates adult human OPC to proliferate. Moreover, to study Shh implication on haOPCs differentiation, cells culture was held for ten days *in vitro* (10 DIV), then tested for the expression of O4 and MBP, two markers characteristic of pre-OLs/immature-OLs, or myelinating OL, respectively. Both conditions presented the two markers expressed in the same cells indicating that in humans, the transition from pre-OL to mature-OL could not follow the same timing as in rodents regarding the protein expression during maturation. Thus, we decided to follow these cells for a more extended maturation period in both conditions. The study of DIV16 to DIV20 cells shown that cells treated with Shh presented more branching compared to the control, however, we cannot conclude that this effect is due to a better differentiation or an indirect influence, as could be a better survival of the OPCs treated with Shh. It is also to consider that we use one sample for the 10DIV assay and a different one for the 16-20DIV assay. Due to the variability of the cultures, the age of the

patients, and the cortex regions, more assays has been done to confirm that Shh could also affect the differentiation of haOPCs.

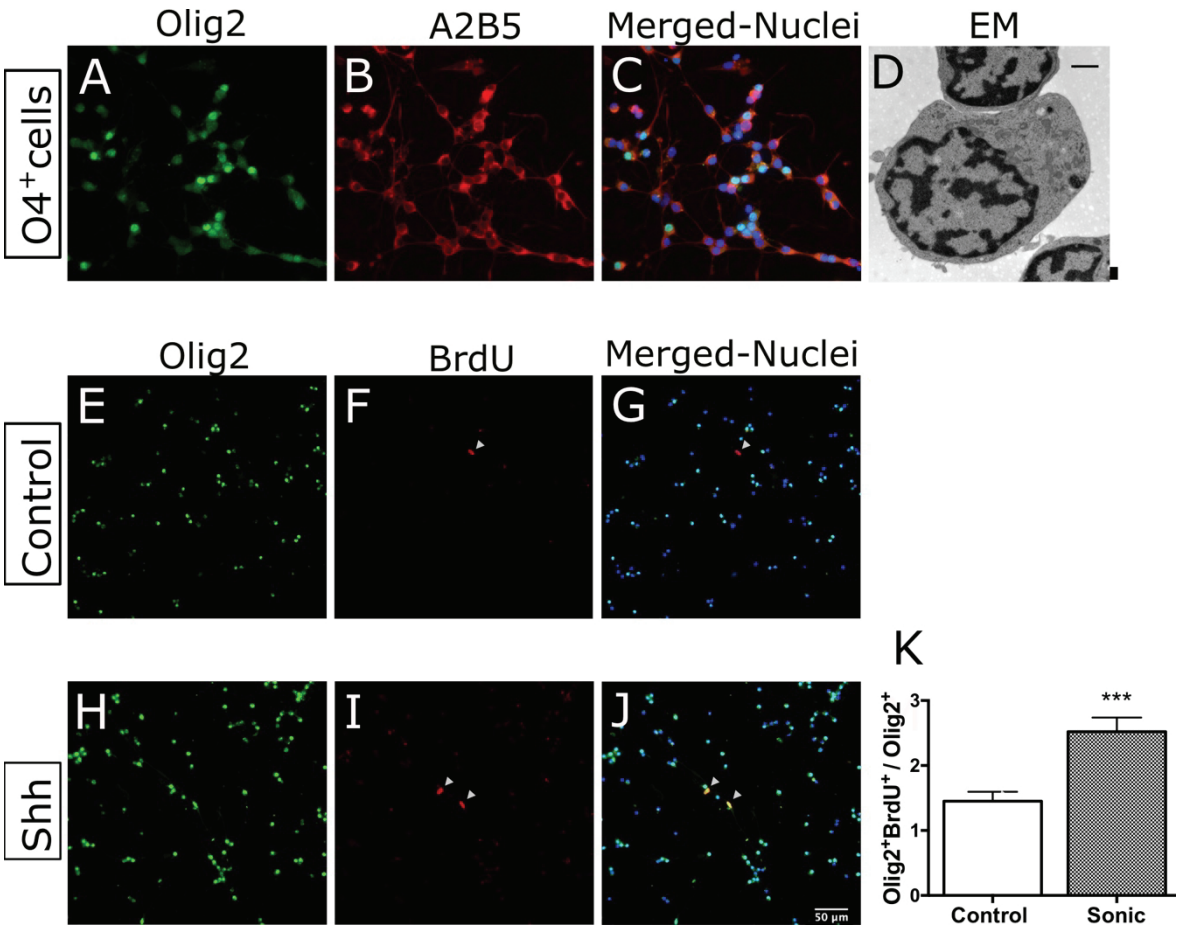

Figure 28 Characterization and proliferation assay of O4⁺ isolated from adult brain cortex. A-C *Representative images of O4⁺cells at DIV6 labeled with Olig2 (green), A2B5 (red). Almost all of the cells show colocalization of the two markers.* ***D*** *Representative EM image shows the typical undifferentiated aspect of OPC.* ***E-J*** *Representative images of O4⁺cells in control conditions and treated with Shh. Cells are labeled with Olig2 (green) and BrdU (red). Arrowheads point to double labeled cells (Olig2⁺BrdU⁺).* ***K*** *Quantification of OPC proliferation rate show increased proliferation after Shh treatment respect to the control. Scale bar 50µm (Scale bar is equal to 26 µm in A-C; Scale bar 1µm in D). The statistical analysis was carried out using the Student's t test, being the statistical level established as ***p < 0.001.*

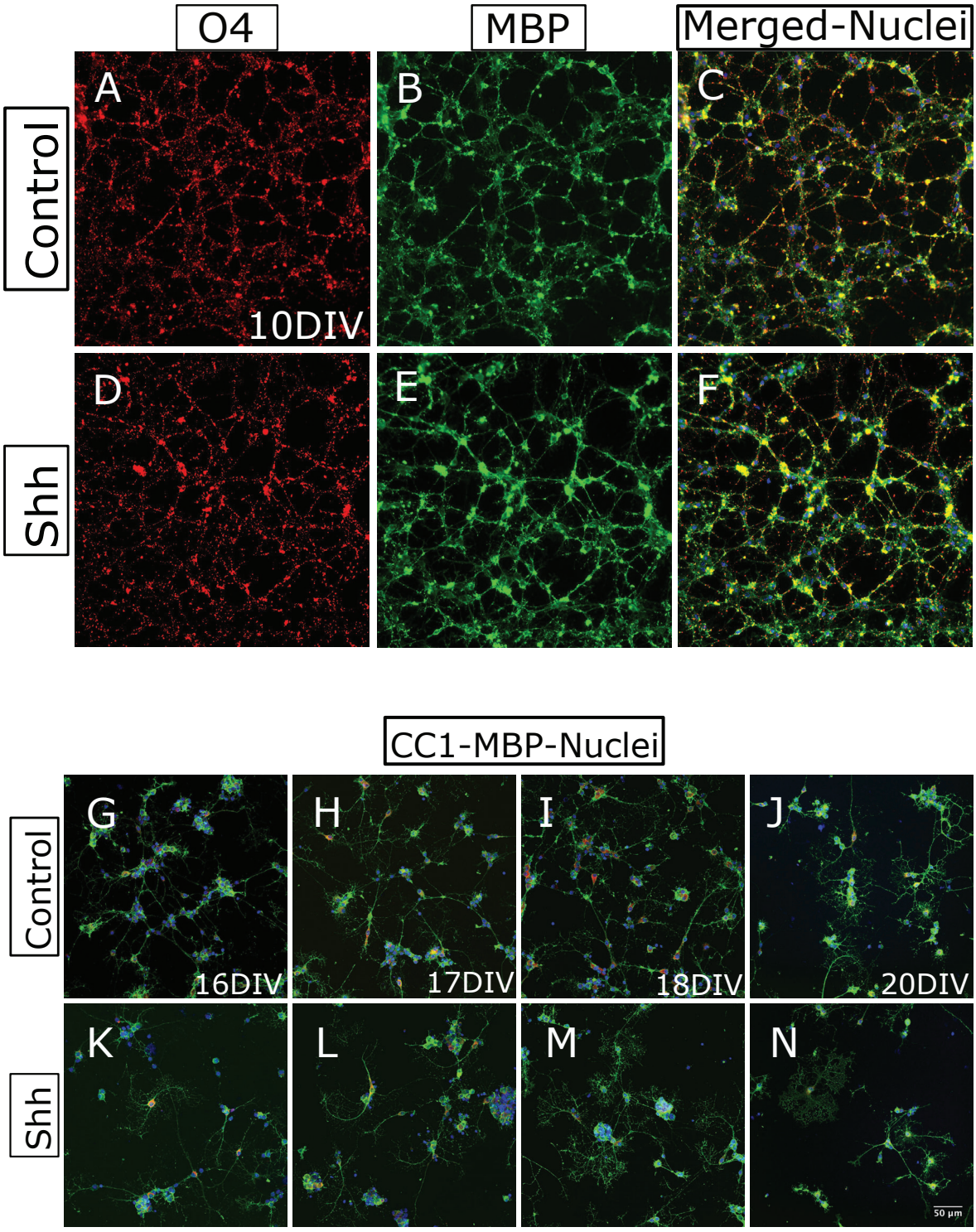

Figure 29 Differentiation assay of OPCs isolated from adult brain cortex. A-F *Representative images of mature OLs at DIV10 labeled with O4 (red), MBP (Green). All the cells show colocalization of the two markers.* ***G-N*** *Representative images of differentiated OLs at 16DIV (G, K), 17DIV (H, L), 18DIV (I, M), 20DIV (J, N). Scale Bar 50µm (Scale bar is equal to 66µm in A-F)*

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DISCUSSION

Discussion

One of our group research lines has been focused for years on Shh signaling as mitogen and chemoattractant of OPCs, as well as in the mechanism controlling the level of Shh available for this cell type to myelinate the optic nerve during development (Merchán et al., 2007; Ortega et al., 2012). Moreover, in the last few years, we extended this research line to the involvement of Shh pathway regulation during MS (data not yet published) too. In the present work, we used in vivo genetic approaches to increase or decrease Shh signaling, and then study how Shh could regulates parenchymal OPCs during postnatal development and repair processes.

Soon after birth, parenchymal OPCs are uniformly distributed throughout the whole brain (grey and white matters), enabling OLs rapid generation. OPCs keep generating OLs also in maturity and, although diminished, even in the aging mice brain (Bergles e Richardson, 2015; Hughes et al., 2013). Moreover, parenchymal OPCs respond locally to demyelination by generating new OLs (Franklin e Ffrench-Constant, 2008; Huang et al., 2014). Several studies involving the morphogen Shh have revealed his contribution during the specification, proliferation, and differentiation of the OPCs at different stages of CNS development (Briscoe e Thérond, 2013). However, the direct role of Hh signaling on the parenchymal OPCs after birth remains unclear.

Postnatal regulation of OPCs proliferation and differentiation by Shh pathway

The morphogen Shh aroused a lot of interest since it was characterized as necessary and sufficient to express the oligodendrocyte transcription factors Olig1 and Olig2 associated with the oligodendroglial lineage in the developing neural tube (Martí et al., 1995; Pringle et al., 1996; Poncet et al., 1996). The morphogen Shh was also found essential for the organization of neuronal subtype identity in the vertebrate CNS during development, as well as it is implicated in the development and postnatal maintenance of SVZ neural stem cells (Machold et al., 2003; Loulier et al., 2006; Balordi e Fishell, 2007b; 2007a; Julien Ferent et al., 2014). In the present study, we examined

the OPCs differentiation status after a conditional increase or decrease Shh cascade in the $NG2^{+}$ cells during postnatal myelination.

Hh family is composed of several proteins whose functions are partially redundantly regarding specific cellular functions. For example, Shh null mutant mice were reported to show normal oligodendrocyte generation, which implies an effective compensation by the other Hh proteins (Nery et al., 2001; Bürglin, 2008). To rule out effects due to partial compensation, we decided to focus our attention on the Smo receptor essential for the transduction of this signaling pathway. Thus, the modulation of Smo gene was used to act both in the canonical and non-canonical pathways through a combination of *in vivo* conditional approaches, which led to the partial loss and gain of function of OPCs. Tamoxifen was administered to the lactating mother at P1-P2, and OPC differentiation was studied at P14, coincident with the peak of postnatal myelination (Parnavelas, 1999; Murtie et al., 2005; Bribián et al., 2008; de Castro e Zalc, 2013; Sánchez e Armstrong, 2018). We found a higher number of recombinant OPCs in the CC of the group where Shh signaling was overactivated with respect to the Control or $Smo^{fl/+}$ groups, concluding that, after the commitment in OL-lineage, upregulation of Smo in OPCs continue to promote their proliferation. The increase of recombined-OPCs can be explained by looking at the downstream proteins activated by the Shh pathway. In the CNS, cell cycle control occurs during the G1 phase transition. Extracellular signals as mitogens drive cells through G1/S transition into the S-phase, entering another cell division round. In the absence of mitogens (or in the presence of anti-mitogenic signals), the cell withdraws from the cell cycle and may start the differentiation program (Casaccia-Bonnefil e Liu, 2003). In the NSC and OPCs, the progression from one phase of the cell cycle to the next one is regulated by cyclins. Several works had shown how Shh pathway leads to the activation of cyclins D and E, which activate cyclin-dependent kinases, CDK4/6 or CDK2, respectively. Both cyclins have been found in several CNS structures as the retina and the spinal cord, increasing the proliferating neural progenitors at the expense of neural differentiation (Casaccia-Bonnefil e Liu, 2003; Lobjois et al., 2004; Yaping Wang et al., 2005; Locker et al., 2006). Avery recent work shows a cross-link between the overactivation of Smo in OLs and the increase of cyclin D1, leading to the enhance of OPCs proliferation (Xu et al., 2020). In our present work, we did not study the rate of proliferating OPCs

