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Investigating the oligodendrocyte progenitor cells and their progeny heterogeneity in the mammalian central nervous system

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Abstract

Oligodendrocytes (OLs) are macroglial cells present in the vertebrate central nervous system (CNS). OLs are responsible for myelinating the neurons, ensuring an efficient propagation of electrical impulses along the axon, through saltatory conduction. Myelination disorders, such as multiple sclerosis, damage the myelin sheath and disrupt the normal performance of neurons. When this kind of event happens, OLs have the capability of remyelinating the affected axons for a certain period. Oligodendrocytes are originated by oligodendrocyte progenitor cells (OPCs), which are highly proliferative, as identified by the expression of the specific molecular marker platelet-derived growth factor receptor alpha (Pdgfra). OPCs proliferate in three phased developmental waves from different domains in the ventricles of the developing brain and spinal cord, giving rise to twelve distinct subpopulations of OLs. These proliferation waves occur in distinct time-points, namely on embryonic (E) days 13.5 and 15.5, and gradually throughout a postnatal (P) wave that starts at birth. Bulk and single-cell RNA-seq analysis performed by the host laboratory suggests that OPCs and OLs present a high degree of heterogeneity at transcriptional level: the gene expression profile of OPCs and progeny varies dramatically during development between and within different areas of the CNS. Thus, it was possible to identify putative cell markers for the twelve different OL subpopulations. To validate these markers in tissue sections, antibodies and RNA probes were tested through immunohistochemistry (IHC) and RNAscope *in situ* hybridization (ISH), respectively. Since the proliferation waves of OPCs occur in three different time points, the developmental origin of the distinctive OL subpopulations was also investigated both in the brain and spinal cord. This subject was addressed by conducting lineage tracing experiments using the PdgfrCreER-RCE mouse line, tagging GFP (green fluorescent protein) reporter with OPCs and their progeny. Recombination of the reporter gene was induced either on E12.5 or P3-5, in order to respectively target the first embryonic and postnatal waves of OPC proliferation and their progeny. Subsequently, brain and spinal cord were collected at different ages (P20 and P60), and IHC and ISH assays were performed to test the putative markers for different subpopulations of OL lineage. In this work, the pattern of expression of antibodies/probes for cellular markers of OL subpopulations was analysed: MOL1 and MOL2 in the juvenile mouse, and MOL5/6, OPCs/COP in the adult mouse. Moreover, colocalization of signal between diverse cellular markers for OL lineage was assessed in the adult mouse, targeting the embryonic and postnatal waves of OPC proliferation and progeny. The results presented in this work contribute for a broader comprehension regarding OL lineage heterogeneity, as well as their developmental origin in the CNS of the mouse.

Keywords: Oligodendrocyte, oligodendrocyte progenitor cell, developmental wave, heterogeneity, cell marker

Resumo

O sistema nervoso é uma complexa rede de células especializadas que comunicam entre si, permitindo ao organismo adquirir e partilhar informação sobre o próprio e o que o rodeia. Isto envolve componentes sensoriais que detectam estímulos ambientais, e componentes motores que comandam os diferentes músculos, bem como secreções glandulares. A rede que constitui o sistema nervoso é composta por neurónios e células especializadas, denominadas células da glia, ou simplesmente glia. A sinalização entre estes diferentes tipos celulares permite a realização de processos cognitivos como o pensamento, comunicação, aprendizagem e formação de memórias, e também a movimentação e sensação. Isto permite a percepção e interação com o mundo, outros indivíduos e si mesmo, moldando o próprio comportamento. Ao longo do desenvolvimento do sistema nervoso, factores de crescimento irão induzir a formação da espinal medula, posteriormente levando à origem do cérebro. A proliferação e diferenciação de células estaminais neurais, que são multipotentes, levará à geração de neurónios e glia no sistema nervoso. Os neurónios são o componente básico para o funcionamento do cérebro, espinal medula e nervos periféricos, sendo o principal tipo celular do sistema nervoso. Estas células comunicam entre si através da condução de impulsos nervosos ao longo do axónio e graças à existência de conexões entre si, denominadas sinapses. Para além disso, as célula da glia têm também papéis cruciais para o correto funcionamento do sistema nervoso central (SNC), incluindo proteção imunológica no caso da microglia, suporte metabólico e recaptação de neurotransmissores no caso dos astrócitos, e os oligodendrócitos são responsáveis pela mielinização dos neurónios.

Os oligodendrócitos (OLs) interagem com axónios adjacentes, através da projeção de diversos processos celulares, envolvendo-os em mielina. A bainha de mielina fornece suporte aos neurónios e facilita a propagação de impulsos eléctricos de forma rápida e eficiente, através de condução saltatória. Tendo em conta que a mielinização neuronal está relacionada com aspectos comportamentais, cognitivos e emocionais, este trata-se de um processo crucial para o correto funcionamento do cérebro humano. Um dos aspectos mais característicos da mielinização é que começa no estado embrionário e estende-se após o nascimento, em humanos ao longo das duas primeiras décadas de vida, e em ratinho até aos dois meses. No entanto, a produção de mielina também pode acontecer noutras alturas da vida, nomeadamente em contexto de doenças que comprometam o funcionamento dos OLs ou danifiquem as bainhas de mielina já existentes, como é o caso da esclerose múltipla. Cada OL tem a capacidade para mielinizar cerca de 60 axónios distintos, dependendo do área do SNC e diâmetro dos axónios. Os OLs são originados por células progenitoras de oligodendrócitos (OPCs), que são altamente migratórios, como identificado pela expressão dos marcadores moleculares específicos proteoglicano NG2 e *platelet-derived growth factor receptor alfa* (Pdgfra). Ao longo do desenvolvimento e durante episódios de remielinização a linhagem dos OLs diferencia-se progressivamente e de forma altamente regulada. Estes processos são controlados por uma multitude de factores moleculares intrínsecos e extrínsecos, que induzem alterações epigenéticas e regulação transcricional e traducional em OPCs e OLs, orquestrando assim a produção de mielina.

A origem dos OPCs ocorre em ondas de proliferação sequenciais, provenientes de domínios dorsais e ventrais do cérebro e espinal medula em desenvolvimento. Em ratinho, a primeira onda de produção de OPCs ocorre sensivelmente no dia embrionário (E) 13.5, seguido por uma segunda onda no dia E15.5. Uma terceira, e última, onda de proliferação ocorre após o nascimento, e gradualmente ao longo de alguns dias. Experiências de *fate-mapping* demonstram que na espinal medula os OPCs têm fundamentalmente origens ventrais, contribuindo em 85-90% para a origem de OLs no ratinho adulto. Por outro lado, no cérebro, a primeira onda de proliferação de OPCs é essencialmente reposta por precursores originados de zonas mais dorsais, numa fase posterior do processo de desenvolvimento.

Curiosamente, a ablação génica de células precursoras tanto de origem ventral como dorsal resulta numa expansão compensatória da população contrária, originando um número final de OLs e níveis de mielinização normais, o que demonstra um grande grau de redundância. Para além disto, os OPCs são também capazes de comunicar com neurónios, através de potenciais de ação, demonstrando funcionalidade equivalente em OPCs originados tanto de zonas ventrais como dorsais. Apesar de a população de OLs aparentar ser altamente homogénea na sua função, os OPCs estão aptos a gerar OLs com potencial de mielinização heterogéneo. No caso da espinal medula, os OLs produzem bainhas de mielina mais espessas e os segmentos internodais nos axónios são mais longos, contrariamente aos OLs corticais. Para além disso, OPCs da matéria branca diferenciam-se em OLs mielinizantes quando transplantados tanto para matéria branca como cinzenta, o que não acontece com OPCs da matéria cinzenta, gerando menos OLs produtores de mielina mesmo quando transplantados para matéria branca. Assim, é preciso clarificar se os OLs se tornam morfologicamente diversificados ao longo da maturação, através de interações com o meio envolvente, ou se possuem heterogeneidade funcional intrinsecamente.

A esclerose múltipla é uma doença crónica autoimune no sistema nervoso central, caracterizada por surtos de desmielinização neuronal com diversos níveis de gravidade. Julga-se que seja causada por diversos factores, nomeadamente susceptibilidade genética, infeção viral, desestabilização no metabolismo e fatores ambientais. Carateriza-se pela perda de força muscular, alterações na coordenação e sensibilidade, visão enevoada e dificuldades no discurso. Normalmente é desencadeada entre os 20 e os 45 anos de idade e, de momento, não tem cura. Na maioria dos pacientes, a esclerose múltipla apresenta estádios iniciais de *deficits* neurológicos reversíveis, quando os sintomas abrandam ou desaparecem, o que corresponde a uma resposta remielinizante dos OPCs. No entanto, ao longo do tempo os OPCs vão perdendo a capacidade de responder de forma eficiente até que acabam por perder a sua função na totalidade. Este padrão designa-se *relapse-remitting* e é o subtipo mais comum da doença. A remielinização normalmente segue-se a uma perda patológica de mielina, de forma a restaurar as propriedades dos neurónios através da produção de novas bainhas de mielina. Este é um processo complexo que envolve factores celulares e moleculares na sua regulação. Numa fase inicial as áreas afectadas são colonizadas pelos OPCs, que depois se diferenciam em OLs mielinizantes, que contactam com os axónios afectados e produzem novas bainhas de mielina funcionais. Foi demonstrado anteriormente que o transplante de OPCs no SNC origina oligodendrogénese e consequente remielinização axonal. Desta forma, os OPCs são a população celular que deve ser focada para o desenvolvimento de novas terapias para doenças desmielinizantes.

Análise de sequenciação de RNA (RNA-seq) em bloco e célula a célula, sugere que os OPCs e OLs possuem um elevado grau de heterogeneidade a nível transcricional. Foram analisados 5072 transcriptomas de células singulares, isolados de 10 regiões específicas do SNC de ratinho juvenil e adulto, sendo identificadas 12 subpopulações distintas da linhagem de OLs. Foi também possível criar um modelo de diferenciação, partindo dos OPCs e identificando as diversas fases dos OLs até atingirem o estado maduro. A existência destes estados distintos de OPCs e OLs sugerem uma possível heterogeneidade funcional. Assim, foi também possível criar uma lista de marcadores celulares putativos para a identificação das diferentes subpopulações de OLs presentes no cérebro e espinal medula do ratinho. De forma a validar estes marcadores em secções de tecido do SNC de ratinho, usaram-se anticorpos e sondas de RNA, testados respetivamente através de técnicas de imuno-histoquímica (IHC) e hibridização *in situ* (ISH). Para além disso, e tendo em conta que as ondas de proliferação de OPCs ocorrem em três fases distintas, também se procedeu à investigação da origem do desenvolvimento das diferentes subpopulações de OLs, tanto no cérebro como na espinal medula. Estes estudos foram feitos recorrendo a *lineage tracing,* utilizando a linhagem de ratinho

PdgfraCreER-RCE, induzindo, através de injeções de tamoxifeno, a expressão do gene repórter GFP (*green fluorescent protein*) nos OPCs e respetiva descendência. Induziu-se a recombinação e expressão de GFP nos dias 12.5 do estado embrionário ou 3-5 após o nascimento do ratinho, pondo em evidência respetivamente a primeira ou última ondas de proliferação de OPCs e descendência. Subsequentemente, o cérebro e espinal medula foram recolhidos em idades distintas, nomeadamente no ratinho juvenil (20 dias) e adulto (60 dias), e realizaram-se ensaios de IHC e ISH. Neste trabalho foi analisado o padrão de expressão de marcadores celulares, através da utilização de anticorpos/sondas específicos, de subpopulações de OLs: MOL1 e MOL2 no ratinho juvenil, e MOL5/6, OPCs/COP no ratinho adulto. Procedeu-se ainda à análise de co-localização de sinal entre diversos marcadores celulares da linhagem de OLs no ratinho adulto. Os resultados apresentados neste trabalho contribuem para uma maior compreensão sobre a heterogeneidade da linhagem de OLs, bem como a sua origem ao longo do desenvolvimento do SNC do ratinho. A aquisição desta informação poderá ser útil no sentido de melhor compreender as alterações moleculares e de diferenciação de OPCs e OLs em eventos de remielinização em contexto de doenças desmielinizantes, como a esclerose múltipla.

Palavras-chave: Oligodendrócito, célula precursora de oligodendrócitos, onda de desenvolvimento, heterogeneidade, marcador celular

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Introduction

Nervous system, a complex and dynamic network

The nervous system is a complex network of specialized cells that communicate with each other, enabling the organism to acquire and share information about its surroundings and itself. This involves sensory components, which detect environmental stimuli, motor components that operate skeletal, cardiac and smooth muscles, and also mechanisms that regulate glandular secretions (Kandel et al., 2013). These extremely intricate processes ensure that the homeostasis of the individual is properly sustained, by managing inputs that come from either an internal or external source, and delivering adjusted outputs to specific areas within the organism. Evolutionarily, higher-class organisms have their nervous system broadly divided in central nervous system (CNS), composed by the brain and spinal cord, and peripheral nervous system (PNS), which branches throughout the body and connects the CNS to limbs and organs (Byme et al., 2014). The network that forms the nervous system is composed of neurons and specialized cells named glial cells or glia, which differ between the CNS and PNS. The signalling between these cell-types and structures enables cognitive processes such as thinking, communicating, learning and memory storing, as well as all function and sensation. This allows the perception and interaction with the world, other individuals and itself, shaping one's own behaviour (Squire et al., 2008).

Due to the high structural and functional complexity of the nervous system, the developmental process that starts during the embryonic stage and prolongs itself throughout adulthood involves tightly regulated cellular and molecular mechanisms. During early embryogenesis, all the tissues and cell-types that will later be formed are originated from three germ layers, namely endoderm, mesoderm and ectoderm, which are generated during a process termed gastrulation (Mtui, E., Gruener, G., & Fitzgerald, 2011; Zuchero and Barres, 2015). Each cell layer gives rise to specific tissues – endoderm forms the digestive tube lining, mesoderm develops into muscle, bone and connective tissue, and ectoderm generates the nervous system, as well as skin and hair. Following gastrulation, the notochord – a flexible, rod-shaped structure that runs along the back of the embryo – has been formed from the mesoderm and then releases molecular signals to the overlying ectoderm. This induces its differentiation into neuroectoderm and the formation of the neural plate, which consists on a strip of neuronal stem cells (NSCs) that originate the nervous system. The neural plate then folds in upon itself, forming the neural tube, which later develops into brain and spinal cord. The PNS develops from the neural crest, located in the dorsal border of the neural tube, presenting cell migration and differentiation at a later stage (Gilbert, 2010; Kandel et al., 2013).

During the development of the spinal cord, growth factors, also known as morphogens, are released and induce the formation of motor areas ventrally and sensory areas dorsally. This differentiation occurs due to the concentration gradients formed by the morphogens, which take part in many processes during embryogenesis and throughout development until adulthood (Decimo et al., 2012). Later on, the brain is formed in a similar fashion, developing from primordial structures – prosencephalon, mesencephalon and rhombencephalon. These three areas progressively change, giving rise to telencephalon and diencephalon, which derive from the prosencephalon and become the forebrain, the mesencephalon develops into midbrain, and lastly the rhombencephalon matures into metencephalon and myelencephalon to form the hindbrain (Gilbert, 2010). Meanwhile, there is proliferation of neural stem cells (NSCs), which are self-renewing, multipotent cells that generate the neurons and glia of the nervous system. During embryonic development of vertebrates, NSCs of the central nervous system are named radial glial cells, and are localized in a transient area known as

ventricular zone (VZ). Both neurons and glial cells differentiate and migrate from this area to other places in the CNS, progressively establishing the neural circuits. The ventricular zone eventually subsides but there are other niches of NSCs that are maintained after birth, such as the subventricular zone (SVZ) and the dentate gyrus of the hippocampus, where neurogenesis still occurs throughout adulthood (Campbell and Götz, 2002; Nadarajah et al., 2003). Therefore, based on chemical signalling and molecular crosstalk, the adult brain has the capability of generating novel neurons and glial cells if needed, which conveys a certain degree of adaptability and plasticity, essential for survival (Decimo et al., 2012).

