STEREOLOGICAL ANALYSIS OF OLIGODENDROCYTE PROGENITOR CELLS IN THE ADULT MOUSE BRAIN

By

Jenna Boulanger

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Contributions of collaborators

For all the articles included in this thesis, I created the experimental design, did most of the data collection, was responsible for data analysis, and wrote the accompanying manuscript. C. Messier served as my thesis supervisor. He helped with experimental design and contributed to manuscript writing and editing as part of his supervisory role. Undergraduate students (volunteers, writing their undergraduate thesis, and NSERC-funded summer students), who include V. LeBlanc and E.-L. Khoo, participated in data collection. W. A. Staines served as an advisor on article 1. J. Liang helped with experimental manipulations.

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GENERAL INTRODUCTION

Oligodendrocyte progenitor cells (OPCs; also known as NG2-glial cells, synantocytes, or polydendrocytes) are a type of glial cell that give rise, as their name suggests, to myelinating oligodendrocytes during early stages of post-natal life (Kang, Fukaya, Yang, Rothstein, & Bergles, 2010; Nishiyama, Watanabe, Yang, & Bu, 2002). OPCs express the platelet-derived growth factor receptor alpha (PDGFRα) and the chondroitin sulfate proteoglycan neuron-glia antigen 2 (NG2), both of which are downregulated in favour of O4 and myelin-specific antigens as the cell transitions from an OPC to an oligodendrocyte phenotype (Butt et al., 1997; Levine, Stincone, & Lee, 1993; Nishiyama, Lin, Giese, Heldin, & Stallcup, 1996; Stallcup, 2002).

Interestingly, a large number of OPCs remain undifferentiated after developmental myelination is completed, and OPCs correspond to 2-9% of the total cell population of the adult brain (Dawson, Polito, Levine, & Reynolds, 2003; Ffrench-Constant & Raff, 1986; Nishiyama, Chang, & Trapp, 1999; Pringle, Mudhar, Collarini, & Richardson, 1992; Wolswijk & Noble, 1989). Adult OPCs are uniformly distributed between the grey and white matter of the central nervous system (CNS), where they keep unique territories through self-avoidance (Hughes, Kang, Fukaya, & Bergles, 2013). While their proliferative activity does decline with age due to a slowing down of the cell cycle, they continue to undergo cell division in the adult, representing the mature brain's most active population of cycling cells (Dawson et al., 2003; Psachoulia, Jamen, Young, & Richardson, 2009; Rivers et al., 2008; Simon, Gotz, & Dimou, 2011). The primary fates of daughter cells of adult OPCs are self-renewal and oligodendrocyte differentiation (Nishiyama, Boshans, Goncalves, Wegrzyn, & Patel, 2016), although there is a debate as to their ability to also differentiate into astrocytes and neurons (Nishiyama et al., 2016; Nishiyama, Komitova, Suzuki, & Zhu, 2009; Vigano & Dimou, 2016; Wigley & Butt, 2009).

Since adult OPCs continue to give rise to oligodendrocytes during adulthood, much of the work on the subject has focused on their response to demyelinating insults. The thesis therefore begins with a review of the factors involved in the activation, recruitment, and differentiation of OPCs into myelinating oligodendrocytes in response to white matter injury in the CNS. This review is published (Boulanger & Messier, 2014) and has received to this day 33 citations. The goal of the review was to provide an overview of the literature available on OPCs, as well as to identify factors that may also play a role in OPC cellular dynamics in the intact brain, and more specifically in a form of plasticity known as white matter plasticity. White matter plasticity corresponds to myelin-related changes that occur after developmental myelination is completed. It encompasses two processes: the *de novo* myelination of previously unmyelinated axons, and the remodeling of existing myelin sheaths (reviewed in (Wang & Young, 2014)). Since the product of white matter plasticity is the optimization of the speed and synchronicity of neural inputs, white matter plasticity and the mechanisms behind it could have important implications for learning and memory (Lillard & Erisir, 2011; Pajevic, Basser, & Fields, 2014; Young et al., 2013).

But the generation of new oligodendrocytes is not the only way by which adult OPCs influence neural networks. Indeed, OPCs establish synaptic contacts with neurons and are equipped with functional glutamatergic and GABAergic receptors that induce cellular depolarization and an intracellular influx of Ca^{2+} upon being activated (Bergles, Roberts, Somogyi, & Jahr, 2000; Bernstein, Lyons, Moller, & Kettenmann, 1996; Gallo, Mangin, Kukley, & Dietrich, 2008; Kirchhoff & Kettenmann, 1992; Lin & Bergles, 2004a, 2004b; Tanaka et al., 2009; Verkhratsky, Trotter, & Kettenmann, 1990). Furthermore, adult OPCs are densely arborized and extend processes to multiple neuronal somata, nodes of Ranvier, and neuron-to-

neuron synapses (Boda & Buffo, 2014; Butt et al., 1999; Gallo et al., 2008; Kukley, Capetillo-Zarate, & Dietrich, 2007). Finally, the soma of some grey matter OPCs are closely apposed to that of a neuron, forming what some have characterized as satellite cells (Boda & Buffo, 2014; Gallo et al., 2008; Mangin, Kunze, Chittajallu, & Gallo, 2008).

Because of these characteristics, adult OPCs have been hypothesized to at least monitor and perhaps even modulate neural networks by integrating the synaptic activity of multiple neurons (Boda & Buffo, 2014; Butt, Hamilton, Hubbard, Pugh, & Ibrahim, 2005; Gallo et al., 2008; Maldonado, Velez-Fort, Levavasseur, & Angulo, 2013; Mangin et al., 2008). It is not surprising, then, that neurotransmitters and experience-dependent neural network activity influence the proliferation and differentiation of adult OPCs (Boda & Buffo, 2014; Fields, 2008, 2015; Gallo et al., 2008; Gibson et al., 2014; Karadottir & Attwell, 2007; Purger, Gibson, & Monje, 2015; Tomlinson, Leiton, & Colognato, 2015; Wake, Lee, & Fields, 2011). However, *in vivo* evidence of the effects of neurotransmission on OPC proliferation and differentiation is limited, and only a few experience-related factors (i.e. exercise, environmental enrichment, behavioural stimulation, and motor learning) have been studied.

The main goal of this study was to further explore the hypothesis that experience-dependent neural network activity and neurotransmission can modulate adult OPC proliferation and differentiation. More specifically, we used stereology to establish whether extensive reference memory training and system-wide administration of GABAergic agonists and antagonists could influence the proliferation and differentiation of adult OPCs, as well as the prevalence of OPC-neuron pairs. Analysis of the effects of reference memory training on OPC proliferation and differentiation corresponds to experiment 2, analysis of the effects of GABAergic agents on OPC proliferation and differentiation corresponds to experiment 3, and

analysis of the effects of both reference memory training and GABAergic agents on OPC-neuron pairs, as well as an histological analysis of these closely apposed cells, corresponds to experiment 4.

To evaluate OPC proliferation, we made use of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU), which is widely used in the study of cellular proliferation. Proliferating cells incorporate BrdU into their DNA by substituting it for thymidine during the S-phase of the cellular cycle, during which DNA is replicated, and pass it on to their daughter cells (Ngwenya, Peters, & Rosene, 2005). BrdU immunohistochemistry (IHC) is then used to label these cells, but the DNA has to be denaturated by heat and acid to allow anti-BrdU antibodies to bind to the BrdU incorporated into the nuclear DNA (Gratzner, 1982). Unfortunately, denaturation can considerably damage the tissue (Ffrench et al., 1994; Moran, Darzynkiewicz, Staiano-Coico, & Melamed, 1985) and reduce or even eliminate, in some cases, immunostaining of several proteins. To prevent such deterioration of immunostaining, we developed a simple histological technique that is presented in experiment 1 (Boulanger et al., 2016).

To evaluate the effects of reference memory training and GABAergic agents on OPC differentiation, we made use of the NG2-CreER^{TM}:EYFP reporter mouse, which allows for genetic fate mapping of OPCs through tamoxifen-induced Cre-recombination in the NG2 locus. When recombination is completed, EYFP expression is activated in NG2+ cells. These EYFP+ cells continue to express EYFP+ even if they are no longer NG2+, therefore enabling lineage tracing of labelled cells (Zhu et al., 2011). Three possible fates were analysed in experiments 3 and 4: OPC, oligodendrocyte, and neuron. In these experiments, a neuronal fate was evaluated through expression of the neuronal markers NeuN and HuCD. Analysis of the expression of an

antigen typically thought of as a selective marker of adult neurogenesis, doublecortin (DCX), was done in experiment 5 (Boulanger & Messier, in review; Brown et al., 2003).

In experiment 1, we show that, when trying to visualize BrdU along with other proteins of interest, doing the immunochemistry in two-steps with the addition of a second post-fixation period preserves immunostaining and extends the range of proteins that can be detected in combination with BrdU. In experiments 2 and 3, we used the technique presented in experiment 1 and stereological analysis of the NG2-CreER^{TM}:EYFP reporter mouse to estimate the total number of OPCs in the corpus callosum and cortex of the adult mouse and determine the proportion of proliferating adult OPCs and their fate. We show that 1) white and grey matter OPCs have different properties, 2) OPCs continue to differentiate into oligodendrocytes into adulthood, and 3) provide evidence for the neuronal differentiation of adult OPCs in cortical grey matter. In experiment 2, we found no evidence to show that reference memory training affects the proliferation and differentiation of adult OPCs. In experiment 3, we provide evidence that $GABA_A$-receptor agonism plays a modulatory role on adult OPC proliferation and differentiation. In experiment 4, we provide a stereological estimate of the total number of OPC-neuron pairs in the cortex of the adult mouse. We show that the neurons in these pairs are most likely GABAergic interneurons, and provide evidence to show that GABAergic neurotransmission affects the total number of OPC-neuron pairs. Finally, in experiment 5, we show that all OPCs express DCX, and that DCX-expression is downregulated as OPC transition to an oligodendrocyte phenotype. We suggest that DCX-expression in OPCs is not associated with a neuronal phenotype, but is instead involved in the migration of these cells over short-distances.

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Running Head: Myelination in the adult brain

REVIEW: FROM PRECURSORS TO MYELINATING OLIGODENDROCYTES: CONTRIBUTIONS OF INTRINSIC AND EXTRINSIC FACTORS TO WHITE MATTER PLASTICITY IN THE ADULT BRAIN

Jenna J. BOULANGER* and Claude MESSIER*

*School of Psychology, University of Ottawa

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Correspondence to: C. Messier, School of Psychology, University of Ottawa, 136 Jean-Jacques Lussier Room 2076A, Ottawa, Ontario, K1N 6N5, CANADA. Tel (613) 562-5800 ext. 4562, Fax: (613) 562-5147; E-mail: cmessier@uottawa.ca.

Abbreviations

In alphabetical order:

AKT1: serine-threonine-specific protein kinase AKT-PKB

BDNF: brain-derived neurotrophic factor

bFGF: basic fibroblast growth factor

bHLH: basic helix-loop-helix transcription factor

BMP: bone morphogenic protein

BMP2: bone morphogenic protein 2

BMP4: bone morphogenic protein 4

Cdk2: cyclin-dependent kinase-2

CNPase: cyclic nucleotid 3-phosphohydrolase

CNS: central nervous system

CNTF: ciliary neurotrophic factor

CREB-1: cAMP response element-binding protein-1

CXC: chemokine of the C-X-C motif

CXCL1: CXC ligand 1

CXCL10: CXC ligand 10

CXCL12: CXC ligand 12

CXCL8: CXC ligand 8

CXCR2: chemokine receptor type 2

CXCR4: chemokine receptor type 4

CXCR7: chemokine receptor type 7

FcRϒ: common gamma chain of immunoglobulin Fc receptors

Fyn: proto-oncogene tyrosine-protein kinase Fyn

GFAP: glial fibrillary acidic protein

GSTπ: glutathione S-transferase pi

Hes1: bHLH transcription factor Hes1

Hes5: bHLH transcription factor and Hes5

HDAC: histone deacetylases

Id2: inhibitor of DNA binding 2

Id4: inhibitors of DNA binding 4

IGF-1: insulin-like growth factor 1

IL-1β: interleukin-1 beta

IL-6: interleukin-6

KLF9: Krueppel-like factor 9

LIF: leukemia inhibitory factor

LIMK-1: Lim domain kinase-1

MAPK: mitogen-activated protein kinase

MBP: myelin basic protein

MMP: matrix metalloproteinase

MS: multiple sclerosis

mTOR: mammalian target of rapamycin

Myt1: myelin transcription factor 1

NG2: chondroitin sulfate proteoglycan neuron-glia antigen 2

NT-3: neurotrophin-3

O4: oligodendrocyte marker O4

Olig1: bHLH transcription factor 1

Olig2: bHLH transcription factor 2

OPC: oligodendrocyte progenitor cell

PDGF: platelet-derived growth factor

PDGFRα: platelet-derived growth factor receptor alpha

PI3K: phosphatidylinositol 3 kinase

PKC: protein kinase C

PLP: proteolipid protein

PPAR: peroxisome proliferator-activated receptor

PSA-NCAM: polysialylated-neural cell adhesion molecule

RhoA: Ras homolog gene family, member A

RXR: retinoid X receptor

RXRϒ: retinoid X receptor gamma

S100B: S100 calcium binding protein B

Sox: SRY-related HMG-box

Sox2: SRY-related HMG-box transcription factor 2

Sox10: SRY-related HMG-box transcription factor 10

Sox17: SRY-related HMG-box transcription factor 17

T3: thyroid hormone triiodothyronine

TG2: tissue transglutaminase-2

TGF-β1: transforming growth factor beta-1

TNFR1: tumor necrosis factor receptor 1

TNFR2: tumor necrosis factor receptor 2

TNFα: tumor necrosis factor alpha

TrkC: neurotrophic tyrosine kinase receptor 3

Zfp: zinc finger protein

Abstract

Oligodendrocyte precursor cells (OPC) are glial cells that metamorphose into myelinating oligodendrocytes during embryogenesis and early stages of post-natal life. OPCs continue to divide throughout adulthood and some eventually differentiate into oligodendrocytes in response to demyelinating lesions. There is growing evidence that OPCs are also involved in activity-driven de novo myelination of previously unmyelinated axons and myelin remodelling in adulthood. In this review, we summarize the interwoven factors and cascades that promote the activation, recruitment and differentiation of OPCs into myelinating oligodendrocytes in the adult brain based mostly on results found in the study of demyelinating diseases. The goal of the review was to draw a complete picture of the transformation of OPCs into mature oligodendrocytes to facilitate the study of this transformation in both the normal and diseased adult brain.

Key Words: myelin, multiple sclerosis, myelin remodelling, oligodendrocyte, plasticity, remyelination

1. Introduction

Oligodendrocyte progenitor cells (OPCs; also known as polydendrocytes, oligodendrocyte precursor cells or NG2 glial cells) are glial cells that give rise, as their name suggests, to myelinating oligodendrocytes (Zhu, Bergles, & Nishiyama, 2008). However, a large number of OPCs maintain an undifferentiated state thereafter and OPCs remain abundant in the adult brain (Dawson, Polito, Levine, & Reynolds, 2003a). Adult OPCs are uniformly distributed between the grey and white matter of the central nervous system (CNS) (Levine & Reynolds, 1999). In vivo imaging studies show that OPCs actively sample their environment using motile filipodia and growth cones and keep unique territories that are maintained through self-avoidance (Hughes, Kang, Fukaya, & Bergles, 2013). While the proliferative activity of OPCs does decline with age (Levine, Stincone, & Lee, 1993; Woodruff, Fruttiger, Richardson, & Franklin, 2004), they continue to undergo cell division (Nishiyama, Watanabe, Yang, & Bu, 2002) and give rise to myelinating oligodendrocytes throughout adulthood (Richardson, Pringle, Mosley, Westermark, & Duboisdalcq, 1988; Rivers et al., 2008; Trotter, Karram, & Nishiyama, 2010; Zhu et al., 2008).

Proliferation and differentiation of OPCs into myelinating oligodendrocytes increase in response to a variety of insults, including demyelination, the pathological hallmark of multiple sclerosis (MS) (Carroll & Jennings, 1994; Keirstead, Levine, & Blakemore, 1998; Levine & Reynolds, 1999; Redwine & Armstrong, 1998; Sim, Zhao, Li, Lakatos, & Franklin, 2002a; Watanabe, Toyama, & Nishiyama, 2002). OPC proliferation and differentiation also underlie a recently identified and novel form of neural plasticity which has been termed white matter plasticity (reviewed in (Wang & Young, 2013)). White matter plasticity in adulthood encompasses both the myelination of previously unmyelinated axons as well as the remodelling

of existing myelin sheaths. White matter plasticity in the healthy adult brain is thought to be modulated by neural activity and has been linked to cognitive functioning (Nunez, Nelson, Pych, Kim, & Juraska, 2000; Stevens, Porta, Haak, Gallo, & Fields, 2002). For example, a recent report has shown that, in mice, the rate of OPC proliferation is increased during sleep in a way that is proportional to the duration of REM sleep. OPC differentiation into a premyelinating oligodendroglial phenotype occurred primarily when animals were awake, though the transcription of myelin-related genes was greater when they were asleep (Bellesi et al., 2013). This therefore suggests that white matter plasticity is associated with normal brain activity and memory consolidation.

It appears that the first form of white matter plasticity would consist in the myelination of previously unmyelinated axons or axonal branches. Approximately 29% of mature oligodendrocytes found in the adult rodent brain are thought to have been generated in advanced stages of post-natal life (Rivers et al., 2008). Similarly, neuroimaging studies suggest that myelination of the human cortex is not complete until the third decade of life (Fuster, 2002). Functional improvements in response to maturation seem to correspond to new or increased myelination of neuronal circuits (Johansen-Berg, 2007; Yasuda et al., 2011). Furthermore, piano playing has been shown to increase white matter volumes in healthy adult subjects, suggesting that training may lead to better performances through improvements in conduction velocity achieved through increased levels of myelination (Bengtsson et al., 2005).

The second form of white matter plasticity is known as myelin remodelling (Young et al., 2013). Myelin remodelling refers to the replacement of a long myelin internode by two or more shorter internodes, thereby modifying the speed of neural impulses (Pajevic, Basser, & Fields, in press; Seidl, in press; Young et al., 2013). For example, internode length and myelin sheath

thickness can be modulated to optimize the temporal correlation of binaural auditory inputs (Seidl, in press). Though it has yet to be demonstrated, it could be hypothesized that myelin remodelling would require the destruction and clearance of myelin debris, making it quite similar to what is seen in response to white matter injury, albeit to a much lesser extent (Kotter, Zhao, van Rooijen, & Franklin, 2005). An additional similarity between demyelinating diseases and myelin remodelling is found in the resulting myelin sheaths, which are generally shorter and thinner than those generated during development (Franklin & Ffrench-Constant, 2008; Franklin, Zhao, & Sim, 2002). Although myelin remodelling is more prominent during brain maturation and aging (Bennett & Madden, in press), it also appears to have a functional role in learning and memory throughout the lifespan (reviewed in (Zatorre, Fields, & Johansen-Berg, 2012)).

While white matter changes are driven by a variety of cognitive-related functions, its implications and mechanisms of action are only now starting to be elucidated. Most of what we know about myelination is derived from studies examining developmental myelination or remyelination in response to injury or disease. Several characteristics specific to adult OPCs and the environment in which adult myelination takes place have led us to believe that some of the molecular processes implicated in remyelination may also be involved in adult white matter plasticity. Before addressing these characteristics and the molecular pathways that could steer the transition of adult OPCs into myelinating oligodendrocytes, we present a brief overview of MS because its study has so far provided the most information on the fate of adult OPCs and their transition into myelinating oligodendrocytes in the adult brain.