directly, but the high number in the GF-Smo mice led us to think that Shh pathway blocking the OPCs in an undifferentiated stage could be promoting their proliferation, affecting their differentiation into mature OL. We confirmed this phenomenon by looking at the percentage of mature OLs: as expected, we found a decrease of $CC1^+$ cells in the GF-Smo mice with respect to the other groups, confirming that the differentiation was inhibited. Although the total density of OPCs was not changed between the Control or $Smo^{fl/+}$ groups, the proportion of the total OPCs with respect to the mature OLs was higher in the GF-group. The apparent divergence between the unaltered OPCs density and the substantial increment in the recombinant-OPCs may be explained by compensatory mechanisms between proliferation and survival, which is fundamental to maintain the correct equilibrium during development. (Emery e Lu, 2015; Bergles e Richardson, 2015). These findings, including our present work, are in conflict with the *in vitro* findings from Wang and Almazán (2016). They manipulated Shh signaling with cyclopamine (Smo inhibitor) and found that inactivation of Shh signaling causes a dose-dependent decrease of MBP^+ in OLs primary culture, while when they agonized Smo with SAG (Smo agonist), they observed reversion (L.-C. Wang e Almazan, 2016). One possible explanation is that the authors observe an increase of arborization in the cells treated with SAG. Since Shh is also implicated in the OPC survival (Traiffort et al., 2016), SAG treatment's effect may be due to an increase in survival more than in the number of differentiated OLs. Moreover, cyclopamine causes many potential side effects that could mask its pro-differentiating power (Rimkus et al., 2016). In line with this, either adenovirus-mediated ablation of Smo or its ectopic activation resulted in, respectively, a reduced or increased generation of mature OLs in the CC of neonate. These results were also confirmed using transgenic mice, where the Smo receptor was genetically inactivated or activated in the dorsal radial glial cells (RGCs; Tong et al., 2015). However, the fact that the authors target the RGCs tell us that decreasing or increasing Shh pathway in these cells leads to minor or major production of fate-OLs respect their control. Thus, a higher number of OLs are available for differentiation, but the study did not elucidate the effect on committed-OPCs. On the other hand, studies in spinal cord development have shown that increasing Shh expression level during the embryonic stages enhanced the generation of precursor cells but blocked their maturation. These findings provided evidence

that proliferation and differentiation can be regulated separately in precursor cells of the spinal cord (Rowitch et al., 1999). The advantage of our present studies is that we could quantify the direct effect of Shh on OPC differentiation in vivo. We can affirm that postnatal OPCs can respond to Shh stimulus, increasing the number of OPCs in the GF-Smo mice, and that increase is reflected in a minor number of mature OLs.

To elucidate how a delay in the OLs differentiation would affect myelination, we focus on the CC myelination of axons and the organization and density of the nodes of Ranvier. Paranodes are sites of tight contact with the myelinating glia, ensuring the electrochemical insulation of the nodal area from the adjacent membrane domains. Changes in white matter structure are often assumed to reflect an altered number of myelin wraps (Savvaki et al., 2008; Ritter et al., 2013; Arancibia-Cárcamo et al., 2017; Luo et al., 2018). Thus, the function of myelinated axons is dependent on the structure of the node of Ranvier. During development, an optimal level of myelination is achieved, and hyper or hypomyelination results in functional deficits that may be reflected in perturbation of neuronal activity resulting from changes in the internodal and/or nodal organization (Richardson et al., 2000; Edgar e Sibille, 2012; Bakiri et al., 2011; Ritter et al., 2013; Murcia-Belmonte et al., 2016; Luo et al., 2018). Consistent with our previous findings, in our present work, we observed a decrease of myelination in the GF-Smo mice with respect to the control, without any change in the axonal content of the CC. Moreover, the density of nodes of Ranvier was also reduced compared to the Control or *Smo*$^{fl/+}$ mice, which suggest that GF-Smo have longer internodes. Those results let us postulate that the saltatory conduction would be also affected.

Our data agree with the work of Samanta et al. 2015, where precocious myelination is observed in 9-days old Gli1 null mutants mice, suggesting that Gli1 (which is an effective readout of canonical Shh pathway) results in a delay of the onset of myelination, respect their control (Samanta et al., 2015). Finally, the work from Xu et al. (2020) in the spinal cord helped to clarify the stage-specific regulation of OL by Smo-mediated signaling. Using transgenic inducible mice to manipulate Smo receptor, the authors found that increasing Smo pathway in the NSC provoked an increment of OPCs proliferation and a precocious OLs differentiation. However, when they act directly on committed-OPCs, the overactivation of Smo promoted proliferation but

negatively regulated their differentiation into myelin-forming phenotypes. Furthermore, when Smo receptors were inactivated in the OPCs, they did not find differences in proliferation or differentiation, suggesting that, after specification, Smo receptor is not required for the terminal differentiation of OPCs (Xu et al., 2020).

As the concentration of Shh exposure is also essential for the induction and proliferation of OPCs (Ribes e Briscoe, 2009; Ortega et al., 2012; Oustah et al., 2014), we genetically inactivated one allele of the Smo receptor to decrease the level of OPCs response to the endogenous Smo activity; then we studied the OPCs differentiation and myelination during the postnatal myelination. As Samanta et al., 2015 with the Gli null mice, we were expecting increased myelination, contrary to the over-activation of Smo. However, we did not find any change with respect to the control, which suggests a possible rescue by the remaining allele or, as demonstrated by Xu et al. (2020) in the spinal cord, that Smo activity is not required for the proliferation and differentiation of OPCs (Samanta et al., 2015; Xu et al., 2020). One of the main differences between these apparently discordant experiments is the embryonal stages on one side, the conditional modulation or postnatal manipulation of the several Hh components on the other side, and the NSC or parenchymal OPCs targeting. It is important to keep in mind that specification and differentiation processes depend on a complex network of extracellular signals and transcriptional factors, as well as epigenetic regulation (Emery e Lu, 2015; Murao et al., 2016; Tiane et al., 2019). Thus, the extracellular environment during embryonal or postnatal stages plays an essential role in the regulation of Shh pathway. Further studies on the complete loss of function (currently underway in our laboratory) will help to elucidate if the inhibition of Shh pathway after birth could play any role in the OPCs differentiation and myelination.

The elucidation of Shh signaling controlling the OLs differentiation during development is a fundamental prerequisite to understanding the molecular mechanisms undergoing myelination and would be useful for studying new therapies for myelin repair. However, the time-window and the concentration in which this morphogen act undoubtedly deserves further investigation.

Shh pathway regulates OPCs proliferation during the demyelination phase of the cuprizone model.

The effects seen during development led our investigation to study the possibility that the Shh pathway could be exploited to therapeutically ameliorate the demyelinating condition and facilitate remyelination in the adult brain. Indeed, remyelination has to be regarded as a regenerative process that shares many features with the myelination mechanisms that occur during development. Suppose remyelination as a recapitulation of what happens during development, sharing many transcription factors and signaling pathways: in that case, the reactivation of the Hh pathway should also be involved during these regeneration events. After discovering its involvement in OPCs proliferation and differentiation (see above), Shh cascade has become the subject of several studies aimed at understanding the role of this pathway during demyelinating diseases such as MS or in its demyelinating murine models.

Wang et al. (2008) investigated Shh expression and some of its downstream proteins in limited human brain samples of MS patients (one active lesion and one chronic-inactive lesion). They show that Shh signaling is upregulated in the active lesions from MS brain samples. On the contrary, Shh cascade was found downregulated in the chronic-active and inactive MS brain samples lesions. The authors confirmed their results using the EAE-murine model: Shh upregulation was found during the onset of the disease and downregulated during the late phases of EAE. Shh transcripts, and their downstream proteins, were also found in the early stages of a lysolecithin-mediated demyelination model, promoting cell proliferation in the CC of mice(Yue Wang et al. 2008; J. Ferent et al. 2013). Since that, many studies have shown how agonizing drugs of Hh pathway are crucial for the promotion of OLs differentiation, while antagonists led to impairment of OLs differentiation and remyelination (Najm et al., 2015; Yao et al., 2016; Del Giovane e Ragnini-Wilson, 2018; Hubler et al., 2018). All these findings suggest that Shh signaling function is activated just at a specific time during the demyelination process. However, none of these studies explain the mechanism by which Shh affects OPC proliferation and differentiation, and if it is due to a direct effect on these cells or is it indirect. To date, no information has been reported about what happens if these signaling pathways are over or downregulated directly on the parenchymal OPCs. To address the consequence of the Smo up or downregulation in

a pathological context, cuprizone demyelination was induced in the same transgenic mice used in our developmental studies. It is important to point out that, due to the heterogeneous processes in MS, the perfect animal model for this disease does not exist, and each model allows to study of distinct aspects of the disease rather than its entire complexity. The appropriate model has to match the pathological feature for which a putative treatment or signaling would be studied. Cuprizone model can induce reliable and consistent demyelination and remyelination in defined white matter tracts without excessive morbidity, mortality and T cell involvement (Stidworthy et al. 2003; Gudi et al. 2014). We chose to inject tamoxifen during or after the cuprizone stimulated demyelination phase and compared the effects with the ones that occurred in the parenchymal OPCs during the progression of postnatal myelination. To understand the role of the Shh pathway in a MS-related pathological contest, we injected tamoxifen simultaneously with the beginning of the cuprizone diet. We then investigated how the Smo receptor modulation affected glia cells response during the demyelination phase. After three weeks of cuprizone diet, microglia and macrophages gained their activation status and phagocytosed the myelin debris generated during demyelination. This is an essential step because myelin and its debris contain proteins that inhibit OPCs differentiation during remyelination (Robinson e Miller, 1999; Syed et al., 2008). Moreover, microglia and astrocytes are crucial in releasing factors implicated in the proliferation of OPCs after demyelinating injury. Indeed, the depletion of macrophagic cells or the pharmacological inhibition during toxin-induced demyelination lead to remyelination impairment (M. R. Kotter et al. 2001; Chari et al. 2006; Pons e Rivest 2020). To confirming the ongoing demyelination, we tested the presence of activated macrophages/microglia cells. As expected, in the CC of all groups, the myelin decrease was accompanied by macrophagic cells increase with respect to the mice that were not treated with cuprizone. Although the $Smo^{fl/+}$ group showed a higher level of macrophages/microglial cells when compared to the control group, there were no differences regarding the number of proliferating OPCs. At the same time, OPCs proliferation was higher in the GF-Smo group. Since the activation of macrophages/microglial is related to the increase of OPCs recruitment (Rawji e Yong, 2013; Gudi et al., 2014; Lampron et al., 2015; Cignarella et al., 2020), we could have expected a more significant OPCs increase in the $Smo^{fl/+}$ group. This apparent

discrepancy can be explained by the fact that we used Iba-1, a marker of both pro-inflammatory and anti-inflammatory (pro-regenerative) macrophages/microglial: while the first one secretes many pro-inflammatory cytokines and free radicals such as and TNF-α, IFN-γ, and nitric oxide associated with excitotoxic neural and OL death. The second one secretes anti-inflammatory cytokines, such as IL-1β, and neurotrophic factors necessary for the survival of OLs and the promotion of remyelination (Elkabes et al., 1996; Mark R. Kotter et al., 2005; Takeuchi et al., 2005; Rawji e Yong, 2013). Nevertheless, the crosstalk between proliferating OPCs and macrophages/microglia was not the aim of our study, as happens with the distinction between the detrimental vs. beneficial effects of activated macrophages/microglia. Instead, we have focused our present work on the proliferation of OPCs, suggesting that the GF-Smo group increase could recapitulate the mechanisms seen during development.

During demyelinating insult, it has been proved an upregulation of several genes involved during oligodendroglial development (Savvaki et al. 2008; Fancy, Zhao, e Franklin 2004). Specifically, growth factors have been identified that can regulate OPC proliferation, differentiation, and survival in demyelinated lesions. The PDGF-A, for example, improves remyelination during the recovery period following cuprizone administration. While acute and chronic demyelination areas have increased expression of FGF2 ligand and FGF receptors (Hinks e Franklin 1999; Frost et al. 2003; Armstrong 2007; Vana et al. 2007). Shh signaling crosstalks with both factors to increase OPCs proliferation during development. Thus, we cannot discard the hypothesis of combining these factors to promote remyelination.

Acting on the Smo receptor modulation, we cannot explain if this signaling promotes proliferation through the canonical or non-canonical pathway. Sánchez et al. (2018) used transgenic mice to label Gli1$^+$cells at P6-9 and examined the fate after acute or chronic cuprizone demyelination. They found that the Shh pathway is implicated in OPC proliferation and contributes to CC remyelination, confirming, in part, our results. But, when they delay the tamoxifen induction to fate Gli1$^+$cells during or after demyelination, no Gli1$^+$cells were found in the CC (table 3). Instead, labeled cells remain restricted to the SVZ, demonstrating the lack of canonical Shh cascade in the CC. However, when they injected SAG directly in the CC after chronic demyelination, they

found increased OPC proliferation and enhanced remyelination without the involvement of Gli1, indicating signaling through the non-canonical Hh pathway (table 3; Sánchez e Armstrong 2018; Sánchez et al., 2018) . Suppose the proliferation effect induced by Smo activation is activated via a non-canonical (Gli-independent) pathway: in that case, our results do not constitute a discordant result with the one seen by the cited authors. We do not know the repercussion of this increase in the recovery phase. Certainly, it represents an interesting clue for complementary studies. Moreover, although all these works have in common the pro-regenerative repercussion induced by Shh pathway, most of them are not able to prove whether it is an indirect effect by acting on cells other than OLs.

Shh pathway influences OPCs differentiation during the demyelination phase.

To address the consequence of the Smo-modulation on the parenchymal OPCs after the demyelinating insult, we delay the tamoxifen injection to the last week of cuprizone diet before mice get back to standard chow, providing cells enough time to undergo recombination and express tdTomato reporter. We examined results after one and two weeks of recovery. Surprisingly, after one week of recovery, the GF-Smo group does not display any difference with respect to the control. In contrast, in the $Smo^{fl/+}$ group, we found an increase in Olig2-tdT^{+}cells density as well as in the density of mature OLs with respect to the GF-Smo group. These differences were more evident after two weeks of recovery. Olig2-tdT^{+}cells density in the $Smo^{fl/+}$ group was higher both in the Control and GF-Smo group, as well as the density of mature OLs was also higher with respect to the Control.