Neurons are the core components of the brain, spinal cord and peripheral nerves, being the main cell-type of the nervous system. These cells generate, process and transmit information by conducting electrical impulses over the axon and by forming connections, named synapses, between themselves and other tissues (Byme et al., 2014). Sensory neurons are responsive to external stimuli, such as touch, sound or light, and send signals to the spinal cord and brain, while motor neurons receive signalling from the CNS, causing muscle contractions and affecting glands (Kandel et al., 2013). Glial cells display different subtypes, whose roles include immunological protection, metabolic and structural support, and neuronal insulation. Glia constitutes 50-60% of the cells in the adult mouse brain and up to 90% in the adult human brain, which is an indicator that glial cells posed crucial roles for the increase of complexity in neurological function throughout evolution (Herculano-Houzel, 2014; Rowitch, 2004). This data joined with on-going investigation has shattered the previous view that glial cells were secondary in the CNS, merely forming a binding support network without any other relevant tasks associated. It is now known that the two groups of glia, namely microglia and macroglia, display important specific roles, morphologies and developmental origin (Rowitch and Kriegstein, 2010).

Oligodendrocytes, myelinating cells of the CNS

Oligodendrocytes (OLs) are glial cells that branch out numerous processes, which interact with nearby axons, enveloping them in myelin (**Fig. 1**). The myelin sheath is an insulation that results from modified plasma membrane, being primarily composed of lipids and proteins (Simons and Nave, 2015). By ensheathing the axon, this fatty structure provides support to neurons and promotes the rapid and efficient propagation of electrical impulses. These impulses are transmitted along the axon by saltatory conduction, where action potentials are regenerated at unmyelinated nodes of Ranvier rather than being propagated in a linear manner (Bauer et al., 2009). Neuronal myelination is linked with behavioural, cognitive and emotional aspects, therefore the efficiency of this process is crucial for the normal performance of the mature human brain.

One of the most striking features about the myelinating process is that it unfolds mainly at a later stage of neuronal development, over a prolonged time frame. In humans, it progresses during the first two decades of life, while in mice it takes place over the first two postnatal months (Lebel et al., 2008; Mitew et al., 2014). However, myelination also occurs throughout life, either to substitute lost OLs and myelin or to myelinate axons that were previously unmyelinated (Bartzokis et al., 2012). There is also evidence of experience-driven plasticity in the myelination process, which includes learning and cognitive stimulation. In fact, extensive long training of motor activities, such as playing a musical instrument or juggling, correlates with an increase in white matter volume in areas of the brain that are active during the performance of these tasks (Bengtsson et al., 2005; Scholz et al., 2009). Thus, the

amount of neuronal activity is another important factor in the modulation of myelin production in the CNS (Ishibashi et al., 2006).

A single OL can myelinate up to 60 different axons, depending on the specific tract and axonal diameter, and the production of adjacent myelin segments in the axon is performed by distinct OLs (Baumann and Pham-Dinh, 2001; Miller, 2002). In contrast, the myelinating cells in the PNS, known as Schwann cells, display a different morphology, consisting of a single myelin segment. They are organized in tandem along the axon and form nodes of Ranvier between them, enabling saltatory conduction of electrical impulses (Baumann and Pham-Dinh, 2001). Most OLs are located in white matter, hence the high levels of myelination in these areas of the CNS. On the contrary, grey matter consists of numerous neuronal cell bodies and fewer axons, myelinated to a lesser degree (Ohtani et al., 2014). Since myelin constitutes up to 50% of the human adult brain volume, injuries or diseases that affect its structure or production, such as multiple sclerosis, can cause loss of neurological function and the life span of the individual might be dramatically affected (Miller, 2002).

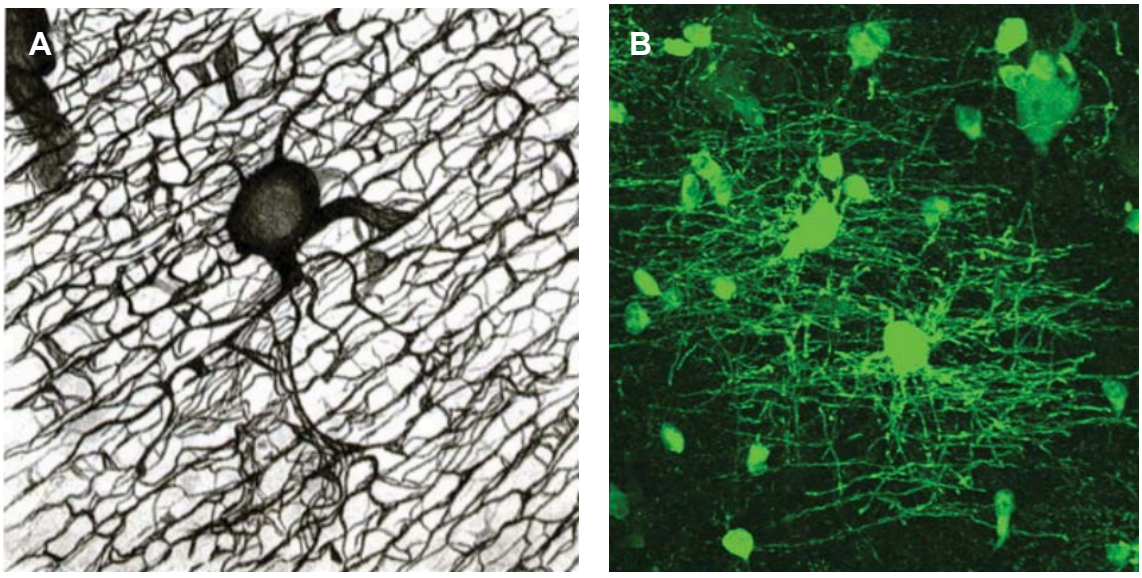

Figure 1. Morphology of oligodendrocyte cells. A) Oligodendrocytes project cellular processes and branches that follow the orientation of nerve fibres, forming complex wraps. B) Fluorescent staining of oligodendrocytes expressing proteolipid protein (PLP) tagged to green fluorescent protein (GFP). (Adapted from Simons and Nave, 2015).

Origin and proliferation of oligodendrocyte progenitor cells

Oligodendrocytes are generated by oligodendrocyte progenitor cells (OPCs), which constitute 5-8% of the total number of cells in the human adult CNS (Richardson et al., 2011). In rodents, the OL developmental program starts with the specification of OPCs derived from neural stem and progenitor cells during late embryonic gestation. OPCs are highly migratory and proliferative, as identified by the expression of specific molecular markers NG2 proteoglycan and platelet-derived growth factor receptor alpha (Pdgfra) (Baumann and Pham-Dinh, 2001; Bercury and Macklin, 2015). Throughout development and during remyelination events, the oligodendrocyte lineage progressively differentiates in a tightly regulated manner. These processes are controlled by a multitude of intrinsic and extrinsic molecular cues, which orchestrate myelination both spatially and temporarily. These signals are mainly growth factors, kinases and extracellular matrix molecules, all of which influence epigenetic

modifications, transcriptional and translational regulation, and cytoskeleton dynamics in OPCs and OLs (**Fig. 2)** (Bercury and Macklin, 2015; Miller, 2002).

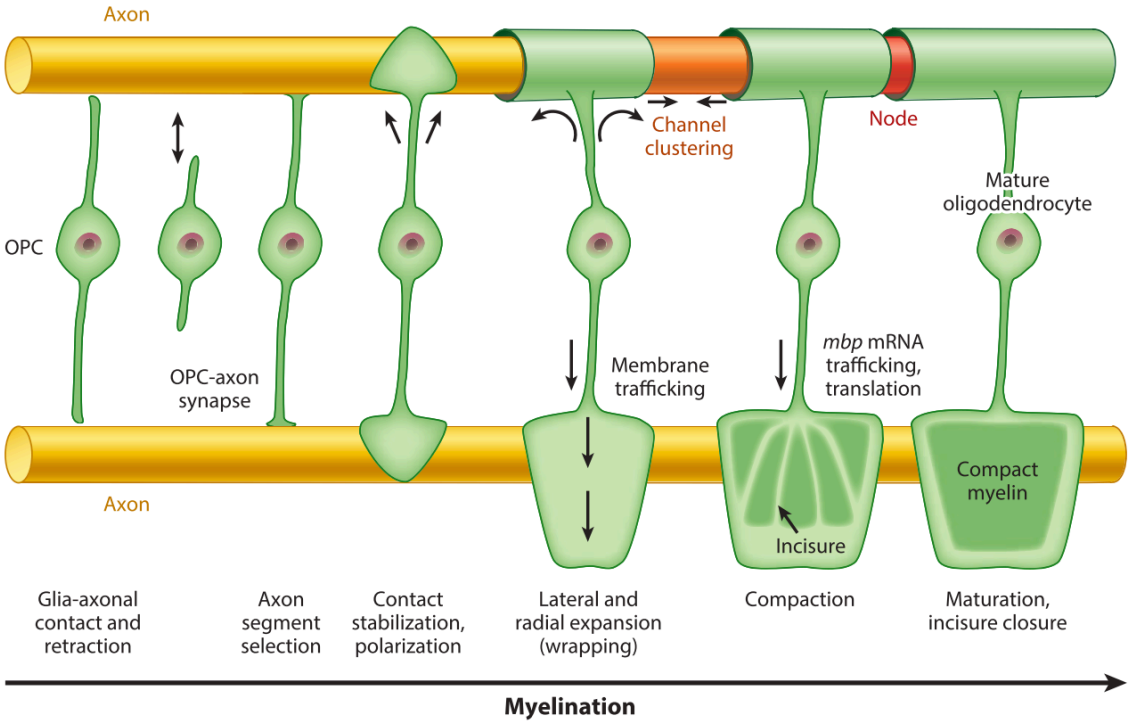

Figure 2. Model of the differentiation of a committed oligodendrocyte progenitor cell (OPC) into a mature myelinating oligodendrocyte. Until initial contacts are stabilized, OPCs extend and retract cellular processes. In the lower half of the figure, the myelin sheath is represented with an unrolled structure. Maturation of the nodes of Ranvier requires the glia-dependent clustering of axonal voltage-gated ion channels, depicted in the upper half of the figure. (Adapted from Nave et. al, 2014)

The developmental origin of OPCs occurs in sequential proliferation waves from different ventral and dorsal domains of the developing brain and spinal cord, influenced by several cellular factors (Richardson et al., 2006) (**Fig. 3**). In mice, the first wave of OPC production takes place around embryonic day (E) 13.5 in the progenitor niche of the ventral neuroepithelium of the developing spinal cord (Lu et al., 2000) and in the medial ganglionic eminence of the ventral ventricular zone of the telencephalon (Tekki-Kessaris et al., 2001). This is followed by a second wave of OPC genesis at E15.5, from the dorsal region of the developing spinal cord (Cai et al., 2005) and from the lateral and caudal ganglionic eminences of the ventral VZ of the telencephalon (Kessaris et al., 2006). A third wave takes place after birth, however its specific origins – whether from progenitor cells around the central canal or from proliferative OPCs – remain unclear in the SC (Rowitch and Kriegstein, 2010) and are originated mainly from the subventricular zone, in the brain (Aguirre et al., 2007). During the postnatal development of the brain, OPCs migrate from the SVZ to other regions, where they stop dividing and differentiate into mature myelinating OLs (Menn et al., 2006).

Fate-mapping experiments show that, in the spinal cord, OPCs have fundamentally ventral origins, with ventrally derived cells accounting for 85-90% of adult OLs. In contrast, the first wave of proliferation in the brain is essentially replaced by more dorsally generated precursors at a later stage of the developmental process. Interestingly, genetic ablation of precursors of both ventral and dorsal

origin results in the compensatory expansion of the opposing population, conveying a regular final number of OLs and myelin, showing a large degree of redundancy (Kessaris et al., 2006; Richardson et al., 2006). Additionally, OPCs are capable of communicating with neurons through action potentials (Maldonado et al., 2013; Tomassy and Fossati, 2014), showing functionality, and both ventrally- and dorsally-generated precursors present similar electrophysiological properties (Tripathi et al., 2011).

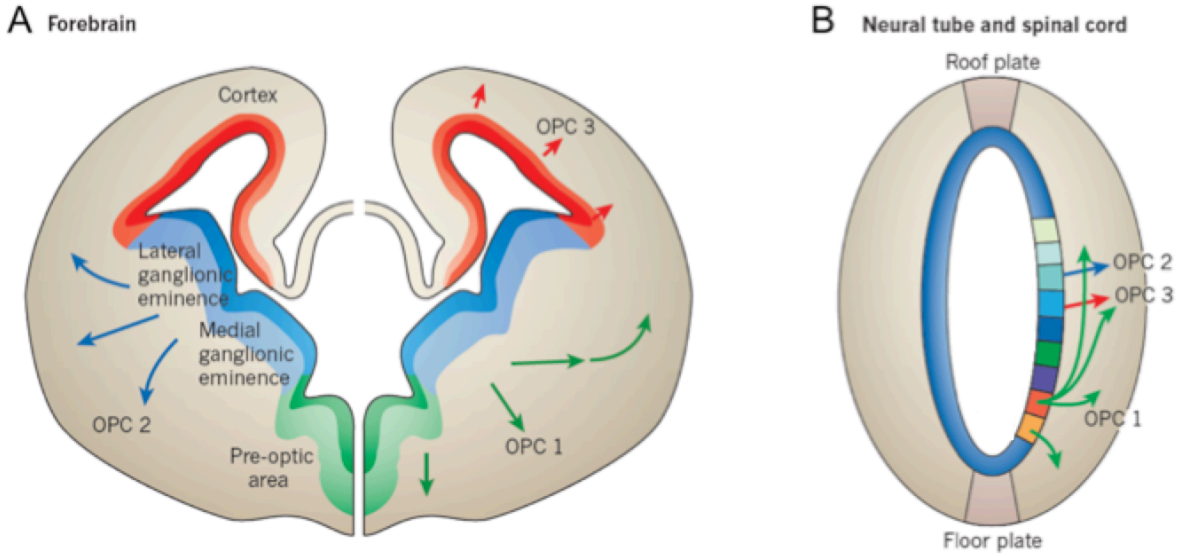

Figure 3. Multiple waves of OPC proliferation during the development of the mammalian CNS. A) Three sequential waves of OPCs (OPC 1, OPC 2 and OPC 3) arise from specific domains in the forebrain ventricular zone. OPC 1 arises at E13.5, OPC 2 starts at E15.5 and OPC 3 begins at birth. B) Two distinct waves of OPCs emanate from the ventral region and dorsal region of the spinal cord (OPC1 – E13.5; OPC 2 – E15.5), while the origins of the third wave remain unclear (OPC3 – starts at birth). (Adapted from Rowitch and Kriegstein, 2010).