1.1 Multiple sclerosis

MS is a progressively debilitating disease that is generally considered to be an autoimmune disorder of the human nervous system (Adams, Poston, & Buk, 1989; Hickey, 1999; Prineas &

Graham, 1981; Sririam & Rodriguez, 1997). While disease course varies greatly across patients, it typically presents in a relapsing/remitting form, with episodes of acute demyelination and neurological dysfunction followed by remyelination and functional recovery (Arnett et al., 2004; Fancy, Chan, Baranzini, Franklin, & Rowitch, 2011; Gold, Linington, & Lassmann, 2006). MS episodes are characterized by a compromised blood-brain barrier, which leads to the infiltration of activated macrophages and of T-cell lymphocytes into the brain parenchyma. These immune cells possess myelin-specific antigens that lead to the destruction of myelin sheaths located in the vicinity of the injury (Adams et al., 1989; Gold et al., 2006; Prineas et al., 1989; Ransohoff, 2012; Sririam & Rodriguez, 1997) (Fig. 1.1-3). Remyelination occurs spontaneously in the first stages of the disease, when lesions are of an acute nature and elicit a robust inflammatory response (Blakemore, 1973; Goldschmidt, Antel, Konig, Bruck, & Kuhlmann, 2009; Lucchinetti et al., 1999; Patani, Balaratnam, Vora, & Reynolds, 2007; Patrikios et al., 2006; Prineas, Barnard, Kwon, Sharer, & Cho, 1993; Prineas & Connell, 1979; Raine & Wu, 1993; Wolswijk, 2002). However, remyelination is often incomplete or inefficient and chronically demyelinated lesions eventually fail to remyelinate (Franklin, 2002; Gensert & Goldman, 1997; Goldschmidt et al., 2009; Patrikios et al., 2006; Prineas et al., 1993; Wolswijk, 1998). Remyelination failure has been attributed to processes associated with aging (Chang, Nishiyama, Peterson, Prineas, & Trapp, 2000; Chang, Tourtellotte, Rudick, & Trapp, 2002; Foote & Blakemore, 2005b; Kuhlmann et al., 2008; Shen, Liu, Li, Wolubah, & Casaccia-Bonnefil, 2008a; Sim et al., 2002a; Wolswijk, 1998, 2000; Woodruff et al., 2004; Zhao, Fancy, Kotter, Li, & Franklin, 2005). Older animals show impairments in the recruitment of macrophages and histone deacetylases (HDAC), both key factors in the road to remyelination. Aging also reduces the sensitivity of OPCs to growth factors, which promote the transition of OPCs into a mature myelinating phenotype. Old

age has also been shown to decrease myelin integrity and white matter volumes in healthy adults, making it a limiting factor for both remyelination and white matter plasticity (Sim, Zhao, Penderis, & Franklin, 2002b; van Wijngaarden & Franklin, 2013).

Successful remyelination is needed to prevent the irreversible cognitive and physical symptoms characteristic of the later stages of MS (Liebetanz & Merkler, 2006). Similarly, disruptions in white matter integrity have been associated with the cognitive decline often observed in the later stages of adulthood (reviewed in (Hinman & Abraham, 2007). Myelin provides axons with trophic signals necessary for the maintenance of their functional and morphological integrity (Lappe-Siefke et al., 2003; Yin et al., 2006). Lack of myelination can cause severe axonal pathology, resulting in atrophy and, ultimately, degeneration (Gensert & Goldman, 1997; Goldschmidt et al., 2009; Lappe-Siefke et al., 2003; Patrikios et al., 2006; Wolswijk, 1998; Yin et al., 2006). Remyelination of demyelinated axons is necessary for functional recovery (Liebetanz & Merkler, 2006) and the task is undertaken by OPCs rather than by pre-existing oligodendrocytes that have survived the injury, further linking remyelination and white matter plasticity (Blakemore & Keirstead, 1999; Dawson et al., 2003a; Gensert & Goldman, 1997; Keirstead & Blakemore, 1997; Keirstead et al., 1998; Levine, Reynolds, & Fawcett, 2001; Watanabe et al., 2002).

1.2 General properties of OPCs

Developmental and adult OPCs respond similarly to naked axons. In both cases, OPCs increase their rate of proliferation (Carroll & Jennings, 1994; Carroll, Jennings, & Ironside, 1998; Ffrench-Constant & Raff, 1986; Gensert & Goldman, 1997; Keirstead et al., 1998; Redwine & Armstrong, 1998), migrate into areas lacking myelination (Franklin & Blakemore, 1997), differentiate into mature myelinating oligodendrocytes (Deloulme et al., 2004; Kuhlmann

et al., 2008), and myelinate axons (Caillava et al., 2011; Fancy et al., 2011). Developmental and adult OPCs also follow the same differentiation and maturation program (Fancy et al., 2011). Indeed, as they mature along the oligodendroglial lineage, both developmental and adult OPCs progressively downregulate the expression of the platelet-derived growth factor receptor alpha (PDGFRα) and the chondroitin sulfate proteoglycan neuron-glia antigen 2 (NG2) in favour of the oligodendrocyte marker O4, which is characteristic of an immature, premyelinating state (Fig. 2 #1, 2, 5) (Nishiyama, 2007; Taupin, 2010). As they progress toward a fully mature oligodendroglial phenotype, immature oligodendrocytes downregulate O4 and start expressing myelin-specific proteins such as myelin oligodendrocyte glycoprotein, proteolipid protein (PLP), myelin basic protein (MBP), cyclic nucleotide 3-phosphohydrolase (CNPase), glutathione S-tranferase pi (GSTπ), and galactocerebroside (Fig. 2 #6) (Deloulme et al., 2004; Labombarda et al., 2009; Nishiyama, 2007; Polito & Reynolds, 2005; Taupin, 2010). As the cells progress through the oligodendrocyte lineage, their morphology also changes, from a bipolar shape at the unactivated OPC stage to a multipolar, branch-like appearance in the later stages of maturation (Fig. 2 #1, 2, 5) (Baer et al., 2009; Watanabe, Hadzic, & Nishiyama, 2004; Xiao et al., 2010). Despite the fact that developmental and adult myelination do share some common features, including the progenitors' response to naked axons and the differentiation program that they take on (as reviewed by (Mitew et al., 2013), characteristics unique to adult OPCs and the mature environment in which they evolve make myelination occurring in adulthood quite different than what is observed in development, as will be discussed in the following sections.

1.3 The adult myelinating environment

Despite the fact that developmental and adult myelination do share some common features, adult OPCs are functionally different from their embryonic and early post-natal

counterparts, the cell cycle of adult OPCs being considerably longer than that of developmental OPCs (Shi, Marinovich, & Barres, 1998; Woodruff et al., 2004). The presence of established neural networks also makes the adult environment very different than the one found in development. For example, several factors involved in developmental myelination negatively affect differentiation of OPCs into mature myelinating oligodendrocytes when they are re-expressed in the context of demyelination (Baer et al., 2009; Kuhn, Winkler, Kempermann, Thal, & Gage, 1997; Miller & Mi, 2007; Piaton, Gould, & Lubetzki, 2010). These factors include hyaluronan, produced by reactive astrocytes in the context of demyelination (Back et al., 2005), as well as the activation of the Notch (John et al., 2002; Stidworthy et al., 2004; Zhang et al., 2009) and Wnt signalling pathways (Fancy et al., 2009; Feigenson, Reid, See, Crenshaw, & Grinspan, 2011). Furthermore, denuded axons may have been altered during the process of demyelination or in its aftermath, which makes them different from the state in which they were during development, and these changes may affect ensheathment efficiency (Piaton et al., 2010).

The demyelinated environment is also different from the developmental one because of the presence of immune and inflammatory factors that are both a cause and a consequence of demyelination in MS and in many experimental models of the disease (Fancy et al., 2011). Such factors may also be involved in white matter plasticity because the presence of T-cell lymphocytes appears to be required for spatial learning and other forms of neural plasticity (Ziv, Avidan, Pluchino, Martino, & Schwartz, 2006). Another distinctive feature of the demyelinated environment is the accumulated myelin debris, which triggers an aggressive macrophage response that contributes to the inflammatory profile of MS lesions (Kotter, Li, Zhao, & Franklin, 2006). Myelin-associated proteins inhibit the maturation and terminal differentiation of OPCs, and clearance of myelin debris is therefore a prerequisite to successful regeneration

(Plemel, Manesh, Sparling, & Tetzlaff, 2013; Robinson & Miller, 1999; Syed et al., 2008). Microglia may play a role similar to that of macrophages in white matter plasticity, as both myelin remodelling and normal ageing are associated with loss of myelin (Tang, Nyengaard, Pakkenberg, & Gundersen, 1997; Young et al., 2013). By effectively clearing myelin debris and secreting a variety of extrinsic factors that are known to modulate OPC dynamics, acute inflammation promotes remyelination, as will be discussed in further detail later (Arnett, Wang, Matsushima, Suzuki, & Ting, 2003; Franklin & Kotter, 2008; Kotter, Setzu, Sim, Van Rooijen, & Franklin, 2001; Kotter, Stadelmann, & Hartung, 2011; Kotter et al., 2005; Larsen, Wells, Stallcup, Opdenakker, & Yong, 2003; Li, Setzu, Zhao, & Franklin, 2005; Loughlin et al., 1997; Sim et al., 2002b).

For all of these reasons, white matter plasticity may be closer to remyelination than to developmental myelination and may thus be influenced by some of the same factors. It could also be hypothesized that OPCs involved in white matter plasticity adopt an intrinsic program similar to the one that steers remyelination. It must be noted that not one single factor is responsible for remyelination and that the absence of one factor is usually compensated by another (Kotter et al., 2011; Talbott et al., 2007). When a demyelinating insult strikes the adult brain, OPCs respond in a three-step sequence regulated by a complex pattern of signalling events that act in a coordinated manner to ensure repair (Franklin, 2002; Talbott et al., 2007; Zhao et al., 2005). Repair is achieved through OPC activation, recruitment, and, finally, differentiation into a mature oligodendroglial phenotype. In the case of white matter plasticity, similar intrinsic and extrinsic signals may influence OPCs to improve, rather than restore, neural transmission and cortical functioning.

2. OPC response to demyelination

2.1 OPC activation

OPC activation is the first step of the remyelination process undertaken by OPCs. OPC activation involves a phenotypic switch allowing progenitors to exit the mitotically quiescent state they adopt in the absence of pro-myelinating factors in favour of a proliferative and responsive one (Fig. 2 #1, 2) (Franklin & Ffrench-Constant, 2008; Franklin & Kotter, 2008). OPC activation is a complex process triggered by the infiltration of blood-borne immune cells within the brain parenchyma and the subsequent demyelination and inflammatory response, consisting in the activation of macrophages, astrocytes and microglial cells (Adams et al., 1989; Glezer, Lapointe, & Rivest, 2006; Prineas & Graham, 1981; Rhodes, Raivich, & Fawcett, 2006; Sririam & Rodriguez, 1997). The factors secreted by reactive astrocytes and microglia activate OPCs in a highly focal manner, with only OPCs found directly in the demyelinated region responding to these signals (Fig. 1 #2, 3) (Reynolds et al., 2002).

OPC activation develops in response to the presence of reactive microglia and astrocytes rather than to inflammation per se (Cenci di Bello, Dawson, Levine, & Reynolds, 1999; Keirstead et al., 1998; Levine & Reynolds, 1999; Nishiyama, Yu, Drazba, & Tuohy, 1997). Consequently, OPC activation can occur in the absence of demyelination (Cenci di Bello et al., 1999; Keirstead et al., 1998; Levine & Reynolds, 1999; Nishiyama et al., 1997). However, the transient influx of blood-derived inflammatory cells that come with demyelination stimulate microglia and reactive astrocytes, along with the recently infiltrated macrophages, to secrete various mitogens and cytokines that activate OPCs, a prerequisite to cell cycle entry (Fig. 1 #4-6) (Bieber, Kerr, & Rodriguez, 2003; Kerschensteiner et al., 1999; Reynolds et al., 2002). Mice lacking or that have lower levels of T-cell lymphocytes do not remyelinate as efficiently as wild

type mice (Bieber et al., 2003; Chari, Zhao, Kotter, Blakemore, & Franklin, 2006; Kerschensteiner et al., 1999; Kotter et al., 2001; Kotter et al., 2005; Li et al., 2005). Furthermore, mice engineered to have T-cell lymphocytes that are not specific for myelin antigens do not remyelinate (Hvilsted Nielsen, Toft-Hansen, Lambertsen, Owens, & Finsen, 2011). Conversely, mice with T-cells specific for the myelin-specific antigen PLP show a 25 percent increase in oligodendrocyte numbers in comparison to lesioned wild type mice (Hvilsted Nielsen et al., 2011). Myelin-specific antibodies infiltrating the brain along with lymphocytes have also been shown to stimulate macrophages to increase myelin debris clearance, a key component of repair (Mosley & Cuzner, 1996). Thus, the cells that are causing demyelination also work to promote repair.

Several genes involved in the differentiation of OPCs into oligodendrocytes during development are also upregulated during the activation phase of remyelination. Examples include NG2 (Levine & Reynolds, 1999; Levine et al., 2001; Nishiyama et al., 1997), the basic helix-loop-helix (bHLH) transcription factors 1 and 2 (Olig1; Olig2) (Fancy, Zhao, & Franklin, 2004; Glezer et al., 2006; Talbott et al., 2005), and the homeodomain transcription factor Nkx2.2 (Fig. 2. #1, 2) (Fancy et al., 2004; Talbott et al., 2005; Watanabe et al., 2004). The phenotypic switch during OPC activation also involves morphological changes, with OPCs increasing in size and gaining more processes once they become activated (Levine & Reynolds, 1999; Nishiyama et al., 1997; Reynolds et al., 2002).

In summary, a demyelinating insult characterized by the infiltration of T-cell lymphocytes triggers a strong inflammatory response. This response includes an increased rate of proliferation and myelin debris clearance by macrophages (Mosley & Cuzner, 1996), as well as an increase in the release of mitogens and cytokines by macrophages, microglia, and reactive

astrocytes (Kerschensteiner et al., 1999; Nathan, 1987). In addition to further activating the glial cells that produced them (Feder & Laskin, 1994; Kerr & Patterson, 2004; Sugiura et al., 2000), mitogens and cytokines upregulate the expression of key transcription factors within OPCs that allow them to re-enter the cell cycle and proceed to the recruitment phase of remyelination (Cui et al., 2004; Fancy et al., 2004; Levine & Reynolds, 1999; Nishiyama et al., 1997). Thus, the initial step of the remyelination process, OPC activation, is highly dependent on the innate immune response to demyelination and proper regulation of this process is essential to repair. Not all immune responses to demyelination are likely to be present in white matter plasticity, but some most likely are. For example, T-cell lymphocytes and activated microglial cells are required for learning- and environmental enrichment-dependent plasticity to take place (Ziv et al., 2006). Inflammatory cells are also involved in the clearance of the generated myelin debris during myelin remodelling. Finally, OPC activation in white matter plasticity may be mediated through neurotransmitter release: OPCs possess receptors for glutamate and GABA and there is evidence that activation of such receptors triggers the intrinsic program allowing OPCs to enter the recruitment and differentiation phases of adult myelination (Bergles, Roberts, Somogyi, & Jahr, 2000; Lin & Bergles, 2004; Luyt et al., 2007).

2.2 The recruitment phase

The recruitment phase of remyelination is two-fold and consists in the proliferation and migration of OPCs into areas of demyelination (Fig. 1 #6, 7) (Hinks & Franklin, 1999; Levine & Reynolds, 1999; Redwine & Armstrong, 1998; Sim et al., 2002b). At the core of this process lies the immune response which triggers the release of growth factors (Hinks & Franklin, 1999), cytokines (Nathan, 1987), and matrix metalloproteinases (MMPs) (DaSilva & Yong, 2008; Larsen et al., 2003; Skuljec et al., 2011; Ulrich et al., 2006; Vos, van Haastert, de Groot, van der

Valk, & de Vries, 2003). Together, these secretory factors recruit a number of activated OPCs into demyelinated areas by increasing their rate of proliferation (Redwine & Armstrong, 1998) and/or by facilitating the migration of the progenitors (Omari, John, Lango, & Raine, 2006; Skuljec et al., 2011). Factors that are more closely related to the demyelinated axons promote this process (Nait Oumesmar et al., 1995; Piaton et al., 2011; Spassky et al., 2002). Immune-mediated lesions cause a localized burst of OPC proliferation, which results in a 3-fold increase in OPC numbers within days of a demyelinating event (Cenci di Bello et al., 1999). The number of new OPCs produced during proliferation approximates the number of OPCs required to remyelinate a given demyelinated area (Levine & Reynolds, 1999; Reynolds et al., 2002). Activated microglial cells are also recruited in response to environmental enrichment and spatial learning (Ziv et al., 2006) and these types of neural and cognitive stimulating activities have been shown to increase OPC proliferation in the healthy adult brain (Ehninger et al., 2011). Thus, the factors secreted by activated glial cells may also be involved in OPC-mediated white matter plasticity.

2.2.1 Growth factors in OPC recruitment

Activated OPCs are more sensitive to mitogens secreted by reactive astrocytes, macrophages, and microglia, and, conversely, mitogens increase the proliferative activity of OPCs (Franklin & Kotter, 2008). Thus, it is growth factors, rather than the innate mitogenic potential of OPCs, that regulate oligodendroglial phenotypic plasticity in adult human cells (Gogate et al., 1994). This could apply to white matter plasticity as exercise, which upregulates growth factor expression, has been shown to increase white matter volumes in adults (Kramer & Erickson, 2007). Decreased levels of growth factors in later stages of adulthood have also been linked to reduced levels of myelination in both the healthy and diseased CNS (Hinks & Franklin,

2000; Tang et al., 1997). In demyelinating disease, exogenous administration of a growth factor cocktail composed of insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and neurotrophin-3 (NT-3) has been shown to promote OPC proliferation and, consequently, repair (Kumar, Biancotti, Yamaguchi, & de Vellis, 2007), thus further supporting the role of growth factors as key regulators of adult OPC recruitment.

PDGF is a growth factor produced specifically by reactive astrocytes following demyelination (Hinks & Franklin, 1999; Kotter et al., 2005). PDGF, its receptor PDGFRα, and NG2 interact to control OPC proliferation in the developing brain as well as in response to experimentally-induced demyelination (Bogler, Wren, Barnett, Land, & Noble, 1990; Frost, Nielsen, Le, & Armstrong, 2003; Mckinnon, Matsui, Duboisdalcq, & Aaronson, 1990; Wolswijk & Noble, 1992). Since PDGF deficiency by itself is sufficient to downregulate OPC repopulation of demyelinated lesions, it appears that PDGF plays a central role in remyelination (Murtie, Zhou, Le, Vana, & Armstrong, 2005). Cultured adult OPCs exposed to PDGF increase their rate of proliferation and also show an increased propensity for migration (Wolswijk & Noble, 1992). Overexpression of PDGF in transgenic mice promotes cell survival during the recovery period of experimentally-induced demyelination and, as a consequence, improves repopulation of demyelinated areas (Vana et al., 2007a). Finally, aged animals experience a delay in PDGF expression that corresponds closely to the delayed repair following a demyelinating event (Hinks & Franklin, 2000; Sim et al., 2002b).

The trophic activity of PDGF is regulated by its receptor, PDGFRα, which is expressed specifically by OPCs (Hart, Richardson, Heldin, Westermark, & Raff, 1989). PDGFRα activation is thought to inhibit the premature differentiation of OPCs, thus promoting

proliferation (Calver et al., 1998; Noble, Murray, Stroobant, Waterfield, & Riddle, 1988). OPC densities are decreased in the absence of PDGFRα (Murtie et al., 2005). Furthermore, PDGFRα overexpression results in increased levels of OPC proliferation in both acute and chronic demyelinated lesions (Vana et al., 2007a; Woodruff et al., 2004). Peak levels of PDGFRα expression are attained when OPCs are at the height of their proliferative activity following a demyelinating event (Redwine & Armstrong, 1998; Woodruff et al., 2004).

PDGF and PDGFRα are thought to regulate OPC proliferation through another OPC marker, NG2 (Asher et al., 2005). NG2 and PDGFRα bind to form a complex within OPCs that efficiently delivers PDGF to its receptor (Nishiyama, Lin, Giese, Heldin, & Stallcup, 1996). The rate of NG2 shedding, which refers to the proteolytic release of the ectodomain of the proteoglycan, is upregulated in parallel with increases in cell density within lesions. While OPCs continue to express PDGFRα as they differentiate, they are no longer sensitive to the trophic effects of its ligand because the absence of NG2 blocks PDGF signalling. OPCs that no longer express NG2 fail to proliferate, even in the presence of PDGF (Asher et al., 2005).

While the rate of proliferation and motility of OPCs are increased in the presence of PDGF, the increase is greater when PDGF is presented in combination with bFGF (Murtie et al., 2005; Wolswijk & Noble, 1992). Expression of both mitogens is greatly increased during the recruitment phase of remyelination (Hinks & Franklin, 1999). Like PDGF, bFGF can also maintain cultured OPCs in an undifferentiated, highly proliferative state (Kotter et al., 2011). Expression of PDGFRα and NG2 by OPCs is increased in the presence of bFGF (Mckinnon et al., 1990). Thus, in addition to having direct trophic effects on OPCs (Frost et al., 2003; Mckinnon et al., 1990; Wolswijk & Noble, 1992), bFGF may promote the proliferation and motility of OPCs by increasing their sensitivity to PDGF (Messersmith, Murtie, Le, Frost, &

Armstrong, 2000). Also, bFGF knock-out does not exacerbate the decreased levels of OPC proliferation and cell density observed in PDGFRα null mice. Thus, PDGF and bFGF may influence OPC proliferation through different pathways (Murtie et al., 2005). Since bFGF knock-out impairs OPC differentiation in experimentally-induced models of demyelination but has no effect on OPC density (Oh et al., 2003), it most likely inhibits OPC differentiation while PDGF acts as the primary regulator of OPC cell division.