Despite the increase of OLs, $Smo^{fl/+}$ mice did not show any change in remyelination, measured by MBP or MOG expression after one week of recovery. However, differences between groups could be masked because once cuprizone is removed, the remyelination specific events, as the analysis of the white matter tracts, pose a difficulty to be quantified by IHC. Moreover, adult CC represents a complicated area for myelin staining due to epitopes masking (Rawji e Yong 2013; Gonsalvez et al. 2019). To overcome these problems, we analyze the distribution of myelinated axons and G-ratio in

the CC by electron microscopy two weeks after cuprizone was withdrawn. G-ratio cannot be measured for denuded axons. However, as myelin sheath is laid down sequentially, wrap by wrap, newly remyelinated axon results in a thinner myelin sheath and exhibit an increased G-ratio when compared with a normally myelinated fiber of equivalent diameter (Stidworthy et al., 2003). The identification of abnormally thin myelin sheaths remains the most reliable means of identifying remyelination (Stidworthy et al. 2003). In our study, all treated mice display higher G-ratio compare to the non-treated mice, but in the $Smo^{fl/+}$ group, G-ratio resulted even higher than control and GF-Smo groups. Furthermore, this finding was reinforced when we sorted remyelinated axon (G-ratio ≥0.8) from pre-existing myelinated axons (<0.8), allowing us to conclude that remyelination is higher in the $Smo^{fl/+}$ mice.

Unlike what was observed during development, during the remyelination phase, the loss of an allele does affect OLs differentiation. This event could be explained by the fact that, during development, Smo mediated signaling cooperates with other growth factors to promote OPC proliferation. Once OPCs reached a suitable concentration, the pathway has to be inhibited in favor of the differentiation to myelinated OLs. In this case, the loss of allele during development could be compensated by all the other factors presented in the developing environment. As in development, a pro-inflammatory demyelinating environment causes the activation of Shh signaling together with other growth factors expressed during development to increase the OPCs available for the myelin restoration (Traiffort et al., 2020). Once demyelinating insults ceased, OPCs reached the appropriate density and the re-expression of growth factors is downregulated; the partial inhibition of Smo receptor could then favor remyelination.

The activity of Shh in precise time-window is known in several regions during CNS development (Merchán et al., 2007; Ortega et al., 2012; Clemente et al., 2013; Traiffort et al., 2016). However, its role after demyelination induced by cuprizone was highlight by the works of Samanta et al. (2015) and (Table 3). First, the authors followed the fate of cells expressing Gli1 during CNS demyelination injury. They found that NSCs in the SVZ are the only cells where Shh pathway is activated (at least the canonical way). These cells are then recruited in the CC, where they give rise to OLs, as shown, also, by Sánchez et al., 2018. When using the same paradigm, Gli1 expression

is abolished in the NSC (Gli ^{null} mice), it leads to an increase of differentiation and remyelination of the CC, similarly to our present results with Smo $^{fl/+}$ paradigm. However, Smo expression is abolished together with a partial loss of Gli1 (Smo$^{fl/fl}$;Gli1$^{+/-}$), there is no increase of OLs, proving that loss of Gli1 give different results than the loss of Smo (Samanta et al., 2015). Contrarily, upregulation of Smo in the Gli1$^{+/-}$ mice leads to increased OPC at the expense of mature OLs, while the upregulation of Smo in the Gli ^{null} mice shows an increase of mature OLs (Samanta et al., 2015; Table 3). Finally, by blocking Gli1 with the inhibitor GANT61, they obtain increased NSC recruitment and differentiation into OLs. They concluded that the inhibition of Gli1 mobilized NSC for remyelination (Table 3; (Samanta et al. 2015).

These results by Samanta et al. (2015) show similarities with our work. The commons points are the increase of OPC parallel with the overactivation of Smo starting with the cuprizone induction on the one hand (evidenced in green on Table 3). And on the other hand, the increase of mature OLs resulting from the loss or partial loss of Gli1 or Smo expression (evidenced in blue on Table 3). However, these works also show many differences. First of all is the target cell: Samanta et al. (2015) target NSC of the SVZ, while we target committed-OPC. Second, we act on different members of the Shh pathway (Smo vs. Gli1), and the differences observed could be due to a different activation of the canonical or non-canonical pathway. Three, the time in which tamoxifen is injected and the cuprizone stages analyzed could also reflect a specific role of Shh pathway that could be different depending on the demyelination or remyelination events, as well as the modified environment in which the OPCs have to respond.

Table 4 Summary of the results obtained using the cuprizone model by the different authors, (including our present work).

Authors	Mice (Cre-reporter)	Tamoxifen (TMX) /or Drug	Cuprizone	Target	Effects
Samanta et al., 2015	Gli1$^{+/-}$ (fate map)	Adult	/	vSVZ	NSCs Gli1^{+}
	Gli1$^{+/-}$ (fate map)	Tamoxifen start with CPZ	5W+2	CC	OLs Gli1^{+}
	Gli1^{NULL}	Tamoxifen start with CPZ	5W+2	CC	↑ mature OLs Gli1^{+}
	Gli1$^{+/-}$ Smo$^{fl/fl}$	Tamoxifen start with CPZ	5W+2	CC	NO OLs Gli1^{+} increase

	$Gli1^{+/-}Smo^{M2}$	Tamoxifen start with CPZ	5W+2	CC	↑ OPCs $Gli1^{+}$ ↓ mature OLs $Gli1^{+}$
	$Gli1^{NULL}Smo^{M2}$	Tamoxifen start with CPZ	5W+2	CC	↑ mature OLs $Gli1^{+}$
	$Gli1^{+/-}$ (fate map)	Tamoxifen start with CPZ + Gli-inhibitor at the late stage of CPZ	5W+2	CC	↑ OPCs and mature OLs $Gli1^{+}$ -No effect on non-recombinant OPC
Sánchez and Armstrong, 2018	$Gli1^{+/-}$ (fate map)	Tamoxifen start at P6-P9	5W+3 12W+6 days	CC	$Gli1^{+}$ remyelinating OLs $Gli1^{+}$proliferating OPCs
Sánchez et al., 2018	$Gli1^{+/-}$ (fate map)	Tamoxifen start after 3 weeks of CPZ	3W+2	CC	No $Gli1^{+}$ cells $Gli1^{+}$ cells in vSVZ
	$Gli1^{+/-}$ (fate map)	Tamoxifen start after 4 weeks of CPZ	5W+3	CC	No $Gli1^{+}$ cells $Gli1^{+}$ cells only in the vSVZ
	$Gli1^{+/-}$ (fate map)	Tamoxifen start at the end of CPZ -+SAG	12W+10 days	CC	↑ OPCs proliferation ↑ remyelination No $Gli1^{+}$ cells $Gli1^{+}$ cells in vSVZ
Our results	*Smo^{M2}*	*Tamoxifen start with CPZ*	*3W*	*CC*	↑ *OPCs proliferation*
	$Smo^{fl/+}$	*Tamoxifen start after 4 weeks of CPZ*	*5W+2*	*CC*	↑ *OLs density* ↑ *remyelination*

Demyelination and remyelination are caused by the complex interaction of different pathophysiologic mechanisms with several possible scenarios and cellular players, both environmental and intrinsic, that guide the behavior of OLs through the various stage of remyelination. We still know little about the physiological dynamics that form and reform the myelin sheath during normal development and in the adult, as well as after demyelinating damage. It is extremely relevant to address when and how these interactions occur because efficient remyelination might depend as much on the precise timing of action as on the presence or absence of these factors.

Smo-modulated OPCs transplantation addresses the role of Shh pathway during myelination.

One of the main problems to compare Smo modulation between development and remyelination is the environment in which OPCs act. *In vitro* approaches using agonists or antagonists could have solved this problem. However, they did not provide all the necessary information required for a complete understanding of this pathway role during up or downregulation in a neutral physiological environment, leading to an erroneous conclusion. We addressed this important question using a model in which OPCs from the transgenic P7 mice cortex were transplanted into adult mice retina. In physiological conditions, OLs are absent from the retina and the intraocular segment of the retinal ganglion cell axons are not myelinated. By contrast, nearly all ganglion cells axons are myelinated once they pass the optic papilla in the distal part of the optic nerve (Ffrench-Constant et al., 1988; Bartsch et al., 1989; Bartsch et al., 1994). Nevertheless, myelination of intraretinal axons segments occurs after transplantation of OPCs into the rodent retina (Laeng et al., 1996; Ader et al., 2000; Setzu et al., 2004; Setzu et al., 2006). In the present study, we transplanted cortical OPCs into the retinas of adult mice to ask whether the modulation of Smo gene could affect the OPCs differentiation into myelin-forming OLs in a particular environment of the host tissue, devoid of growth factors present during development or demyelinating injury. Intraretinally transplanted OPCs differentiated into myelin-forming OLs, as demonstrated by the presence of MBP positive cells located in the region of the nerve fiber layer. Transplantation of OPCs from Smo-modulated and control mice were also confirmed by the tdT reporter associated with the cells bodies or myelinated axons. The analysis of host retinas after 5 weeks of OPCs transplantation revealed a significant increase in the amount of myelin in the $Smo^{fl/+}$ compared with the *GF-Smo* group. These observations indicate that differentiation of Smo-modulated OPC is influenced by local environmental cues of the host tissue, eventually giving rise to the appropriate response for the particular developmental stage. Moreover, these results could explain the lack differences observed in the $Smo^{fl/+}$ group during development or in the 3 weeks cuprizone-demyelinating model, where the upregulation of growth

factors could compensate the haploinsufficiency of Smo. On the other hand, similarly to what observed after demyelinating injury, the Smo haploinsufficiency in the transplanted OPCs into adults' mice retinas is not influenced by local host-derived signals (or at least they are not the same to the ones found during development/demyelination) confirming an effect of local environmental cues on the OPCs differentiation. Furthermore, as described above, Samanta et al., 2015 reported an increase of mature OPCs $Gli1^+$ after the injection of Gli-inhibitor in the late stages of cuprizone. Targeting Smo receptor we choose to act both on canonical and no-canonical pathway, however, we do not know if and how this two signaling could interact in response to the surrounding stimuli.

Shh signaling exerts its effects on primary OPC human culture

The significance of the Shh pathway in human brain development is highlighted by the dramatic consequences of mutations causing the disruption of this pathway (Nanni et al. 1999; Heussler et al. 2002; Santiago et al. 2006; Currier, Polk, e Reeves 2012). Thus, dramatic effects are the proof for the relative power of this morphogen, as well as why Shh is so timely-restricted/controlled during development.

As observed in other species, Shh signaling was found involved in the expression of the transcriptional factor Olig2 in human embryonic stem cells. Following Olig2 expression, progenitors reached the OPC phenotype co-expressing SOX10 and PDGFRα. Moreover, FGF2 promotes both embryonal or adult hOPC proliferation (Hu et al., 2009; Bribián et al., 2020), and the activation of Smo in embryonal hOPC synergize with the FGF2 factor increasing proliferation (Hu et al., 2009).

Despite the growing knowledge about the role of Shh pathway in OPC proliferation, differentiation, and myelination from rodent in vitro studies, experimental animal models, or human tissue samples, molecular mechanisms underlying its role in human OL are only partly understood. This result might be explained by the limited availability of primary OLs from patients with myelin disease to perform functional studies or drug screens. The method developed in the present work made it possible to isolate adult hOPC from the brain of adult humans undergoing surgical resections. Only cerebral cortex hOPC was used to avoid any variability introduced by anatomical

origin or location (Bribián et al. ,2020). Isolated hOPC expressed the same markers as the murine-derived OPC (A2B5, Olig2). Moreover, our new protocol allowed the culture of these cells for longer periods than is described with other isolating methods (Cui et al., 2013; Medina-Rodríguez et al., 2013; Bribián et al., 2020). This is a breakthrough since very little is known about adult hOPC differentiation *in vitro*. The study of hOPC cells behavior and their comparison with their mouse counterparts is crucial since the progression of murine OPC is not the same as that of humans. For example, a significant difference found was the culture time: human culture requires a longer time to study proliferation and differentiation, making it hard to understand the switch from OPC to mature or myelinating OL. Our study provides evidence that adult hOPC retains the competence to respond to Shh ligand stimulus, promoting cell proliferation. However, the proliferation rate decreases with respect to the ones studied in the correspondent murine OPC (Medina-Rodríguez et al. 2013; Bribián et al. 2020). These differences agree with previous lab work showing that the OPCs heterogeneity depends on the anatomical region from which they are isolated and from species, the stage in the life cycle, and the functional system they pertain (Bribian et al., 2020). While regional differences are generally accepted, the recent report of our group has demonstrated the relevance of species and the age of the source in determining OPC heterogeneity Shh treated and control cells did not show changes in the number of differentiated cells at DIV10. However, from DIV16 to DIV20, Shh treated cells seem to show more branches. One possible explanation could be that Shh pathway has been reported neuroprotection properties (Chechneva et al., 2014). Undoubtedly, more in-depth studies are needed to understand the role of this pathway in human OLs. The use of both agonist and antagonist, for example, could clarify if the branching increase could be due to a better differentiation or neuroprotectio

Conclusions

1- Shh pathway through the Smo receptor can regulate the proliferation and differentiation of parenchymal OPC at the CC during development: The upregulation of the Smo receptor maintains OPC in an undifferentiated stage, allowing them to enter another cell division round.

2- The upregulation of the Smo receptor negatively influences the OPC ability to undergo differentiation affecting, in this way, the normal myelination of the CC during development.

3- The partial downregulation of Smo does not affect development, showing OPC differentiation and myelination comparable to the control ones.

4- Shh pathway through Smo receptor can be upregulated to promote OPC proliferation during demyelination but does not affect once the demyelinated insult with cuprizone is terminated.

5- The partial inhibition of the Smo receptor does not show any change during cuprizone-mediated demyelination events, as observed during normal development. On the contrary, after demyelinating insult, the downregulation of Smo leads to an increase in OPC differentiation and remyelination.

6- The improvement of differentiation through Smo downregulation is abolished by the expression of growth or transcriptional factors, as the ones found during development or demyelination (e.g. FG2, PDGFRα). This ability is reestablished once these genes are downregulated, as observed during remyelination or after the transplantation of Smo-downregulated OPC in the retina of adult mice.