Remyelination during multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune inflammatory neurological disease of the CNS that attacks neurons and destroys myelin to various degrees (Lassmann et al., 2001). Even though the cause is unknown, it appears to involve a combination of genetic susceptibility and non-genetic triggers, such as viral infections, metabolic disorders or environmental factors. The symptoms include muscle weakness, changes in coordination and sensibility, blurred vision, speech difficulties, fatigue, among others (Compston and Coles, 2008). Its onset usually happens in adults between 20 and 45 years of age, and occasionally during childhood or late middle age. At the moment, there is not a cure for MS and the available treatments are based on the improvement of function after an attack and preventing new occurrences (Chari, 2007; Goldenberg, 2012).

Besides inflammation and loss of myelin, MS lesions also present different levels of astrogliosis, phagocytic activity, oligodendroglial loss and axonal pathology (Lassmann et al., 2001). However, in most patients, MS initially presents stages of reversible neurological deficits, when symptoms improve or disappear, corresponding to a local response from OPCs and remyelination to a certain degree. Eventually, as disease episodes recur over time, remyelination occurs with increasingly lower efficiency and eventually fails in the majority of the cases. This pattern is known as relapsing-remitting MS, a sub-type of the disease that affects about 85% of patients (Barnett and Prineas, 2004; Bjartmar and Trapp, 2001).

Remyelination usually follows a pathological loss of myelin, in order to restore the properties of neurons by producing new myelin sheaths around damaged axons of the CNS. This is a complex process that involves interdependent cellular and molecular factors in its regulation and occurs in two major phases. Affected areas are colonized by OPCs, which then differentiate into myelinating OLs that contact impaired axons and produce novel functional myelin sheaths (Chari, 2007). It has been previously shown that transplantation of OPCs in the CNS results in oligodendrogenesis and consequential axonal remyelination, while transplanted mature OLs fail to remyelinate the axons in the new surrounding area (Hans S Keirstead, 1999). This demonstrates that OPCs are the cell population that should be targeted in order to achieve remyelination, presenting a novel therapy for demyelinating diseases.

Oligodendrocytes display heterogeneity

It is well established that OPCs and OLs are extremely important in order to sustain a proper axonal myelination. Even though the OL population in the CNS is thought to be highly homogeneous in function (Kessaris et al., 2006), these cells were firstly described as morphologically heterogeneous (del Río-Hortega, 1928). Indeed, OPCs are capable of generating OLs with distinct innate heterogeneous myelination potential, as OLs in the spinal cord produce thicker myelin sheets and longer internode segments compared to cortical OLs (Bechler et al., 2015). Furthermore, while white matter OPCs differentiate into myelinating OLs when transplanted both in the white or grey matters, grey matter OPCs do not generate as many myelinating OLs, even when transplanted into white matter. Therefore, it is not clear whether OLs become morphologically diversified throughout maturation from interactions within the local environment or whether there is an intrinsic functional heterogeneity (Bechler et al., 2015; Tomassy and Fossati, 2014).

Thus, while cells are proliferating they undergo continuous morphological and functional changes, which are orchestrated by an intricate programming of gene expression and a myriad of internal and external signalling molecules. Even though this biological dynamism makes defining a cell-type a delicate task, the host laboratory tackled this issue by using single-cell RNA-seq. This enabled the acquisition of the transcriptional background of thousands of individual cells and identification of existing RNA molecules. Some of these molecules are only present in a given number of cells, which were analysed and categorized as a certain subpopulation (Trapnell, 2015; Zeisel et al., 2015).

Bulk and single-cell RNA-seq analysis of the host laboratory suggest that OPCs and OLs present a high degree of heterogeneity at transcriptional level: the gene expression profile of OPCs varies dramatically during development between and within different parts of the CNS. Moreover, cells of the oligodendrocyte lineage present distinct transcriptional profiles within the same brain region (Marques et al., 2016; Zeisel et al., 2015). The host group analyzed 5072 transcriptomes of single cells expressing molecular markers from the OL lineage, which were isolated from 10 specific regions of the mouse juvenile and adult CNS, identifying 13 distinct cell populations (**Fig. 4**). Moreover, *t*-distributed stochastic neighbour embedding (t-SNE), supported by pseudotime analysis, presented a narrow differentiation path, establishing a connection between OPCs and myelin-forming oligodendrocytes, which then diversify into six mature states (**Fig. 5)** (Marques et al., 2016).

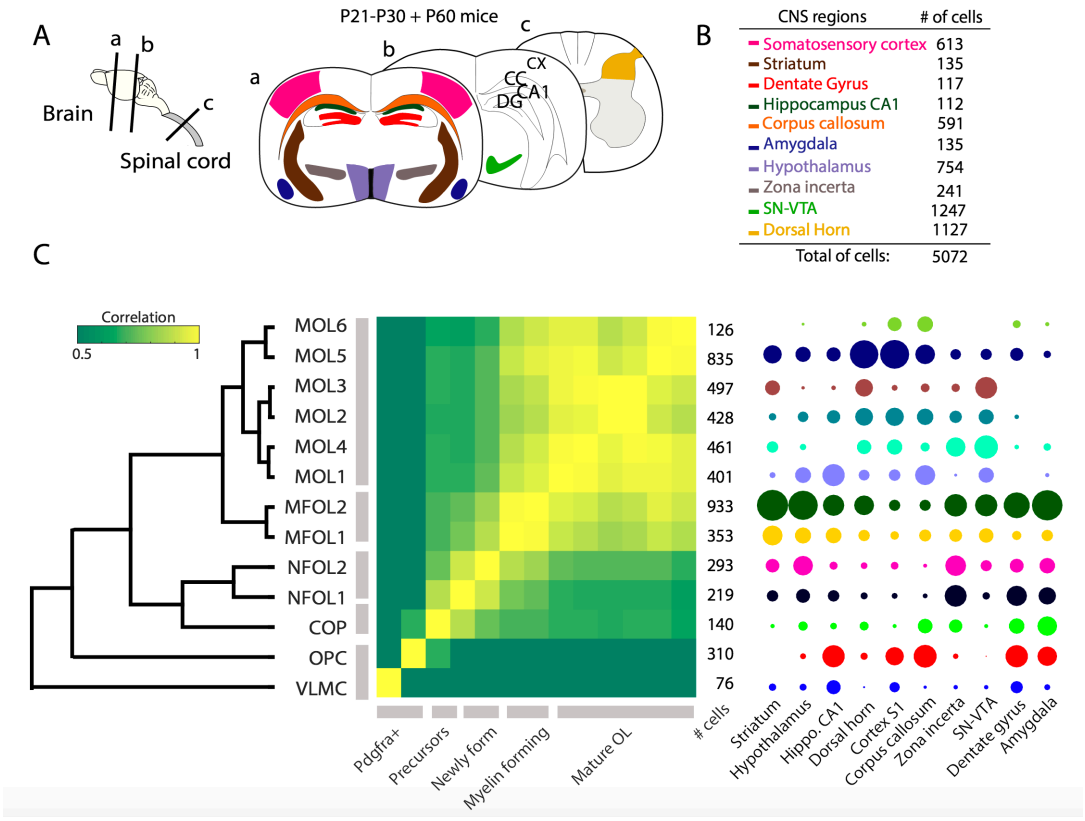

Figure 4. Single cell RNA-Seq reveals 12 subpopulations of OLs and a VLMC population with markers of the oligodendrocyte lineage in ten regions of the mouse CNS. A) Illustration of dissection scheme for the ten CNS regions analysed by single cell RNA-Seq. B) Table enumerating the number of cells analysed (after exclusion) for each CNS region. C) Dendrogram of OL populations as reconstructed by linkage clustering (left panel). Heatmap described the correlation between each pair of clusters (middle panel). Abundance of each of the subclasses along the different CNS regions is represented by the area of the circles (right panel). (Adapted from Marques et al., 2016).

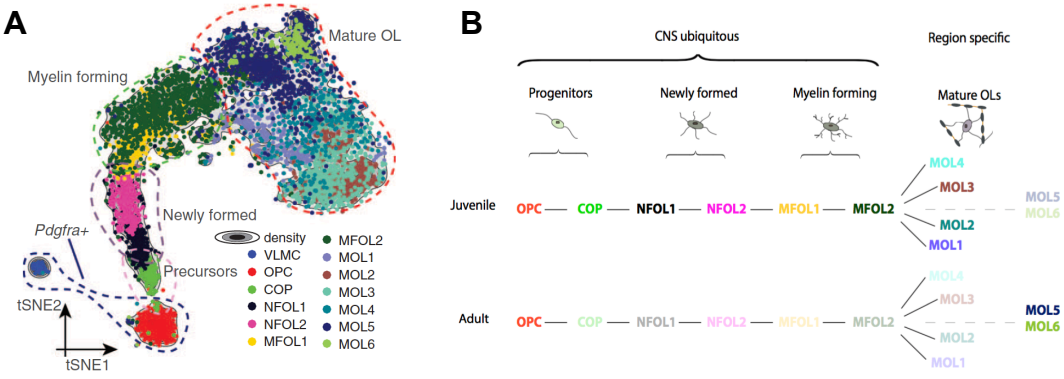

Figure 5. Model of oligodendrocyte lineage progression from precursors to mature cells. A) t-Distributed stochastic neighbor embedding projection displaying the trajectory from OPCs to mature OLs. B) Oligodendrocyte maturation process is sequential, continuous and uniform along CNS until the myelin-forming stage, while mature populations show heterogeneity and region specificity. MOL1-4 are more common in the juvenile mouse, while MOL5/6 can be found mostly in the adult mouse. (Adapted from Marques et al., 2016).

The oligodendrocyte lineage arises from OPCs, which express the cell-marker Pdgfra. Differentiation-committed oligodendrocyte precursors (COPs) are distinct from OPCs because they lack the expression of Pdgfra, as all subpopulations from here on, and express genes involved in maintaining an OL undifferentiated state. COPs also express migratory genes and low levels of cell cycle markers. Newly formed oligodendrocytes (NFOL1 and 2) express genes that induce differentiation, and are followed by myelin-forming oligodendrocytes (MFOL1 and 2), which express genes responsible for myelin formation. Finally, mature oligodendrocytes (MOL1-6) express late oligodendrocyte differentiation genes and genes present in myelinating cells. A second Pdgfra^{+} population was identified, namely vascular and leptomeningeal cells (VLMCs), which are distinct from OPCs and the rest of OL lineage. Moreover, VLMCs exhibit markers from the pericyte lineage and are present in blood vessels and meninges. The existence of these distinctive OPC and OL states suggests possible functional heterogeneity (Marques et al., 2016).

Exploring oligodendrocyte lineage heterogeneity and developmental origin

The integration of bulk transcriptomics and single-cell RNA-seq data provide rigorous data sets describing the transcriptional OPC and OL heterogeneity from different areas of the CNS and at different developmental stages. From these data sets, the host lab short-listed genes that are potentially important in defining the functional heterogeneity of OPCs in different areas of the CNS. Seven of the short-listed genes are transcription factors or regulators, involved in cell fate specification, differentiation, migration, or maintenance of the progenitor state, but that have not been associated with the regulation of OPC cell state (Marques et al., 2016).

To assess the specificity and validate these markers in brain and spinal cord sections, antibodies and RNA probes were tested through immunohistochemistry (IHC) and RNAscope *in situ* hybridization (ISH), respectively. Genes specifically expressed by the identified 12 OL subpopulations were selected and antibodies raised against their encoded proteins were acquired. Those antibodies were tested through IHC firstly on non-experimental mice for protocol optimization, followed by the application of those protocols on experimental (PdgfraCreER-RCE) juvenile (P20 – 20 days old) and adult mice (P60 – 60 days old). Additionally, the detection of co-expression of the mRNAs and proteins labeled by the antibodies was intended, as indeed the putative cell markers were selected based on mRNA data. However, mRNAs and proteins can have differential temporal patterns, an issue tackled by combining and assessing fluorescent ISH with IHC on brain and spinal cord sections from juvenile and adult experimental mice.

Moreover, it was hypothesized that cortical and spinal cord OPCs have a distinct differentiation potential, as the percentage of the 12 OL subpopulations is differentially represented in these two regions of the CNS. Also, as the proliferation waves of OPCs occur in three different time points, the developmental origin of the distinctive OL subpopulations was also investigated both in the brain and spinal cord. This subject was addressed by conducting lineage-tracing experiments using the tamoxifen-inducible PdgfraCreER-RCE mouse line, selectively expressing GFP (green fluorescent protein) reporter in OPCs and their progeny. Recombination of the reporter gene was induced either on E12.5 (embryonic day E12.5) or P3-5 (postnatal days 3-5), in order to respectively target the first embryonic and postnatal waves of OPC proliferation and their progeny. Subsequently, brain and spinal cord were collected at different ages (P20 and P60), and IHC and ISH assays were performed to test the putative markers for different subpopulations of OL lineage.

Previously unpublished data together with published reports support the hypothesis that the oligodendrocyte lineage might display functional heterogeneity and distinct subpopulations might be differentially originated from developmental waves. The present work focuses on these issues and, therefore, we aimed at:

1. Validation of antibodies and RNA probes for detection of putative cell markers through immunohistochemistry and fluorescent *in situ* hybridization (RNAscope);

2. Determination of the developmental origin of distinct oligodendrocyte subpopulations in the juvenile and adult mice

Materials and methods

Animals

All experimental procedures performed followed the guidelines and recommendations of local animal protection legislation and were approved by the local committee for ethical experiments on laboratory animals (Stockholms Norra Djurförsöksetiska, Sweden). Tamoxifen inducible Pdgfra-CreER/RCE mice with a C57BL/6 genetic background are a strain expressing Cre recombinase under the control of a Pdgfra genomic promoter. Moreover, this strain has a loxP-flanked STOP cassette and a downstream GFP reporter gene, both under the control of a ubiquitous ROSA26 *locus*. When tamoxifen is administered it activates Cre recombinase, which will recognize both loxP sites and excise the STOP sequence, allowing the transcription of the GFP gene in Pdgfra+ cells. This Cre-Lox system enables the labelling of OPCs and their progeny in recombinant mice. In order to obtain this mouse line within one generation, B6N.Cg-Tg (Pdgfra-CreERT)467Dbe/J (Jackson Laboratories, CA, USA), also referred to as Pdgfra-CreER mice, were crossed with the reporter mice line Gt(ROSA)26Sor$^{tm1.1(CAG-EGFP)Fsh}$/Mmjax (Jackson Laboratories, CA, USA), also referred to as RCE:loxP-GFP. Pdgfra-CreER (non-inducible Cre) mice were also used for optimization of immunohistochemistry and fluorescent *in situ* hybridization protocols.

Genotyping

Experimental mice carrying both Pdgfra-CreER and RCE:loxP-GFP constructs were identified visually by GFP fluorescence under a UV light source between birth (P0) and the third postnatal day (P3). The full genotypes were confirmed by PCR using the following primers: Pdgfra-CreER (forward – 5' TCA GCC TTA AGC TGG GAC AT 3'; reverse – 5' ATG TTT AGC TGG CCC AAA TG 3'), RCE (mutant – 5' CCA GGC GGG CCA TTT ACC GTA AG 3'; common – 5' AAA GTC GCT CTG AGT TGT TAT 3'; wild type – 5' GGA GCG GGA GAA ATG GAT ATG 3'), GFP (mutant forward – 5' GAT CAC TCA CGG CAT GGA C 3'; mutant reverse – 5' CGG GCC TCT TCG CTA TTA C 3'; wild type forward – 5' AAG GGA GCT GCA GTG GAG TA 3'; wild type reverse – 5' GAG CGG GAG AAA TGG ATA TG 3'). PCR protocols were performed according to Jackson Laboratories instructions (**Table 1**).