While PDGF and bFGF have garnered a lot of interest due to their action on the OPC-specific receptor PDGFRα, other classes of growth factors have also been shown to modulate OPC recruitment. Neurotrophins are a family of growth factors that include, amongst others, the brain-derived neurotrophic factor (BDNF) and NT-3. BDNF and NT-3 have both been shown to facilitate proliferation and differentiation of OPCs (Barres et al., 1994b; Barres, Schmid, Sendnter, & Raff, 1993; McTigue, Horner, Stokes, & Gage, 1998). However, only BDNF is increased in response to a demyelinating insult (Hinks & Franklin, 1999; Ikeda et al., 2001). T-cell lymphocytes have been found to be required for BDNF expression in the healthy adult brain during spatial learning. Since BDNF is thought to be associated with the molecular and cellular changes required for learning to take place, it is probable that it is also involved in white matter plasticity (Ziv et al., 2006). It is likely that NT-3 exerts its trophic effects on OPCs through the cAMP response element-binding protein 1 (CREB-1). Prolonged activation of CREB-1 has been observed in response to a growth factor cocktail containing NT-3 in experimentally-induced models of demyelination. The increase in proliferation was partially attributed to NT-3 because it has the ability to phosphorylate the neurotrophic tyrosine kinase receptor 3 (TrkC) which is found on OPCs (Kumar et al., 2007). TrkC phosphorylation is thought to activate the mitogen-activated protein kinase (MAPK)/protein kinase C (PKC) pathway, which then activates CREB-

1. Activation of CREB-1 favours DNA synthesis and, thus, proliferation of OPCs (Johnson, Chu, & Sato-Bigbee, 2000).

IGF-1 also appears to be implicated in OPC recruitment following a demyelinating insult. IGF-1 mRNA and protein levels are upregulated early in response to experimentally-induced demyelination, and this increase coincides with the recruitment of OPCs within areas of demyelination. IGF-1 increases the proliferation of OPCs both *in vitro* (Heerens, Ye, & D'Ercole, 2002) and in response to demyelination *in vivo* (Mason et al., 2000; Ye, Li, Richards, DiAugustine, & D'Ercole, 2002). IGF- 1 may also have a protective effect on the brain as experimental knockout of the receptor IGFR1 increases the levels of OPC apoptosis in response to a demyelinating injury (Barres et al., 1993; Mason, Xuan, Dragatsis, Efstratiadis, & Goldman, 2003; Ness & Wood, 2002; Ye & D'Ercole, 1999). It is hypothesized that IGF-1's pro-survival effect counterbalances the cytotoxic activity of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα): IGF-1 has been shown to inhibit TNFα-induced OPC cell death *in vitro* (Ye & D'Ercole, 1999). In addition to lending support to the pro-remyelination properties of IGF-1, this evidence further supports the notion that remyelination requires a finely tuned orchestration of the numerous factors present in the lesion environment to be successful.

2.2.2 Cytokines in OPC recruitment

Cytokines are signalling molecules that, when secreted by immune cells in response to insults such as demyelination, trigger inflammatory responses (Fig. 1 #4) (Franklin & Kotter, 2008; Zhao, Li, & Franklin, 2006). Once activated, microglial cells can produce proinflammatory cytokines of their own (Mason, Suzuki, Chaplin, & Matsushima, 2001; Nathan, 1987). Cytokine expression is upregulated in the cerebrospinal fluid of MS patients (Ip, Wiegand, Morse, & Rudge, 1993) and remyelination is impaired in their absence (Arnett et al.,

2001; Glezer et al., 2006; Mason et al., 2001). Inflammatory cytokines are believed to be key players in the promyelinating environment required for repair in response to acute MS lesions. Specifically, they are thought to be responsible for OPC mobilization within demyelinated lesions (Arnett et al., 2001; Glezer et al., 2006; Mason et al., 2001).

Like many proinflammatory cytokines, TNFα is believed to have both positive and negative effects on regeneration following demyelination, the type of effect depending on the activated TNFα receptor (Armstrong, 2007; Locklsey, Killeen, & Lenardo, 2001). TNFα is thought to promote remyelination by inducing OPC activation, recruitment, and proliferation through TNF receptor 2 (TNFR2) activation in acute models of demyelination (Akassoglou et al., 1998; Arnett et al., 2001; Declercq, Denecker, Fiers, & Vandenabeele, 1998; Eugster et al., 1999; Haridas, Darnay, Natarajan, Renu, & Aggarwal, 1998; Suvannavejh et al., 2000; Weiss et al., 1998). TNFα also mediates demyelination by inducing cell death through TNF receptor 1 (TNFR1) activation (Akassoglou et al., 1998; Arnett et al., 2001; Declercq et al., 1998; Eugster et al., 1999; Haridas et al., 1998; Suvannavejh et al., 2000; Weiss et al., 1998). TNFR2 null mice show lower levels of remyelination, similar to those of TNFα null mice in response to experimentally-induced demyelination, while remyelination in TNFR1 null mice is comparable to that of wild type mice (Arnett et al., 2001; Eugster et al., 1999; Liu et al., 1998; Suvannavejh et al., 2000). TNFα, along with TNFR2 but not TNFR1, is upregulated in response to demyelination and during the early stages of remyelination in experimentally-induced models of demyelination (Arnett et al., 2001; Voss et al., 2012). Elevated levels of TNFα are also found within and in proportion to the severity of active MS lesions (Beck et al., 1988; Maimone, Gregory, Arnason, & Reder, 1991; Selmaj, Raine, Cannella, & Brosnan, 1991; Sharief, Phil, & Hentges, 1991). While TNFα has overwhelmingly been associated with tissue injury, it has also

been found to be upregulated in response to physiological activity. The cytokine has specifically been hypothesized to regulate the synaptic plasticity associated with exercise (Albensi & Mattson, 2000), thereby suggesting that it may also play a role in exercise-induced white matter plasticity (Kramer & Erickson, 2007).

The IL-6 superfamily of cytokines includes such factors as the leukemia inhibitory factor (LIF) and the ciliary neurotrophic factor (CNTF). While IL-6 levels are very low in the healthy adult brain, they are upregulated in response to inflammation, thus implicating the cytokine in demyelinating disease and possibly also myelin remodelling (Brown, Metcalf, & Gough, 1994). LIF and CNTF enhance the proliferation, differentiation, and maturation of cultured OPCs into mature myelinating oligodendrocytes (Barres et al., 1996; Mayer, Bhakoo, & Noble, 1994; Talbott et al., 2007). They also promote the survival of cultured OPCs (Albrecht et al., 2003; Barres et al., 1993; Mayer et al., 1994) and oligodendrocytes (Barres et al., 1993; Kerr & Patterson, 2005; Talbott et al., 2007). While LIF can exert these same effects *in vivo*, exogenously administered CNTF does not replicate the *in vitro* findings (Deverman & Patterson, 2012; Talbott et al., 2007). Furthermore, the increase in the rate of proliferation produced by those cytokines is only observed in the presence of PDGF (Barres et al., 1996; Deverman & Patterson, 2012). Thus, IL-6 cytokines might promote OPC cell division by increasing their sensitivity to growth factors or by activating them, rather than by acting directly on cell cycle machinery (Barres et al., 1996; Deverman & Patterson, 2012). CNTF in particular appears to influence remyelination rather indirectly because it is only upregulated concurrently with bFGF and contributes to repair through activation of the IGFR1 on oligodendroglial cells (Jiang, Levison, & Wood, 1999). Even so, experimental demyelination is severe in CNTF null mice and

they show a large decrease in the number of proliferating OPCs and a significant increase in the rate of oligodendroglial apoptosis (Linker et al., 2002).

Chemokines, which constitute a superfamily of small (8-10 kDa) proinflammatory cytokines secreted primarily by reactive astrocytes, also play an important role in the recruitment phase of remyelination (Glezer et al., 2006; Omari, Lutz, Santambrogio, Lira, & Raine, 2009). While astrocytes in MS lesions have been shown to express many chemokines of the C-X-C motif (CXC) including CXC ligand 1 (CXCL1), CXC ligand 8 (CXCL8), and CXC ligand 10 (CXCL10), only CXCL1 has been shown to functionally influence OPCs through chemokine receptor type 2 (CXCR2) signalling (Tsai et al., 2002). CXCR2 expression on OPCs is upregulated during de- and remyelination in MS tissue, as well as in response to experimentally-induced white matter insults (Glabinski, O'Bryant, Selmaj, & Ransohoff, 2000; Lindner et al., 2008; Nguyen & Stangel, 2001; Tsai et al., 2002; Williams, Piaton, & Lubetzki, 2007b). The increase in CXCL1-positive astrocytes at the lesion margin leads to the accumulation and recruitment of CXCR2-positive OPCs toward MS lesions by way of chemoattraction, which ultimately facilitates repair (Fig. 1 #4, 6, 7) (Omari et al., 2006; Omari, John, Sealfon, & Raine, 2005; Omari et al., 2009). CXCL1 has also been shown to promote the proliferation of OPCs in culture in the presence of PDGF (Robinson, Tani, Strieter, Ransohoff, & Miller, 1998). Overexpression of CXCL1 has been shown to have the same effect (Omari et al., 2009). However, CXCR2 null mice show normal remyelination in response to cuprizone treatment (Lindner et al., 2008), thereby suggesting the presence of receptor redundancy in chemokine signalling.

2.2.3 MMPs and the extracellular matrix in OPC recruitment

In addition to mitogens and cytokines, activated microglia and macrophages also secrete MMPs in response to demyelination (Fig. 1 #4, 7) (DaSilva & Yong, 2008; Larsen et al., 2003; Skuljec et al., 2011; Ulrich et al., 2006; Vos et al., 2003). The proteolytic properties of MMPs have both negative and positive effects on adult myelination. MMPs exacerbate demyelination through breakdown of the blood-brain barrier and degradation of myelin-specific proteins (Shiryaev et al., 2009; Skuljec et al., 2011; Vos et al., 2000; Yong, Power, Forsyth, & Edwards, 2001). On the other hand, MMPs create an environment favourable to remyelination and, possibly, white matter plasticity through cleavage of extracellular matrix components, thereby facilitating the migration of phagocytic macrophages and OPCs (Larsen et al., 2003; Li et al., 2005; Skuljec et al., 2011). Cleavage of myelin proteins by MMPs also facilitates myelin debris clearance by phagocytic macrophages, which positively influences maturation along the oligodendroglial lineage (Kotter et al., 2006; Shiryaev et al., 2009). Furthermore, the proteolytic activity of MMP-3 has been specifically implicated in the release of growth factors as it works to increase the bioavailability of free, intact forms of mitogens such as IGF (Dubois-Dalcq & Murray, 2000; Fowlkes et al., 2004; McCawley & Matrisian, 2001).

Extracellular matrix molecules are expressed at high levels during regeneration and integrins are their principal receptors (Hynes, 1992; Zhao, Fancy, Franklin, & ffrench-Constant, 2009). Integrins are transmembrane receptors expressed by OPCs and ligand binding creates a signalling complex that affects multiple levels of cell functioning (Hynes, 2002; Zhao et al., 2009). OPCs upregulate αVintegrin expression in response to a demyelinating insult and activation of the receptor contributes to the migration, proliferation, differentiation, and maturation of OPCs along the oligodendrocyte cell lineage (Blaschuk, Frost, & ffrench-Constant,

2000; Milner, Edwards, Streuli, & Ffrench-Constant, 1996; Zhao et al., 2009). For example, αVintegrins interact with growth factor receptors to regulate migration and proliferation of OPCs (Baron, Colognato, & Ffrench-Constant, 2005). αVintegrins also form a complex with the myelin-specific protein PLP that mediates OPC migration. This has direct implications for white matter plasticity because αVintegrin/PLP-mediated OPC migration is stimulated by glutamate receptor activation, which is directly linked to neural activity (Gudz, Komuro, & Macklin, 2006).

Osteopontin is a secreted extracellular matrix glycoprotein that acts as a modulator of immune function (Chabas et al., 2001; Hur et al., 2007; Zhao, Fancy, ffrench-Constant, & Franklin, 2008). In experimental models of demyelination, osteopontin is secreted by activated microglia and macrophages, reactive astrocytes, and some neurons (Chabas et al., 2001; Selvaraju et al., 2004; Zhao et al., 2008). Like other extracellular matrix-associated molecules, it has been linked with exacerbation of disease severity as it promotes the survival of T-cell lymphocytes (Hur et al., 2007). However, its chemotactic properties contribute to repair because osteopontin can recruit macrophages and astrocytes into lesion sites where they will secrete mitogens and other factors necessary for OPC-mediated regeneration (Selvaraju et al., 2004). The chemotactic properties of osteopontin also promote OPC migration and, consequently, the repopulation of demyelinated areas by OPCs (Selvaraju et al., 2004). Osteopontin accumulates more slowly in older animals in response to toxin-induced demyelination (Selvaraju et al., 2004), which supports the hypothesis that it is also involved in OPC recruitment (Zhao et al., 2008).

2.2.4 Axonal signals in OPC recruitment

Factors related to axons are also thought to play a role in the repair process undertaken by OPCs in response to demyelination (Fig. 1 #6, 7). Semaphorins are a class of secreted and membrane proteins that primarily act as guides to axonal growth cones (Chisholm & Tessier-

Lavigne, 1999). They also have co-receptors on oligodendroglial cells (Spassky et al., 2002). This, along with the fact that they have higher levels of expression in plastic regions of the adult brain, points to a potential role of semaphorins in white matter plasticity (de Wit & Verhaagen, 2003). Class 3 semaphorin levels are increased in actively demyelinating lesions (Piaton et al., 2011; Williams et al., 2007a). Semaphorin 3A is thought to be deleterious to remyelination as it inhibits OPC migration (Spassky et al., 2002), differentiation (Syed et al., 2008; Syed et al., 2011), and the formation axon-process contacts required for axonal ensheathment (Syed et al., 2011). However, semaphorin 3F is thought to counterbalance these effects by acting as a chemo-attractant and mitogenic factor to activated OPCs (Boyd, Zhang, & Williams, 2013; Piaton et al., 2011; Spassky et al., 2002; Sugimoto et al., 2001). OPC recruitment and, consequently remyelination, is increased by semaphorin 3F overexpression and semaphorin 3A downregulation (Boyd et al., 2013; Piaton et al., 2011). Semaphorin 3F is thus considered to be an important factor in promoting remyelination following a demyelinating insult and proper regulation of class 3 semaphorins appears to be crucial to repair (Boyd et al., 2013).

The polysialylated-neural cell adhesion molecule (PSA-NCAM) is expressed by axons devoid of myelin sheaths during development and in response to demyelination (Charles et al., 2002). During development, PSA-NCAM is thought to act as a negative regulator of myelination as it prevents oligodendrocyte processes from attaching to axons, thus making loss of PSA-NCAM expression a prerequisite to myelination (Charles et al., 2002). While this may also apply to adult myelination, the pro-migratory properties of PSA-NCAM have been hypothesized to promote repair by recruiting OPCs and other neural progenitor cells into sites of injury, thereby facilitating repopulation of demyelinated areas (Nait Oumesmar et al., 1995; Picard-Riera et al., 2002). Specifically, post-natal PSA-NCAM is thought to guide OPCs toward sources of PDGF

(Zhang, Vutskits, Calaora, Durbec, & Kiss, 2004), a role that could also apply to white matter plasticity.

2.2.5 Cyclin-dependent kinases in OPC recruitment

Cyclin-dependent kinases act as the core regulators of cell cycle machinery, responding to a variety of intra- and extra-cellular signals that both initiate and promote cell cycle progression (Caillava & Baron-Van Evercooren, 2012). Since PDGF induces OPCs to undergo a certain number of cell divisions, it may be responsible for the formation cyclin-dependent kinase complexes that regulate the proliferation of activated OPCs (Paez, Garcia, Soto, & Pasquini, 2006; Tang, Tokumoto, Apperly, Lloyd, & Raff, 2001). bFGF may also be involved in this process (Paez et al., 2006). Activation of cyclin-dependent kinases in the G1 phase of mitosis triggers the phosphorylation of the retinoblastoma protein, which in turn promotes the transcription of genes responsible for S phase entry (Caillava & Baron-Van Evercooren, 2012). Cyclin-dependent kinase-2 (Cdk2) specifically regulates cell cycle progression in OPCs (Fig. 2 #2) (Belachew et al., 2002). OPC proliferation is reduced in the absence of Cdk2 and this decrease correlates with enhanced cell cycle exit, with cells prematurely committing to the OPC or mature oligodendrocyte lineage (Belachew et al., 2002; Caillava et al., 2011; Jablonska et al., 2007). However, remyelination in Cdk2 null mice is delayed and not entirely blocked (Caillava et al., 2011). Thus, Cdk2 appears to increase OPC sensitivity to injury-induced signals of proliferation thereby modulating the timing of their differentiation into myelin-producing oligodendrocytes.

Clearly, the recruitment phase of the remyelinating process taken on by OPCs involves a large number of factors with interlocking influences that must work in a coordinated manner to ensure efficient repair. There is reason to believe that a number of these factors are also involved

in white matter plasticity. For example, the growth factors released in response to a variety of neural activities may encourage DNA synthesis and increase the sensitivity of OPCs to a number of recruitment-promoting extrinsic signals (Caillava et al., 2011; Paez et al., 2006). The actions of the many factors listed above, together with the added influence of others, trigger the differentiation phase when actively proliferating OPCs become mature, myelinating oligodendrocytes.

2.3 The differentiation phase

The differentiation phase corresponds to the final phase of the myelination process when recruited OPCs exit the cell cycle to adopt an oligodendroglial phenotype and establish contacts with naked axons (Fig. 2 #5-7) (Fancy et al., 2011; Fancy et al., 2010; Miron, Kuhlmann, & Antel, 2011; Zhao et al., 2005). Once contact is established, the oligodendroglial processes ensheath the axons by wrapping themselves around them several times (Fancy et al., 2011; Fancy et al., 2010; Franklin, 2002; Miron et al., 2011). This is followed by the compaction of the myelin sheath, which enables proper saltatory conduction and restores the functional integrity of the axon (Fig. 2 #7) (Fancy et al., 2011; Fancy et al., 2010).

Differentiation of OPCs is controlled by a closely regulated intrinsic program dependent on a cell cycle timer that measures the elapsed time and number of cell divisions undergone by the cell. The cell cycle timer, while intrinsic, is not entirely cell autonomous (Tokumoto, Durand, & Raff, 1999). Extrinsic factors, specifically the thyroid hormone triiodothyronine (T3) and the withdrawal of PDGF, trigger cell cycle exit and initiate differentiation (Dugas, Ibrahim, & Barres, 2012; Temple & Raff, 1986; Tokumoto, Apperly, Gao, & Raff, 2002; Tokumoto et al., 1999; Tokumoto, Tang, & Raff, 2001). The Kip family of cyclin-dependent kinase inhibitors, along with p18/INK, most likely triggers cell-cycle exit and differentiation by inhibiting the

activity of Cdk2, which keeps OPCs in a proliferative state (Fig. 2 #3, 4) (Sherr & Roberts, 1999).Cyclin-dependent kinase inhibitors such as p57/Kip2, p27/Kip1, and p18/INK regulate OPC maturation along the oligodendroglial lineage (Dugas, Ibrahim, & Barres, 2007; Durand, Gao, & Raff, 1997; Kremer et al., 2009; Pfeifenbring et al., 2013). Indeed, *in vitro* findings show that p57/Kip2, p18/INK, and p27/Kip1 levels increase over time as OPCs proliferate, and levels of these cyclin-dependent kinase inhibitors regulate the number of cell divisions OPCs can go through before undergoing terminal differentiation (Dugas et al., 2007; Durand et al., 1997; Tokumoto et al., 2002). Thus, levels of p57/Kip2, p18/INK, and p27/Kip1 determine the length of the proliferative phase. When maximal levels are attained, these cyclin-dependent kinase inhibitors are downregulated, thereby triggering the onset of the differentiation phase of myelinating process. Consequently, a decrease in p57/Kip2 expression is associated with the first signs of repair in experimentally-induced demyelination (Kremer et al., 2009; Pfeifenbring et al., 2013). The Kip family of cyclin-dependent kinase inhibitors most likely triggers cell-cycle exit and differentiation by inhibiting the activity of Cdk2, which keeps OPCs in a proliferative state (Fig. 2 #3, 4) (Sherr & Roberts, 1999).

By increasing the expression of cyclin-dependent kinase inhibitors, both T3 and PDGF withdrawal can terminate the proliferative process (Fig. 2 #4). While thyroid hormone triggers an increase in a multitude of cell cycle inhibitory proteins, PDGF withdrawal only increases one of those proteins, p27/Kip1. Both T3 and PDGF withdrawal decrease cyclin levels, a family of proteins that interact with cyclin-dependent kinases to regulate cell cycle progression, but they influence cyclin expression in different ways (Tokumoto et al., 2002; Tokumoto et al., 1999; Tokumoto et al., 2001). Furthermore, PDGF withdrawal decreases the expression of two immediate early genes while T3 treatment does not (Tokumoto et al., 1999). Expression of

immediate early genes is thought to mediate the proliferative response to growth factors, thus making them key players in the recruitment, highly proliferative phase of the repair process (Angel & Karin, 1991; Tokumoto et al., 1999). They could also contribute to white matter plasticity since immediate early gene expression in adult OPCs are modulated by a variety of extrinsic stimuli that modify the electrical status of cells (Johnson et al., 2000; Tian & Bishop, 2002). Thus, T3- and growth factor-induced differentiation depend on different molecular pathways.