7- Shh pathway maintains its ability to induce changes compatible with (re)myelinatioin *in vitro.*

Bibliography

Absinta, Martina, Pascal Sati, Federica Masuzzo, Govind Nair, Varun Sethi, Hadar Kolb, Joan Ohayon, Tianxia Wu, Irene C. M. Cortese, e Daniel S. Reich. 2019. «Association of Chronic Active Multiple Sclerosis Lesions With Disability In Vivo». *JAMA Neurology* 76 (12): 1474–83. https://doi.org/10.1001/jamaneurol.2019.2399.

Ader, Marius, Jinhong Meng, Melitta Schachner, e Udo Bartsch. 2000. «Formation of Myelin after Transplantation of Neural Precursor Cells into the Retina of Young Postnatal Mice». *Glia* 30 (3): 301–10. https://doi.org/10.1002/(SICI)1098-1136(200005)30:3<301::AID-GLIA9>3.0.CO;2-S.

Aguirre, Adan, Jeff L. Dupree, J. M. Mangin, e Vittorio Gallo. 2007. «A Functional Role for EGFR Signaling in Myelination and Remyelination». *Nature Neuroscience* 10 (8): 990–1002. https://doi.org/10.1038/nn1938.

Arancibia-Cárcamo, I. Lorena, Marc C. Ford, Lee Cossell, Kinji Ishida, Koujiro Tohyama, e David Attwell. 2017. «Node of Ranvier Length as a Potential Regulator of Myelinated Axon Conduction Speed». *ELife* 6. https://doi.org/10.7554/eLife.23329.

Armstrong, Regina C. 2007. «Growth factor regulation of remyelination: behind the growing interest in endogenous cell repair of the CNS». *Future neurology* 2 (6): 689–97. https://doi.org/10.2217/14796708.2.6.689.

Arnett, Heather A., Stephen P. J. Fancy, John A. Alberta, Chao Zhao, Sheila R. Plant, Sovann Kaing, Cedric S. Raine, David H. Rowitch, Robin J. M. Franklin, e Charles D. Stiles. 2004. «BHLH Transcription Factor Olig1 Is Required to Repair Demyelinated Lesions in the CNS». *Science (New York, N.Y.)* 306 (5704): 2111–15. https://doi.org/10.1126/science.1103709.

Aubert-Broche, B., V. Fonov, I. Leppert, G. B. Pike, e D. L. Collins. 2008. «Human Brain Myelination from Birth to 4.5 Years». *Medical Image Computing and Computer-Assisted Intervention: MICCAI ... International Conference on Medical Image Computing and Computer-Assisted Intervention* 11 (Pt 2): 180–87. https://doi.org/10.1007/978-3-540-85990-1_22.

Bai, C. Brian, Wojtek Auerbach, Joon S. Lee, Daniel Stephen, e Alexandra L. Joyner. 2002. «Gli2, but Not Gli1, Is Required for Initial Shh Signaling and Ectopic Activation of the Shh Pathway». *Development (Cambridge, England)* 129 (20): 4753–61.

Bakiri, Yamina, Ragnhildur Káradóttir, Lee Cossell, e David Attwell. 2011. «Morphological and electrical properties of oligodendrocytes in the white matter of the corpus callosum and cerebellum». *The Journal of Physiology* 589 (Pt 3): 559–73. https://doi.org/10.1113/jphysiol.2010.201376.

Balaskas, Nikolaos, Ana Ribeiro, Jasmina Panovska, Eric Dessaud, Noriaki Sasai, Karen M. Page, James Briscoe, e Vanessa Ribes. 2012. «Gene Regulatory Logic for Reading the Sonic Hedgehog Signaling Gradient in the Vertebrate Neural Tube». *Cell* 148 (1–2): 273–84. https://doi.org/10.1016/j.cell.2011.10.047.

Balordi, Francesca, e Gord Fishell. 2007a. «Hedgehog Signaling in the Subventricular Zone Is Required for Both the Maintenance of Stem Cells and the Migration of Newborn Neurons». *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 27 (22): 5936–47. https://doi.org/10.1523/JNEUROSCI.1040-07.2007.

———. 2007b. «Mosaic Removal of Hedgehog Signaling in the Adult SVZ Reveals That the Residual Wild-Type Stem Cells Have a Limited Capacity for Self-Renewal». *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 27 (52): 14248–59. https://doi.org/10.1523/JNEUROSCI.4531-07.2007.

Barkovich, A. J., e B. O. Kjos. 1988. «Normal Postnatal Development of the Corpus Callosum as Demonstrated by MR Imaging». *AJNR. American Journal of Neuroradiology* 9 (3): 487–91.

Bartsch, U, A Faissner, J Trotter, U Dorries, S Bartsch, H Mohajeri, e M Schachner. 1994. «Tenascin demarcates the boundary between the myelinated and nonmyelinated part of retinal ganglion cell axons in the developing and adult mouse». *The Journal of Neuroscience* 14 (8): 4756–68. https://doi.org/10.1523/JNEUROSCI.14-08-04756.1994.

Bartsch, U., F. Kirchhoff, e M. Schachner. 1989. «Immunohistological Localization of the Adhesion Molecules L1, N-CAM, and MAG in the Developing and Adult Optic Nerve of Mice». *The Journal of Comparative Neurology* 284 (3): 451–62. https://doi.org/10.1002/cne.902840310.

Baxi, Emily G., Joseph DeBruin, Jing Jin, Hayley J. Strasburger, Matthew D. Smith, Jennifer L. Orthmann-Murphy, Jason T. Schott, Amanda N. Fairchild, Dwight E. Bergles, e Peter A. Calabresi. 2017. «Lineage Tracing Reveals Dynamic Changes in Oligodendrocyte Precursor Cells Following Cuprizone-Induced Demyelination». *Glia* 65 (12): 2087–98. https://doi.org/10.1002/glia.23229.

Bengtsson, Sara L., Zoltán Nagy, Stefan Skare, Lea Forsman, Hans Forssberg, e Fredrik Ullén. 2005. «Extensive Piano Practicing Has Regionally Specific Effects on White Matter Development». *Nature Neuroscience* 8 (9): 1148–50. https://doi.org/10.1038/nn1516.

Bergles, Dwight E., e William D. Richardson. 2015. «Oligodendrocyte Development and Plasticity». *Cold Spring Harbor Perspectives in Biology* 8 (2): a020453. https://doi.org/10.1101/cshperspect.a020453.

Blaess, Sandra, Jomichelle D. Corrales, e Alexandra L. Joyner. 2006. «Sonic Hedgehog Regulates Gli Activator and Repressor Functions with Spatial and Temporal Precision in the Mid/Hindbrain Region». *Development (Cambridge, England)* 133 (9): 1799–1809. https://doi.org/10.1242/dev.02339.

Boullerne, Anne Isabelle. 2016. «The History of Myelin». *Experimental Neurology* 283 (Pt B): 431–45. https://doi.org/10.1016/j.expneurol.2016.06.005.

Bradl, Monika, e Hans Lassmann. 2010. «Oligodendrocytes: Biology and Pathology». *Acta Neuropathologica* 119 (1): 37–53. https://doi.org/10.1007/s00401-009-0601-5.

Bribián, Ana, María José Barallobre, Nadia Soussi-Yanicostas, e Fernando de Castro. 2006. «Anosmin-1 Modulates the FGF-2-Dependent Migration of Oligodendrocyte Precursors in the Developing Optic Nerve». *Molecular and Cellular Neurosciences* 33 (1): 2–14. https://doi.org/10.1016/j.mcn.2006.05.009.

Bribián, Ana, Pedro F. Esteban, Diego Clemente, Nadia Soussi-Yanicostas, Jean-Léon Thomas, Bernard Zalc, e Fernando de Castro. 2008. «A Novel Role for Anosmin-1 in the Adhesion and Migration of Oligodendrocyte Precursors». *Developmental Neurobiology* 68 (13): 1503–16. https://doi.org/10.1002/dneu.20678.

Bribián, Ana, Eva M. Medina-Rodríguez, Fernando Josa-Prado, Isabel García-Álvarez, Isabel Machín-Díaz, Pedro F. Esteban, Verónica Murcia-Belmonte, et al. 2020. «Functional Heterogeneity of Mouse and Human Brain OPCs: Relevance for Preclinical Studies in Multiple Sclerosis». *Journal of Clinical Medicine* 9 (6). https://doi.org/10.3390/jcm9061681.

Briscoe, J., A. Pierani, T. M. Jessell, e J. Ericson. 2000. «A Homeodomain Protein Code Specifies Progenitor Cell Identity and Neuronal Fate in the Ventral Neural Tube». *Cell* 101 (4): 435–45. https://doi.org/10.1016/s0092-8674(00)80853-3.

Briscoe, James, e Pascal P. Thérond. 2013. «The Mechanisms of Hedgehog Signalling and Its Roles in Development and Disease». *Nature Reviews. Molecular Cell Biology* 14 (7): 416–29. https://doi.org/10.1038/nrm3598.

Bürglin, Thomas R. 2008. «The Hedgehog Protein Family». *Genome Biology* 9 (11): 241.

https://doi.org/10.1186/gb-2008-9-11-241.

Carballo, Gabriela Basile, Jéssica Ribeiro Honorato, Giselle Pinto Farias de Lopes, e Tania Cristina Leite de Sampaio E. Spohr. 2018. «A Highlight on Sonic Hedgehog Pathway». *Cell Communication and Signaling: CCS* 16 (1): 11. https://doi.org/10.1186/s12964-018-0220-7.

Casaccia-Bonnefil, Patrizia, e Aixiao Liu. 2003. «Relationship between Cell Cycle Molecules and Onset of Oligodendrocyte Differentiation». *Journal of Neuroscience Research* 72 (1): 1–11. https://doi.org/10.1002/jnr.10565.

Castro, Fernando de, e Ana Bribián. 2005. «The Molecular Orchestra of the Migration of Oligodendrocyte Precursors during Development». *Brain Research. Brain Research Reviews* 49 (2): 227–41. https://doi.org/10.1016/j.brainresrev.2004.12.034.

Castro, Fernando de, e Bernard Zalc. 2013. *Migration of Myelin-Forming Cells in the CNS. In: Comprehensive Developmental Neuroscience: Cellular Migration and Formation of Neuronal Connections, volume 2.* 1st ed. Amsterdam: Elsevier.

Chari, Divya M., Chao Zhao, Mark R. Kotter, William F. Blakemore, e Robin J. M. Franklin. 2006. «Corticosteroids Delay Remyelination of Experimental Demyelination in the Rodent Central Nervous System». *Journal of Neuroscience Research* 83 (4): 594–605. https://doi.org/10.1002/jnr.20763.

Chechneva, O V, F Mayrhofer, D J Daugherty, R G Krishnamurty, P Bannerman, D E Pleasure, e W Deng. 2014. «A Smoothened receptor agonist is neuroprotective and promotes regeneration after ischemic brain injury». *Cell Death & Disease* 5 (10): e1481. https://doi.org/10.1038/cddis.2014.446.

Chojnacki, Andrew, e Samuel Weiss. 2004. «Isolation of a Novel Platelet-Derived Growth Factor-Responsive Precursor from the Embryonic Ventral Forebrain». *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 24 (48): 10888–99. https://doi.org/10.1523/JNEUROSCI.3302-04.2004.

Cignarella, Francesca, Fabia Filipello, Bryan Bollman, Claudia Cantoni, Alberto Locca, Robert Mikesell, Melissa Manis, et al. 2020. «TREM2 activation on microglia promotes myelin debris clearance and remyelination in a model of multiple sclerosis». *Acta Neuropathologica* 140 (4): 513–34. https://doi.org/10.1007/s00401-020-02193-z.

Clemente, Diego, María Cristina Ortega, Carolina Melero-Jerez, e Fernando de Castro. 2013. «The Effect of Glia-Glia Interactions on Oligodendrocyte Precursor Cell Biology during Development and in Demyelinating Diseases». *Frontiers in Cellular Neuroscience* 7 (dicembre): 268. https://doi.org/10.3389/fncel.2013.00268.

Compston, Alastair, e Alasdair Coles. 2008. «Multiple Sclerosis». *Lancet (London, England)* 372 (9648): 1502–17. https://doi.org/10.1016/S0140-6736(08)61620-7.

Crawford, A. H., J. H. Stockley, R. B. Tripathi, W. D. Richardson, e R. J. M. Franklin. 2014. «Oligodendrocyte Progenitors: Adult Stem Cells of the Central Nervous System?» *Experimental Neurology*, Special Issue: Stem cells: Newly emerging concepts in the nervous system, 260 (ottobre): 50–55. https://doi.org/10.1016/j.expneurol.2014.04.027.

Cui, Qiao-Ling, Tanja Kuhlmann, Veronique E. Miron, Soo Yuen Leong, Jun Fang, Pavel Gris, Timothy E. Kennedy, Guillermina Almazan, e Jack Antel. 2013. «Oligodendrocyte Progenitor Cell Susceptibility to Injury in Multiple Sclerosis». *The American Journal of Pathology* 183 (2): 516–25. https://doi.org/10.1016/j.ajpath.2013.04.016.

Currier, Duane G., Renita C. Polk, e Roger H. Reeves. 2012. «A Sonic Hedgehog (Shh) Response Deficit in Trisomic Cells May Be a Common Denominator for Multiple Features of Down Syndrome». *Progress in Brain Research* 197: 223–36. https://doi.org/10.1016/B978-0-444-54299-1.00011-X.

Dawson, Mary R. L., Annabella Polito, Joel M. Levine, e Richard Reynolds. 2003. «NG2-Expressing Glial Progenitor Cells: An Abundant and Widespread Population of Cycling Cells in the Adult Rat CNS». *Molecular and Cellular Neurosciences* 24 (2): 476–88. https://doi.org/10.1016/s1044-7431(03)00210-0.

Del Giovane, Alice, e Antonella Ragnini-Wilson. 2018. «Targeting Smoothened as a New Frontier in the Functional Recovery of Central Nervous System Demyelinating Pathologies». *International Journal of Molecular Sciences* 19 (11). https://doi.org/10.3390/ijms19113677.