PCR mix (volumes for 1 reaction):

1) 12,5 µl Dream Taq Mastermix (2x, Thermo Scientific)
2) 0,6 µl primer forward (from 10 µM stock, 0,25 µM final concentration)
3) 0,6 µl primer reverse (from 10 µM stock, 0,25 µM final concentration)
4) H_2O (total volume of 24 µl)
5) 0,5-1 µl DNA template

Step	Temperature	Time
Denaturation	94 °C	1 min
Amplification (35 cycles)	94 °C	45 sec
	58 °C	45 sec
	72 °C	1 min
Elongation	72 °C	5 min
Hold	10 °C	

Table 1. PCR conditions for genotyping

Lineage tracing

For Cre-Lox recombination at E12.5, experimental female mice were given 1mg tamoxifen (10mg/ml in corn oil) intraperitoneal injections at pregnancy day 12.5, GFP-labelling the first wave of OPC proliferation and their progeny. For Cre-Lox recombination at P3-5, experimental progenitor female mice were given daily 2mg tamoxifen (20mg/ml in corn oil) intraperitoneal injections after the litter was delivered, from day 3 to 5, GFP-labelling the third wave of OPC proliferation and their progeny.

Tissue preparation

After inducing recombination, experimental mice at post-natal day 20 or 60 were anesthetized with a ketamine/xylazine mixture (up to 80 mg/kg body weight ketamine and 10 mg/kg body weight xylazine) via intraperitoneal injection. Animals were tested for unresponsiveness through toe-pinch method. They were then perfused intracardially with PBS followed by PFA (paraformaldehyde) 4%. Brain and spinal cord were dissected and transferred to 4% PFA and incubated overnight at 4ºC. The tissue was then rinsed with PBS and maintained in 30% sucrose/ 0.1M PBS overnight at 4ºC, it was then stored at -80ºC. For cryosectioning, experimental tissue was embedded in optimal cutting temperature (OCT) compound, placed in a cryostat, coronal sections of 14-16µm thickness were collected on glass slides and stored at -80ºC.

Immunostaining

Oligodendrocyte subpopulations' developmental origin and distribution were analysed by using specific antibodies for putative cell markers (**Table 2**). This task was performed through two distinct immunohistochemistry protocols.

Standard protocol

Tissue sections were thawed for 15 min, washed in PBS-0.05% Tween-20 (PBS-T) for 10 min, treated with blocking solution (5-10% donkey serum, 0.3% Triton X-100 in PBS-T) for 1 h and then incubated overnight with primary antibody diluted in 2-3% donkey serum solution. Tissue sections were then washed three times in PBS-T (10 min per wash), treated with secondary antibody Alexa Fluor-conjugated diluted in 2-3% donkey serum solution for 1 h, washed again three times in PBS-T (10 min per wash), incubated with DAPI (nuclear counterstain) for 1-2 min and washed once again for 10 min in PBS-T. Slides were coverslipped in mounting medium (Dako) and stored in a cold room (~4ºC) until further microscopic analysis. All steps were performed at room temperature (20-25ºC).

Streptavidin enhancement protocol

Tissue sections were thawed for 15 min, washed in PBS-0.05% Tween-20 (PBS-T) for 10 min, treated with blocking solution (5-10% donkey serum, 0.3% Triton X-100 in PBS-T) for 1 h and then incubated overnight with primary antibody diluted in 2-3% donkey serum solution. Tissue sections were then washed three times in PBS-T (10 min per wash), treated with biotinylated secondary antibody diluted in 2-3% donkey serum solution for 1 h, washed again three times in PBS-T (10 min per wash), incubated with streptavidin Alexa Fluor-conjugated diluted in 2-3% donkey serum solution for 1 h, washed again three times in PBS-T (10 min per wash), incubated with DAPI (nuclear counterstain) for 1-2 min and washed once again for 10 min in PBS-T. Slides were coverslipped in mounting medium (Dako) and stored in a cold room (~4ºC) until further microscopic analysis. All steps were performed at room temperature (20-25ºC).

Antibody	Host	Dilution for IHC	Source
GFP (Green fluorescent protein)	Chicken	1:500 (IHC and ISH)	Abcam
Egr2 (Early growth response 2)	Rabbit	1:200	Abcam
FosB (FBJ osteosarcoma oncogene B)	Rabbit	1:500	Abcam
HOPX (Homeodomain only protein)	Rabbit	1:100	Abcam
Ptgds (Prostaglandin D2 synthase)	Rabbit	1:50	Abcam
Alexa Fluor 488-conjugated antibody to chicken IgG	Donkey	1:500 (IHC and ISH)	Invitrogen
Alexa Fluor 568-conjugated antibody to rabbit IgG	Donkey	1:500	Invitrogen
Alexa Fluor 647-conjugated antibody to rabbit IgG	Donkey	1:500	Invitrogen
Byotinilated antibody to rabbit IgG	Donkey	1:500	Invitrogen
Streptavidin Alexa Fluor 568-conjugated	Donkey	1:500	Invitrogen

Table 2. Antibody sources and concentrations

Immunostaining coupled with RNAscope

RNAscope fluorescent *in situ* hybridization (ISH) assay was coupled with immunohistochemistry for identification of single RNA transcripts and target proteins. Tissue sections were thawed for 15 min, placed in a hot plate (100ºC) while 1x target retrieval at 98-100ºC was continuously added for 5 min. Sections were transferred to a staining dish containing distilled water (RNAse free) and washed twice for 2 min, followed by a 2 min wash of EtOH 100%. Protease IV was applied to the sections for 20 min at room temperature (20-25ºC), which were posteriorly transferred to a dish containing distilled water (RNAse free) and washed twice for 2 min. RNAscope Multiplex Fluorescent Assay v2 (Advanced Cell Diagnostic) assay followed, consisting on cycles of hybridization of the target probe, signal amplification and HRP (horse radish peroxidase) signal development, according to the manufacturer's protocol instructions. Standard immunohistochemistry protocol was then performed.

The probes used in these studies were against Egr2, HOPX, Ptgds, Ptprz1 (Protein tyrosine phosphatase receptor type Z) and Sox10 (stock concentrations from kit). The primary antibody used in these studies was against GFP. Secondary antibody was Alexa Fluor 488-conjugated donkey antibody to chicken IgG.

Image acquisition and analysis

Representative images with a combination of DAPI, GFP (Alexa 488), Alexa 555 and/or Alexa 647 staining were obtained in a Zeiss LSM800 Confocal with ZEN software interface. The regions of interest in the brain were the somatosensory cortex and corpus callosum, while in the spinal cord were the dorsal horn and corticospinal tract. The tissue was photographed by performing a Z-stack of 5-14 stacks per image, representing 3-7 µm of tissue depth, with a 40x objective.

Images for quantification were photographed by performing a Z-stack of 2-4 stacks per image, representing 1.5-3 µm of tissue depth, with a 20x objective. A tiling method was also performed, with the number of acquired tiles depending on the region of interest (corpus callosum – 3 tiles; cortex, dorsal horn and corticospinal tract – 4 tiles each). For each time-point (P20 or P60) 3 animals were used and 3 tissue sections were photographed per animal. The cell counting was normalized to the area analysed in each photo (CellProfiler).

Results

Validation of antibodies against cell markers for oligodendrocyte lineage in the juvenile mouse

For the detection of mature oligodendrocytes 1 (MOL1) subpopulation in the brain and spinal cord of the mouse, we assessed an antibody against Egr2. We targeted the postnatal wave of OPCs ($Pdgfra^+$) and their progeny by conducting lineage-tracing experiments and identifying these cells as GFP^+. To achieve this, experimental mice (PdgfraCreER-RCE) were recombined on postnatal day 3 to 5 (P3-5) and tissue was collected on postnatal day 20 (P20), followed by a standard immunohistochemistry protocol. The antibody used for the detection of GFP is reliable, consistently displaying a strong and clear signal localized within the cells throughout all the experiments presented in this work. Contrarily, Egr2 expression pattern lacks specificity, displaying a broad background signal without overlap between GFP^+ cells in the cortex, corpus callosum and corticospinal tract. Nevertheless, it is possible to detect the outline of some $Egr2^+$ cells in the dorsal horn appearing to colocalize with GFP^+ cells, although given the unspecific signal in the remaining analysed areas it is not possible to be completely certain (**Supplementary fig. 1**). Negative controls, with the omission of the primary antibody, were performed and assessed visually throughout all IHC experiments in this work (images not shown). Thus, a negative control for Egr2 was analysed, in which unspecific background fluorescence was absent (images not shown). Additionally, we performed a staining in the same areas of mice recombined on P3-5 and collected the tissue on P60, and Egr2 fluorescent signal was absent both in the brain and spinal cord (images not shown). As potential MOL1 were detected in the dorsal horn, the protocol for this antibody requires further optimization, in order to improve the fluorescent signal in the remaining areas.

Since the detection of MOL1 could not be fully accomplished by using the antibody against Egr2, we targeted FosB, another marker for this OL subpopulation. This marker is also present in vascular and leptomeningeal cells (VLMC), as indicated by single-cell RNA-seq, and consequently the antibody against FosB detects both subpopulations. VLMCs are described as a secondary $Pdgfra^+$ subpopulation, alongside OPCs, being also labelled by the GFP antibody. To test this antibody we recombined experimental mice on P3-5, targeting the postnatal wave of OPC and progeny (GFP^+ cells), and collected brain and spinal cord on P20, followed by a standard immunohistochemistry procedure. $FosB^+$ cells present a clear staining, with the signal distributed within the cell nuclei, in the cortex, dorsal horn and corticospinal tract (**Supplementary fig. 2**). Contrarily, the signal of $FosB^+$ cells in the corpus callosum was non-existent. The negative control did not show any fluorescent signal. Double positive FosB/GFP cells presence is displayed in the cortex and dorsal horn while it is absent in the corticospinal tract, where a smaller number of $FosB^+$ cells was detected. Therefore, a number of cells generated from the postnatal wave of proliferation are present in the juvenile mouse as $FosB^+$ cells, requiring further identification as either MOL1 or VLMC.

In order to detect mature oligodendrocytes 2 (MOL2) subpopulation in the CNS of the mouse, we tested an antibody against HOPX. In this lineage tracing experiment we recombined experimental mice on P3-5, in order to target the postnatal wave of OPC and their progeny (GFP^+ cells), collected tissue on P20 and performed a standard immunohistochemistry protocol. $HOPX^+$ cells display a strong signal, which seems to be present mostly around the nuclei. The staining puts the cells in evidence even though there is a certain degree of background fluorescent signal, which was not detected in the negative control (images not shown). Moreover, there is colocalization between GFP^+ cells and $HOPX^+$ cells, both in the brain and spinal cord (**Fig. 6**). This shows that a number of MOL2 are originated from OPCs that proliferate from the postnatal wave and are present in the juvenile mouse.

We performed an additional staining in mice recombined on P3-5, whose tissue was collected on P60, and we did not detect any HOPX$^+$ cells (images not shown). Thus, based on its relatively clear and strong signal and overlap with GFP$^+$ cells, HOPX is a potentially reliable antibody for the identification and lineage tracing of MOL2 in the juvenile mouse.

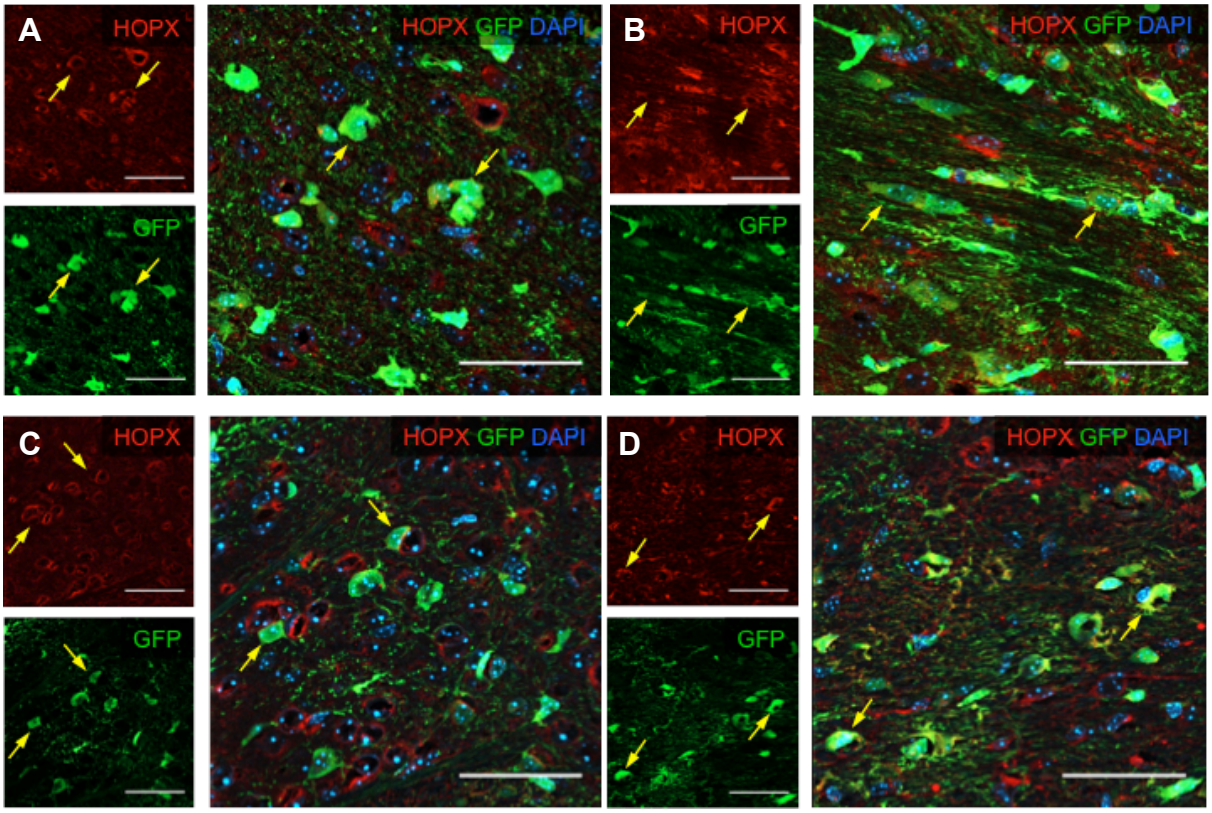

Figure 6. HOPX$^+$ cells colocalize with GFP$^+$ cells originated from the postnatal developmental wave in the juvenile mouse. Immunohistochemistry targeting MOL2 (HOPX) and Pdgfra$^+$ cells and progeny (GFP) in cortex (A), corpus callosum (B), dorsal horn (C) and corticospinal tract (D). Experimental mice were recombined on P3-5 and tissue was collected on P20. Yellow arrows indicate signal colocalization between HOPX$^+$ cells and GFP$^+$ cells. Scale bars represent 50μm.