2.3.1 T3 in OPC differentiation

T3, which is thought to be regulated by immune factors (Pallinger & Csaba, 2008), puts an end to the timing component of the myelinating process by acting on nuclear receptors to override trophic activity in favour of differentiation, even in the presence of saturating levels of growth factors (Baas, Legrand, Samarut, & Flamant, 2002; Barres, Lazar, & Raff, 1994a; Durand & Raff, 2000). Thyroid hormone treatment upregulates the number of pre-oligodendrocytes, immature oligodendrocytes, and mature oligodendrocytes found within demyelinated lesions and accelerates remyelination (Calza, Fernandez, & Giardino, 2010; Calza et al., 2005; Fernandez et al., 2004; Franco, Silvestroff, Soto, & Pasquini, 2008; Silvestroff, Bartucci, Pasquini, & Franco, 2012). Similarly, OPCs in mice devoid of the thyroid receptor α1 are unable to exit the cell cycle to undergo terminal differentiation (Billon, Jolicoeur, Tokumoto, Vennstrom, & Raff, 2002). Thyroid hormone administration has specifically been shown to alter the expression of cell cycle clock-related genes *in vitro* (Dugas et al., 2012; Tokumoto et al., 2001). One such gene, the Krueppel-like factor 9 (KLF9), increases the expression of the myelin-specific proteins CNPase and MBP (Dugas et al., 2012).

Retinoid X receptors (RXRs) are transcription factors that form homodimers and heterodimers with other nuclear receptors, including thyroid hormone receptors (Huang & Franklin, 2011). Thus, RXRs may be one of the target receptors through which T3 triggers OPC differentiation. Treatment with retinoic acid has been shown to improve remyelination following toxin-induced demyelination. Specifically, RXR-gamma (RXRϒ) expression is upregulated in response to demyelination and throughout the process of remyelination in oligodendrocyte lineage cells. Conversely, differentiation is impaired when cultured OPCs are exposed to RXR antagonists and repair is delayed in RXRϒ null mice. Interestingly, location of RXRϒ varies according to phenotype of oligodendroglial cells. Cytosolic RXRϒ is thought to inhibit differentiation of activated OPCs while translocation to the nucleus leads to maturation along the oligodendrocyte lineage. Furthermore, localization of RXRϒ corresponds with lesion status in MS, with more nuclear RXRϒ being found in active, remyelinating lesions than in chronically demyelinated ones (Huang et al., 2011). T3 may influence white matter plasticity through RXR signalling in two ways. First, retinoic acid is implicated in the maturation of the post-natal cerebral cortex, a process that correlates with de novo and increased myelination in some areas of the brain (Wagner, Luo, & Drager, 2002). Secondly, expression of RXR-related proteins is higher in plastic regions of the brain where white matter plasticity may be at play (Haskell, Maynard, Shatzmiller, & Lamantia, 2002; Wagner, Luo, Sakai, Parada, & Drager, 2006).

Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors for thyroid hormones (Issemann & Green, 1990). PPARs and RXRs are known to act in concert to inhibit microglial activation and promote remyelination in MS (Hanafy & Sloane, 2011). Furthermore, PPAR expression is modulated by the proinflammatory cytokine TNFα, which is released as part of the immune reaction to demyelination (Cimini, Bernardo, Cifone, Di

Marzio, & Di Loreto, 2003), as well as in response to physiological activity, thereby suggesting that PPAR signalling may also be involved in white matter plasticity (Albensi & Mattson, 2000). PPAR-mediated regulation of target transcription genes requires heterodimerization by RXRs (Schulman, Shao, & Heyman, 1998). Specifically, heterodimerization of PPARs is involved in cellular maturation and process extension by oligodendroglial cells in experimental models of demyelination (Bernardo, Bianchi, Magnaghi, & Minghetti, 2009; Roth et al., 2003; Saluja, Granneman, & Skoff, 2001).

Thus, T3 contributes to both phases of the differentiation process. First, it modulates the expression of Cdk2 and overrides the expression of growth factors, thereby putting an end to the recruitment phase and triggering the start of final phase of adult myelination. After that, T3 stimulates the transcription of myelin-specific genes and, through downstream activation of PPARs, promotes formation of myelin sheaths proper by facilitating process extension, the first step toward axonal ensheathment.

2.3.2 Growth factors and cytokines in the differentiation phase

Several growth factors are involved in triggering the differentiation phase of remyelination (Hinks & Franklin, 2000). For example, in the absence of PDGF, OPCs rapidly stop dividing to undergo terminal differentiation (Barres et al., 1994a). Furthermore, since peak levels of IGF-1 expression coincide with both the accumulation of recruited OPCs and the first signs of myelin-specific proteins, IGF-1 has been hypothesized to act as a differentiation factor for activated adult OPCs (Hinks & Franklin, 1999; McMorris, Smith, DeSalvo, & Furlanetto, 1986; Saneto, Low, Melner, & de Vellis, 1988; Woodruff & Franklin, 1999). Further supporting this hypothesis is the observation that IGF-1 also promotes the differentiation of perinatal OPCs and of adult neural progenitor cells (Hsieh et al., 2004; McMorris & Dubois-Dalcq, 1988).

Because peak levels of expression of transforming growth factor beta-1 (TGF-β1) are also reached at the onset of the differentiation phase, it is thought to have a role similar to that IGF-1 (Hinks & Franklin, 1999). TGF-β1 is believed to promote cell-cycle exit and, consequently, the differentiation of activated OPCs (Franklin, 2002; McKinnon, Piras, Ida, & Dubois-Dalcq, 1993). In support of this hypothesis, an *in vitro* study has shown that in the presence of PDGF, TGF-β1 decreases OPC proliferation in favour of differentiation along the oligodendrocyte lineage (McKinnon et al., 1993). On the other hand, TGF-β1 expression is thought to have some deleterious effects on remyelination as bone morphogenic proteins (BMPs), the largest subclass of the TGF-β superfamily, have been shown to favour OPC differentiation into astrocytes rather than along the oligodendrocyte lineage (Cheng et al., 2007; Grinspan et al., 2000). Olig1, in collaboration with Olig2, is thought to counteract the deleterious effects of BMP signalling by interrupting the formation of a nuclear complex between BMPs and the glial fibrillary acidic protein (GFAP) promoter, thereby preventing the formation of a glial scar and promoting repair (Cheng et al., 2007).

The differential activity of cytokines is also thought to be involved in triggering and accelerating the differentiation process (Gottle et al., 2010; Hinks & Franklin, 2000; Kotter et al., 2011). In addition to its prominent role in the recruitment phase of remyelination, CXCL1 also participates in the differentiation process by stimulating the synthesis of MBP, a myelin-specific protein (Kadi et al., 2006). However, CXCL12 is the chemokine that is most directly involved in the differentiation phase of remyelination as it promotes the maturation of OPCs along the oligodendrocyte lineage (Carbajal, Miranda, Tsukamoto, & Lane, 2011; Patel, McCandless, Dorsey, & Klein, 2010). Upregulation of CXCL12 occurs in response to TNFR2 activation (Patel et al., 2012) and signalling through CXCR4 is essential in triggering the differentiation of

actively proliferating OPCs into mature myelinating oligodendrocytes in the cuprizone model of demyelination (Patel et al., 2010). In the absence of CXCR4 signalling, OPC numbers are increased but numbers of cells expressing the myelin-specific proteins CNPase and GSTπ are decreased, thus supporting the role of CXCL12/CXCR4 signalling in maturation along the oligodendrocyte lineage (Carbajal et al., 2011; Patel et al., 2010). CXCL12 can also bind to CXCR7, which has similar levels of expression in both the healthy and the lesioned brain. While the expression of CXCR4 is downregulated following the initiation of differentiation phase in OPCs, CXCR7 expression steadily increases as cells mature and is maintained in myelinating oligodendrocytes. This suggests that CXCR4 and CXCR7 are both functionally involved in the pro-maturation effects of CXCL12 on differentiating OPCs (Gottle et al., 2010).

2.3.3 Kinases and Ras homolog gene family, member A (RhoA) signalling in the differentiation phase

Various kinase proteins, which play crucial roles in gene regulation, are involved in initiating and promoting the maturation and terminal differentiation of OPCs into myelinating oligodendrocytes. Specifically, increased myelin production has been shown to occur when the serine-threonine-specific protein kinase AKT-PKB (AKT1) is phosphorylated by phosphatidylinositol 3 kinase (PI3K) (Flores et al., 2008; Taveggia, Feltri, & Wrabetz, 2010; Taveggia et al., 2005). PI3K is activated by neuregulin-1 type III, a signal produced by naked axons (Taveggia et al., 2005) and the PI3K-AKT1 pathway is activated by growth factors and extracellular matrix molecules (Tyler et al., 2009). Downstream PI3K-AKT1 signalling activates the kinase mammalian target of rapamycin (mTOR), which is responsible for inducing the terminal differentiation of OPCs into myelin-forming oligodendrocytes. mTOR promotes cell growth and protein translation through mTOR1 signalling and cytoskeleton organization via

mTOR2 (Tyler et al., 2009). Inhibition of mTOR signalling stops the differentiation process at the pre-oligodendrocyte stage of maturation (Tyler et al., 2009).

RhoA is a GTPase protein that acts on the actin cytoskeleton of cells to promote the formation of myelin membranes by oligodendrocytes. RhoA activation is hypothesized to be required for differentiated OPCs to undergo the morphological changes necessary to establish functional myelin sheaths, the final step in the repair process (Czopka et al., 2009). The actions of the cyclin-dependent kinase inhibitor p57/Kip2 are thought to be mediated through a downstream target of RhoA signalling, the Lim domain kinase-1 (LIMK-1) (Kremer et al., 2009; Lingor et al., 2007). Downregulation of p57/Kip2 is thought to induce nuclear translocation of LIMK-1 and cytosolic LIMK-1 has been shown to promote and stabilize process outgrowth by maturing oligodendroglial cells (Kremer et al., 2009).

Tissue transglutaminase-2 (TG2), a member of the Ca^{2+}-dependent protein cross-linking family of enzymes (Lorand & Graham, 2003), enhances RhoA signalling through transamidation in demyelinating disease (Singh et al., 2003). Demyelination triggers a strong calcium influx within OPCs, leading to TG2 expression (Chattopadhyay et al., 2008; Paez et al., 2009; Soliven, 2001). TG2 null mice show impaired remyelination in response to experimentally-induced demyelination, and this correlates with decreased levels of differentiation and myelin sheath formation (Van Strien et al., 2011). Depolarization of OPCs through glutamate or GABA receptor activation also triggers a strong calcium influx within cells that could implicate TG2 and RhoA signalling in white matter plasticity, particularly since RhoA has been implicated in structural remodelling in response to experience-induced cortical plasticity (Berger, Schnitzer, Orkand, & Kettenmann, 1992; Cerri et al., 2011; Gallo, Mangin, Kukley, & Dietrich, 2008).

The proto-oncogene tyrosine-protein kinase Fyn (Fyn), a member of the protein-tyrosine kinase oncogene family present in the signalling pathway of integrins, is involved in kinase- and RhoA signalling in the differentiation phase of remyelination. Fyn binds to Fc receptors on microglial cells, which stimulates phagocytosis and myelin debris clearance (Nakahara & Aiso, 2006). In addition to being expressed by microglial cells, Fc receptors, more specifically the common gamma chain of immunoglobulin Fc receptors (FcRϒ), are also found on OPCs, both in the healthy brain and in MS lesions undergoing repair (Kremer, Aktas, Hartung, & Kury, 2011; Nakahara & Aiso, 2006). Activation of Fyn signalling in OPCs is achieved through glutamate-mediated synaptic activity (Wake, Lee, & Fields, 2011). Fyn kinase-dependent signalling is thought to be essential to OPC differentiation along the oligodendrocyte lineage (Nakahara, Aiso, & Suzuki, 2009; Nakahara et al., 2003; Umemori, Sato, Yagi, Aizawa, & Yamamoto, 1994) and to the transcription of the myelin-specific gene MBP (Nakahara et al., 2003; Umemori et al., 1999; White et al., 2008). Fyn has also been shown to interact with cytoskeletal proteins such as RhoA to induce the morphological changes associated with a mature oligodendroglial phenotype (Belkadi & LoPresti, 2008; Klein et al., 2002; Liang, Draghi, & Resh, 2004; Rajasekharan et al., 2009).

Extracellular matrix components modulate RhoA signalling and have been found to promote successful remyelination (Czopka, Von Holst, Schmidt, Ffrench-Constant, & Faissner, 2009). Extracellular matrix molecules appear to be directly involved in OPC differentiation, as shown by recombinant treatment of cortical cultures with osteopontin. Osteopontin binds to αVintegrins and promotes MBP production, which ultimately leads to increased levels of myelination (Selvaraju et al., 2004; Zhao et al., 2008). Furthermore, cleavage of key components of the extracellular matrix by MMPs facilitates process outgrowth and the establishment of

contacts between newly differentiated oligodendrocytes and previously de- or unmyelinated axons (Li et al., 2005; Oh et al., 1999). The proteolytic properties of MMPs are also involved in the shedding and clearance of NG2 by differentiating OPCs. NG2 accumulates in the absence of MMP-9 and has an inhibitory effect on maturation along the oligodendrocyte lineage (Larsen et al., 2003). Together, these results indicate that target genes both upstream and downstream from RhoA are involved in the differentiation process, thus making it an intrinsic factor integral to myelin sheath formation in repair and possibly also in white matter plasticity.

2.3.4 Transcription factors in the differentiation phase

Finally, a number of transcription factors regulate the differentiation and maturation of OPCs into myelinating oligodendrocytes. In order for the differentiation process to begin, a number of transcription factors that act as differentiation inhibitors in the Notch and Wnt signalling pathways must be downregulated. These transcription factors include the bHLH transcription factors Hes1 and Hes5, the inhibitors of DNA binding (Id) Id2 and Id4, and the SRY-related HMG-box (SOX) transcription factor Sox2 (Cunliffe & Casaccia-Bonnefil, 2006; Fancy et al., 2010; Hanafy & Sloane, 2011; Nelson & Nusse, 2004; Shen et al., 2008b). Transcription factor downregulation is achieved through the deacetylation of histones found in the promoter regions of these genes. Histone deacetylation changes the conformation of chromatin in a way that supresses gene expression. It is undertaken by histone deacetylases (HDACs) in the CNS (Shen, Li, & Casaccia-Bonnefil, 2005). Recruitment of HDACs is less efficient in later stages of adulthood, which may account for the lower levels of myelination observed in older adults and of remyelination when they are subjected to experimental demyelination (Shen et al., 2008a; Tang et al., 1997). Higher levels of histone acetylation have also been observed in animals subjected to social isolation, which correlates with thinner myelin

sheaths and impaired white matter plasticity in adulthood (Liu et al., 2012). Alternatively, recruited OPCs that do not differentiate into myelinating oligodendrocytes transiently express S100 calcium binding protein B (S100B), which marks the return of the slow-dividing or non-activated adult OPC phenotype (Deloulme et al., 2004).

Olig1 and Olig2 are bHLH transcription factors expressed by most cells along the oligodendrocyte lineage, including OPCs (Fig. 2 #1, 2, 5) (Arnett et al., 2004; Cheng et al., 2007; Ligon et al., 2006). Shortly following the induction of a white matter insult, the expression of Olig1 and Olig2 is strongly upregulated at the lesion border (Fig. 1 #2) (Glezer et al., 2006). While Olig1 is not thought to be necessary for developmental myelination, it is essential to remyelination, because Olig1 null mice only show a subtle delay in developmental myelination but completely fail to remyelinate in response to experimentally-induced demyelination (Arnett et al., 2004; Cheng et al., 2007). Nuclear translocation of Olig1 has been involved with the generation of oligodendrocytes in adulthood and, more specifically, in the repair process following demyelinating injuries and disease (Arnett et al., 2004). Location of Olig1 proteins varies throughout development, being located in the nucleus of neonatal OPCs and in the cytoplasm of post-natal and adult OPCs (Arnett et al., 2004). Olig1 is also increasingly cytoplasmic as cells mature along the oligodendrocyte lineage where it promotes the expansion and maturation of the membranes of oligodendrocytes (Arnett et al., 2004; Niu et al., 2012). In the early phases of adult myelination, Olig1 protein levels are upregulated and located in the nucleus rather than in the cytoplasm of OPCs (Arnett et al., 2004; Yu et al., 2010). This process is thought to promote the differentiation phase of adult myelination by inducing the transcription of myelin-specific genes (Arnett et al., 2004; Niu et al., 2012; Xin et al., 2005; Yu et al., 2010).

Conversely, Olig2 has been found to contribute to repair rather indirectly by modulating the expression of downstream factors such as Nkx2.2, Sox10, and Sox17, which are all more closely implicated in the differentiation of OPCs (Moll et al., 2013; Soundarapandian et al., 2011; Stolt et al., 2002; Zhou, Choi, & Anderson, 2001; Zhou, Wang, & Anderson, 2000). Both Olig2 and Nkx2.2 have been found to be essential to oligodendrocyte lineage specification during development, but because Olig2 is still detectable in mature myelinating oligodendrocytes, Nkx2.2 has been associated with lineage specification in adult myelination (Fancy et al., 2004; Watanabe et al., 2004). Contrary to what is seen with bHLH transcription factors, Nkx2.2 expression is increased in proliferating OPCs that have been recruited to lesion sites but is rapidly downregulated as cells transition to a mature oligodendroglial phenotype (Watanabe et al., 2004; Zhao et al., 2005). Consequently, Nkx2.2 is considered to be a marker of activated, proliferating OPCs (Fig. 2 #1, 2, 5) (Fancy et al., 2004; Syed et al., 2011) and is specifically implicated in the initial phases of remyelination (Watanabe et al., 2004). Nevertheless, Nkx2.2 has consensus binding sites on the promoter regions of two myelin-specific proteins, PLP and MBP, where it promotes or represses, respectively, the expression of these proteins in activated OPCs (Gokhan et al., 2005; Labombarda et al., 2009; Qi et al., 2001; Wei, Miskimins, & Miskimins, 2005). The presence of these consensus binding sites further supports the role of Nkx2.2 in triggering the terminal differentiation of OPCs.

Gtx is also a member of the homeodomain family of transcription factors and it is only expressed in mature oligodendrocytes (Sim, Hinks, & Franklin, 2000). Peak levels of Gtx expression are reached when oligodendroglial cells complete differentiation, which occurs together with the expression of the myelin-specific proteins MBP and PLP (Awatramani et al., 1997; Sim et al., 2000). Thus, Gtx is believed to ensure proper termination of maturation along

the oligodendrocyte lineage, allowing differentiated OPCs to reach a mature, myelinating phenotype (Fig. 2 #6).

Myelin transcription factor (Myt1) is a zinc-finger protein (Zfp) also involved in the terminal differentiation of OPCs through regulation of the myelin-specific gene PLP (Armstrong, Kim, & Hudson, 1995). In experimental models of demyelination, increases in the density of Myt1-positive OPCs were shown to be associated with the first signs of remyelination (Vana, Lucchinetti, Le, & Armstrong, 2007b). Since downregulation of nucleic Myt1 in immature oligodendroglial cells is associated with the appearance of mature myelinating oligodendrocytes, it is thought to be required for the maturation and terminal differentiation of oligodendrocyte lineage cells (Armstrong et al., 1995; Hanafy & Sloane, 2011; Vana et al., 2007b).

Differentiation of OPCs is a complex, mostly intrinsically regulated process that can be triggered either by T3 (Barres et al., 1994a; Dugas et al., 2012) or by the downregulation of trophic factors that promote the proliferation and inhibit differentiation of OPCs (Durand & Raff, 2000). T3 and growth factor withdrawal both modulate, through different pathways, the expression of proteins related to the cell cycle timer and initiate the sequence of events leading to the adoption of a mature oligodendroglial fate by former OPCs (Asher et al., 2005; Caillava & Baron-Van Evercooren, 2012; Dugas et al., 2007; Durand & Raff, 2000; Kremer et al., 2009). RhoA signalling and reconfiguration of the extracellular matrix by secreted MMPs also play a role in the differentiation phase as they allow for the reorganization of the cytoskeleton of myelin-producing cells and enable the extension of their processes (Czopka et al., 2009; Oh et al., 1999). The ensheathment and compaction of such processes around axons will lead to enhanced neural functioning in both white matter plasticity and disease, a property that is characteristic of maturation and the early stages of MS (Liebetanz & Merkler, 2006).