DEL RIO-HORTEGA, P. 1919. «El tercer elemento de los centros nerviosos.I.La microglia en estado normal.II.Intervencion de la microglia en los procesos patologicos.III.Naturaleza probable de la microglia». *Boll.Socieded Esp.Biol.* 9: 69–120.

Dessaud, Eric, Andrew P. McMahon, e James Briscoe. 2008. «Pattern Formation in the Vertebrate Neural Tube: A Sonic Hedgehog Morphogen-Regulated Transcriptional Network». *Development (Cambridge, England)* 135 (15): 2489–2503. https://doi.org/10.1242/dev.009324.

Dimou, Leda, Christiane Simon, Frank Kirchhoff, Hirohide Takebayashi, e Magdalena Götz. 2008. «Progeny of Olig2-Expressing Progenitors in the Gray and White Matter of the Adult Mouse Cerebral Cortex». *Journal of Neuroscience* 28 (41): 10434–42. https://doi.org/10.1523/JNEUROSCI.2831-08.2008.

Dincman, Toros A., Jason E. Beare, Sujata Saraswat Ohri, e Scott R. Whittemore. 2012. «Isolation of Cortical Mouse Oligodendrocyte Precursor Cells». *Journal of Neuroscience Methods* 209 (1): 219–26. https://doi.org/10.1016/j.jneumeth.2012.06.017.

Edgar, N., e E. Sibille. 2012. «A Putative Functional Role for Oligodendrocytes in Mood Regulation». *Translational Psychiatry* 2 (maggio): e109. https://doi.org/10.1038/tp.2012.34.

Elkabes, S., E. M. DiCicco-Bloom, e I. B. Black. 1996. «Brain Microglia/Macrophages Express Neurotrophins That Selectively Regulate Microglial Proliferation and Function». *Journal of Neuroscience* 16 (8): 2508–21. https://doi.org/10.1523/JNEUROSCI.16-08-02508.1996.

Emery, Ben. 2010. «Regulation of Oligodendrocyte Differentiation and Myelination». *Science (New York, N.Y.)* 330 (6005): 779–82. https://doi.org/10.1126/science.1190927.

Emery, Ben, e Q. Richard Lu. 2015. «Transcriptional and Epigenetic Regulation of Oligodendrocyte Development and Myelination in the Central Nervous System». *Cold Spring Harbor Perspectives in Biology* 7 (9): a020461. https://doi.org/10.1101/cshperspect.a020461.

Fancy, Stephen P. J., Emily P. Harrington, Tracy J. Yuen, John C. Silbereis, Chao Zhao, Sergio E. Baranzini, Charlotte C. Bruce, et al. 2011. «Axin2 as Regulatory and Therapeutic Target in Newborn Brain Injury and Remyelination». *Nature Neuroscience* 14 (8): 1009–16. https://doi.org/10.1038/nn.2855.

Fancy, Stephen P. J., Chao Zhao, e Robin J. M. Franklin. 2004. «Increased Expression of Nkx2.2 and Olig2 Identifies Reactive Oligodendrocyte Progenitor Cells Responding to Demyelination in the Adult CNS». *Molecular and Cellular Neurosciences* 27 (3): 247–54. https://doi.org/10.1016/j.mcn.2004.06.015.

Feil, R., J. Brocard, B. Mascrez, M. LeMeur, D. Metzger, e P. Chambon. 1996. «Ligand-Activated Site-Specific Recombination in Mice». *Proceedings of the National Academy of Sciences of the United States of America* 93 (20): 10887–90. https://doi.org/10.1073/pnas.93.20.10887.

Feil, Susanne, Nadejda Valtcheva, e Robert Feil. 2009. «Inducible Cre Mice». *Methods in Molecular Biology (Clifton, N.J.)* 530: 343–63. https://doi.org/10.1007/978-1-59745-471-1_18.

Ferent, J., C. Zimmer, P. Durbec, M. Ruat, e E. Traiffort. 2013. «Sonic Hedgehog Signaling Is a Positive Oligodendrocyte Regulator during Demyelination». *Journal of Neuroscience* 33 (5): 1759–72. https://doi.org/10.1523/JNEUROSCI.3334-12.2013.

Ferent, Julien, Loïc Cochard, Hélène Faure, Maurizio Taddei, Heidi Hahn, Martial Ruat, e Elisabeth

Traiffort. 2014. «Genetic Activation of Hedgehog Signaling Unbalances the Rate of Neural Stem Cell Renewal by Increasing Symmetric Divisions». *Stem Cell Reports* 3 (2): 312–23. https://doi.org/10.1016/j.stemcr.2014.05.016.

Fernandez-Castaneda, Anthony, e Alban Gaultier. 2016. «Adult Oligodendrocyte Progenitor Cells - Multifaceted Regulators of the CNS in Health and Disease». *Brain, Behavior, and Immunity* 57 (ottobre): 1–7. https://doi.org/10.1016/j.bbi.2016.01.005.

Ffrench-Constant, C., R. H. Miller, J. F. Burne, e M. C. Raff. 1988. «Evidence That Migratory Oligodendrocyte-Type-2 Astrocyte (O-2A) Progenitor Cells Are Kept out of the Rat Retina by a Barrier at the Eye-End of the Optic Nerve». *Journal of Neurocytology* 17 (1): 13–25. https://doi.org/10.1007/BF01735374.

Fields, R. Douglas. 2008. «White Matter in Learning, Cognition and Psychiatric Disorders». *Trends in Neurosciences* 31 (7): 361–70. https://doi.org/10.1016/j.tins.2008.04.001.

———. 2014. «Myelin Formation and Remodeling». *Cell* 156 (1–2): 15–17. https://doi.org/10.1016/j.cell.2013.12.038.

Franklin, Robin J. M., Charles ffrench-Constant, Julia M. Edgar, e Kenneth J. Smith. 2012. «Neuroprotection and Repair in Multiple Sclerosis». *Nature Reviews. Neurology* 8 (11): 624–34. https://doi.org/10.1038/nrneurol.2012.200.

Franklin, Robin J. M., e Charles Ffrench-Constant. 2008. «Remyelination in the CNS: From Biology to Therapy». *Nature Reviews. Neuroscience* 9 (11): 839–55. https://doi.org/10.1038/nrn2480.

Franklin, Robin J. M., e Steven A. Goldman. 2015. «Glia Disease and Repair-Remyelination». *Cold Spring Harbor Perspectives in Biology* 7 (7): a020594. https://doi.org/10.1101/cshperspect.a020594.

Frischer, Josa M., Stephen D. Weigand, Yong Guo, Nilufer Kale, Joseph E. Parisi, Istvan Pirko, Jay Mandrekar, et al. 2015. «Clinical and Pathological Insights into the Dynamic Nature of the White Matter Multiple Sclerosis Plaque». *Annals of Neurology* 78 (5): 710–21. https://doi.org/10.1002/ana.24497.

Frohman, Elliot M., Michael K. Racke, e Cedric S. Raine. 2006. «Multiple Sclerosis--the Plaque and Its Pathogenesis». *The New England Journal of Medicine* 354 (9): 942–55. https://doi.org/10.1056/NEJMra052130.

Frost, Emma E., Joseph A. Nielsen, Tuan Q. Le, e Regina C. Armstrong. 2003. «PDGF and FGF2 regulate oligodendrocyte progenitor responses to demyelination». *Journal of Neurobiology* 54 (3): 457–72. https://doi.org/10.1002/neu.10158.

Fünfschilling, Ursula, Lotti M. Supplie, Don Mahad, Susann Boretius, Aiman S. Saab, Julia Edgar, Bastian G. Brinkmann, Celia M. Kassmann, Iva D. Tzvetanova, e Wiebke Möbius. 2012. «Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity». *Nature* 485 (7399): 517–521.

Furusho, Miki, Yoshimi Kaga, Akihiro Ishii, Jean M. Hébert, e Rashmi Bansal. 2011. «Fibroblast Growth Factor Signaling Is Required for the Generation of Oligodendrocyte Progenitors from the Embryonic Forebrain». *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 31 (13): 5055–66. https://doi.org/10.1523/JNEUROSCI.4800-10.2011.

Goldman, Steven A., Maiken Nedergaard, e Martha S. Windrem. 2012. «Glial Progenitor Cell–Based Treatment and Modeling of Neurological Disease». *Science* 338 (6106): 491–95. https://doi.org/10.1126/science.1218071.

Gonsalvez, David G., SangWon Yoo, Jessica L. Fletcher, Rhiannon J. Wood, Georgina A. Craig, Simon S. Murray, e Junhua Xiao. 2019. «Imaging and Quantification of Myelin Integrity After Injury With Spectral Confocal Reflectance Microscopy». *Frontiers in Molecular Neuroscience* 12: 275. https://doi.org/10.3389/fnmol.2019.00275.

Green, Ari J., Jeffrey M. Gelfand, Bruce A. Cree, Carolyn Bevan, W. John Boscardin, Feng Mei, Justin Inman, et al. 2017. «Clemastine Fumarate as a Remyelinating Therapy for Multiple Sclerosis (ReBUILD): A Randomised, Controlled, Double-Blind, Crossover Trial». *Lancet (London, England)* 390 (10111): 2481–89. https://doi.org/10.1016/S0140-6736(17)32346-2.

Grzybowski, Andrzej, e Krzysztof Pietrzak. 2013. «Robert Remak (1815-1865)». *Journal of Neurology* 260 (6): 1696–97. https://doi.org/10.1007/s00415-012-6761-6.

Gudi, Viktoria, Stefan Gingele, Thomas Skripuletz, e Martin Stangel. 2014. «Glial Response during Cuprizone-Induced de- and Remyelination in the CNS: Lessons Learned». *Frontiers in Cellular Neuroscience* 8: 73. https://doi.org/10.3389/fncel.2014.00073.

Gurdon, J. B., e P. Y. Bourillot. 2001. «Morphogen Gradient Interpretation». *Nature* 413 (6858): 797–803. https://doi.org/10.1038/35101500.

Han, Young-Goo, Nathalie Spassky, Miriam Romaguera-Ros, Jose-Manuel Garcia-Verdugo, Andrea Aguilar, Sylvie Schneider-Maunoury, e Arturo Alvarez-Buylla. 2008. «Hedgehog Signaling and Primary Cilia Are Required for the Formation of Adult Neural Stem Cells». *Nature Neuroscience* 11 (3): 277–84. https://doi.org/10.1038/nn2059.

Hayakawa, Kazuhide, Loc-Duyen D. Pham, Angel T. Som, Brian J. Lee, Shuzhen Guo, Eng H. Lo, e Ken Arai. 2011. «Vascular Endothelial Growth Factor Regulates the Migration of Oligodendrocyte Precursor Cells». *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 31 (29): 10666–70. https://doi.org/10.1523/JNEUROSCI.1944-11.2011.

Heffner, Caleb S., C. Herbert Pratt, Randal P. Babiuk, Yashoda Sharma, Stephen F. Rockwood, Leah R. Donahue, Janan T. Eppig, e Stephen A. Murray. 2012. «Supporting Conditional Mouse Mutagenesis with a Comprehensive Cre Characterization Resource». *Nature Communications* 3: 1218. https://doi.org/10.1038/ncomms2186.

Heussler, H. S., M. Suri, I. D. Young, e M. Muenke. 2002. «Extreme Variability of Expression of a Sonic Hedgehog Mutation: Attention Difficulties and Holoprosencephaly». *Archives of Disease in Childhood* 86 (4): 293–96. https://doi.org/10.1136/adc.86.4.293.

Hinks, G. L., e R. J. Franklin. 1999. «Distinctive Patterns of PDGF-A, FGF-2, IGF-I, and TGF-Beta1 Gene Expression during Remyelination of Experimentally-Induced Spinal Cord Demyelination». *Molecular and Cellular Neurosciences* 14 (2): 153–68. https://doi.org/10.1006/mcne.1999.0771.

Hiremath, M. M., Y. Saito, G. W. Knapp, J. P. Ting, K. Suzuki, e G. K. Matsushima. 1998. «Microglial/Macrophage Accumulation during Cuprizone-Induced Demyelination in C57BL/6 Mice». *Journal of Neuroimmunology* 92 (1–2): 38–49. https://doi.org/10.1016/s0165-5728(98)00168-4.

Horner, Philip J., Michaela Thallmair, e Fred H. Gage. 2002. «Defining the NG2-Expressing Cell of the Adult CNS». *Journal of Neurocytology* 31 (6–7): 469–80. https://doi.org/10.1023/a:1025739630398.

Hu, Bao-Yang, Zhong-Wei Du, Xue-Jun Li, Melvin Ayala, e Su-Chun Zhang. 2009. «Human Oligodendrocytes from Embryonic Stem Cells: Conserved SHH Signaling Networks and Divergent FGF Effects». *Development (Cambridge, England)* 136 (9): 1443–52. https://doi.org/10.1242/dev.029447.

Huang, Wenhui, Na Zhao, Xianshu Bai, Khalad Karram, Jacqueline Trotter, Sandra Goebbels, Anja Scheller, e Frank Kirchhoff. 2014. «Novel NG2-CreERT2 Knock-in Mice Demonstrate Heterogeneous Differentiation Potential of NG2 Glia during Development». *Glia* 62 (6): 896–913. https://doi.org/10.1002/glia.22648.

Hubler, Zita, Dharmaraja Allimuthu, Ilya Bederman, Matthew S. Elitt, Mayur Madhavan, Kevin C. Allan, H. Elizabeth Shick, et al. 2018. «Accumulation of 8,9-Unsaturated Sterols Drives Oligodendrocyte Formation and Remyelination». *Nature* 560 (7718): 372–76. https://doi.org/10.1038/s41586-018-0360-

3.
Hughes, Ethan G., Shin H. Kang, Masahiro Fukaya, e Dwight E. Bergles. 2013. «Oligodendrocyte Progenitors Balance Growth with Self-Repulsion to Achieve Homeostasis in the Adult Brain». *Nature Neuroscience* 16 (6): 668–76. https://doi.org/10.1038/nn.3390.