Validation of RNA probes against cell markers for oligodendrocyte lineage in the juvenile mouse

Following these results, we performed a fluorescent staining *in situ* hybridization coupled with immunohistochemistry, labelling single RNA molecules and proteins, respectively. Here we used an Egr2 RNA probe to identify MOL1, and the regular GFP antibody, thereby assessing the colocalization of signal within cells. Negative controls for probes were performed, according to instructions of the manufacturer, and assessed visually throughout all ISH coupled with IHC experiments in this work (images not shown). In order to target the postnatal developmental wave, experimental mice were recombined on P3-5 and the tissue was collected on P20. Egr2 probe displays a scattered fluorescent signal and the RNA molecules are localized in the cell body and cellular processes. There is colocalization between GFP$^+$ cells and Egr2 RNA in all analysed areas (**Supplementary fig. 3**), though quantification is challenging since the signal of the probe is too dispersed. Nevertheless, this experiment shows that a number of MOL1 are originated from the postnatal wave of OPC proliferation and are present in the analysed areas of the CNS in the juvenile mouse. Moreover, we performed a similar experiment targeting MOL2 by using a RNA probe for HOPX, aiming to establish a comparison with the expression pattern of the HOPX antibody. However,

while the probe was effective and displayed a clear signal, GFP labelling presented some issues, most likely due to deficient blocking, displaying a fluorescence pattern that did not allow the proper identification of OPCs and progeny (images not shown).

Validation of antibodies against cell markers for oligodendrocyte lineage in the adult mouse

For the detection of mature oligodendrocytes 5 and 6 (MOL5/6) in the adult mouse we assessed an antibody against Ptgds. Experimental mice were recombined on P3-5 for lineage tracing, targeting the postnatal wave of OPCs and their progeny and the tissue was collected on P60. Here we firstly used a standard immunohistochemistry protocol, which proved to be non-efficient, since the Ptgds antibody signal was too faint (images not shown). Consequently, we enhanced the fluorescent signal of the antibody by using a streptavidin system, which gave a considerably clearer image of the expression pattern. Ptgds$^+$ cells display a strong signal, distributed within the cell body and cellular processes, as well as a degree of colocalization with GFP$^+$ cells in the analysed areas of the brain and spinal cord (**Fig. 7**). This demonstrates that a number of MOL5/6 are originated from the postnatal wave of proliferation, being present in the cortex, corpus callosum, dorsal horn and corticospinal tract in the adult mouse.

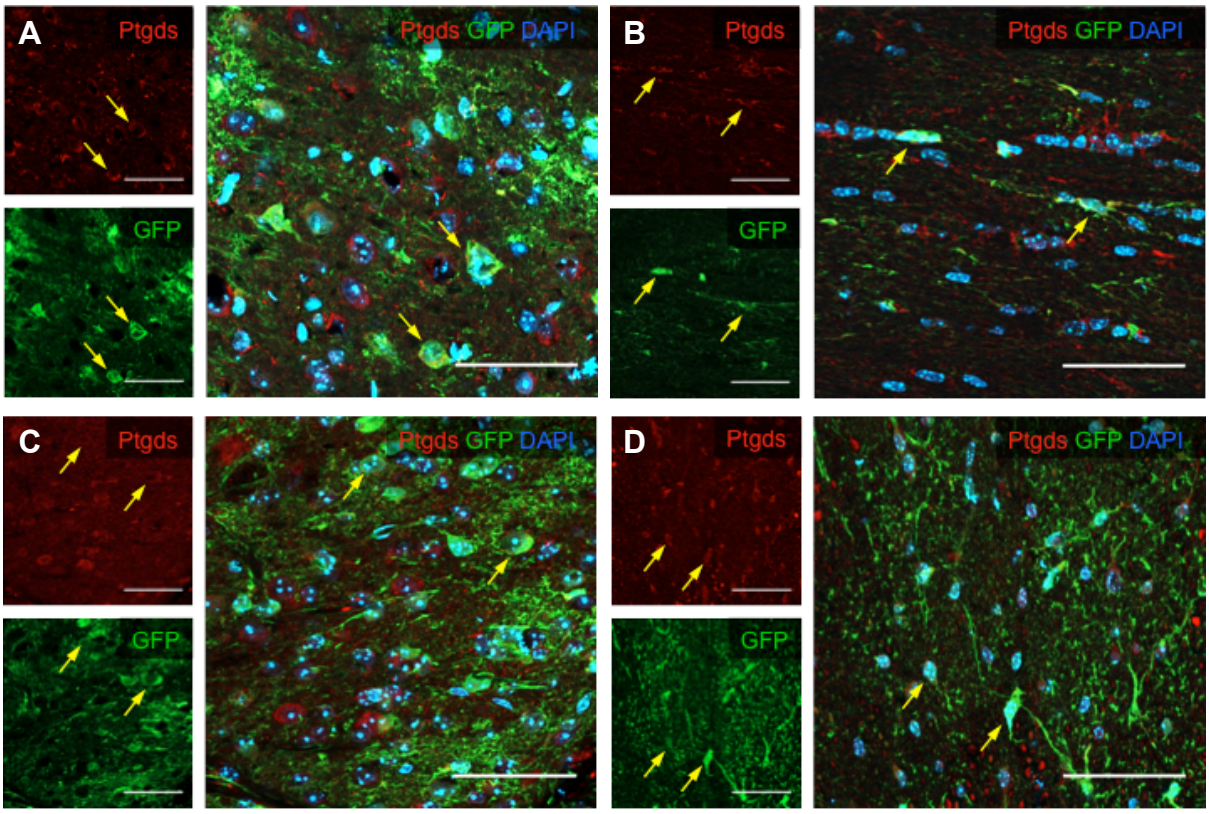

Figure 7. Ptgds$^+$ cells colocalize with GFP$^+$ cells originated from the postnatal developmental wave in the adult mouse. Immunohistochemistry targeting MOL5 and 6 (Ptgds) and Pdgfra$^+$ cells and progeny (GFP) in cortex (A), corpus callosum (B), dorsal horn (C) and corticospinal tract (D). Experimental mice were recombined on P3-5 and tissue was collected on P60. Yellow arrows indicate signal colocalization between Ptgds$^+$ cells and GFP$^+$ cells. Scale bars represent 50µm.

Validation of RNA probes against cell markers for oligodendrocyte lineage in the adult mouse

Following these results, we performed fluorescent staining coupling *in situ* hybridization and immunohistochemistry, labelling RNA molecules and proteins, respectively. To do this we used a Ptgds RNA probe and the mentioned Ptgds antibody, assessing signal colocalization within cells, as to validate the antibody efficiency for the detection of MOL5/6. Here, as in the previous experiment, we recombined experimental mice on P3-5, targeting the postnatal wave of OPC and their progeny and collected the tissue on P60. Fluorescent imaging showed that both probe and antibody signal is clear and located within the cell body. However, while colocalization was evidenced in the corpus callosum, it was absent in the cortex and dorsal horn (images of corticospinal tract not acquired) (**Supplementary fig. 4**). Additionally, a larger number of cells were labelled by the antibody, in comparison to those identified by the probe. We proceeded with experiments for validation of subpopulation-specific cell markers by using RNA probes, since these results are a closer approach to the initial data acquired through single-cell RNA-seq due to the assessment of similar molecules by both techniques.

We performed another *in situ* hybridization coupled with immunohistochemistry, labelling Ptgds RNA molecules and GFP. Here we used two sets of mice, recombined either on E12.5 or P3-5 for lineage tracing, respectively targeting the embryonic and postnatal developmental waves, and collected the tissue on P60. In order to make a time-wise efficient analysis, we imaged cortex, corpus callosum and dorsal horn under both recombination conditions, dismissing the corticospinal tract. As established in the previous experiment, the fluorescent signal of the probe is clear and distributed within the cell body. Moreover, colocalization between $Ptgds^{+}$ cells and GFP^{+} cells is seen in all analysed areas from mice recombined on both time-points (**Fig. 8A-F**). This demonstrates that a number of MOL5/6 are originated from the first embryonic wave of OPC proliferation, while others differentiate from the postnatal wave and cells from both origins are present in the adult mouse.

To better assess the percentage of MOL5/6 that are originated from each wave of OPC proliferation, we performed image quantification (**Fig. 8G**). In the cortex, the percentage of cells originated from the first embryonic wave that become MOL5/6 is ~20%, while ~40% of cells originated from the postnatal wave differentiate into these subpopulations. Both waves similarly originate MOL5/6 in the corpus callosum, which accounts for ~80% of lineage traced cells from each wave of proliferation. The percentage of lineage traced MOL5/6 in the dorsal horn originated from the embryonic wave accounts for ~30%, while ~50% of the cells that derive from the postnatal wave give rise to MOL5/6. Thus, in the corpus callosum the majority of cells originated from the embryonic and postnatal developmental waves give rise to MOL5/6 in the adult mouse. Moreover, the differentiation potential is balanced between both waves, as each of them originates a similar percentage of MOL5/6. In contrast, in the cortex and dorsal horn a lesser percentage of cells differentiate into MOL5/6, where the postnatal wave originates a higher percentage of MOL5/6 in comparison with the embryonic wave. Additionally, we quantified the percentage of MOL5/6 out of all cells present in the analysed areas of the mature mouse (**Supplementary fig. 5**). These subpopulation constitute ~10% of the cells in the cortex, ~50% in the corpus callosum and ~20% in the dorsal horn.

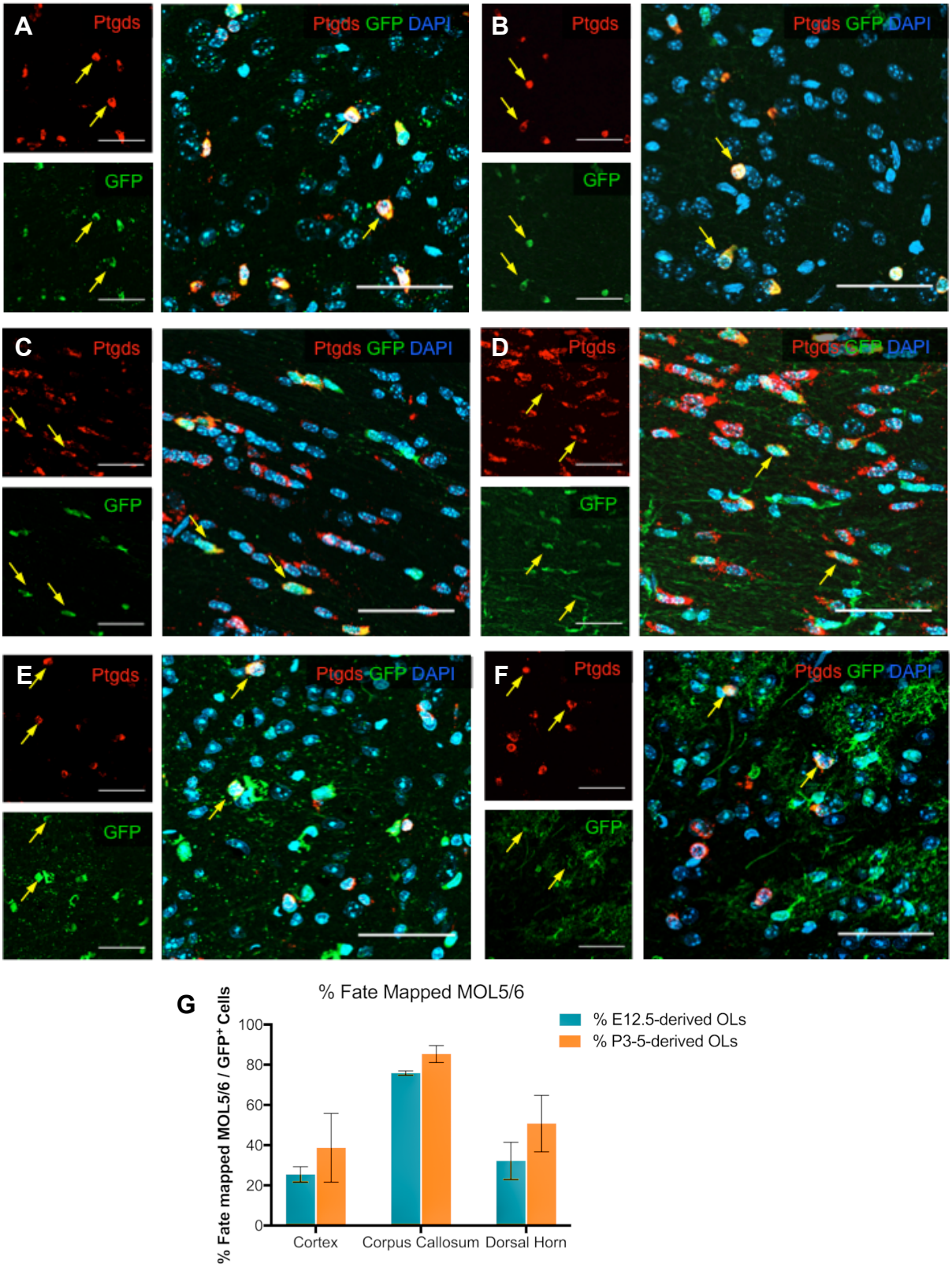

Figure 8. Ptgds^{+} cells colocalize with GFP^{+} cells originated from the embryonic and postnatal developmental waves in the adult mouse RNAscope *in situ* hybridization combined with standard immunohistochemistry targeting MOL5 and 6 (Ptgds - probe) and Pdgfra^{+} cells and progeny (GFP - antibody) in cortex (A, B), corpus callosum (C, D) dorsal horn (E, F). Experimental mice were recombined on E12.5 (A, C, E) and P3-5 (B, D, F) and tissue was collected on P60. Yellow arrows indicate signal colocalization between Ptgds RNA molecules and GFP^{+} cells. Scale bars represent 50µm. Percentage of fate mapped MOL5/6 originated from the embryonic and postnatal developmental waves (G). Data reported as mean ± standard error of the mean (SEM), n=3.

Validation of RNA probes against cell markers for OPCs and COP in the adult mouse

Ptprz1 is a cell marker for OPCs and differentiation-committed oligodendrocyte precursors (COP), as evidenced by single-cell RNA-seq. We analysed the level of colocalization between $Ptprz1^+$ cells and GFP^+ cells, and the percentage of OPCs and COP that derive from the embryonic and postnatal waves. For lineage tracing, mice were recombined either on E12.5 or P3-5, targeting the embryonic and postnatal waves of proliferation, and tissue was collected on P60. We performed *in situ* hybridization coupled with immunohistochemistry, labelling Ptprz1 RNA molecules and GFP. The fluorescent signal of the probe displayed a scattered pattern, distributed in the cell body and cellular processes. Colocalization between $Ptprz1^+$ cells and GFP^+ cells can be seen in all analysed regions, namely cortex, corpus callosum and dorsal horn, in both recombination conditions (**Fig. 9A-F**). These results demonstrate that OPCs and COP are present in the adult mouse and result from both developmental waves.

In order to evaluate the percentage of OPCs and COP that arise from each wave of proliferation, we performed image quantification (**Fig. 9G**). In the cortex, both waves seem to contribute for the proliferation and differentiation of OPCs and COP by ~10% each. A similar result was gathered in the corpus callosum, where the amount of cells that derive from each wave is ~10% of OPCs and COP. In the dorsal horn, ~30% of cells from the first wave are OPCs and COP, while less than 10% of those that derive from the postnatal wave are OPCs and COP. Thus, in the adult mouse a larger percentage of cells derived from the first embryonic wave are maintained as OPCs and COP, in comparison to the postnatal wave, in the dorsal horn. By contrast, in the cortex and corpus callosum both developmental waves contribute equally for the presence of OPCs and COP. Moreover, the percentage of OPCs and COP out of all cells present in the analysed areas of the mature mouse was quantified (**Supplementary fig. 5**). OPCs and COP constitute ~10% of the cells in the cortex, ~30% in the corpus callosum and ~30% in the dorsal horn.