It must be noted that the fine tuning of this phase of repair is particularly important because it is the primary cause of remyelination failure in MS (Chang et al., 2000; Chang et al., 2002; Foote & Blakemore, 2005a; Kuhlmann et al., 2008; Wolswijk, 1998, 2000). Even though a considerable amount of OPCs and premyelinating oligodendrocytes can be found in proximity of demyelinated axons in chronic MS lesions (Chang et al., 2000; Chang et al., 2002; Kuhlmann et al., 2008; Reynolds et al., 2002; Sloane et al., 2010; Wolswijk, 1998), these cells appear to be unable to differentiate into mature myelin-forming oligodendrocytes (Chang et al., 2000; Chang et al., 2002; Foote & Blakemore, 2005a; Kuhlmann et al., 2008; Wolswijk, 1998, 2000). It might be useful to determine if this phase is also altered during aging, as age-related changes in learning and memory can be attributed to loss of white matter integrity and volume, both of which being related to impairments in white matter plasticity (Madden et al., 2009).

3. Concluding remarks

The widespread distribution and continued proliferative activity of OPCs have raised questions as to their role in the adult CNS beyond regeneration in demyelinating diseases such as MS (Dawson, Polito, Levine, & Reynolds, 2003b). An increasing number of reports suggest that they may be involved in a form of plasticity that has long been overlooked: white matter plasticity (Fields, 2010). White matter plasticity increases levels of myelination and of temporal summation between axons within a network, thereby reducing conduction times and presumably improving cognitive functioning (Fields, 2005). Like remyelination in the case of injury, de novo myelination and myelin remodelling, the two main subtypes of white matter plasticity, are most likely achieved through the production of new myelinating oligodendrocytes by adult OPCs (Rivers et al., 2008; Young et al., 2013).

Little is known about the dynamics of white matter plasticity in the healthy adult brain. Because this process takes place in the context of pre-existing neural networks and since adult OPCs are inherently different than their developmental counterparts (Shi et al., 1998; Woodruff et al., 2004), we have hypothesized that white matter plasticity may share some common features with remyelination in MS and other forms of demyelinating injury. An important component of remyelination is the inflammatory response, which may also be present in white matter plasticity, if only because myelin debris have to be cleared by macrophages during myelin remodelling (Kotter et al., 2006). Consistent with this notion, environmental enrichment and other forms of cognitive stimulation that lead to white matter plasticity have also been found to recruit and require inflammatory cells (Ehninger et al., 2011; Ziv et al., 2006). Inflammatory cells secrete the growth factors and cytokines largely responsible for triggering the intrinsic program taken on by OPCs on their way to a mature oligodendroglial phenotype (Bieber et al., 2003; Kerschensteiner et al., 1999; Reynolds et al., 2002). Growth factors are also generated in response to various forms of activity linked to white matter plasticity, an example of which is exercise (Kramer & Erickson, 2007). MMPs, secreted by inflammatory cells in disease and necessary for synaptic plasticity (DaSilva & Yong, 2008; Larsen et al., 2003; Meighan et al., 2006; Skuljec et al., 2011; Ulrich et al., 2006; Vos et al., 2003), likely play a role in both white matter plasticity and injury because their proteolytic activity allows for the migration of oligodendroglial cells and the extension of their processes through the crowded ECM of the mature adult brain (Larsen et al., 2003; Li et al., 2005; Oh et al., 1999; Skuljec et al., 2011). All of these shared features between white matter plasticity and remyelination suggest that similar factors could drive the differentiation of OPCs into myelinating oligodendrocytes in the adult brain, may it be healthy or injured.

Much work has been dedicated to the factors that cause remyelination to fail in demyelinating diseases (reviewed in (Fancy et al., 2010; Franklin, 2002; Franklin & Ffrench-Constant, 2008). The same can be said of the factors needed for developmental myelination (reviewed in (Ahrendsen & Macklin, 2013; Huang, Zhao, Zheng, & Qiu, 2013)). However, more research needs to be done on the factors that are specifically involved in promoting adult myelination, both in the injured and in the non-injured brain. A better understanding of the factors modulating the transition of OPCs from a progenitor to a myelinating oligodendrocyte phenotype specifically in the adult could yield useful information for MS treatment, while also shedding light on the extent to which axonal myelination contributes to the plastic reorganization of the brain in response to learning and memory (Fields, 2010; Richardson, Young, Tripathi, & McKenzie, 2011; Zatorre et al., 2012). Modulation of these factors could lead to better treatment outcomes for those plagued with MS and to improved cognitive performance, especially in the later stages of adult life, where both white matter plasticity and remyelination seem to fail (Sim et al., 2002b; van Wijngaarden & Franklin, 2013).

Tables

Table 1.1. Extrinsic factors involved in the recruitment phase of remyelination.

Extrinsic factors involved in the recruitment phase of remyelination

PDGF	Secreted by inflammatory cells, this growth factor is the primary regulator of OPC proliferation through activation of Cdk2.
bFGF	Secreted by inflammatory cells, this growth factor increases sensitivity of OPCs to PDGF; primary inhibitor of OPC differentiation.
Neurotrophins (NT-3 and BDNF)	Secreted by inflammatory cells, these growth factors activate CREB-1, thus favouring OPC proliferation.
IGF-1	Secreted by inflammatory cells, this growth factor promotes OPC survival.
TNF_{α}	Secreted by inflammatory cells, this cytokine promotes OPC activation and recruitment through TNFR2 signalling.
IL-6	Secreted by inflammatory cells, these cytokines activate and increase OPC sensitivity to PDGF.
CXCL1/CXCR2	Secreted by reactive astrocytes, the cytokine CXCL1 promotes OPC migration by way of chemoattraction.
MMPs	Secreted by inflammatory cells, these proteases breakdown components of the extracellular matrix, thereby facilitating OPC migration.
OPC and other extracellular matrix molecules	Secreted by inflammatory cells, these molecules recruit macrophages, activated microglia, reactive astrocytes, and OPCs to lesion sites by way of chemoattraction.
Semaphorin 3F	Axonal signal involved in the recruitment of OPCs to lesion sites by way of chemoattraction.
PSA-NCAM	Axonal signal involved in the recruitment of OPCs to lesion sites by way of chemoattraction.

Table 1.2. Intrinsic factors involved in the recruitment phase of remyelination.

Intrinsic factors involved in the recruitment phase of remyelination

$PDGFR_{\alpha}$	Delivers PDGF to OPCs.
NG2	Forms a complex to deliver PDGF to its receptor.
Cdk2	Activated by PDGF, triggers proliferation and regulates cell cycle progression in OPCs.

Table 2.1. Extrinsic factors involved in the differentiation phase of remyelination.

Extrinsic factors involved in the differentiation phase of remyelination

T3	Regulated by immune-related factors, this thyroid hormone triggers cell cycle exit.
PDGF	Withdrawal of this growth factor acts on intracellular factors to trigger cell cycle exit.
IGF-1	Growth factor that promotes cell cycle exit and differentiation of OPCs.
TGF-β1	Cytokine that promotes cell cycle exit and differentiation of OPCs.
CXCL12/CXCR4	CXCL12 signalling through CXCR4 triggers cell cycle exit and OPC differentiation.
CXCL12/CXCR7	CXCL12 signalling through CXCR7 promotes maturation along the oligodendroglial lineage.
MMPs	Breakdown of the extracellular matrix facilitates process outgrowth by differentiated OPCs.

Table 2.2. Intrinsic factors involved in the differentiation phase of remyelination.

Intrinsic factors involved in the differentiation phase of remyelination

CdkIs	Increased as cells proliferate, maximal levels trigger cell cycle exit and terminal differentiation of OPCs.
RXRs	Target nuclear receptor for T3's actions; translocation of the nucleus promotes OPC maturation along the oligodendroglial lineage.
PPARs	Forms heterodimers with RXRs which serves to regulate the transcription of differentiation-related gene.
PI3K-AKT1 pathway	Activated by growth factors and extracellular matrix molecules, activates mTOR.
mTOR	Induces terminal differentiation of OPCs into myelin-forming oligodendrocytes.
LIMK-1	Translocated to the nucleus in response to CdkI downregulation where it acts on the cytoskeleton to promote process outgrowth.
RhoA	Acts on the cytoskeleton to promote the formation of functional myelin sheaths.
TG2	Activated in response to demyelination, increases the activity of RhoA.
Fyn	Activates the transcription of myelin-specific genes.
HDACs	Downregulate the expression of differentiation inhibitors.
Olig1	Upregulated and translocated to the nucleus in response to demyelination; promotes the transcription of myelin-specific genes.
Olig2	Upregulated in response to demyelination; modulates the expression of Nkx2.2.
Nkx2.2	Upregulated in response to demyelination; required for lineage specification; promotes the transcription of myelin-specific genes.

Myt1	Regulates the expression of myelin-specific proteins.
Gtx	Promotes maturation and assures proper termination of differentiation along the oligodendrocyte lineage.

Figure legends

Fig 1. Demyelination and oligodendrocyte precursor cells activation and recruitment. 1) Breakdown of the blood-brain barrier leads to the infiltration of macrophages and of T-cell lymphocytes into the brain parenchyma; 2) Infiltrated T-cell lymphocytes attack surrounding myelin sheaths; 3) Resulting myelin debris activates microglial cells and astrocytes. Macrophages and microglia work to clear the myelin debris; 4) Macrophages, activated microglia, and reactive astrocytes produce and secrete growth factors, cytokines, and matrix metalloproteinases, which begin to breakdown the extracellular matrix; 5) Secreted growth factors and cytokines activate non-activated OPCs; 6) Activated OPCs have an increased sensitivity to the mitogenic effects of growth factors and cytokines, thus increasing the rate of OPC proliferation. They also answer to pro-migratory signals secreted by the denuded axon; 7) Proliferating OPCs migrate toward areas lacking myelination, where they differentiate into myelinating oligodendrocytes and repair demyelinated lesions.

Fig 2. Differentiation of oligodendrocyte precursor cells into myelinating oligodendrocytes. 1) Non-activated OPCs can be identified through expression of the proteoglycan NG2 and the growth factor receptor PDGFR$_\alpha$. They also express the transcription factors Olig1 and Olig2, with Olig1 being specifically expressed in the nucleus of these cells; 2) Activated OPCs increase the expression of NG2, PDGFR$_\alpha$, Olig2, and Olig1, which begins to translocate to the cell's cytoplasm. Activated OPCs also begin to express the transcription factor Nkx2.2 as well the cyclin-dependent kinase 2 (Cdk2), which triggers cell cycle entry; 3) Proliferating OPCs progressively increase the expression of cyclin-dependent kinase inhibitors (CdkIs); 4) Cells exit the cell cycle when maximal levels of CdkIs are reached or when T3 is present or when the growth factor PDGF is withdrawn from the cell's environment; 5) Cells that have begun to differentiate decrease the expression of Nkx2.2 and no longer express NG2 or PDGFR$_\alpha$. They rather express the transcription factor Myt1 as well as the premyelinating oligodendrocyte-specific antigen O4; 6) Mature oligodendrocytes express the transcription factor Gtx as well as myelin-specific antigens such as MBP, GST$_\pi$, and CNPase; 7) Mature myelinating oligodendrocytes establish contacts with naked axons and ensheath them by wrapping their processes around them several times.

Figures

Figure 1.

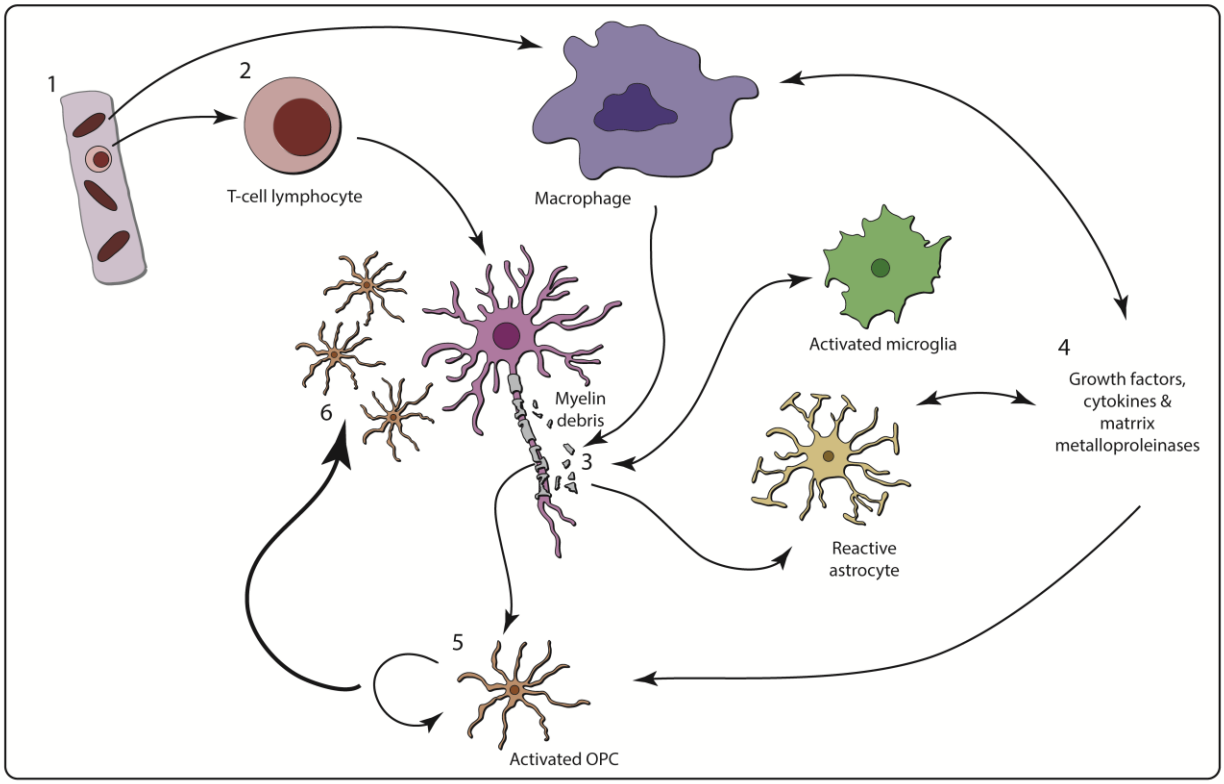
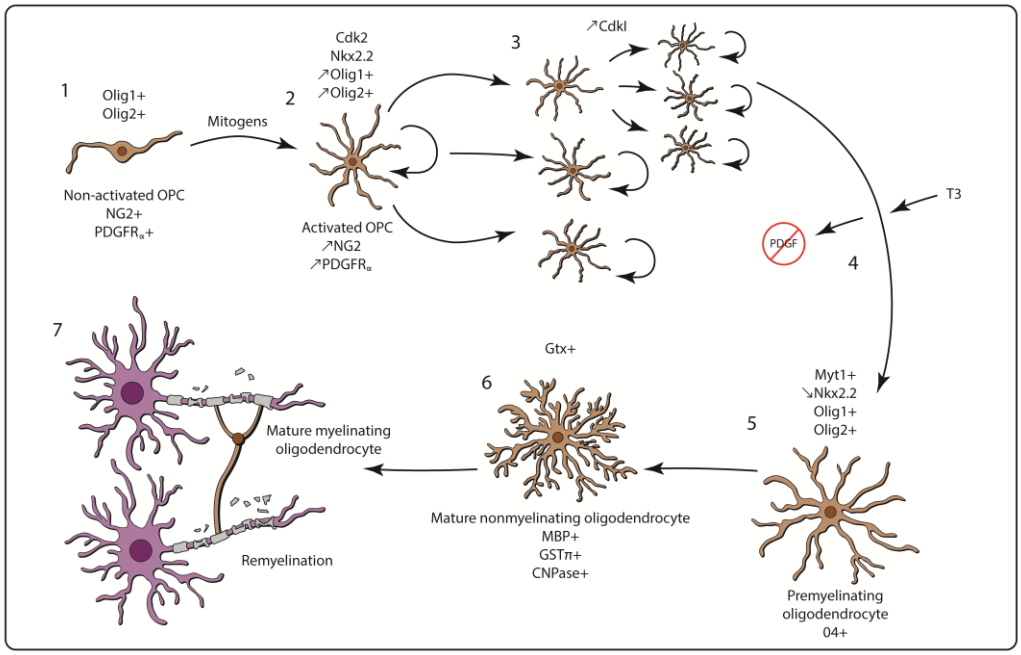


Figure 2.


1
Olig1+
Olig2+
Non-activated OPC
NG2+
PDGFRα+
Mitogens
2
Cdk2
Nkx2.2
↗Olig1+
↗Olig2+
Activated OPC
↗NG2
↗PDGFRα
3
↗Cdkl
PDGF
T3
4
5
Myt1+
↘Nkx2.2
Olig1+
Olig2+
Premyelinating
oligodendrocyte
04+
6
Gtx+
Mature nonmyelinating oligodendrocyte
MBP+
GSTπ+
CNPase+
7
Mature myelinating
oligodendrocyte
Remyelination

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Running Head: Improving immunostaining denaturation

EXPERIMENT 1: A SIMPLE HISTOLOGICAL TECHNIQUE TO IMPROVE IMMUNOSTAINING WHEN USING DNA DENATURATION FOR BRDU LABELLING

Jenna J. Boulanger, William A. Staines[1], Véronique LeBlanc, Eve-Ling Khoo, Jacky Liang, and Claude Messier

School of Psychology, University of Ottawa

[1] Department of Cellular and Molecular Medicine, University of Ottawa.

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Correspondence to: C. Messier, School of Psychology, University of Ottawa, 136 Jean-Jacques Lussier Room 2076A, Ottawa, Ontario, K1N 6N5, CANADA. Tel (613) 562-5800 ext. 4562, Fax: (613) 562-5147; E-mail: cmessier@uottwa.ca.

Abbreviations

In alphabetical order:

BrdU: 5-bromo-2'-deoxyuridine

DCX: doublecortin

EdU: 5-ethynyl-2’-deoxyuridine

EYFP: enhanced fluorescent yellow protein

GFP: green fluorescent protein

GST-pi: glutathione S-transferase pi

MBP: myelin basic protein

NeuN: neuronal nuclear protein, also known as Fox-3 or Rbfox3

NG2: chondroitin sulfate proteoglycan neuron-glia antigen 2

OPC: oligodendrocyte progenitor cell

PDGFRα: platelet-derived growth factor receptor alpha

Sox10: SRY-related HMG-box transcription factor 10

Abstract

The typical immunohistochemistry technique used to reveal 5-bromo-2'-deoxyuridine (BrdU) incorporation requires denaturation of the DNA by heat and acid to permeabilize the cell nucleus. This treatment can damage tissue and reduce the antigenicity of several proteins, which then leads to weak immunostaining and/or false negatives. We show that an overnight post-fixation step following immunohistochemistry for antigens of interest protects immunostaining during the acid/heat denaturation treatment for subsequent BrdU staining. We used this technique to study the differentiation of recently divided oligodendrocyte progenitor cells in NG2CreER:EYFP reporter mice. We used a GFP anti-EYFP antibody to maximize visualization of the EYFP-containing oligodendrocyte progenitor cells, Olig1, and GST-pi to confirm the cell phenotype. Immunostaining for GFP, Olig1, and GST-pi is reduced by DNA denaturation. We found that incorporating a post-fixation step after double immunostaining for GFP/Olig1 and GFP/GST-pi prior to DNA denaturation prevented the fading and false negatives associated with this treatment. This simple addition to BrdU immunohistochemistry protocols extends the range of proteins that can be detected in combination with BrdU, along with the number of antibodies that can be used successfully in the study of cell proliferation.

Keywords: immunohistochemistry, denaturation, BrdU, neurogenesis, gliogenesis

Introduction

5-bromo-2'-deoxyuridine (BrdU) is an analog of the nucleoside thymidine widely used in the study of cell proliferation. In pulse-chase labelling, BrdU (the pulse) is administered to an animal either through intraperitoneal (i.p.) injections or through the animal's drinking water (Taupin, 2007). Proliferating cells incorporate BrdU into their DNA by substituting it for thymidine during the S-phase of the cellular cycle, during which DNA is replicated, and pass it on to their daughter cells (Ngwenya et al., 2005). Once cells have incorporated BrdU, animals are euthanized after a predetermined period of time (the chase period) (Kee et al., 2002; Taupin, 2007). BrdU immunohistochemistry (IHC) is then used to label these cells to provide evidence of division (Gratzner, 1982). DNA has to be denaturated by heat and acid to allow anti-BrdU antibodies to bind to the BrdU incorporated into the nuclear DNA. Unfortunately, denaturation can considerably damage the tissue (Ffrench et al., 1994; Moran et al., 1985) and reduce or even eliminate, in some cases, immunostaining of several proteins. Other alternatives have been proposed such as heating in sodium citrate buffer (Tang et al., 2007), fixation of cell cultures in cold methanol, for 30 min followed by brief immersion in NaOH for 10-15 s (Campana et al., 1988) or the use of protease for resin-embedded sections (Matsuura and Suzuki, 1997). The present technique differs from those alternatives by first performing the typical immunohistochemistry for antigens of interest (other than BrdU), followed by an overnight paraformaldehyde fixation step. The typical denaturation step (heat/acid) and BrdU immunohistochemistry is then performed. The addition of a paraformaldehyde fixation step after the initial immunohistochemistry protects the staining from the heat/acid treatment.