Huxley, A. F., e R. Stampfli. 1949. «Saltatory transmission of the nervous impulse». *Archives des Sciences Physiologiques* 3 (4): 435–448.

Ihrie, Rebecca A., Jugal K. Shah, Corey C. Harwell, Jacob H. Levine, Cristina D. Guinto, Melissa Lezameta, Arnold R. Kriegstein, e Arturo Alvarez-Buylla. 2011. «Persistent Sonic Hedgehog Signaling in Adult Brain Determines Neural Stem Cell Positional Identity». *Neuron* 71 (2): 250–62. https://doi.org/10.1016/j.neuron.2011.05.018.

Kang, Shin H., Masahiro Fukaya, Jason K. Yang, Jeffrey D. Rothstein, e Dwight E. Bergles. 2010. «NG2+ CNS Glial Progenitors Remain Committed to the Oligodendrocyte Lineage in Postnatal Life and Following Neurodegeneration». *Neuron* 68 (4): 668–81. https://doi.org/10.1016/j.neuron.2010.09.009.

Kessaris, Nicoletta, Matthew Fogarty, Palma Iannarelli, Matthew Grist, Michael Wegner, e William D. Richardson. 2006. «Competing Waves of Oligodendrocytes in the Forebrain and Postnatal Elimination of an Embryonic Lineage». *Nature Neuroscience* 9 (2): 173–79. https://doi.org/10.1038/nn1620.

Kessaris, Nicoletta, Françoise Jamen, Lee L. Rubin, e William D. Richardson. 2004. «Cooperation between Sonic Hedgehog and Fibroblast Growth Factor/MAPK Signalling Pathways in Neocortical Precursors». *Development (Cambridge, England)* 131 (6): 1289–98. https://doi.org/10.1242/dev.01027.

Kondo, T., e M. Raff. 2000. «Oligodendrocyte Precursor Cells Reprogrammed to Become Multipotential CNS Stem Cells». *Science (New York, N.Y.)* 289 (5485): 1754–57. https://doi.org/10.1126/science.289.5485.1754.

Kotter, M. R., A. Setzu, F. J. Sim, N. Van Rooijen, e R. J. Franklin. 2001. «Macrophage Depletion Impairs Oligodendrocyte Remyelination Following Lysolecithin-Induced Demyelination». *Glia* 35 (3): 204–12. https://doi.org/10.1002/glia.1085.

Kotter, Mark R., Chao Zhao, Nico van Rooijen, e Robin J. M. Franklin. 2005. «Macrophage-Depletion Induced Impairment of Experimental CNS Remyelination Is Associated with a Reduced Oligodendrocyte Progenitor Cell Response and Altered Growth Factor Expression». *Neurobiology of Disease* 18 (1): 166–75. https://doi.org/10.1016/j.nbd.2004.09.019.

Kremer, David, Orhan Aktas, Hans-Peter Hartung, e Patrick Küry. 2011. «The Complex World of Oligodendroglial Differentiation Inhibitors». *Annals of Neurology* 69 (4): 602–18. https://doi.org/10.1002/ana.22415.

Laeng, Pascal, Martin Molthagen, Ester Gui-Xia Yu, e Udo Bartsch. 1996. «Transplantation of Oligodendrocyte Progenitor Cells into the Rat Retina: Extensive Myelination of Retinal Ganglion Cell Axons». *Glia* 18 (3): 200–210. https://doi.org/10.1002/(SICI)1098-1136(199611)18:3<200::AID-GLIA4>3.0.CO;2-2.

Lai, Karen, Brian K. Kaspar, Fred H. Gage, e David V. Schaffer. 2003. «Sonic Hedgehog Regulates Adult Neural Progenitor Proliferation in Vitro and in Vivo». *Nature Neuroscience* 6 (1): 21–27. https://doi.org/10.1038/nn983.

Lampron, Antoine, Antoine Larochelle, Nathalie Laflamme, Paul Préfontaine, Marie-Michèle Plante, Maria Gabriela Sánchez, V. Wee Yong, Peter K. Stys, Marie-Ève Tremblay, e Serge Rivest. 2015. «Inefficient clearance of myelin debris by microglia impairs remyelinating processes». *The Journal of Experimental Medicine* 212 (4): 481–95. https://doi.org/10.1084/jem.20141656.

Lassmann, Hans. 2018. «Multiple Sclerosis Pathology». *Cold Spring Harbor Perspectives in Medicine* 8 (3). https://doi.org/10.1101/cshperspect.a028936.

Lee, Youngjin, Brett M. Morrison, Yun Li, Sylvain Lengacher, Mohamed H. Farah, Paul N. Hoffman, Yiting Liu, Akivaga Tsingalia, Lin Jin, e Ping-Wu Zhang. 2012. «Oligodendroglia metabolically support axons and contribute to neurodegeneration». *Nature* 487 (7408): 443–448.

Lelievre, Vincent, Cristina A. Ghiani, Akop Seksenyan, Pierre Gressens, Jean de Vellis, e James A. Waschek. 2006. «Growth Factor-Dependent Actions of PACAP on Oligodendrocyte Progenitor Proliferation». *Regulatory Peptides* 137 (1–2): 58–66. https://doi.org/10.1016/j.regpep.2006.04.024.

Levine, J. M., e R. Reynolds. 1999. «Activation and Proliferation of Endogenous Oligodendrocyte Precursor Cells during Ethidium Bromide-Induced Demyelination». *Experimental Neurology* 160 (2): 333–47. https://doi.org/10.1006/exnr.1999.7224.

Li, Huiliang, Ye He, William D. Richardson, e Patrizia Casaccia. 2009. «Two-Tier Transcriptional Control of Oligodendrocyte Differentiation». *Current Opinion in Neurobiology* 19 (5): 479–85. https://doi.org/10.1016/j.conb.2009.08.004.

Li, Shun, Li-Xiang Chen, Xiu-Hua Peng, Chao Wang, Bo-Yin Qin, Dan Tan, Cheng-Xiao Han, et al. 2018. «Overview of the Reporter Genes and Reporter Mouse Models». *Animal Models and Experimental Medicine* 1 (1): 29–35. https://doi.org/10.1002/ame2.12008.

Ligon, Keith L., Santosh Kesari, Masaaki Kitada, Tao Sun, Heather A. Arnett, John A. Alberta, David J. Anderson, Charles D. Stiles, e David H. Rowitch. 2006. «Development of NG2 Neural Progenitor Cells Requires Olig Gene Function». *Proceedings of the National Academy of Sciences of the United States of America* 103 (20): 7853–58. https://doi.org/10.1073/pnas.0511001103.

Lobjois, Valérie, Bertrand Benazeraf, Nicolas Bertrand, François Medevielle, e Fabienne Pituello. 2004. «Specific Regulation of Cyclins D1 and D2 by FGF and Shh Signaling Coordinates Cell Cycle Progression, Patterning, and Differentiation during Early Steps of Spinal Cord Development». *Developmental Biology* 273 (2): 195–209. https://doi.org/10.1016/j.ydbio.2004.05.031.

Locker, Morgane, Michalis Agathocleous, Marcos A. Amato, Karine Parain, William A. Harris, e Muriel Perron. 2006. «Hedgehog Signaling and the Retina: Insights into the Mechanisms Controlling the Proliferative Properties of Neural Precursors». *Genes & Development* 20 (21): 3036–48. https://doi.org/10.1101/gad.391106.

Loulier, Karine, Martial Ruat, e Elisabeth Traiffort. 2006. «Increase of Proliferating Oligodendroglial Progenitors in the Adult Mouse Brain upon Sonic Hedgehog Delivery in the Lateral Ventricle». *Journal of Neurochemistry* 98 (2): 530–42. https://doi.org/10.1111/j.1471-4159.2006.03896.x.

Lu, Q. Richard, Tao Sun, Zhimin Zhu, Nan Ma, Meritxell Garcia, Charles D. Stiles, e David H. Rowitch. 2002. «Common Developmental Requirement for Olig Function Indicates a Motor Neuron/Oligodendrocyte Connection». *Cell* 109 (1): 75–86. https://doi.org/10.1016/s0092-8674(02)00678-5.

Luo, Fucheng, Jessie Zhang, Kathryn Burke, Rita R. Romito-DiGiacomo, Robert H. Miller, e Yan Yang. 2018. «Oligodendrocyte-specific loss of Cdk5 disrupts the architecture of nodes of Ranvier as well as learning and memory». *Experimental neurology* 306 (agosto): 92–104. https://doi.org/10.1016/j.expneurol.2018.05.001.

Machold, Robert, Shigemi Hayashi, Michael Rutlin, Mandar D. Muzumdar, Susana Nery, Joshua G. Corbin, Amel Gritli-Linde, et al. 2003. «Sonic Hedgehog Is Required for Progenitor Cell Maintenance in Telencephalic Stem Cell Niches». *Neuron* 39 (6): 937–50. https://doi.org/10.1016/s0896-6273(03)00561-0.

Madisen, Linda, Theresa A. Zwingman, Susan M. Sunkin, Seung Wook Oh, Hatim A. Zariwala, Hong Gu, Lydia L. Ng, et al. 2010. «A Robust and High-Throughput Cre Reporting and Characterization System for the Whole Mouse Brain». *Nature Neuroscience* 13 (1): 133–40. https://doi.org/10.1038/nn.2467.

Maire, Cécile L., Amélie Wegener, Christophe Kerninon, e Brahim Nait Oumesmar. 2010. «Gain-of-Function of Olig Transcription Factors Enhances Oligodendrogenesis and Myelination». *Stem Cells (Dayton, Ohio)* 28 (9): 1611–22. https://doi.org/10.1002/stem.480.

Mangin, Jean-Marie, e Vittorio Gallo. 2011. «The Curious Case of NG2 Cells: Transient Trend or Game Changer?» *ASN Neuro* 3 (1): e00052. https://doi.org/10.1042/AN20110001.

Marie, Corentine, Adrien Clavairoly, Magali Frah, Hatem Hmidan, Jun Yan, Chuntao Zhao, Juliette Van Steenwinckel, et al. 2018. «Oligodendrocyte Precursor Survival and Differentiation Requires Chromatin Remodeling by Chd7 and Chd8». *Proceedings of the National Academy of Sciences of the United States of America* 115 (35): E8246–55. https://doi.org/10.1073/pnas.1802620115.

Martí, E., R. Takada, D. A. Bumcrot, H. Sasaki, e A. P. McMahon. 1995. «Distribution of Sonic Hedgehog Peptides in the Developing Chick and Mouse Embryo». *Development (Cambridge, England)* 121 (8): 2537–47.

McTigue, D. M., P. Wei, e B. T. Stokes. 2001. «Proliferation of NG2-Positive Cells and Altered Oligodendrocyte Numbers in the Contused Rat Spinal Cord». *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 21 (10): 3392–3400.

Medina-Rodríguez, E. M., F. J. Arenzana, J. Pastor, M. Redondo, V. Palomo, R. García de Sola, C. Gil, A. Martínez, A. Bribián, e F. de Castro. 2013. «Inhibition of Endogenous Phosphodiesterase 7 Promotes Oligodendrocyte Precursor Differentiation and Survival». *Cellular and Molecular Life Sciences: CMLS* 70 (18): 3449–62. https://doi.org/10.1007/s00018-013-1340-2.

Medina-Rodríguez, Eva María, Ana Bribián, Amanda Boyd, Valle Palomo, Jesús Pastor, Alfonso Lagares, Carmen Gil, Ana Martínez, Anna Williams, e Fernando de Castro. 2017. «Promoting in Vivo Remyelination with Small Molecules: A Neuroreparative Pharmacological Treatment for Multiple Sclerosis». *Scientific Reports* 7: 43545. https://doi.org/10.1038/srep43545.

Merchán, Paloma, Ana Bribián, Cristina Sánchez-Camacho, Melissa Lezameta, Paola Bovolenta, e Fernando de Castro. 2007. «Sonic Hedgehog Promotes the Migration and Proliferation of Optic Nerve Oligodendrocyte Precursors». *Molecular and Cellular Neurosciences* 36 (3): 355–68. https://doi.org/10.1016/j.mcn.2007.07.012.

Messersmith, D. J., J. C. Murtie, T. Q. Le, E. E. Frost, e R. C. Armstrong. 2000. «Fibroblast Growth Factor 2 (FGF2) and FGF Receptor Expression in an Experimental Demyelinating Disease with Extensive Remyelination». *Journal of Neuroscience Research* 62 (2): 241–56. https://doi.org/10.1002/1097-4547(20001015)62:2<241::AID-JNR9>3.0.CO;2-D.

Meyer, Niklas, Nadine Richter, Zoya Fan, Gabrielle Siemonsmeier, Tatyana Pivneva, Philipp Jordan, Christian Steinhäuser, Marcus Semtner, Christiane Nolte, e Helmut Kettenmann. 2018. «Oligodendrocytes in the Mouse Corpus Callosum Maintain Axonal Function by Delivery of Glucose». *Cell Reports* 22 (9): 2383–94. https://doi.org/10.1016/j.celrep.2018.02.022.

Michailov, Galin V., Michael W. Sereda, Bastian G. Brinkmann, Tobias M. Fischer, Bernhard Haug, Carmen Birchmeier, Lorna Role, Cary Lai, Markus H. Schwab, e Klaus-Armin Nave. 2004. «Axonal Neuregulin-1 Regulates Myelin Sheath Thickness». *Science (New York, N.Y.)* 304 (5671): 700–703. https://doi.org/10.1126/science.1095862.

Miller, Daniel J., Tetyana Duka, Cheryl D. Stimpson, Steven J. Schapiro, Wallace B. Baze, Mark J. McArthur, Archibald J. Fobbs, et al. 2012. «Prolonged Myelination in Human Neocortical Evolution». *Proceedings of the National Academy of Sciences of the United States of America* 109 (41): 16480–85. https://doi.org/10.1073/pnas.1117943109.

Miller, David H., Frederik Barkhof, Joseph A. Frank, Geoffrey J. M. Parker, e Alan J. Thompson. 2002. «Measurement of Atrophy in Multiple Sclerosis: Pathological Basis, Methodological Aspects and Clinical Relevance». *Brain* 125 (8): 1676–95. https://doi.org/10.1093/brain/awf177.