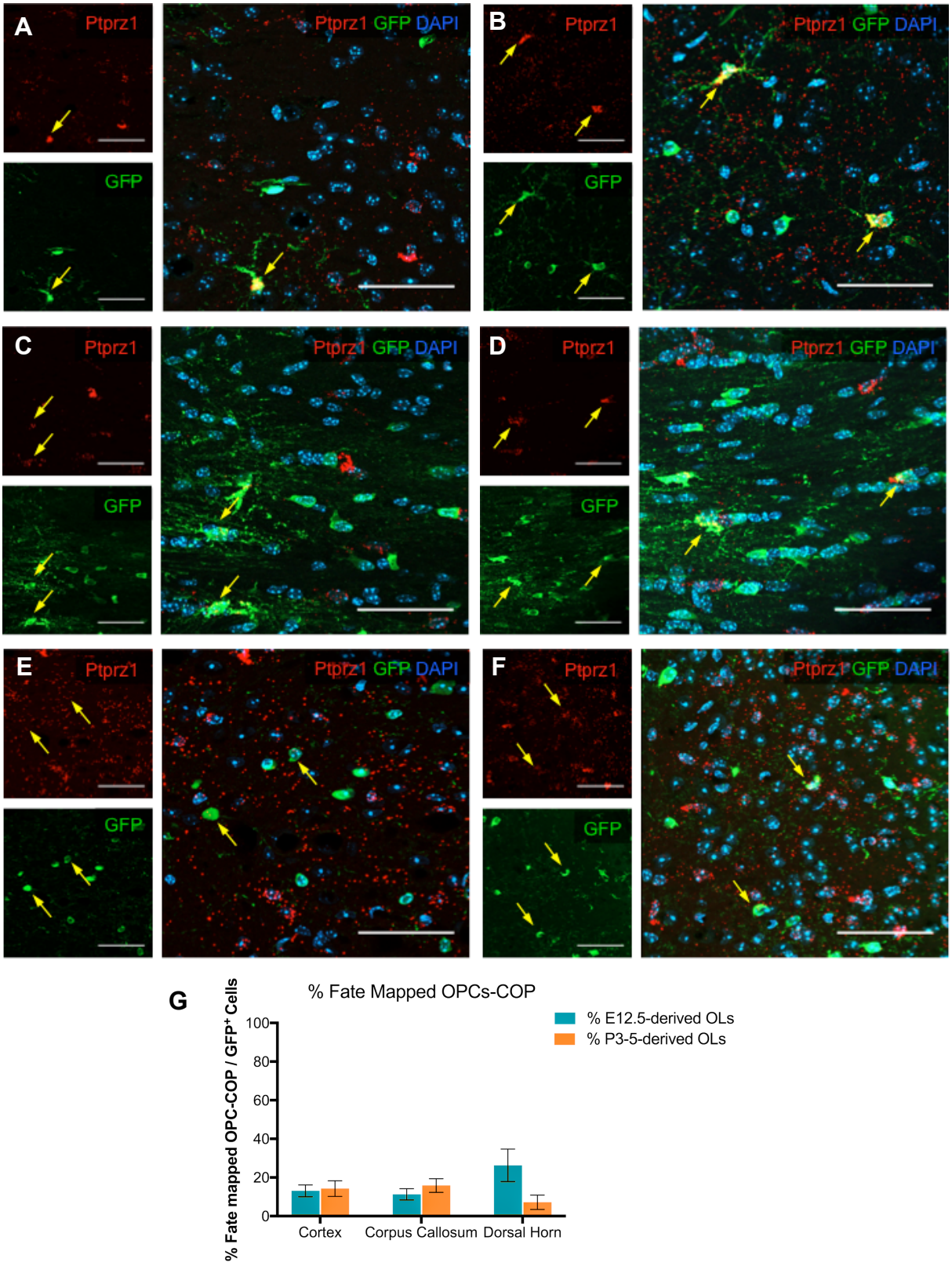

Figure 9. Ptprz1^{+} cells colocalize with GFP^{+} cells originated from the embryonic and postnatal developmental waves in the adult mouse. RNAscope *in situ* hybridization combined with standard immunohistochemistry targeting OPCs and COP (Ptprz1 - probe) and Pdgfra^{+} cells and progeny (GFP - antibody) in cortex (A, B), corpus callosum (C, D) dorsal horn (E, F). Experimental mice were recombined on E12.5 (A, C, E) and P3-5 (B, D, F) and tissue was collected on P60. Yellow arrows indicate signal colocalization between Ptprz1 RNA molecules and GFP^{+} cells. Scale bars represent 50µm. Percentage of fate mapped OPCs and COP originated from the embryonic and postnatal developmental waves (G). Data reported as mean ± standard error of the mean (SEM), *n*=3.

Postnatal developmental wave originates the predominant population of OPCs and progeny in the adult mouse

As a final experiment we performed a triple staining targeting MOL5/6 (Ptgds), fate-mapped embryonic and postnatal waves of OPC and progeny (GFP), and OL lineage (Sox10). Sox10 is an established marker for detection of OL lineage, which includes OPCs and other OL subpopulations altogether. Here we analyse colocalization between double positive cells (Ptgds/Sox10 and GFP/Sox10) and triple positive cells (Ptgds/GFP/Sox10). To do this, mice were recombined either on E12.5 or P3-5, targeting the embryonic and postnatal developmental waves, and tissue was collected on P60. We performed *in situ* hybridization coupled with immunohistochemistry, targeting Ptgds and Sox10 RNA molecules and GFP. The fluorescent signal of Ptgds probe is clear and distributed within the cell body, as previously shown, and Sox10 displays a similar expression pattern throughout the cortex, corpus callosum and dorsal horn (**Fig. 11**). Double and triple colocalization between the different markers can be seen in all analysed areas, in both recombination conditions. These results confirm that Ptgds$^+$ cells (MOL5/6) and GFP$^+$ cells (fate-mapped OPCs and progeny) are also Sox10$^+$ and are indeed part of the OL lineage, thereby validating the specificity of the markers used.

Based on these results we performed the quantification analysis in three distinct branches. Firstly, we analysed the percentage of MOL5/6 per Sox10$^+$ cells in the imaged areas of the CNS (**Fig. 10A**). In the cortex 50-60% of the OL lineage cells are MOL5/6, while in the corpus callosum they constitute ~90% and in the dorsal horn ~70%. Following, we analysed the percentage of fate-mapped OPCs and progeny originated from the embryonic and postnatal waves per Sox10$^+$ cells (**Fig. 10B**). In the cortex, both waves seem to originate ~60% of the Sox10$^+$ cells. Contrarily, in the corpus callosum and spinal cord the embryonic wave seems to originate the least amount of cells, contributing with ~20% on one case and 30-40% on the other, while the postnatal wave gives rise to ~50% and ~40% of cells, respectively. Lastly, the percentage of OLs per total number of cells in the analysed areas was also quantified (**Fig. 10C**). Out of all cells in the corpus callosum ~50% belong to the OL lineage, with the postnatal developmental wave being the main contributor. Dorsal horn follows, with ~25% of its cells being OPCs or OLs and both the embryonic and postnatal waves contributing equally for the their presence. The cortex is the analysed area with the smallest percentage of OL lineage cells, accounting for ~15% of cells present in this area, where the postnatal wave originates a slightly higher number of cells in comparison with the embryonic wave.

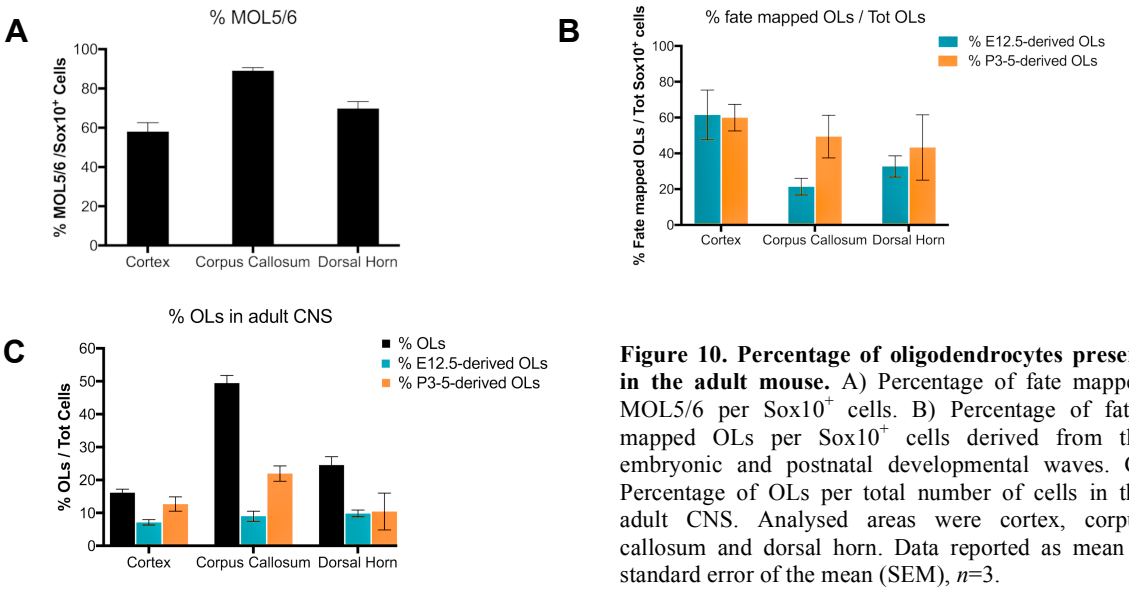

Figure 10. Percentage of oligodendrocytes present in the adult mouse. A) Percentage of fate mapped MOL5/6 per Sox10$^+$ cells. B) Percentage of fate-mapped OLs per Sox10$^+$ cells derived from the embryonic and postnatal developmental waves. C) Percentage of OLs per total number of cells in the adult CNS. Analysed areas were cortex, corpus callosum and dorsal horn. Data reported as mean ± standard error of the mean (SEM), *n*=3.

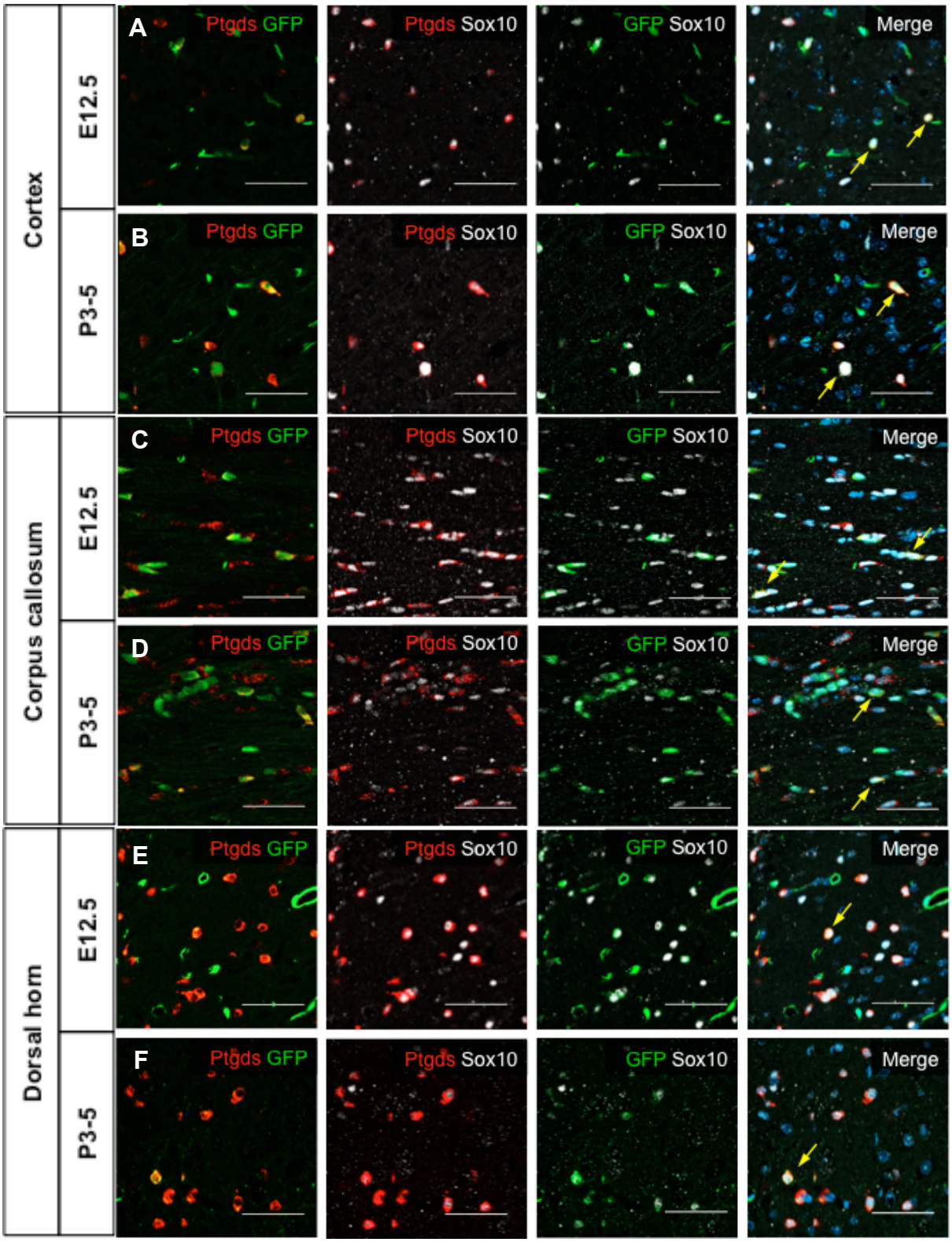

Figure 11. Positive Ptgds/GFP/Sox10 cells are originated from the first embryonic and postnatal developmental waves and are present in the adult mouse. RNAscope *in situ* hybridization combined with standard immunohistochemistry targeting MOL5 and 6 (Ptgds - probe), OL lineage (Sox10 - probe) and Pdgfra^{+} cells and progeny (GFP - antibody) in cortex (A, B), corpus callosum (C, D) and dorsal horn (E, F). Experimental mice were recombined on E12.5 (A, C, E) and P3-5 (B, D, F) and tissue was collected on P60. Merge images display Ptgds, GFP, Sox10 and DAPI. Yellow arrows indicate signal colocalization between Ptgds and Sox10 RNA molecules and GFP^{+} cells. Scale bars represent 50µm.

Discussion

In this work we performed immunohistochemistry (IHC) and *in situ* hybridization (ISH) assays by using antibodies and probes specific for cell markers of subpopulations from OL lineage in order to identify them in areas of the CNS of the juvenile (P20) and adult mice (P60). Moreover, we assessed the developmental origin of OPCs and progeny by performing lineage-tracing experiments. Since the existence of transcriptional heterogeneity in OL lineage was recently unveiled by single-cell RNA-seq data, most antibodies and probes used in this study represent a novelty for the identification of OL subpopulations. By performing IHC assays targeting putative cell-markers of OL lineage in juvenile and adult mice tissue sections, a number of antibodies display potentially specific fluorescent signal. The results obtained by using these antibodies present evidence about the distribution and developmental origin of OL subpopulations. Protocol optimization was performed in the cases where antibody fluorescent signal was not as strong and clear as desirable. This enabled the improvement of signal in some cases, while in others this was not possible. Nevertheless, the antibodies shown here require further validation as to confirm their specificity, which can be verified through several ways.