Alternatives techniques to BrdU exist. For example, 5-ethynyl-2'-deoxyuridine (EdU), also a thymidine analog, is visualized through copper-catalyzed (3+2) cycloaddition (Click

reaction), a protocol that does not require DNA denaturation. However, it remains very costly and therefore doesn't lend itself well to studies with large groups of animals (Zeng et al., 2010). Another alternative, immunostaining with Ki-67, a protein involved in all active phases of the cell cycle, only provides a snapshot of the proliferative activity at the time of death, thereby preventing tracing of the fate of newly divided cells (Kee et al., 2002).

Sample fixation modulates the impact of the denaturation step (Carbajo et al., 1995). Adequate tissue fixation is reached when a balance between preservation of tissue morphology and immunoreactivity of antigens of interest is attained while maintaining sufficient tissue penetrability to ensure IHC labelling (Berod et al., 1981; Schutte et al., 1987). This balance varies across antigens (and primary antibodies): some antigens require short fixation times to maximize immunostaining while others are improved or unaffected by longer fixation periods (Mori et al., 2009). In BrdU immunochemistry, short fixation times are problematic because the extent of tissue damage produced by heat and acid denaturation increases when tissues are lightly fixed (Berod et al., 1981; Miller and Nowakowski, 1988; Mori et al., 2009). Since long fixation times do not reduce BrdU immunostaining (Wojtowicz and Kee, 2006), we thought that introducing a second post-fixation period, prior to DNA denaturation but after immunohistochemistry for proteins that are altered by acid and/or heat could improve immunostaining, while preserving BrdU visualization.

Materials and method

Animals

All mice used were individually housed in a 21+/- 1 ºC vivarium, maintained on a 12-hour light/dark cycle, and had *ad libitum* access to food and water. All animal procedures were done in accordance with the recommendations of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

NG2CreER BAC transgenic mice on a C57BL/6J background (Jackson Laboratory, Bar Harbour, Maine, USA; described in (Zhu et al., 2011)) were bred in house with R26-stop-EYFP transgenic mice on a C57BL/6J background (Jackson Laboratory, Bar Harbour, Maine, USA) to create an NG2-CreER:EYFP reporter mouse. Three to five-month old animals (n=29) received i.p injections of 6 mg of tamoxifen per day, over a period of five days. Mice showing signs of tamoxifen-induced toxicity (i.e. body weight loss, lethargy, ruffled fur) received a reduced dose of 4-5 mg of tamoxifen per injection to maintain health. Six days after the last tamoxifen injection, the animals' drinking water was replaced with BrdU in sweetened water (100 ml water + 0.125 g saccharine + 3 g dextrose + 0.1 g BrdU) for a total of eight days. Mice were perfused either 36-hours (n=20, n=10 males and n=10 females), two weeks (n=4 males) or eight (n=5 males) weeks following the end of BrdU exposure.

Tissue Processing

Anesthetized mice were perfused transcardially with saline followed by Lana's fixative (4% paraformaldehyde-picric acid; modified from (Zamboni, 1967)). Brains were post-fixed in this fixative for one hour before being incubated in 10% sucrose in sodium phosphate buffer (0.1 M, pH 7.2) overnight at 4ºC. Brains were then frozen using CO_2 dry ice and cut into 14µm sagittal sections using a cryostat.

Immunohistochemistry Procedures

Table 1 lists the antibodies used in this study. Note that a GFP anti-EYFP antibody was used to label the NG2-CreER:EYFP-positive OPCs as it considerably enhances visualization of the EYFP NG2 reporter. There were 4 different procedures used to demonstrate the efficacy of the additional post-fixation step.

1) IHC without additional post-fixation step and without acid denaturation: this procedure demonstrates the staining obtained with antigen/antibody combinations in the absence of denaturation.

1. Slides are incubated in a humid chamber with goat, mouse, or rabbit antibodies (IgG) for OPC, oligodendrocyte, or neuronal phenotype and rat anti-BrdU dissolved in PBS (100 mM phosphate buffered normal saline) with 0.3% Triton-X in the dark for 3 hours at room temperature.
2. Slides are rinsed in PBS 3 times for 5 minutes.
3. Slides are incubated in a humid chamber with the appropriate secondary antibodies dissolved in PBS with 0.3% Triton-X for 30 minutes at 37ºC.
4. Slides are rinsed in PBS 3 times for 5 minutes.
5. Sections are treated with custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

2) IHC without additional post-fixation step and with acid denaturation: This procedure shows the labelling obtained following the usual denaturation steps and incubating the BrdU antibody together with the other primary antibodies of interest.

1. Slides are incubated with HCl 2N for 30 minutes at 37ºC.
2. Slides are rinsed 3 times for 5 minutes in 0.1M borate buffer pH 8.5.
3. Slides are rinsed in PBS 3 times for 5 minutes.
4. Slides are incubated in a humid chamber with antibodies for an OPC, oligodendrocyte, or neuronal phenotype and rat anti-BrdU dissolved in PBS with 0.3% Triton-X in the dark for 3 hours at room temperature.
5. Slides are rinsed in PBS 3 times for 5 minutes.
6. Slides are incubated in a humid chamber with the appropriate secondary antibodies dissolved in PBS with 0.3% Triton-X for 30 minutes at 37ºC.
7. Slides are rinsed in PBS 3 times for 5 minutes.
8. Sections are treated with custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

3) Pre-denaturation IHC without additional post-fixation step and with acid denaturation: This procedure examined if pre-staining the slides with the primary antibodies of interest prior to denaturation improved overall staining.

1. Slides are incubated in a humid chamber with goat, mouse, or rabbit antibodies (IgG) for OPC, oligodendrocyte, or neuronal phenotype dissolved in PBS with 0.3% Triton-X in the dark for 3 hours at room temperature.
2. Slides are rinsed in PBS 3 times for 5 minutes.
3. Slides are incubated in a humid chamber with the appropriate secondary antibodies dissolved in PBS with 0.3% Triton-X for 30 minutes at 37ºC.
4. Slides are rinsed in PBS 3 times for 5 minutes.
5. Slides are incubated in HCl 2N for 30 minutes at 37ºC.
6. Slides are rinsed 3 times for 5 minutes in 0.1M borate buffer pH 8.5.
7. Slides are rinsed in PBS 3 times for 5 minutes.
8. Slides are incubated in a humid chamber with rat anti-BrdU dissolved in PBS with 0.3% Triton-X overnight at 4 ºC.
9. Slides are rinsed in PBS 3 times for 5 minutes.
10. Slides are incubated in a humid chamber with donkey anti-rat secondary antibody dissolved in PBS with 0.3% Triton-X for 3 hours at room temperature.
11. Slides are rinsed in PBS 3 times for 5 minutes.
12. Sections are treated with custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

4) IHC with post-fixation prior to acid denaturation: This is the proposed procedure in which antigens of interest were labelled first followed by an overnight post-fixation step before the acid denaturation and BrdU staining.

1. Slides are incubated in a humid chamber with goat, mouse, or rabbit antibodies (IgG) for OPC, oligodendrocyte, or neuronal phenotype dissolved in PBS with 0.3% Triton-X in the dark for 3 hours at room temperature.
2. Slides are rinsed in PBS 3 times for 5 minutes.
3. Slides are incubated in a humid chamber with the appropriate secondary antibodies dissolved in PBS with 0.3% Triton-X for 30 minutes at 37ºC.
4. Slides are rinsed in PBS 3 times for 5 minutes.
5. Slides are incubated in a humid chamber with Lana's fixative overnight at 4 ºC.
6. Slides are rinsed in PBS 3 times for 5 minutes.
7. Slides are incubated in HCl 2N for 30 minutes at 37ºC.
8. Slides are rinsed 3 times for 5 minutes in 0.1M borate buffer pH 8.5.
9. Slides are rinsed in PBS 3 times for 5 minutes.

10. Slides are incubated in a humid chamber with rat anti-BrdU dissolved in PBS with 0.3% Triton-X in the dark for 3 hours at room temperature.
11. Slides are rinsed in PBS 3 times for 5 minutes.
12. Slides are incubated in a humid chamber with a donkey anti-rat secondary antibody dissolved in PBS with 0.3% Triton-X for 30 minutes at 37ºC.
13. Slides are rinsed in PBS 3 times for 5 minutes.
14. Sections are treated with custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

Microscopy

Immunofluorescence results were visualized using an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) attached to a ProgRes MF Scan camera (Jenoptik, Jena, Thuringe, Germany). Digital images of immunostainings were captured using the ProgRes CapturePro 2.5 software (Jenoptik, Jena, Thuringe, Germany). High-resolution observations on the quality of IHC labellings were carried out on an Olympus FV1000 BX61 laser scan confocal microscope (Olympus Corporation, Tokyo, Japan) and images were captured using the Olympus Fluoview software (Olympus Corporation, Tokyo, Japan).

Results

Recombination

To answer questions regarding the pluripotency of OPCs, we used a transgenic mouse line that utilizes the Cre recombinase gene under the control of the NG2 promoter. These mice were bred with ones containing an Enhanced Yellow Fluorescent Protein gene (EYFP) inserted into the *Gt(ROSA)26Sor* locus. When tamoxifen is intraperitoneally injected into the progeny of these mice, Cre excises the STOP sequence of the NG2 gene and EYFP expression is observed. This enables the tracing of the lineage of once NG2-positive OPCs at different time points throughout the life of the animal, as cells that were NG2-positive at the time of tamoxifen administration

will remain EYFP regardless of their phenotype at the time of death. We assessed the efficacy of the NG2-CreER:EYFP reporter by measuring the number of EYFP/GFP labeled cells that also expressed platelet-derived growth factor alpha ($PDGFR_{\alpha}$), a marker for oligodendrocyte precursor cells. Animals were euthanized 36 hours after the end of the tamoxifen treatment. Reporter efficacy was measured in fixed slices that were not acid-denaturated nor post-fixed. Cells were counted in both the cortex and corpus callosum. Results showed that Cre induction efficiency was 72.0% (n=20, SEM=18.50%).

Immunohistochemistry

We examined a number of different antigens: The first one was GFP to visualize reporter EYFP/GFP labeled cells. Two anti-GFP primary antibodies were used: one of them (Abcam ab1218) showed the best improvement following the additional post-fixation. The second one was doublecortin (DCX), a microtubule protein found in migrating neuroblasts (Brown et al., 2003) and in OPCs in low levels (Tamura et al., 2007). The third one, oligodendrocyte transcription factor 1 (Olig1), was labeled to identify OPCs. We also used a GST-pi antibody to label the cell body of mature oligodendrocytes and a MBP (myelin basic protein) antibody to visualize their processes. Visual inspection of the IHC without post-fixation and with acid denaturation revealed that staining for GFP, DCX and Olig1 was not visible in oligodendrocyte precursor cells following DNA denaturation (Fig. 1A, 1D, and 1G). The intensity of DCX immunostaining found in the subgranular layer of the dentate gyrus was significantly decreased following DNA denaturation (Fig. 4A). However, immunostaining for $PDGFR_{\alpha}$, and myelin basic protein (MBP) was unaffected by DNA denaturation (Figures 5-6). These results illustrate the effects of denaturation which range from the conservation of certain antigen/antibody

labeling, an attenuation of staining for others and to the disappearance of labelling for certain antigens.

IHC that included a post-fixation step prior to DNA denaturation produced GFP, DCX, Olig1, and GST-pi immunolabelling comparable to those obtained without DNA denaturation (Fig. 1C, 1F, 1I, 3C, 4C). BrdU immunostaining was unaffected by the introduction of this post-fixation step (Fig. 7B, 7F). We also verified that performing the IHC for the antigens of interest prior to denaturation (and BrdU IHC) was not the primary cause of the labeling improvement. Figure 2 shows that the results of this sequential procedure was better than performing IHC for antigens of interest after denaturation but still worse than the labeling obtained with the addition of the post-fixation step. Shorter post-fixation times (15 minutes at room temperature) were also tested (not shown): They also resulted in improved immunostaining, albeit to a lesser extent than what was observed with the overnight post-fixation presented here.

Discussion

The addition of a post-fixation step after immunohistochemistry allows for the preserved detection of antigens that lose antigenicity after the DNA denaturation step necessary for BrdU immunostaining. It also preserves endogenous fluorescent proteins in reporter mice and can reduce false negative staining in the context of cell lineage studies. While a similar technique has been briefly mentioned (Mori et al., 2009), it was never fully described and systematically examined. The additional post-fixation step describe here increases the range of antigen/antibody combinations that can be examined in the study of cell proliferation using BrdU immunostaining. The technique also reduces the likelihood of false negative results in the case of the acid/heat treatment. Additionally, the technique could be used to perform selective antibody stripping. For

example when a first antigen labelling needs to be preserved when it is followed two sequential staining using two antibodies raised in rabbit when an acid treatment is used to strip the first rabbit antibody.

Tables

Table 1. List of antibodies used and whether or not the staining efficiency improved or remained the same when cells were labelled using a sequential IHC with acid denaturation followed by an additional post-fixation.

Host	Target	Concentration	Company	Better or same with IHC with post-fixation and with acid treatment?
Primary Antibodies				
Mouse	GFP	1/500	Abcam (ab1218)	Better
Rabbit	GFP	1/1000	Abcam (ab290)	Slightly Better
Rabbit	PDGFRa	1/250	Santa Cruz (sc-338)	Same
Mouse	Olig1	1/1000	Millipore (MAB5540)	Better
Rabbit	GST-pi	1/500	MBL (311-h)	Better
Goat	DCX	1/100	Santa Cruz (sc-8066)	Better
Mouse	Rbfox3	1/500	Millipore (MAB377)	Same
Rabbit	MBP	1/200	Abcam (ab40390)	Same
Rat	BrdU	1/500	Abcam (ab6326)	N/A
Secondary Antibodies				
Donkey	Anti-Rabbit Alexa 488	1/1000	Invitrogen	-
Donkey	Anti-Mouse Alexa 488	1/1000	Invitrogen	-
Donkey	Anti-Goat Alexa 488	1/1000	Invitrogen	-
Donkey	Anti-Rabbit Alexa 594	1/500	Invitrogen	-
Donkey	Anti-Mouse Alexa 594	1/1000	Invitrogen	-

Donkey	Anti-Goat Alexa 594	1/1000	Invitrogen	-
Donkey	Anti-Rat Alexa 594	1/1000	Invitrogen	-
Donkey	Anti-Rabbit Alexa 680	1/500	Invitrogen	-
Donkey	Anti-Mouse Alexa 680	1/800	Invitrogen	-
Donkey	Anti-Goat Alexa 680	1/500	Invitrogen	-
Donkey	Anti-Rat Alexa 647	1/100	Jackson Immuno Research	-
Donkey	Anti-Rat Alexa 680	1/500	Abcam	-

Figure legends

Fig. 1. Comparison of IHC for doublecortin (DCX), Olig1, and GST-pi with acid denaturation (Acid: A, D, G), IHC for DCX, Olig1, and GST-pi without acid denaturation (No acid: B, E, H), IHC with a post-fixation step that followed immunohistochemistry for DCX, Olig1, and GST-pi and preceded acid denaturation (Post fixation before acid: C, F, I). DCX pictures in the parenchyma (A, B, C), Olig1 in corpus callosum and parenchyma (D, E, F), and GST-pi in corpus callosum and parenchyma (G, H, I). Typical IHC for BrdU that includes acid denaturation significantly reduces DCX, Olig1, and GST-pi immunostaining (A, D, and G). The addition of a post-fixation step before acid denaturation allows the visualization of DCX, Olig1, and GST-pi (C, F, and I) at a similar intensity as with no acid denaturation (B, E, H). Pictures were taken at 20X using a fluorescence microscope.

Fig. 2. Performing the immunohistochemistry for DCX (A), Olig1 (B), and GST-pi (C) before acid denaturation but without the post-fixation step presented in Fig. 1 led to a small improvement of immunostaining but did not approach the intensity observed after the inclusion of the post-fixation step. Pictures were taken at 20X using a fluorescence microscope.

Fig. 3. Comparison of two GFP primary antibodies. IHC using the rabbit GFP with post-fixation before acid treatment (C) provides a slightly better staining of GFP than with acid treatment but without post-fixation (A) and is comparable to the staining without acid (B). IHC using the mouse GFP with with post-fixation before acid treatment (F) provides a slightly better staining of GFP than with acid treatment but without post-fixation (D) and is comparable to the staining without acid (E). Pictures were taken at 60X using a confocal microscope in the sensorimotor cortex.

Fig. 4. IHC with acid denaturation (A) attenuates DCX immunostaining in the dentate gyrus of the hippocampus when compared with IHC without acid denaturation (B) and IHC with post-fixation before acid denaturation (C). Pictures were taken at 20X using a fluorescence microscope.

Fig. 5. PDGFRa immunostaining is unaffected by acid treatment. Pictures were taken at 60X using a confocal microscope in the cortex.

Fig. 6. MBP immunostaining is unaffected by acid treatment. Pictures were taken at 60X using a confocal microscope in the cortex.

Fig. 7. IHC with post-fixation and with acid denaturation reveals the presence of triple labelling between GFP (A), BrdU (B), and DCX (C; merged in D); GFP (E), BrdU (F), and Olig1 (G; merged in H); GFP (I), BrdU (J), and PDGFRalpha (K; merged in L); and GFP (M), BrdU (N), GSTpi (O; merged in P). Pictures were taken at 60X using a confocal microscope in the sensorimotor cortex.

Figures

Figure 1.

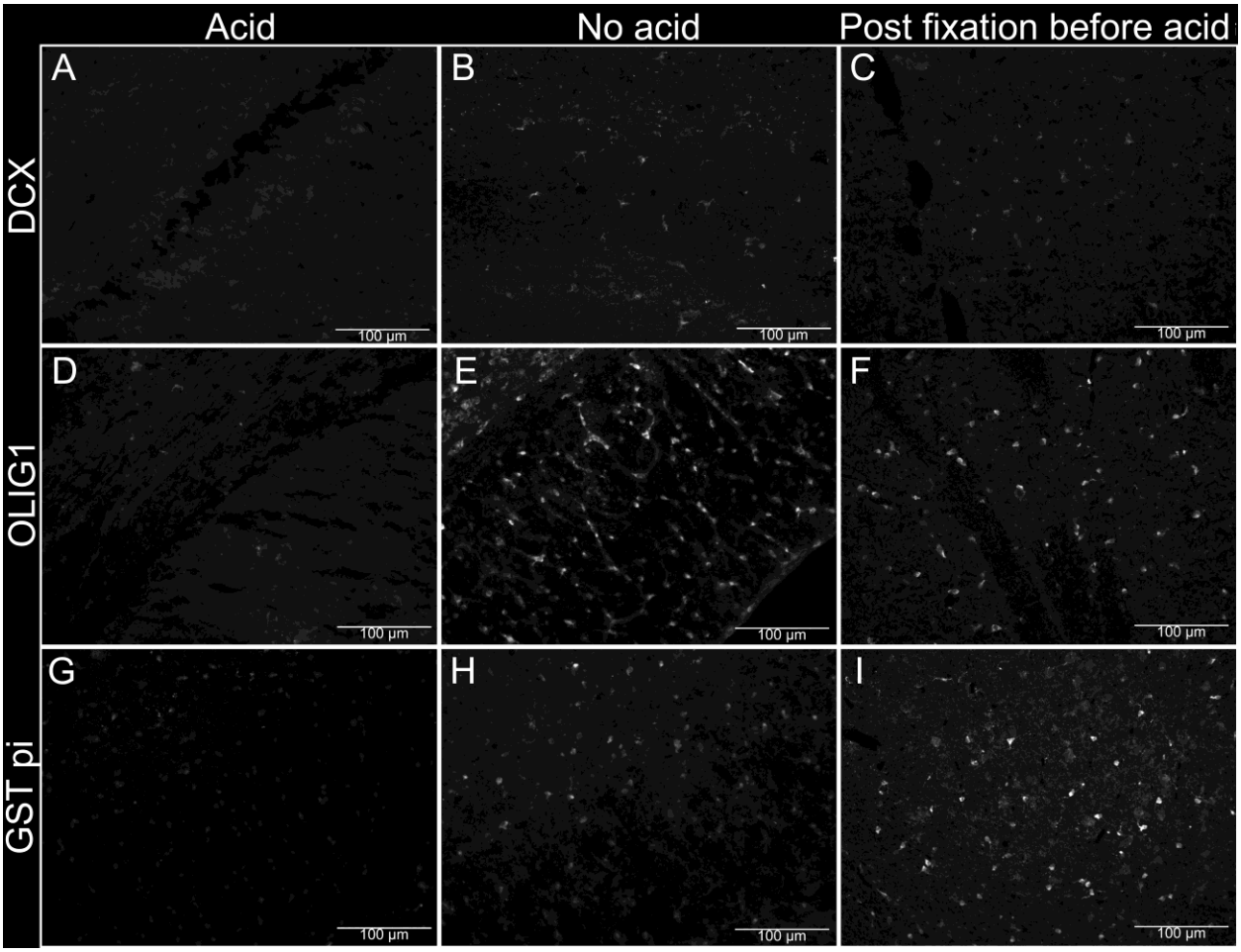


Figure 2.