Murao, Naoya, Hirofumi Noguchi, e Kinichi Nakashima. 2016. «Epigenetic Regulation of Neural Stem Cell Property from Embryo to Adult». *Neuroepigenetics* 5 (marzo): 1–10. https://doi.org/10.1016/j.nepig.2016.01.001.

Murcia-Belmonte, Verónica, Verónica Astillero-López, e Pedro F. Esteban. 2016. «Anosmin 1 Interacts with the Prokineticin Receptor 2 In Vitro Indicating a Molecular Link Between Both Proteins in the Pathogenesis of Kallmann Syndrome». *Protein and Peptide Letters* 23 (7): 650–55. https://doi.org/10.2174/0929866523666160517123331.

Murray, Andrew W. 2004. «Recycling the Cell Cycle: Cyclins Revisited». *Cell* 116 (2): 221–34. https://doi.org/10.1016/s0092-8674(03)01080-8.

Murtie, Joshua C., Yong-Xing Zhou, Tuan Q. Le, e Regina C. Armstrong. 2005. «In Vivo Analysis of Oligodendrocyte Lineage Development in Postnatal FGF2 Null Mice». *Glia* 49 (4): 542–54. https://doi.org/10.1002/glia.20142.

Najm, Fadi J., Mayur Madhavan, Anita Zaremba, Elizabeth Shick, Robert T. Karl, Daniel C. Factor, Tyler E. Miller, et al. 2015. «Drug-Based Modulation of Endogenous Stem Cells Promotes Functional Remyelination in Vivo». *Nature* 522 (7555): 216–20. https://doi.org/10.1038/nature14335.

Nanni, L., J. E. Ming, M. Bocian, K. Steinhaus, D. W. Bianchi, C. Die-Smulders, A. Giannotti, et al. 1999. «The Mutational Spectrum of the Sonic Hedgehog Gene in Holoprosencephaly: SHH Mutations Cause a Significant Proportion of Autosomal Dominant Holoprosencephaly». *Human Molecular Genetics* 8 (13): 2479–88. https://doi.org/10.1093/hmg/8.13.2479.

Naruse, Masae, Yasuki Ishizaki, Kazuhiro Ikenaka, Aoi Tanaka, e Seiji Hitoshi. 2017. «Origin of oligodendrocytes in mammalian forebrains: a revised perspective». *The Journal of Physiological Sciences* 67 (1): 63–70.

Nery, S., H. Wichterle, e G. Fishell. 2001. «Sonic Hedgehog Contributes to Oligodendrocyte Specification in the Mammalian Forebrain». *Development (Cambridge, England)* 128 (4): 527–40.

Okada, Atsumasa, Mitsutoshi Tominaga, Makoto Horiuchi, e Yasuhiro Tomooka. 2007. «Plexin-A4 is expressed in oligodendrocyte precursor cells and acts as a mediator of semaphorin signals». *Biochemical and biophysical research communications* 352 (1): 158–163.

Orentas, D. M., J. E. Hayes, K. L. Dyer, e R. H. Miller. 1999. «Sonic Hedgehog Signaling Is Required during the Appearance of Spinal Cord Oligodendrocyte Precursors». *Development (Cambridge, England)* 126 (11): 2419–29.
Ortega, María Cristina, Olivier Cases, Paloma Merchán, Renata Kozyraki, Diego Clemente, e Fernando de Castro. 2012. «Megalin Mediates the Influence of Sonic Hedgehog on Oligodendrocyte Precursor Cell Migration and Proliferation during Development». *Glia* 60 (6): 851–66. https://doi.org/10.1002/glia.22316.

Oustah, Amir Al, Cathy Danesin, Nagham Khouri-Farah, Marie-Amélie Farreny, Nathalie Escalas, Philippe Cochard, Bruno Glise, e Cathy Soula. 2014. «Dynamics of Sonic Hedgehog Signaling in the Ventral Spinal Cord Are Controlled by Intrinsic Changes in Source Cells Requiring Sulfatase 1». *Development* 141 (6): 1392–1403. https://doi.org/10.1242/dev.101717.

Parnavelas, J. G. 1999. «Glial Cell Lineages in the Rat Cerebral Cortex». *Experimental Neurology* 156 (2): 418–29. https://doi.org/10.1006/exnr.1999.7044.

Pérez-Cerdá, Fernando, María Victoria Sánchez-Gómez, e Carlos Matute. 2015. «Pío Del Río Hortega and the Discovery of the Oligodendrocytes». *Frontiers in Neuroanatomy* 9: 92. https://doi.org/10.3389/fnana.2015.00092.

Perlman, Kelly, Charles P. Couturier, Moein Yaqubi, Arnaud Tanti, Qiao-Ling Cui, Florian Pernin, Jo

Anne Stratton, et al. 2020. «Developmental Trajectory of Oligodendrocyte Progenitor Cells in the Human Brain Revealed by Single Cell RNA Sequencing». *Glia* 68 (6): 1291–1303. https://doi.org/10.1002/glia.23777.

Perry, V. H., e R. D. Lund. 1990. «Evidence That the Lamina Cribrosa Prevents Intraretinal Myelination of Retinal Ganglion Cell Axons». *Journal of Neurocytology* 19 (2): 265–72. https://doi.org/10.1007/BF01217304.

Pfeiffer, S. E., A. E. Warrington, e R. Bansal. 1993. «The Oligodendrocyte and Its Many Cellular Processes». *Trends in Cell Biology* 3 (6): 191–97. https://doi.org/10.1016/0962-8924(93)90213-k.
Poncet, C., C. Soula, F. Trousse, P. Kan, E. Hirsinger, O. Pourquié, A. M. Duprat, e P. Cochard. 1996. «Induction of Oligodendrocyte Progenitors in the Trunk Neural Tube by Ventralizing Signals: Effects of Notochord and Floor Plate Grafts, and of Sonic Hedgehog». *Mechanisms of Development* 60 (1): 13–32. https://doi.org/10.1016/s0925-4773(96)00595-3.

Pons, Vincent, e Serge Rivest. 2020. «Beneficial Roles of Microglia and Growth Factors in MS, a Brief Review». *Frontiers in Cellular Neuroscience* 14. https://doi.org/10.3389/fncel.2020.00284.

Pringle, N. P., W. P. Yu, S. Guthrie, H. Roelink, A. Lumsden, A. C. Peterson, e W. D. Richardson. 1996. «Determination of Neuroepithelial Cell Fate: Induction of the Oligodendrocyte Lineage by Ventral Midline Cells and Sonic Hedgehog». *Developmental Biology* 177 (1): 30–42. https://doi.org/10.1006/dbio.1996.0142.

Procaccini, Claudio, Veronica De Rosa, Valentina Pucino, Luigi Formisano, e Giuseppe Matarese. 2015. «Animal Models of Multiple Sclerosis». *European Journal of Pharmacology* 759 (luglio): 182–91. https://doi.org/10.1016/j.ejphar.2015.03.042.

Rawji, Khalil S., e V. Wee Yong. 2013. «The Benefits and Detriments of Macrophages/Microglia in Models of Multiple Sclerosis». *Clinical and Developmental Immunology* 2013. https://doi.org/10.1155/2013/948976.

Reynolds, Richard, Mary Dawson, Dimitrios Papadopoulos, Annabella Polito, Isabelle Cenci Di Bello, Danielle Pham-Dinh, e Joel Levine. 2002. «The Response of NG2-Expressing Oligodendrocyte Progenitors to Demyelination in MOG-EAE and MS». *Journal of Neurocytology* 31 (6–7): 523–36. https://doi.org/10.1023/a:1025747832215.

Ribes, Vanessa, e James Briscoe. 2009. «Establishing and Interpreting Graded Sonic Hedgehog Signaling during Vertebrate Neural Tube Patterning: The Role of Negative Feedback». *Cold Spring Harbor Perspectives in Biology* 1 (2): a002014. https://doi.org/10.1101/cshperspect.a002014.

Richardson, A. G., C. C. McIntyre, e W. M. Grill. 2000. «Modelling the Effects of Electric Fields on Nerve Fibres: Influence of the Myelin Sheath». *Medical and Biological Engineering and Computing* 38 (4): 438–46. https://doi.org/10.1007/BF02345014.

Richardson, William D., Nicoletta Kessaris, e Nigel Pringle. 2006. «Oligodendrocyte Wars». *Nature Reviews. Neuroscience* 7 (1): 11–18. https://doi.org/10.1038/nrn1826.

Rimkus, Tadas K., Richard L. Carpenter, Shadi Qasem, Michael Chan, e Hui-Wen Lo. 2016. «Targeting the Sonic Hedgehog Signaling Pathway: Review of Smoothened and GLI Inhibitors». *Cancers* 8 (2). https://doi.org/10.3390/cancers8020022.

Río Hortega. 1928. «Tercera Aportación Al Conocimiento Morfológico E Interpretación Funcional De La Oligodendroglía.» *Tercera Aportación Al Conocimiento Morfológico E Interpretación Funcional De La Oligodendroglía.*, 1928.

Rio-Hortega, Pio del. 2012. «Are the glia with very few processes homologous with Schwann cells? by Pío del Río-Hortega. 1922.» *Clinical neuropathology* 31 (6): 460.

Ritter, Jonathan, Thomas Schmitz, Li-Jin Chew, Christoph Bührer, Wiebke Möbius, Marzieh Zonouzi, e Vittorio Gallo. 2013. «Neonatal Hyperoxia Exposure Disrupts Axon-Oligodendrocyte Integrity in the Subcortical White Matter». *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 33 (21): 8990–9002. https://doi.org/10.1523/JNEUROSCI.5528-12.2013.

Robinson, S., e R. H. Miller. 1999. «Contact with Central Nervous System Myelin Inhibits Oligodendrocyte Progenitor Maturation». *Developmental Biology* 216 (1): 359–68. https://doi.org/10.1006/dbio.1999.9466.

Rowitch, David H., e Arnold R. Kriegstein. 2010. «Developmental Genetics of Vertebrate Glial-Cell Specification». *Nature* 468 (7321): 214–22. https://doi.org/10.1038/nature09611.

Rowitch, David H., Benoit St.-Jacques, Scott M. K. Lee, Jonathon D. Flax, Evan Y. Snyder, e Andrew P. McMahon. 1999. «Sonic Hedgehog Regulates Proliferation and Inhibits Differentiation of CNS Precursor Cells». *Journal of Neuroscience* 19 (20): 8954–65. https://doi.org/10.1523/JNEUROSCI.19-20-08954.1999.

Rusznák, Zoltán, Willem Henskens, Emma Schofield, Woojin S. Kim, e YuHong Fu. 2016. «Adult Neurogenesis and Gliogenesis: Possible Mechanisms for Neurorestoration». *Experimental Neurobiology* 25 (3): 103–12. https://doi.org/10.5607/en.2016.25.3.103.

Samanta, Jayshree, Ethan M. Grund, Hernandez M. Silva, Juan J. Lafaille, Gord Fishell, e James L. Salzer. 2015. «Inhibition of Gli1 Mobilizes Endogenous Neural Stem Cells for Remyelination». *Nature* 526 (7573): 448–52. https://doi.org/10.1038/nature14957.

Sánchez, Maria A., e Regina C. Armstrong. 2018. «Postnatal Sonic Hedgehog (Shh) Responsive Cells Give Rise to Oligodendrocyte Lineage Cells during Myelination and in Adulthood Contribute to Remyelination». *Experimental Neurology* 299 (Pt A): 122–36. https://doi.org/10.1016/j.expneurol.2017.10.010.

Sánchez, Maria A., Genevieve M. Sullivan, e Regina C. Armstrong. 2018. «Genetic Detection of Sonic Hedgehog (Shh) Expression and Cellular Response in the Progression of Acute through Chronic Demyelination and Remyelination». *Neurobiology of Disease* 115: 145–56. https://doi.org/10.1016/j.nbd.2018.04.003.

Santiago, Giselda, Dagma Venturini Marques Abramides, Luciana Paula Maximino De-Vitto, Lucilene Arilho Ribeiro, Silvio Garcia Meira, e Antonio Richieri-Costa. 2006. «Language Skills and Neuropsychological Performance in Patients with SHH Mutations and a Holoprosencephaly-like Phenotype». *American Journal of Medical Genetics. Part A* 140 (19): 2085–90. https://doi.org/10.1002/ajmg.a.31311.

Savvaki, Maria, Theofanis Panagiotaropoulos, Antonis Stamatakis, Irene Sargiannidou, Pinelopi Karatzioula, Kazutada Watanabe, Fotini Stylianopoulou, Domna Karagogeos, e Kleopas A. Kleopa. 2008. «Impairment of Learning and Memory in TAG-1 Deficient Mice Associated with Shorter CNS Internodes and Disrupted Juxtaparanodes». *Molecular and Cellular Neurosciences* 39 (3): 478–90. https://doi.org/10.1016/j.mcn.2008.07.025.

Schindelin, Johannes, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, et al. 2012. «Fiji: An Open-Source Platform for Biological-Image Analysis». *Nature Methods* 9 (7): 676–82. https://doi.org/10.1038/nmeth.2019.

Schlegel, Alexander A., Justin J. Rudelson, e Peter U. Tse. 2012. «White Matter Structure Changes as Adults Learn a Second Language». *Journal of Cognitive Neuroscience* 24 (8): 1664–70. https://doi.org/10.1162/jocn_a_00240.

Setzu, Anna, Charles Ffrench-Constant, e Robin J. M. Franklin. 2004. «CNS Axons Retain Their Competence for Myelination throughout Life». *Glia* 45 (3): 307–11. https://doi.org/10.1002/glia.10321.

Setzu, Anna, Justin D. Lathia, Chao Zhao, Karen Wells, Mahendra S. Rao, Charles Ffrench-Constant, e

Robin J. M. Franklin. 2006. «Inflammation Stimulates Myelination by Transplanted Oligodendrocyte Precursor Cells». *Glia* 54 (4): 297–303. https://doi.org/10.1002/glia.20371.