In general, antibodies can perform well in a given context and inadequately in other due to differences in protein conformation and target accessibility, for instance (Bordeaux et al., 2010). A highly specific antibody recognizes its target with minimal off-target binding (cross-reactivity). However, this is not always the case, and IHC results often present issues regarding the confirmation of the efficiency of antibodies, which can be tackled by several methods. Genetic strategies assess antibody specificity by measuring signal in control cells or tissue in which the target gene or epitope has been knocked out or knocked down. Thus, the expression of the target protein is eliminated or dramatically decreased, and the presence of signal indicates cross-reactivity. Another approach consists on using two or more independent antibodies that recognize different regions of the target protein. If the expression pattern generated by the antibodies is correlated it means they are binding to distinct epitopes of the same protein, minimizing the probability of cross-reactivity. Antibodies can also be validated by expressing a protein that contains a reporter, such as GFP or an affinity tag. This allows the detection of colocalization between antibody and reporter signals, thereby validating the specificity of the antibody. An additional method is adsorption, in which the antigen is pre-incubated with the antibody preceding the assay. Though it shows target binding, this approach will not exclude cross-reactivity with proteins that possess similar epitopes as the intended target (Howat et al., 2014; Uhlen et al., 2016). Therefore, at least one of these methods should be used in order to claim that a given antibody has been validated, and the use of more than one strategy strengthens its reliability.

Complementing IHC studies in the mouse, we used RNAscope ISH to identify different OL subpopulations in the CNS. This novel technique enables single-molecule visualization in individual cells by using an amplification system associated with the probe. By enhancing the signal without amplifying the background, this method improves both sensitivity and specificity in comparison to regular RNA ISH. This technique is a closer association with the single-cell RNA-seq data, from which OL lineage markers were identified, since it also evaluates the presence of RNA molecules in the cells. Moreover, RNAscope adds information about spatial distribution of molecules within the cells and localization of cells that incorporate fluorescent signal in different tissue areas (Wang et al., 2012). An important notion to retain in this work is that mRNA levels do not always correlate with the corresponding protein levels in a given cell-type, as this correlation depends on several biological factors. Here we analysed cell-types that undergo dynamic differentiation processes and compared the presence of both molecules. Therefore, the delay between transcription and translation should be taken

into consideration, as well as post-transcriptional processes that might lead to greater deviations from an ideal correlation (Edfors et al., 2016; Liu et al., 2016).

For the detection of MOL1 we performed immunohistochemistry, using an antibody against Egr2 (Early growth response 2), which is a sequence-specific DNA-binding transcription factor present mostly in the nucleus. It is described as a regulator of a diverse array of genes required for peripheral nerve myelination (Jang et al., 2006). Egr2 antibody displayed a fluorescent signal widely distributed in the tissue of the juvenile mouse and apparently unspecific, since the outline of $Egr2^+$ cells could only be dimly detected in the dorsal horn. In this area of the spinal cord, some $Egr2^+$ cells also appear to colocalize with GFP^+ cells, thus potentially being MOL1. A negative control was performed and, therefore, this signal is not due to unspecific binding of the secondary antibody. Consequently, there are several possible reasons why the antibody did not display the desired fluorescent signal. Since the background fluorescence is very strong, the primary antibody might be binding unspecifically to other molecules. A way to counter this would be to increase the concentration of the blocking solution, for instance, thereby reducing cross-reactivity. Also, since there are Egr2 labelled cells that appear to colocalize with GFP^+ cells in the dorsal horn, lowering the concentration of the primary antibody might decrease the background fluorescence and put in evidence $Egr2^+$ cells in the remaining areas. Additionally, ISH coupled with IHC displayed the presence of Egr2 mRNA molecules within GFP^+ cells, specifically in the cellular bodies and processes, not only in the dorsal horn, as the antibody, but in the other analysed areas as well. These results suggest that the levels of mRNA and protein do not correlate or that probably exists a temporal mismatch between their expression in the cell (Edfors et al., 2016). Since the antibody did not display signal in experimental P60 brain and spinal cord, the presence of Egr2 mRNA should be tested with ISH, as to detect the eventual presence of MOL1 in the adult mouse. As stated by Marques et al., some mature oligodendrocyte subpopulations might be present only during the juvenile state and disappear as the organism further develops. Thus, the absence of antibody signal in the tissue of adult mouse might indeed suggest the absence of MOL1.

Furthermore, we used an antibody against FosB (FBJ osteosarcoma oncogene B), a marker for both MOL1 and VLMCs, described as a transcription factor that interacts with Jun proteins and enhances their DNA-binding activity, playing a role in cell proliferation and differentiation (Gruda et al., 1996). FosB antibody exhibited a clear signal distributed within the cell nuclei, in the cortex, dorsal horn and corticospinal tract. While a number of $FosB^+$ cells colocalize with GFP^+ cells present in the cortex and dorsal horn, double positive cells are absent in the corticospinal tract. Even though MOL1 and VLMCs are morphologically distinct, it is not possible to distinguish them simply by labelling with FosB. As VLMCs and OPCs that will give rise to the remaining OL lineage both are $Pdgfra^+$, they also cannot be told apart by GFP antibody. Thus, a way to tackle this problem would be to use another antibody specific for VLMCs, such as Col1a1, and image it on a different fluorescent channel of the confocal microscope. Subsequently, $FosB^+/Col1a1^+$ cells would be VLMCs and $FosB^+/Col1a1^-$ cells would be MOL1. An equivalent result could be achieved by using an antibody against Sox10, a marker for the OL lineage, instead of Col1a1. This way, $FosB^+/Sox10^+$ cells would be MOL1 and $FosB^+/Sox10^-$ cell would correspond to VLMCs (Marques et al., 2016). Although we did not perform the latter experiments, they can be considered in the future if MOL1 or VLMCs are to be imaged through immunohistochemistry.

For MOL2 detection, we used an antibody against HOPX (Homeodomain only protein), the smallest known member of the homeodomain-containing protein family, which is atypically unable to bind DNA. It is involved in the regulation of cell proliferation and differentiation (Mariotto et al., 2016). The signal of the antibody is strong and is present mostly around the nuclei, displaying colocalization

with GFP^+ cells in the brain and spinal cord. Moreover, we tested the antibody in P60 brain and spinal cord but did not detect fluorescent signal, which suggests MOL2 might be absent in the adult mouse CNS. Therefore, the signal appears to be specific in the juvenile mouse tissue, but the antibody requires further validation, which we tried to perform by using a probe specific for HOPX mRNA molecules through RNAscope. The probe signal was clear and distributed within the cell bodies, but the GFP antibody signal was broadly distributed in an unspecific manner, most likely due to an issue with the blocking step. Further experiments should be performed using HOPX antibody and probe in juvenile and adult tissue sections, as to validate the antibody specificity and confirm the presence of HOPX mRNA in GFP^+ cells. Nevertheless, if the antibody is validated it signifies that a number of MOL2 are originated from the postnatal developmental wave and are present in the cortex, corpus callosum, dorsal horn and corticospinal tract of the juvenile mouse.

In the case of MOL5 and 6, mostly present in the adult mouse, the antibody used for their detection was against Ptgds (Prostaglandin D2 synthase). This marker is described as having an anti-apoptotic role in oligodendrocytes as well as other maturation and maintenance functions in the CNS (Evans et al., 2013). After streptavidin enhancement, the antibody displayed a clear signal, colocalizing with postnatal GFP^+ cells both in the brain and spinal cord. Additionally, we simultaneously labelled Ptgds mRNA molecules and proteins in tissue sections by coupling IHC and RNAscope, respectively. Since the initial classification of the 12 OL subpopulations was based on single-cell RNA-seq data, a closer association can be achieved through ISH, as this technique assesses the presence of RNA molecules in the tissue as well. Therefore, a large amount of cellular colocalization between Ptgds probe and antibody would validate the antibody efficiency for the detection of MOL5/6. Nevertheless, colocalization was only present in corpus callosum and there was a higher number of cells that displayed antibody signal, in comparison to those that shown probe signal. These results suggest the existence of temporal disparities between the expression of Ptgds mRNA and protein in the cells. ISH coupled with IHC demonstrated that Ptgds probe displays colocalization with a degree of GFP^+ cells originated from the embryonic and postnatal waves, in the cortex, corpus callosum and dorsal horn. Cellular quantification showed that around half of the cells of the corpus callosum of the adult mouse are MOL5/6, which is in accordance to previous studies that claim a large number of OLs are located in white matter, as these are highly myelinated areas of the CNS (Miller, 2002). Moreover, in the adult mouse, most cells originated from the embryonic and postnatal developmental waves develop into MOL5/6. On the other hand, grey matter areas display a smaller number of oligodendrocytes, as well as lower myelination levels (Dimou et al., 2008). These studies corroborate our results, as a lower percentage of cells in the cortex and dorsal horn were identified as MOL5/6. Additionally, in these areas a slightly higher percentage of MOL5/6 are originated from the postnatal developmental wave, in comparison with the embryonic wave. Cell colocalization analysis between fluorescent signal of probes detecting Ptgds mRNA and Sox10 mRNA, which is an established marker for the entire OL lineage, demonstrates that MOL5 and 6 are indeed the predominant subpopulations from OL lineage (60-90%) in corpus callosum, cortex and dorsal horn. These results correlate with RNA-seq data, which point out that MOL5/6 are the most abundant cells from OL lineage in the analysed areas of the adult mouse (Marques et al., 2016). Together these results demonstrate that MOL5 and 6 are crucial for an efficient axonal myelination, ensuring a proper performance of the CNS in the adult mouse.

Ptprz1 (Protein tyrosine phosphatase receptor type Z) was used for detection of OPCs and COP in the brain and spinal cord of the adult mouse. Ptprz1 is a receptor present in the membrane of OL lineage cells, crucial in regulation of differentiation and myelin production throughout development and during remyelination events (Kuboyama et al., 2015). Probe signal is distributed in the cell bodies and cellular processes, displaying colocalization with GFP^+ cells. While most mRNA molecules are

translated in the rough endoplasmic reticulum, Ptprz1 mRNA molecules seem to be transported to other areas of the cell beforehand, i.e. distal processes. Studies show that some mRNAs contain a localization signal and are transported to the cellular processes, such is the case of myelin basic protein (MBP) mRNA (Jansen, 2001). In these cases, mRNA molecules are incorporated in transport granules and carried along microtubules, only being translated at their destination (Smith, 2004). Therefore, this process is most likely involved in the delivery and translation of Ptprz1 in the cellular processes, influencing thereby the first phases of axonal contact and myelination. COP present slight molecular differences compared to OPCs, as the former lack Pdgfra and express genes involved in migration but also others involved in keeping OLs undifferentiated, while they also present low levels of cell cycle markers. Therefore COP represents a migratory cell state that will later initiate differentiation into other OL subpopulations (Marques et al., 2016). Cellular quantification showed that the corpus callosum and dorsal horn retain a similar amount of OPCs/COP, ~30% of the cells, while the cortex displays only ~10%. Therefore, in the brain there is a slightly larger amount of OPCs/COP in the white matter, in comparison to grey matter. Since we did not acquire images of the white matter in the spinal cord, it is not possible to compare the percentage viewed in the dorsal horn. Moreover, the developmental origin of these OL lineage subpopulations is rather balanced in the brain, with each wave maintaining ~10% of their cells as OPCs/COP. On the other hand, in the dorsal horn the embryonic wave contributes with the major amount of OPCs/COP.

Finally, we used a probe against Sox10, which is a widely used marker for the identification of the entire OL lineage, and analysed colocalization with GFP^+ cells in the adult mouse. Ptgds probe is validated in this experiment, as it colocalizes with Sox10 probe signal and GFP^+ cells as well, showing MOL5/6 are present in the adult mouse CNS and are originated from the embryonic and postnatal waves. In the corpus callosum, around half of the total number of cells in this area belong to OL lineage, with the majority of OLs being originated from the postnatal wave of proliferation. This relates to previous studies, as white matter is largely populated by myelinating OLs (Miller, 2002; Simons and Nave, 2015). Contrarily, the cortex is populated by a small amount of cells from the OL lineage, and their origin is more evenly shared between both developmental waves. Around a quarter of the total amount of cells belongs to OL lineage in the dorsal horn, with the cells derived from both developmental waves contributing equally for these numbers. These results are also in line with preceding studies, as grey matter consists of a lower number of myelinating cells (Hofmann et al., 2017).

The presence of OPCs in multiple sclerosis (MS) lesions is well established, as they respond in the context of demyelination by exhibiting remyelination potential, which has been evidenced by the use of animal models such as experimental autoimmune encephalomyelitis (EAE) mice (Goldenberg, 2012). Therefore understanding OPC origin and response to demyelinating insults is fundamental for the development of cell-based therapies. Studies have shown that OPCs respond to molecular cues and proliferate towards areas that have been injured, thereby differentiating and promoting recovery (Chari, 2007; Compston and Coles, 2008). OPCs are converted to a regenerative phenotype by several transcription factors that play important roles in OL development, and it is observed that this response generally occurs during acute phases of myelination and during early remyelination. This indicates that OPC regenerative phenotype is a transition that recapitulates developmental programs (Gallo and Deneen, 2014; Lu et al., 2002). Since these molecular changes are what lead OPCs to differentiation and consequent remyelination in disease context, the results in this work might be informative in the manipulation and drive of these processes. Here we show molecular singularities in OPCs and myelinating subpopulations of OL lineage, some of them including transcription factors. Moreover, their distribution is described in areas of the brain and spinal cord, also giving information about their

developmental origin. By integrating this information it might be possible to trace a path of differentiation and target areas where demyelination is happening, ensuring a more efficient way of remyelination. Moreover, OPCs display distinct myelination potential based on their location, either in the brain or spinal cord. Indeed, OPCs generate myelin sheath lengths that are a reflection of their *in vivo* origin, demonstrating a regional identity prior to differentiation. Additionally, there are differences in the settling positions assumed by OL lineage cells based on their ventral versus dorsal origin (Bechler et al., 2015; Tripathi et al., 2011). Also, the remyelinating potential of OPCs decreases from early postnatal development towards adulthood (Gallo and Deneen, 2014). Therefore, the manipulation of the transcription and differentiation profile of OPCs is of extreme relevance in order to bring forth a strategy for inducing remyelination in older individuals suffering from MS. Future epigenetic studies, joined with imaging and immunological approaches, have the potential to further understand the mechanisms that regulate remyelination and thereby manipulate this intricate process to some extent.