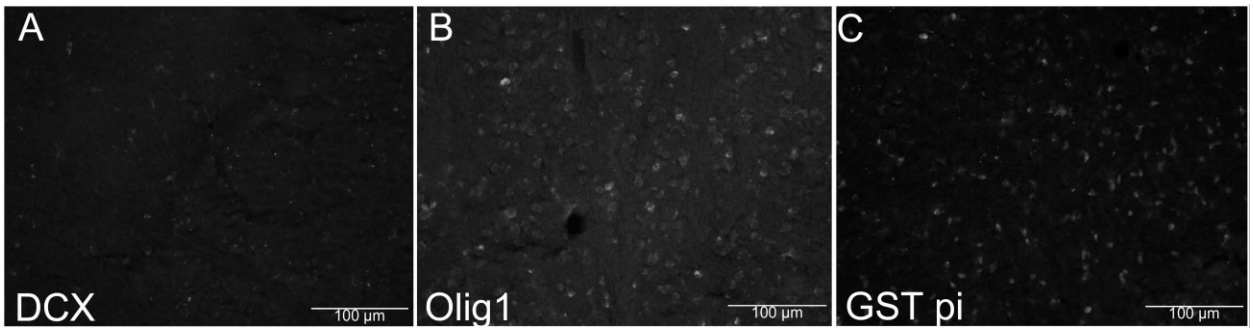


Figure 3.

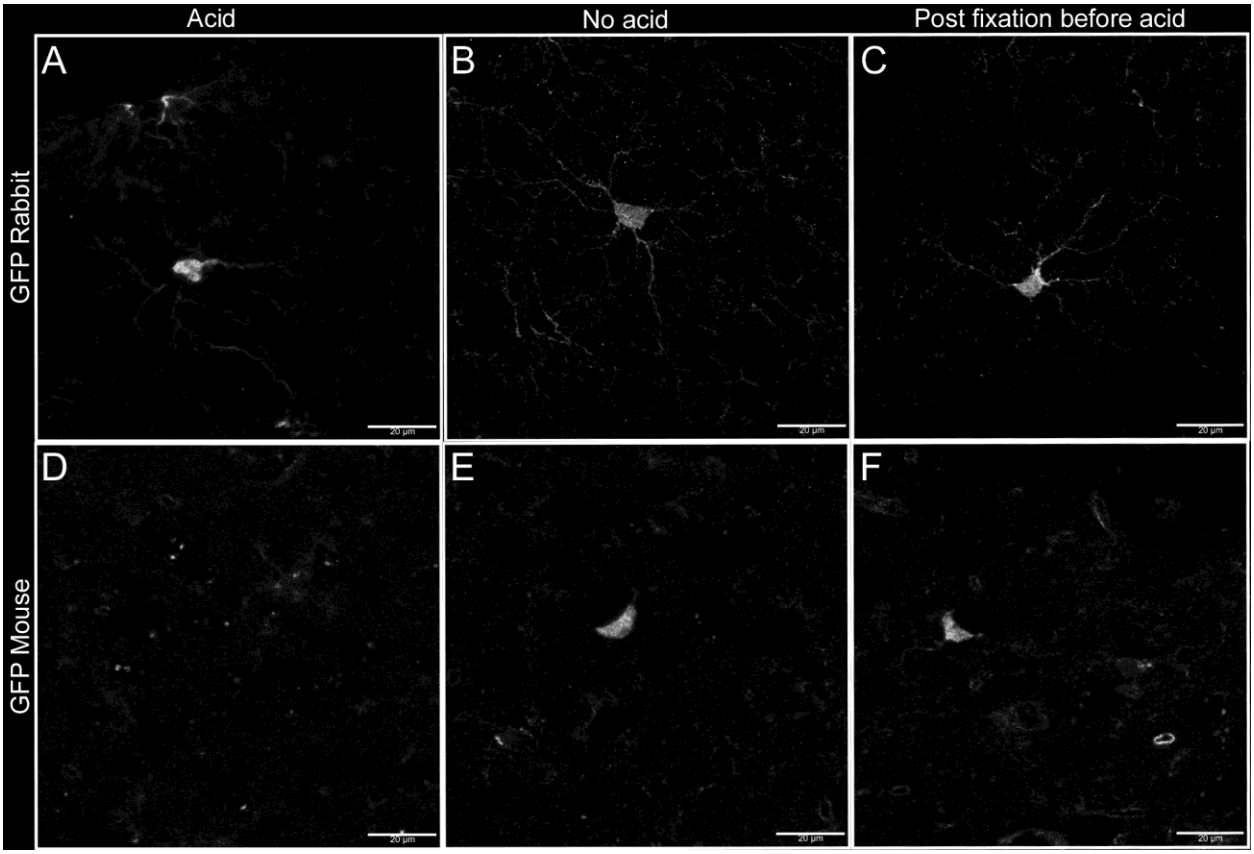

Acid
No acid
Post fixation before acid
GFP Rabbit
GFP Mouse
A
B
C
D
E
F
20 µm

Figure 4.

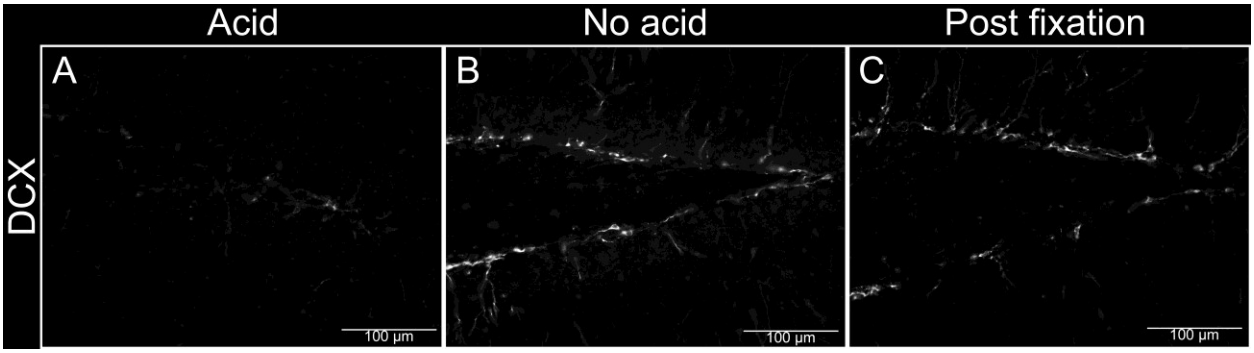


Figure 5.

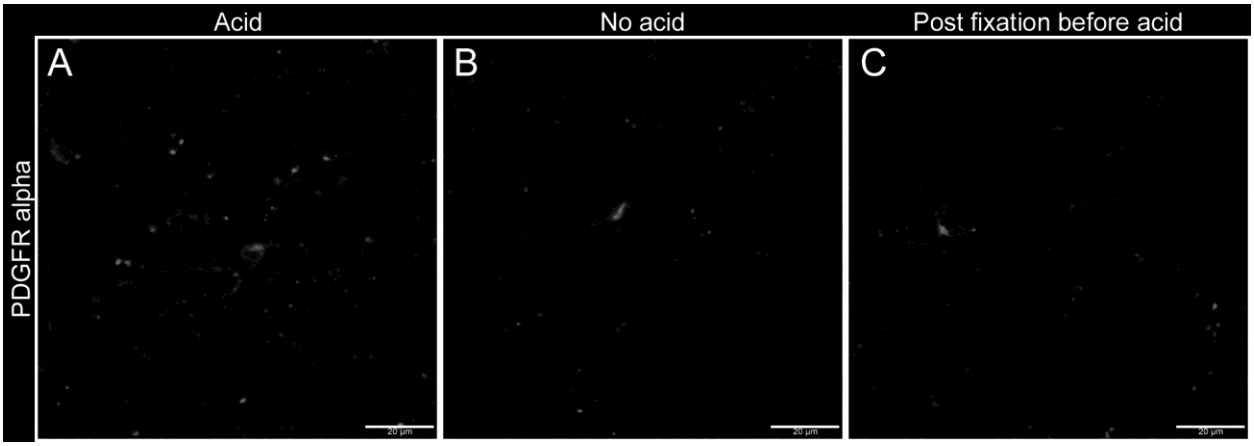

Acid
No acid
Post fixation before acid
A
B
C
PDGFR alpha
20 µm
20 µm
20 µm

Figure 6.

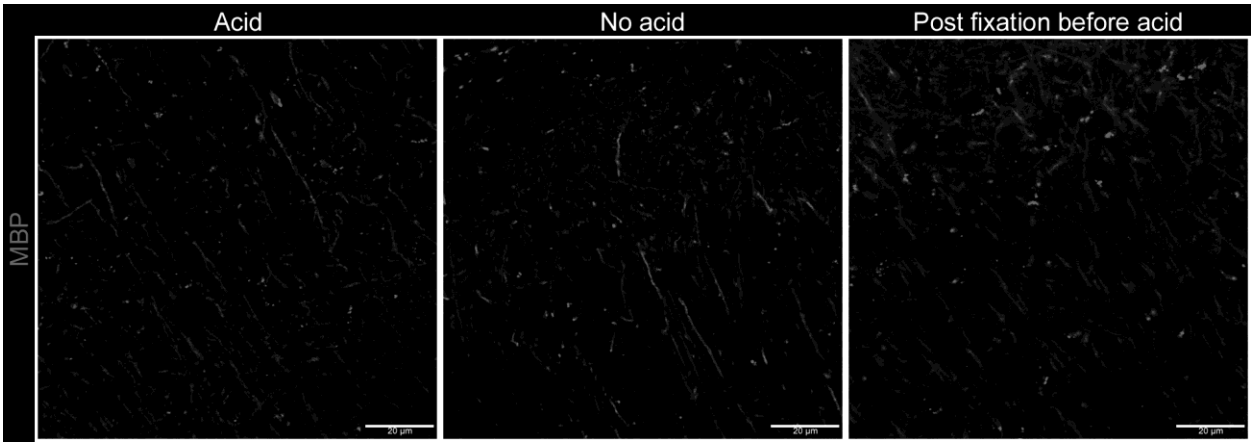
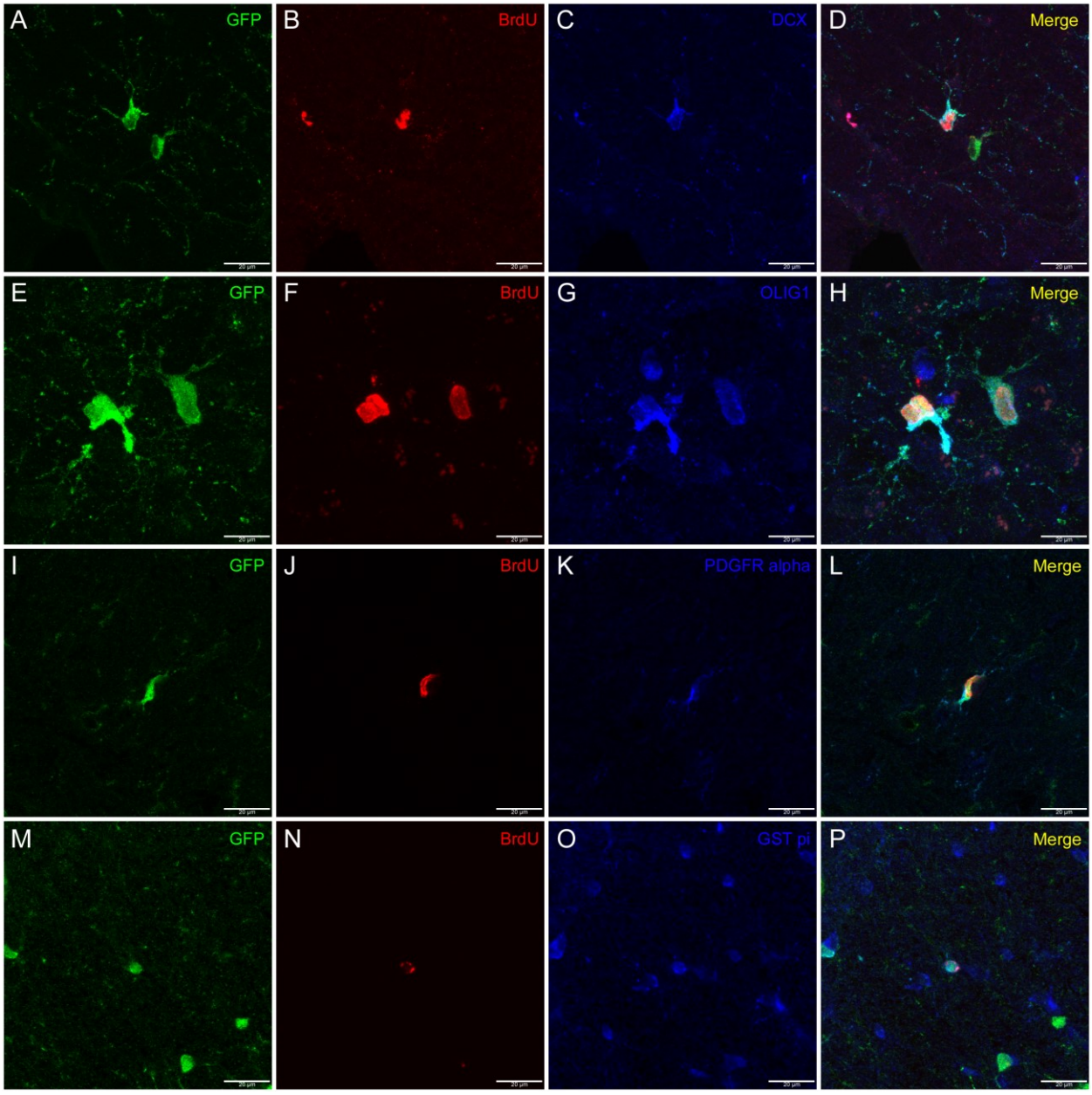


Figure 7.


A
GFP
B
BrdU
C
DCX
D
Merge
E
GFP
F
BrdU
G
OLIG1
H
Merge
I
GFP
J
BrdU
K
PDGFR alpha
L
Merge
M
GFP
N
BrdU
O
GST pi
P
Merge

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EXPERIMENT 2: UNBIASED STEREOLOGICAL ANALYSIS OF THE FATE OF OLIGODENDROCYTE PROGENITOR CELLS IN THE ADULT MOUSE BRAIN AND EFFECT OF REFERENCE MEMORY TRAINING

Jenna J. BOULANGER and Claude MESSIER

School of Psychology, University of Ottawa

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Correspondence to: C. Messier, School of Psychology, University of Ottawa, 136 Jean-Jacques Lussier Room 2076A, Ottawa, Ontario, K1N 6N5, CANADA. Tel (613) 562-5800 ext. 4562, Fax: (613) 562-5147; E-mail: cmessier@uottawa.ca.

Abbreviations

In alphabetical order:

BrdU: 5-bromo-2’-deoxyuridine

CNPase: 2’,3’-cyclic nucleotide 3’-phosphodiesterase

DAPI: 4’,6-diamidino-2-phenylindole

EYFP: enhanced fluorescent yellow protein

GFP: green fluorescent protein

GST-pi: glutathione S-transferase pi

NeuN: Neuronal nuclear protein, also known as Fox-3 or Rbfox3

NG2: chondroitin sulfate proteoglycan neuron-glia antigen 2

OPC: oligodendrocyte progenitor cell

PDGFRα: platelet-derived growth factor receptor alpha

Abstract

Oligodendrocyte progenitor cells (OPC) are glial cells that differentiate into myelinating oligodendrocytes during early stages of post-natal life. However, OPCs persist beyond developmental myelination and represent an important population of cycling cells in the grey and white matter of the adult brain. Here, we used unbiased systematic stereological analysis to determine the total number of OPCs in the neocortex and corpus callosum of the adult mouse. We found that the ratio of OPCs to neurons is of 1 : 10 in the adult neocortex. Likewise, the ratio of OPCs to oligodendrocytes is of 1 : 1 in the cortex and 1 : 7 in the corpus callosum. We also used BrdU labelling and the NG2-CreER^{TM}:EYFP reporter mouse to determine the proportion of proliferating adult OPCs and their fate. We show that OPCs continue to differentiate into oligodendrocytes in adulthood, with white matter OPCs being more likely to differentiate into an oligodendrocyte phenotype than grey matter OPCs. The differentiation of OPCs into an oligodendrocyte phenotype can occur either directly from a spontaneous differentiation by an OPC or following OPC cell division. We also provide evidence for the neuronal differentiation of adult OPCs in the cortical grey matter. Although activity-dependent neural network activity has been hypothesized to serve as a modulator of OPC proliferation and differentiation, we found that reference memory training did not affect the proportion of proliferating and differentiated OPCs in the adult mouse brain.

Key Words: myelin, adult neurogenesis, myelin remodelling, oligodendrocyte, plasticity, gliogenesis, oligodendrocyte progenitor cells, stereology

Introduction

Oligodendrocyte progenitor cells (OPCs; also known as NG2-glial cells, synantocytes, or polydendrocytes) are a type of glial cell that give rise, as their name suggests, to myelinating oligodendrocytes during early stages of post-natal life (Kang, Fukaya, Yang, Rothstein, & Bergles, 2010; Nishiyama, Watanabe, Yang, & Bu, 2002). OPCs specifically express the platelet-derived growth factor receptor alpha (PDGFRα) and the chondroitin sulfate proteoglycan neuron-glia antigen 2 (NG2), both of which are downregulated in favour of O4 and myelin-specific antigens as the cell transitions from an OPC to an oligodendrocyte phenotype (Butt et al., 1997; Levine, Stincone, & Lee, 1993; Nishiyama, Lin, Giese, Heldin, & Stallcup, 1996; Stallcup, 2002).

Interestingly, a large number of OPCs remain undifferentiated after developmental myelination is completed, and OPCs correspond to 2-9% of the total cell population of the adult brain (Dawson, Polito, Levine, & Reynolds, 2003; Ffrench-Constant & Raff, 1986; Nishiyama, Chang, & Trapp, 1999; Pringle, Mudhar, Collarini, & Richardson, 1992; Wolswijk & Noble, 1989). Adult OPCs are uniformly distributed between the grey and white matter of the central nervous system (CNS) and, while their proliferative activity does decline with age due to a slowing down of the cell cycle, they continue to undergo cell division in the adult, representing the mature brain's most active population of cycling cells (Dawson et al., 2003; Psachoulia, Jamen, Young, & Richardson, 2009; Rivers et al., 2008; Simon, Gotz, & Dimou, 2011). The primary fates of daughter cells of adult OPCs are self-renewal and oligodendrocyte differentiation (Nishiyama, Boshans, Goncalves, Wegrzyn, & Patel, 2016), although there is a debate as to their ability to also differentiate into astrocytes and neurons (Nishiyama et al., 2016; Nishiyama, Komitova, Suzuki, & Zhu, 2009; Vigano & Dimou, 2016; Wigley & Butt, 2009). If

these latter fates are possible, they are classified as minor compared to self-renewal and differentiation along the oligodendrocyte lineage (Nishiyama et al., 2016; Robins et al., 2013b).

Since adult OPCs give rise to functional oligodendrocytes, research has primarily examined the proliferation and fate of OPCs following demyelination, an example of which is multiple sclerosis (reviewed in (Boulanger & Messier, 2014)). However, much less is known about the dynamics of OPC proliferation and differentiation under normal conditions in the adult brain (Boda & Buffo, 2014). Neural network activation has been hypothesized to influence OPC proliferation and differentiation (Fields, 2005, 2008; Gibson et al., 2014; Matsumoto et al., 2011; Purger, Gibson, & Monje, 2015; Tomlinson, Leiton, & Colognato, 2015). Several observations support this hypothesis. First, OPCs appear to respond to electrical activity in neurons by extending processes to nodes of Ranvier and synapses (Butt et al., 1999; Kukley, Capetillo-Zarate, & Dietrich, 2007; Ong & Levine, 1999; Ziskin, Nishiyama, Rubio, Fukaya, & Bergles, 2007). Second, OPCs form synapses directly with neurons and have functional glutamate (AMPA and NMDA) and Gamma-Aminobutyric Acid (GABA) receptors (Bergles, Roberts, Somogyi, & Jahr, 2000; Gallo, Mangin, Kukley, & Dietrich, 2008; Luyt et al., 2007; Velez-Fort, Maldonado, Butt, Audinat, & Angulo, 2010). Finally, excitatory synapses between OPCs and neurons undergo activity-dependent changes analogous to long-term potentiation (Ge et al., 2006). These observations, both anatomical and functional, strongly suggest that neurons and OPCs influence each other, even though the exact nature of that interaction remains to be determined.

External factors that activate neural network activity and that appear to act as modulators of adult OPC proliferation and differentiation include physical activity, environmental enrichment, behavioural stimulation, and motor learning. The proposed effects of these external

factors vary across studies. For instance, while Ehninger et al. (2011) found that voluntary wheel running increases OPC proliferation, Simon et al. (2011) observed that it instead favours cell cycle exit and rapid differentiation into an oligodendrocyte phenotype. Supporting the latter is a recent study by Xiao et al. (2016) in which learning a new motor skill like running on a complex wheel was shown to induce the rapid production of new oligodendrocytes by OPCs that had divided prior to the animal being exposed to the wheel. Since Ehninger et al. (2011) looked at the amygdala while Simon et al. (2011) and (Xiao et al., 2016) focused on the somatosensory and motor cortices, it is also possible that the effects of wheel running and motor learning vary by region.