Siegel, Allan, e Hreday Sapru. 2006. In *Essential Neuroscience*, 2006º ed. Lippincott Williams & Wilkins.

Sim, Fraser J., Jennifer K. Lang, Ben Waldau, Neeta S. Roy, Theodore E. Schwartz, Webster H. Pilcher, Karen J. Chandross, et al. 2006. «Complementary Patterns of Gene Expression by Human Oligodendrocyte Progenitors and Their Environment Predict Determinants of Progenitor Maintenance and Differentiation». *Annals of Neurology* 59 (5): 763–79. https://doi.org/10.1002/ana.20812.

Soula, C., C. Danesin, P. Kan, M. Grob, C. Poncet, e P. Cochard. 2001. «Distinct Sites of Origin of Oligodendrocytes and Somatic Motoneurons in the Chick Spinal Cord: Oligodendrocytes Arise from Nkx2.2-Expressing Progenitors by a Shh-Dependent Mechanism». *Development (Cambridge, England)* 128 (8): 1369–79.

Spassky, Nathalie, Fernando de Castro, Barbara Le Bras, Katharina Heydon, Françoise Quéraud-LeSaux, Evelyne Bloch-Gallego, Alain Chédotal, Bernard Zalc, e Jean-Léon Thomas. 2002a. «Directional Guidance of Oligodendroglial Migration by Class 3 Semaphorins and Netrin-1». *Journal of Neuroscience* 22 (14): 5992–6004. https://doi.org/10.1523/JNEUROSCI.22-14-05992.2002.

———. 2002b. «Directional Guidance of Oligodendroglial Migration by Class 3 Semaphorins and Netrin-1». *Journal of Neuroscience* 22 (14): 5992–6004. https://doi.org/10.1523/JNEUROSCI.22-14-05992.2002.

Stallcup, William B. 2002. «The NG2 Proteoglycan: Past Insights and Future Prospects». *Journal of Neurocytology* 31 (6–7): 423–35. https://doi.org/10.1023/a:1025731428581.

Steinman, L., P. J. Utz, e W. H. Robinson. 2005. «Suppression of Autoimmunity via Microbial Mimics of Altered Peptide Ligands». *Current Topics in Microbiology and Immunology* 296: 55–63. https://doi.org/10.1007/3-540-30791-5_4.

Stidworthy, Mark F., Stephane Genoud, Ueli Suter, Ned Mantei, e Robin J. M. Franklin. 2003. «Quantifying the Early Stages of Remyelination Following Cuprizone-Induced Demyelination». *Brain Pathology (Zurich, Switzerland)* 13 (3): 329–39. https://doi.org/10.1111/j.1750-3639.2003.tb00032.x.

Stolt, C. Claus, Petra Lommes, Ralf P. Friedrich, e Michael Wegner. 2004. «Transcription Factors Sox8 and Sox10 Perform Non-Equivalent Roles during Oligodendrocyte Development despite Functional Redundancy». *Development (Cambridge, England)* 131 (10): 2349–58. https://doi.org/10.1242/dev.01114.

Suzuki, Nobuharu, Kaori Sekimoto, Chikako Hayashi, Yo Mabuchi, Tetsuya Nakamura, e Chihiro Akazawa. 2017. «Differentiation of Oligodendrocyte Precursor Cells from Sox10-Venus Mice to Oligodendrocytes and Astrocytes». *Scientific Reports* 7 (1): 14133. https://doi.org/10.1038/s41598-017-14207-0.

Syed, Yasir A., Alexandra S. Baer, Gert Lubec, Harald Hoeger, Georg Widhalm, e Mark R. Kotter. 2008. «Inhibition of Oligodendrocyte Precursor Cell Differentiation by Myelin-Associated Proteins». *Neurosurgical Focus* 24 (3–4): E5. https://doi.org/10.3171/FOC/2008/24/3-4/E4.

Takeuchi, Hideyuki, Tetsuya Mizuno, Guiqin Zhang, Jinyan Wang, Jun Kawanokuchi, Reiko Kuno, e Akio Suzumura. 2005. «Neuritic Beading Induced by Activated Microglia Is an Early Feature of Neuronal Dysfunction Toward Neuronal Death by Inhibition of Mitochondrial Respiration and Axonal Transport». *Journal of Biological Chemistry* 280 (11): 10444–54. https://doi.org/10.1074/jbc.M413863200.

Takizawa, C. G., e D. O. Morgan. 2000. «Control of Mitosis by Changes in the Subcellular Location of Cyclin-B1-Cdk1 and Cdc25C». *Current Opinion in Cell Biology* 12 (6): 658–65. https://doi.org/10.1016/s0955-0674(00)00149-6.

Taylor, Lorelei C., Wendy Gilmore, Jenny P.-Y. Ting, e Glenn K. Matsushima. 2010. «Cuprizone Induces Similar Demyelination in Male and Female C57BL/6 Mice and Results in Disruption of the Estrous Cycle». *Journal of Neuroscience Research* 88 (2): 391–402. https://doi.org/10.1002/jnr.22215.

Tiane, Assia, Melissa Schepers, Ben Rombaut, Raymond Hupperts, Jos Prickaerts, Niels Hellings, Daniel van den Hove, e Tim Vanmierlo. 2019. «From OPC to Oligodendrocyte: An Epigenetic Journey». *Cells* 8 (10). https://doi.org/10.3390/cells8101236.

Tong, Cheuk Ka, Luis C. Fuentealba, Jugal K. Shah, Robert A. Lindquist, Rebecca A. Ihrie, Cristina D. Guinto, Jose L. Rodas-Rodriguez, e Arturo Alvarez-Buylla. 2015. «A Dorsal SHH-Dependent Domain in the V-SVZ Produces Large Numbers of Oligodendroglial Lineage Cells in the Postnatal Brain». *Stem Cell Reports* 5 (4): 461–70. https://doi.org/10.1016/j.stemcr.2015.08.013.

Torre-Fuentes, L., L. Moreno-Jiménez, V. Pytel, J. A. Matías-Guiu, U. Gómez-Pinedo, e J. Matías-Guiu. 2020. «Experimental Models of Demyelination and Remyelination». *Neurologia (Barcelona, Spain)* 35 (1): 32–39. https://doi.org/10.1016/j.nrl.2017.07.002.

Traiffort, Elisabeth, Abdelmoumen Kassoussi, Amina Zahaf, e Yousra Laouarem. 2020. «Astrocytes and Microglia as Major Players of Myelin Production in Normal and Pathological Conditions». *Frontiers in Cellular Neuroscience* 14 (aprile). https://doi.org/10.3389/fncel.2020.00079.

Traiffort, Elisabeth, Mary Zakaria, Yousra Laouarem, e Julien Ferent. 2016. «Hedgehog: A Key Signaling in the Development of the Oligodendrocyte Lineage». *Journal of Developmental Biology* 4 (3). https://doi.org/10.3390/jdb4030028.

Tsai, Hui-Hsin, Emma Frost, Vivien To, Shenandoah Robinson, Charles ffrench-Constant, Robert Geertman, Richard M. Ransohoff, e Robert H. Miller. 2002. «The Chemokine Receptor CXCR2 Controls Positioning of Oligodendrocyte Precursors in Developing Spinal Cord by Arresting Their Migration». *Cell* 110 (3): 373–83. https://doi.org/10.1016/S0092-8674(02)00838-3.

Tsai, Hui-Hsin, Jianqin Niu, Roeben Munji, Dimitrios Davalos, Junlei Chang, Haijing Zhang, An-Chi Tien, et al. 2016. «Oligodendrocyte Precursors Migrate along Vasculature in the Developing Nervous System». *Science* 351 (6271): 379–84. https://doi.org/10.1126/science.aad3839.

Ulloa, Fausto, e James Briscoe. 2007. «Morphogens and the Control of Cell Proliferation and Patterning in the Spinal Cord». *Cell Cycle (Georgetown, Tex.)* 6 (21): 2640–49. https://doi.org/10.4161/cc.6.21.4822.

Vana, Adam C., Nicole C. Flint, Norah E. Harwood, Tuan Q. Le, Marcus Fruttiger, e Regina C. Armstrong. 2007. «Platelet-Derived Growth Factor Promotes Repair of Chronically Demyelinated White Matter». *Journal of Neuropathology and Experimental Neurology* 66 (11): 975–88. https://doi.org/10.1097/NEN.0b013e3181587d46.

Viganò, Francesca, Sarah Schneider, Mauro Cimino, Elisabetta Bonfanti, Paolo Gelosa, Luigi Sironi, Maria P. Abbracchio, e Leda Dimou. 2016. «GPR17 Expressing NG2-Glia: Oligodendrocyte Progenitors Serving as a Reserve Pool after Injury». *Glia* 64 (2): 287–99. https://doi.org/10.1002/glia.22929.

Wang, Li-Chun, e Guillermina Almazan. 2016. «Role of Sonic Hedgehog Signaling in Oligodendrocyte Differentiation». *Neurochemical Research* 41 (12): 3289–99. https://doi.org/10.1007/s11064-016-2061-3.

Wang, Yaping, Gabriel D. Dakubo, Sherry Thurig, Chantal J. Mazerolle, e Valerie A. Wallace. 2005. «Retinal Ganglion Cell-Derived Sonic Hedgehog Locally Controls Proliferation and the Timing of RGC Development in the Embryonic Mouse Retina». *Development (Cambridge, England)* 132 (22): 5103–13. https://doi.org/10.1242/dev.02096.

Wang, Yue, Jaime Imitola, Stine Rasmussen, Kevin C. O'Connor, e Samia J. Khoury. 2008. «Paradoxical Dysregulation of the Neural Stem Cell Pathway Sonic Hedgehog-Gli1 in Autoimmune

Encephalomyelitis and Multiple Sclerosis». *Annals of Neurology* 64 (4): 417–27. https://doi.org/10.1002/ana.21457.

Watanabe, Masahiko, Tarik Hadzic, e Akiko Nishiyama. 2004. «Transient Upregulation of Nkx2.2 Expression in Oligodendrocyte Lineage Cells during Remyelination». *Glia* 46 (3): 311–22. https://doi.org/10.1002/glia.20006.

Xu, Qing, Lihua Guo, Holly Moore, Ronald R. Waclaw, Kenneth Campbell, e Stewart A. Anderson. 2010. «Sonic Hedgehog Signaling Confers Ventral Telencephalic Progenitors with Distinct Cortical Interneuron Fates». *Neuron* 65 (3): 328–40. https://doi.org/10.1016/j.neuron.2010.01.004.

Xu, Xiaofeng, Qian Yu, Minxi Fang, Min Yi, Aifen Yang, Binghua Xie, Junlin Yang, Zunyi Zhang, Zhongmin Dai, e Mengsheng Qiu. 2020. «Stage-specific regulation of oligodendrocyte development by Hedgehog signaling in the spinal cord». *Glia* 68 (2): 422–34. https://doi.org/10.1002/glia.23729.

Yamaguchi, Masahiro, Tatsunori Seki, Itaru Imayoshi, Nobuaki Tamamaki, Yoshitaka Hayashi, Yoshitaka Tatebayashi, e Seiji Hitoshi. 2016. «Neural stem cells and neuro/gliogenesis in the central nervous system: understanding the structural and functional plasticity of the developing, mature, and diseased brain». *The Journal of Physiological Sciences* 66 (3): 197–206.

Yao, Xiaoming, Tao Su, e A. S. Verkman. 2016. «Clobetasol Promotes Remyelination in a Mouse Model of Neuromyelitis Optica». *Acta Neuropathologica Communications* 4 (1): 42. https://doi.org/10.1186/s40478-016-0309-4.

Young, Kaylene M., Konstantina Psachoulia, Richa B. Tripathi, Sara-Jane Dunn, Lee Cossell, David Attwell, Koujiro Tohyama, e William D. Richardson. 2013. «Oligodendrocyte Dynamics in the Healthy Adult CNS: Evidence for Myelin Remodeling». *Neuron* 77 (5): 873–85. https://doi.org/10.1016/j.neuron.2013.01.006.

Zalc, B., D. Goujet, e D. Colman. 2008. «The Origin of the Myelination Program in Vertebrates». *Current Biology: CB* 18 (12): R511-512. https://doi.org/10.1016/j.cub.2008.04.010.

Zhang, H., L. Vutskits, V. Calaora, P. Durbec, e J. Z. Kiss. 2004. «A Role for the Polysialic Acid – Neural Cell Adhesion Molecule in PDGF-Induced Chemotaxis of Oligodendrocyte Precursor Cells». *Journal of Cell Science* 117 (1): 93–103. https://doi.org/10.1242/jcs.00827.

Zhang, Ruilan, Michael Chopp, e Zheng Gang Zhang. 2013. «Oligodendrogenesis after Cerebral Ischemia». *Frontiers in Cellular Neuroscience* 7 (ottobre): 201. https://doi.org/10.3389/fncel.2013.00201.

Zhou, Yong-Xing, Ravinder Pannu, Tuan Q. Le, e Regina C. Armstrong. 2012. «Fibroblast Growth Factor 1 (FGFR1) Modulation Regulates Repair Capacity of Oligodendrocyte Progenitor Cells Following Chronic Demyelination». *Neurobiology of Disease* 45 (1): 196–205. https://doi.org/10.1016/j.nbd.2011.08.004.

Zhu, Xiaoqin, Robert A. Hill, Dirk Dietrich, Mila Komitova, Ryusuke Suzuki, e Akiko Nishiyama. 2011. «Age-Dependent Fate and Lineage Restriction of Single NG2 Cells». *Development (Cambridge, England)* 138 (4): 745–53. https://doi.org/10.1242/dev.047951.

Zuchero, J. Bradley, e Ben A. Barres. 2015. «Glia in Mammalian Development and Disease». *Development (Cambridge, England)* 142 (22): 3805–9. https://doi.org/10.1242/dev.129304.

Appendix

Table 5 Summary of the number of animals used in each objective (N.d= non determined)

Aim	Use	Age	Gender	Number
1	Myelination study	P14	N.d	3-5/genotype
2	Cuprizone	8 Weeks Old	Male	16/genotype
3	OPC culture	P7	N.d	5-7/genotype
	Transplantated	8 Weeks Old	Female	5 WT