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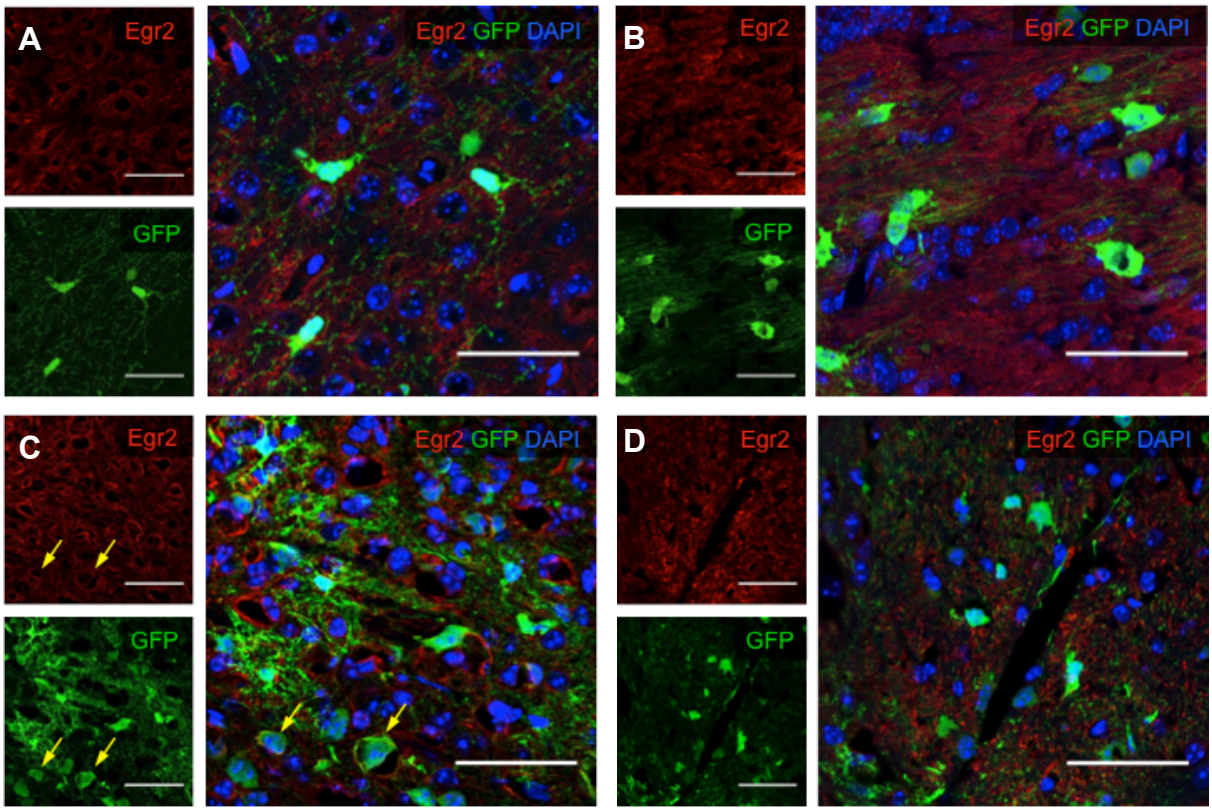
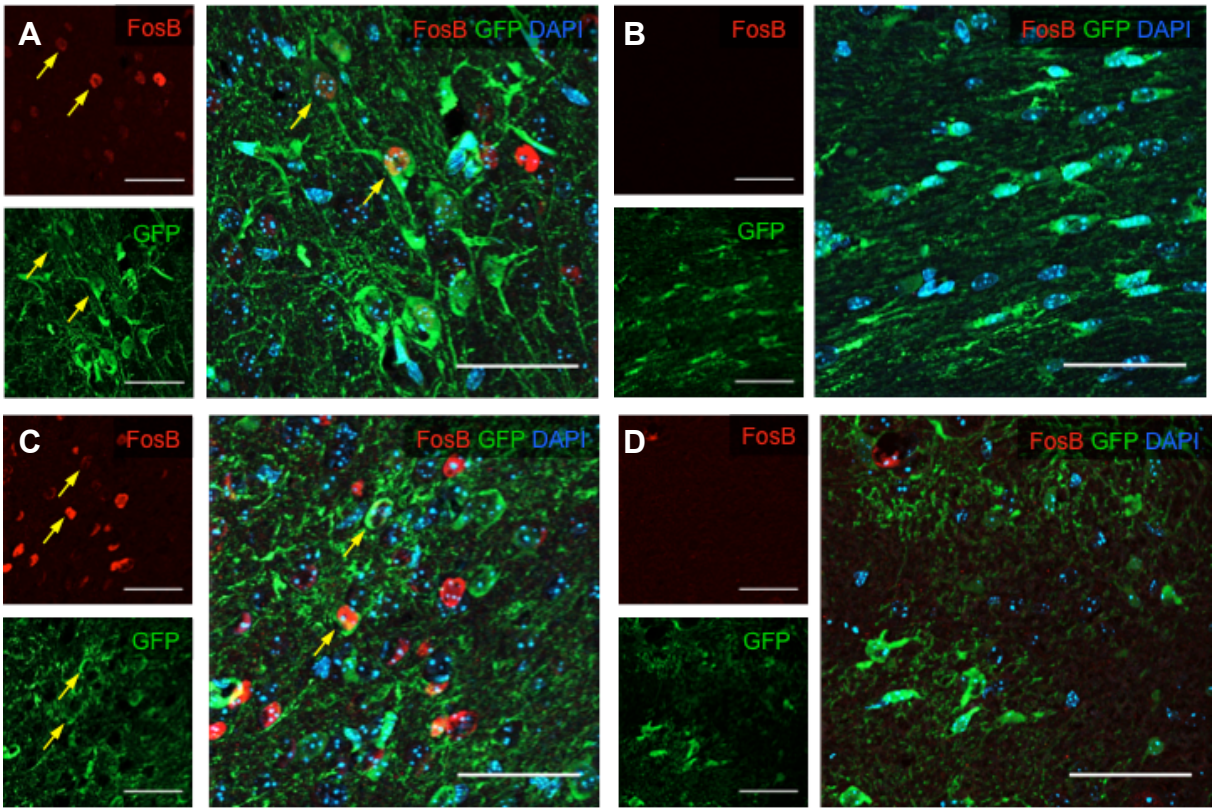
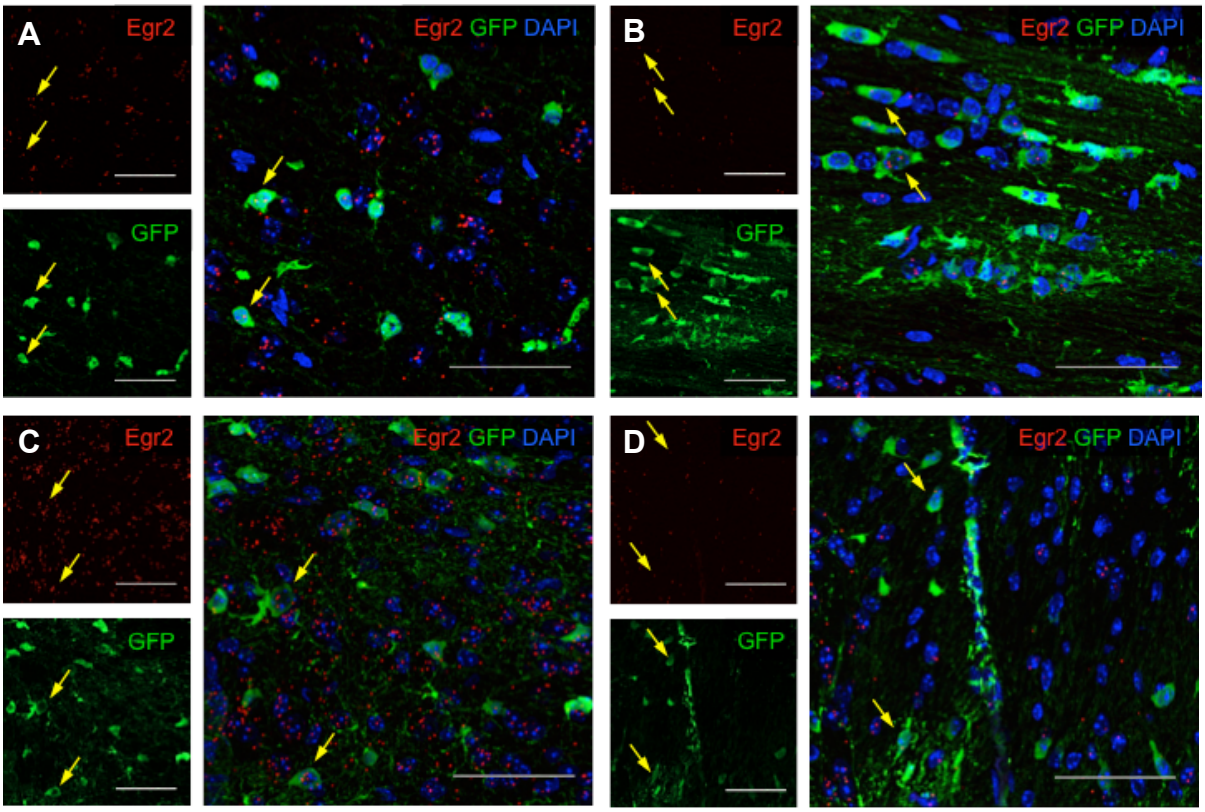
Supplementary data



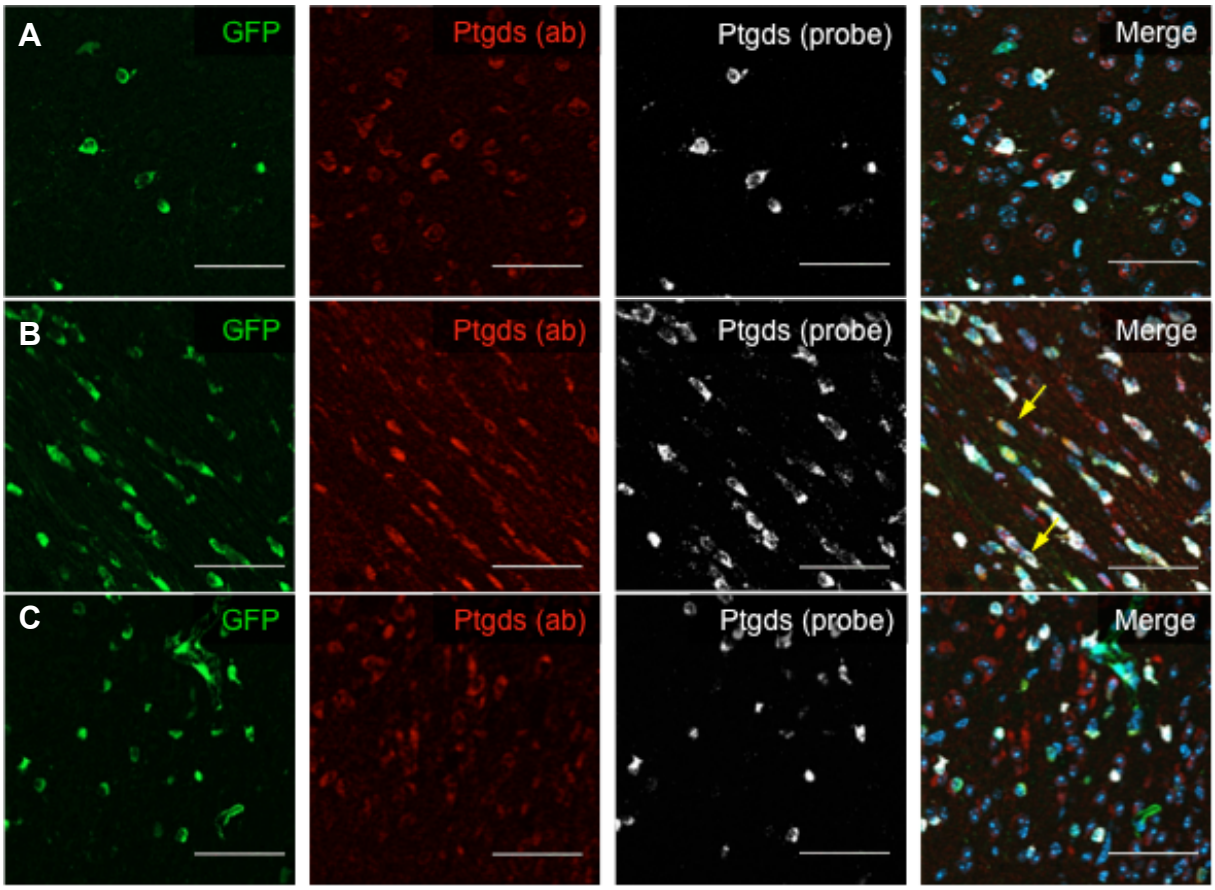
Supplementary figure 1. Standard immunohistochemistry staining for Egr2$^+$ cells and GFP$^+$ cells in the juvenile mouse. Immunohistochemistry targeting MOL1 (Egr2) and Pdgfra$^+$ cells and progeny (GFP) in cortex (A), corpus callosum (B), dorsal horn (C) and corticospinal tract (D). Experimental mice were recombined on P3-5 and tissue was collected on P20. Yellow arrows indicate signal colocalization between Egr2$^+$ cells and GFP$^+$ cells. Scale bars represent 50μm.



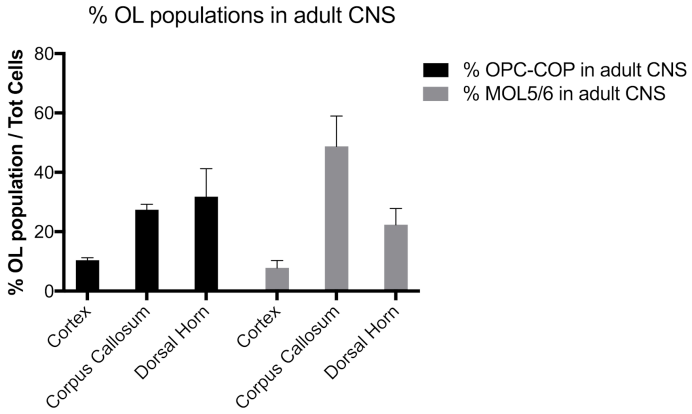
Supplementary figure 2. Standard immunohistochemistry staining for FosB$^+$ cells and GFP$^+$ cells in the juvenile mouse. Immunohistochemistry targeting MOL1 and VLMCs (FosB), and Pdgfra$^+$ cells and progeny (GFP) in cortex (A), corpus callosum (B), dorsal horn (C) and corticospinal tract (D). Experimental mice were recombined on P3-5 and tissue was collected on P20. Yellow arrows indicate signal colocalization between FosB$^+$ cells and GFP$^+$ cells. Scale bars represent 50μm.



Supplementary figure 3. *In situ* hybridization coupled with immunohistochemistry staining for Egr2 (probe) and GFP$^+$ cells in the adult mouse *In situ* hybridization targeting MOL1 (Egr2), and Pdgfra$^+$ cells and progeny (GFP) in cortex (A), corpus callosum (B), dorsal horn (C) and corticospinal tract (D). Experimental mice were recombined on P3-5 and tissue was collected on P20. Yellow arrows indicate signal colocalization between Egr2 RNA molecules and GFP$^+$ cells. Scale bars represent 50µm.



Supplementary figure 4. *In situ* hybridization coupled with immunohistochemistry staining for Ptgds (antibody and probe) and GFP$^+$ cells in the adult mouse. RNAscope *in situ* hybridization combined with standard immunohistochemistry targeting MOL5 and 6 (Ptgds - antibody and probe) and Pdgfra$^+$ cells and progeny (GFP - antibody) in cortex (A), corpus callosum (B) and dorsal horn (C). Experimental mice were recombined P3-5 and tissue was collected on P60. Yellow arrows indicate signal colocalization between Ptgds RNA probe/antibody and GFP$^+$ cells. Scale bars represent 50µm.



Supplementary figure 5. Percentage of MOL5/6 and OPCs-COP in the adult mouse CNS. Percentage of fate mapped MOL5/6 and OPCs-COP originated from the embryonic and postnatal developmental waves. Data reported as mean ± standard error of the mean (SEM), *n*=3.