In addition to studying voluntary exercise, Ehninger et al. (2011) also looked at the effects of behavioural stimulation and environmental enrichment and found that they too increase the proliferation of OPCs located in the amygdala. However, the increases in OPC proliferation observed by Ehninger et al. (2011) did not appear to be accompanied by an increase in the expression of the mature oligodendrocyte marker 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). Contrary to previous results (Ehninger et al., 2011), a study found an increase in the number of CNPase-positive cells in the corpus callosum of middle-aged rats exposed to environmental enrichment for 4 months (Zhao et al., 2011). The cortical white matter of middle-aged and aged rats exposed to an enriched environmental for 4 months also displayed an increase in the lengths of myelinated fibres, axon volumes, and myelin sheath volumes (Yang et al., 2013). Similar changes were also observed in the motor cortex of adult mice in response to motor learning (McKenzie et al., 2014). Localized increases in the density of myelinated pathways have also been observed in human subjects that have undergone various forms of extensive training, such as practicing a musical instrument, learning a complex visuo-motor task

such as juggling or who have increased their levels of aerobic exercise (Bengtsson et al., 2005; Scholz, Klein, Behrens, & Johansen-Berg, 2009; Steele, Bailey, Zatorre, & Penhune, 2013; Voss et al., 2013; Zatorre, Fields, & Johansen-Berg, 2012).

Changes in myelin organization occurring in adulthood correspond to a form of neural plasticity known as white matter plasticity (reviewed in (Wang & Young, 2014)). White matter plasticity encompasses two mechanisms that could both benefit from activity-dependent changes in adult OPC dynamics: the *de novo* myelination of previously unmyelinated axons and myelin remodeling, where new oligodendrocytes replace those that have been lost and/or insert themselves into pre-exiting myelin sheaths (Young et al., 2013). Independent of the mechanism used to achieve it, white matter plasticity is thought to facilitate axonal transmission and/or modulate the synchronicity of axonal inputs, which ultimately optimizes neural communication (Lillard & Erisir, 2011; Pajevic, Basser, & Fields, 2014; Young et al., 2013).

In the present experiment, we used unbiased systematic stereological analysis to examine the proliferation and differentiation of OPCs located in the neocortex and corpus callosum of the adult mouse. We also examined the effects of a different type of external factor, reference memory training, on the proliferation and differentiation of adult OPCs. We found that OPCs are numerous in the adult brain, where they continue to proliferate and differentiate into oligodendrocytes. We show that OPCs can differentiate into oligodendrocytes directly (i.e. without a prior cell division), and that white matter OPCs are more likely to differentiate into oligodendrocytes than grey matter OPCs. We also observed a small number of OPCs that had differentiated into a neuronal phenotype in the cortical grey matter of the adult mouse. Finally, we found that reference memory training does not produce overall changes in the proliferation and differentiation of OPCs located in the corpus callosum or the neocortex of the adult mouse.

Methods

Animals

NG2CreER^{TM} BAC transgenic mice (Jackson Laboratory, Bar Harbour, Maine, USA; described in (Zhu et al., 2011)) were bred in house with gtROSA26-EYFP transgenic mice on a C57BL/6J background (Jackson Laboratory, Bar Harbour, Maine, USA) to generate the NG2-CreER^{TM}:EYFP reporter mouse. The NG2-CreER^{TM}:EYFP reporter mouse allows for genetic fate mapping of OPCs through tamoxifen-induced Cre-recombination in the NG2 locus, which activates EYFP expression in NG2+ cells. These EYFP+ cells continue to express EYFP+ even if they are no longer NG2+, therefore enabling lineage tracing of labelled cells. Figure 1 presents the timeline of the procedure. Offspring aged 4-5 months were separated into 2 groups. In the control group (n=3), the mice stayed in their cages throughout the entire length of the study. The animals in the experimental group (n=5) were trained in a Barnes Maze task. All the animals used in this study were individually housed in a 21+/- 1ºC vivarium, maintained on a reverse 12-hour light/dark cycle and had *ad libitum* access to food and water. All animal procedures were done in accordance with the recommendations of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Procedures

Drug administration. Tamoxifen was dissolved in corn oil at a concentration of 10 mg/ml. Animals received a 0.4 ml i.p. injection of tamoxifen once a day for 5 days. 5 days following the last tamoxifen injection and 3 days prior to the first day of training, the animals' drinking water was replaced with sweetened water (100 ml water + 0.125 g saccharine + 3 g dextrose) to habituate them to the new taste and reduce neophobia. The sweetened water was replaced with BrdU sweetened water (100 ml water + 0.125 g saccharine + 3 g dextrose + 0.1 g

BrdU) 7 days following the last tamoxifen injection and 24 hours prior to the experimental group's first trial on the Barnes Maze. BrdU is an analog of the nucleoside thymidine that is incorporated into the DNA of cells during the S phase of the cellular cycle, making it an useful marker of proliferated cells (Gratzner, 1982). BrdU sweetened water was replaced with regular drinking water 48 hours following the experimental group's second-to-last trial on the Barnes Maze. Mice were perfused eight weeks following the end of BrdU exposure.

Barnes maze. The protocol was adapted from Sunyer, Patil, Hoger, and Lubec (2007). The Barnes Maze consisted in a circular platform with 18 equally spaced holes elevated 105 cm above the floor. A bright spotlight was placed above the maze so that mice would be motivated to escape into a dark chamber (escape box) that could be accessed through one of the holes (target hole). A curtain onto which visual cues were hanged surrounded the maze.

Mice were exposed to the maze three times a day for 4 days. Each trial of a specific day was separated from the next trial by 15-20 minutes. The maze was cleaned with 70% ethanol after each animal. At the beginning of each trial, the lights in the room were switched off and the mouse was placed inside a cylindrical black start chamber at the center of the maze. The flood light was then turned on and the animal remained in the start chamber for 10 seconds. The first trial of the first day consisted in an adaptation period where the animal was guided to the target hole and encouraged to enter the escape box. Once the mouse was inside the escape box, the floodlight was turned off and the mouse remained in the escape box for 1 minutes. If the mouse tried to escape the box, it was gently guided back inside.

In the spatial acquisition trials that followed, the animals were allowed to explore the maze for three minutes. If three minutes had elapsed and the mouse had not yet entered the target hole, it was gently guided to it. Mice were allowed to stay in the escape box for 30 seconds

before being returned to their cage. The fifth day consisted in a probe trial evaluating short-term memory where the escape box was removed from the maze. A similar probe trial to evaluate long-term learning was conducted on day 26 (7 days following the short-term probe). For each probe session, the total number of errors (nose pokes inside a hole other than the target hole) and escape latency (time elapsed before the mouse entered the escape box) were recorded.

Tissue Processing

Anesthetized mice were transcardially perfused with saline followed by Lana's fixative (4% paraformaldehyde-picric acid; modified from Zamboni and Demartin (1967)). Brains were post-fixed in this fixative for one hour before being incubated in 10% sucrose in sodium phosphate buffer (0.1 M, pH 7.2) overnight at 4ºC. Brains were then frozen using CO_2 and cut in 15 µm-thick sagittal sections using a cryostat.

Immunochemistry

Antibodies used and dilutions are presented in Table 1. The sections were stained for the following combinations of primary antibodies: GFP/BrdU/PDGFRα to evaluate OPC proliferation, OPC self-renewal and overall number of OPCs; GFP/BrdU/GST-pi to evaluate OPC proliferation and differentiation into oligodendrocytes; and GFP/BrdU/NeuN to evaluate OPC proliferation, OPC differentiation into neurons and the overall number of neurons to validate the stereological method.

Primary Antibodies. Primary antibody solutions were diluted in 0.3% Triton in 10X PBS. Tissue sections were covered with 50 µl of the primary antibody solution and parafilm was placed on top to prevent evaporation. Slides were incubated at room temperature in a humidified chamber for three hours. Following incubation, slides were washed successively three times for

5 minutes in 10X PBS. An anti-GFP antibody that also recognizes EYFP was used to maximize the visualization of EYFP+ NG2 reporter cells.

Secondary antibodies. Secondary antibody solutions were diluted in 0.3% Triton in 10X PBS. Tissue sections were covered with 50µl of the secondary antibody solution and parafilm was placed on top to prevent evaporation. Slides were incubated in a humidified chamber for 30 minutes at 37ºC. Following incubation, slides were washed successively three times for 5 minutes in 10X PBS.

BrdU immunostaining. To preserve immunostaining during the acid/heat denaturation step required for BrdU labeling, a previously described protocol was used (Boulanger et al., 2016). Immunohistochemistry for GFP and PDGFRα, GST-pi, or NeuN was conducted first (primary and secondary antibodies). This was followed by an overnight post-fixation step where slides were incubated in a humid chamber with Lana's fixative overnight at 4 ºC. Slides were then rinsed in 10X PBS 3 times for 5 minutes. This was followed by DNA denaturation, where slides were incubated in HCl 2N for 30 minutes at 37 ºC. Slides were then rinsed three times for 5 minutes in a 0.1M borate buffer pH 8 and three times for 5 minutes in 10X PBS. For BrdU immunohistochemistry, slides were incubated in a humid chamber with a rat anti-BrdU antibody dissolved in PBS with 0.3% Triton-X in the dark for 3 hours at room temperature. Slides were then rinsed three times for 5 minutes in 10X PBS and then incubated in a humid chamber with a donkey anti-rat secondary antibody dissolved in PBS with 0.3% Triton-X for 30 minutes at 37ºC. Slides were then rinsed in 10X PBS 3 times for 5 minutes, treated with custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

Microscopy

High-resolution digital images were obtained using an Olympus FV1000 BX61 laser scan confocal microscope (Olympus Corporation, Tokyo, Japan). Images were captured using the Olympus Fluoview software (Olympus Corporation, Tokyo, Japan).

Stereology

Stereological counts were made in one cortical hemisphere and in the corpus callosum of that hemisphere using the optical fractionator method of the StereoInvestigator software (StereoInvestigator v.11; MBF Bioscience, VT, USA) on a Leica DMR microscope (Leica Microsystems, Germany). To determine the appropriate number of sections that should be evaluated in this study, the sample over-sample method was carried out on seventeen sections separated by 112µm and originating from one hemisphere of a given animal (starting approximately at lateral 0.36mm and ending approximately at lateral 2.52mm). The data suggested that the estimated number of GFP+, GFP+/BrdU+, GFP+/PDGFRα+ and GFP+/PDGFRα+/BrdU+ cells would show similar values had they been counted at intervals of 336µm instead of 112µm (data not shown). Therefore, in this study, the section evaluation interval was 336µm, with five sections being evaluated for each animal. The starting section of each series was randomly selected to ensure that all areas between lateral 0.36mm and lateral 2.52mm had an equal chance of being examined.

The surface area of the cortex and corpus callosum was manually traced at a low magnification using a 10X objective lens. The optical fractionator method created an equally spaced sampling grid that was superimposed onto the traced areas. The size of the grid boxes was 1475µm x 382µm for the cortex and 900µm x 315µm for the corpus callosum. Based on the

size of the traced area, the StereoInvestigator software randomly selected equally spaced grid boxes inside of which a 200µm x 200µm counting frame (i.e. dissector) was placed in the top left corner. The software selected an average 52 sampling sites in the corpus callosum and 82 sampling sites in the cortex for each set of 5 slides. The optical dissector height (thickness) was 15µm with a 1µm top guard zone. Therefore, for each sampling site, the volume examined was 200µm x 200µm x 15µm.

All cells within the counting frames were imaged using a 40X objective lens. Stained cell bodies found within the randomly chosen sampling sites were manually counted with the help of the software. The estimated number of each cell type within the analyzed region (between lateral 0.36mm and lateral 2.52mm of one hemisphere) was calculated using the total number of cells counted at the dissectors, the section sampling fraction (number of sections/total area), the area sampling fraction (area of section sampled/total area), and the thickness sampling fraction (mean section thickness measured at each dissector site/dissector height).

Data analysis

Statistical analyses were performed using SPSS (v.24; IBM Corporation, USA). The global parameters of OPC proliferation and differentiation were analyzed by combining the results of the stereological analysis of all animals taking part in the study. To compare the properties of white matter- and grey matter OPCs, independent samples two-tailed t-tests were conducted. To determine how reference memory training affected OPC proliferation and differentiation, differences among the experimental group trained in the Barnes maze and the control group were evaluated using independent samples two-tailed t-tests that looked at white and grey matter regions separately. For all statistical analyses, significance was accepted at p value of 0.05. Values are expressed as mean ± standard error of the mean (SEM). Pearson

correlations between performance on the reference memory task and the various cell phenotypes evaluated in the context of this study were also performed. Correlations above .500 were reported.

Results

Validation of the stereological method

To validate our stereology protocol, we compared the estimated total number of cortical NeuN+ cells to previously published results. The average number of NeuN+ cells found in all mice tested was 900,654±121,001 for a 2.16mm-thick portion of the cerebral cortex obtained between lateral 0.36mm and lateral 2.52mm of one hemisphere (n = 8). This portion of the cortex represents roughly 50.4% of the total cortical tissue of one cerebral hemisphere (estimated using coordinates from Paxinos and Franklin (2004)). Similarly, we estimated that our 2.16mm-thick sample contained 63.8% of the corpus callosum found in one hemisphere. Based on the stereological estimate obtained from that portion of the cortex, there would be approximately 1,800,000 NeuN+ cells per cerebral hemisphere, or 3,600,000 NeuN+ cells in the entire cerebral cortex of the mouse. This is comparable to previously obtained estimates in mice, stating that the mouse cerebral cortex contains approximately 4,000,000 neurons (Haug, 1987; Roth & Dicke, 2005). The concordance of this estimate and ours validates our stereological methodology and the estimates that will be presented concerning OPCs.

Validation of our stereological protocol is also provided by the coefficient of error (CE), a parameter of within sample variation generated by the StereoInvestigator software. A CE below 10% is considered sufficient for a valid stereological analysis and suggests that enough cells per probe were counted in the study (Coggeshall & Lekan, 1996; Gundersen, 1986;

Pakkenberg & Gundersen, 1988). We obtained a CE of 6.88% for all combinations of cells counted in this study, suggesting that the stereological parameters chosen were appropriate (Gundersen, Jensen, Kieu, & Nielsen, 1999).

Total number of OPCs in the cerebral cortex and corpus callosum and validation of the NG2-CreER^{TM}:EYFP reporter mouse model

PDGFRα is selectively expressed by OPCs (Butt et al., 1997; Nishiyama et al., 1996). To determine the total number of OPCs in the cerebral cortex and corpus callosum of adult mice, we calculated the estimated total number of PDGFRα+ cells located between lateral 0.36mm and lateral 2.52mm in those regions (Figure 2A). Our results suggest that an average of 25,428±7,218 PDGFRα+ cells are located between those coordinates in the corpus callosum and 94,207±33,516 in the cortex (n = 8). Using the approximation presented in the previous section, (i.e. 50.4% of the cortex in one hemisphere and 63.8% of the corpus callosum), we propose that approximately 80,000 PDGFRα+ cells are located in the adult mouse corpus callosum and 375,000 in the adult mouse cerebral cortex.

Like PDGFRα, NG2 is also a selective OPC marker (Nishiyama et al., 1996; Stallcup, 2002). To study the fate of adult OPCs, we used the NG2-CreER^{TM}:EYFP reporter mouse, in which NG2+ cells come to express EYFP upon tamoxifen-induced Cre recombination (Zhu et al., 2011). To optimize the visualization of reporter cells, we used a GFP antibody that recognizes EYFP. Henceforth, NG2+ reporter cells will be referred to as GFP+ cells. To verify the effectiveness of the NG2-CreER^{TM}:EYFP transgene methodology at identifying OPCs, we compared the estimated total number of GFP+ cells and the estimated total number of PDGFRα+ cells in the portion of the cortex and corpus callosum analyzed in this study. The estimated total number of GFP+ cells was lower than the estimated total number of PDGFRα+ cells, with an average of 20,692±6,760 GFP+ cells in the corpus callosum and 78,431±30,943 GFP+ cells in

the cerebral cortex (n = 8). Since the density of OPCs has been shown to remain stable across the lifetime (Dawson et al., 2003; Rivers et al., 2008), we estimate Cre recombination to have been at least 81.4% and 83.3% successful in the corpus callosum and cortex respectively (i.e. total estimated number GFP+ cells / total estimated number PDGFRα+ cells). The estimated number of GFP+ cells is necessarily lower than the estimated number of PDGFRα+ cells because Cre recombination is rarely complete (i.e. not all NG2+ cells at the time of tamoxifen administration underwent Cre recombination and came to express EYFP; (Hayashi & McMahon, 2002; Zhu et al., 2011)).

Although the estimated number of GFP+ cells in the neocortex and corpus callosum reflect those provided by other reports (Dawson et al., 2003), we used relative values (i.e. proportion of cells of a particular phenotype relative to the total estimated number of GFP+ cells) to evaluate the remaining questions. This will control for variability between animals caused by the efficiency of Cre recombination, the relative success of immunostaining, and, possibly, the relative impact of behavioural manipulations.

A small proportion of adult GFP+ cells are mitotically active during a seven-day period in 4-5-month-old animals

To examine the proliferative activity of OPCs in 4-5-month-old animals, we estimated the proportion of GFP+ cells that were also BrdU+. Since GFP and BrdU immunostaining were present in all three combinations of antibodies used in this study, we combined the results of all three analyses to increase statistical power (Figure 2B). Results show that 7.96±1.93% of GFP+ cells in the corpus callosum and 5.23±1.26% of GFP+ cells in the cortex were also BrdU+. The difference in the proliferative activity of GFP+ cells between regions was not statistically significant (two sample $t(46) = 1.183$, $p = .243$).

Reference memory training does not affect the proliferation of GFP+ cells

Environmental enrichment and voluntary wheel running have been shown to increase the proliferation of OPCs located in the amygdala (Ehninger et al., 2011). To determine whether reference memory training could change the level of BrdU incorporation in the neocortex and corpus callosum, we compared the proportion of BrdU+ reporter cells in trained and untrained animals (Figure 2C). In the corpus callosum, the proportion of GFP+/BrdU+ cells was approximately 5.90±1.39% in the trained group and 11.39±4.55% in the control group. This difference was not statistically significant (two tailed $t(22) = 1.403$, $p = .175$). The results were similar in the cortex, where approximately 3.66±0.65% of GFP+ had incorporated BrdU in the trained group and 7.84±3.10% had done so in the control group. Again, this difference was not statistically significant (two tailed $t(22) = 1.667$, $p = .110$).

About 80% of GFP+ cells in the corpus callosum and cortex have an OPC phenotype

To determine the number of reporter cells that had an OPC phenotype at the end of the study, we calculated the proportion of GFP+ cells that co-expressed PDGFRα in all mice studied (Figure 3A and Figure 4). In the corpus callosum, 79.45±5.36% of all GFP+ cells were PDGFRα+, while 81.69±3.38% of GFP+ cells were PDGFRα+ in the cortex (n = 8). There was no statistically significant difference between the two regions analyzed (two tailed $t(14) = .354$, $p = .729$).

Reference memory training does not alter the proportion of GFP+ cells with an OPC phenotype in the cortex or the corpus callosum

According to Ehninger et al. (2011), while physical exercise and environmental enrichment increased the proliferation of OPCs located in the amygdala, they did not have a marked effect on OPC differentiation. To determine the effect of reference memory training on the proportion of OPCs with an undifferentiated phenotype, we compared the proportion of

PDGFRα+ reporter cells in both the corpus callosum and cortex of trained and untrained animals (Figure 3B). No significant differences in the proportion of GFP+/PDGFRα+ cells were found between groups in both regions analyzed (corpus callosum – control group: n = 3, mean = 90.64 ±9.36%, experimental group: n = 5, mean = 72.74±4.90%, two-tailed t(6) = 1.894, p = .107; cortex – control group: n = 3, mean = 84.39±13.05%, experimental group: n = 5, mean = 80.07±3.64%, two-tailed t(6) = .589, p = .578).

Only a small proportion of GFP+ undergo self-renewal in adulthood

Self-renewal refers to OPCs that have divided but remain in an undifferentiated state following cell cycle exit (i.e. keep an OPC phenotype). To determine the amount of reporter cells that underwent self-renewal, we estimated the proportion of GFP+/PDGFRα+ cells that were co-localized with BrdU (Figure 3C and Figure 5). In the corpus callosum, 6.38±2.46% (n=8) of all GFP+/PDGFRα+ cells were BrdU+, while only 2.83±0.99% (n=8) of GFP+/PDGFRα+ cells were BrdU+ in the cortex. This difference in the proportion of self-renewed OPCs between regions was not statistically significant (two tailed t(14) = 1.341, p = .201).

Reference memory training does not influence the proportion of GFP+ cells that undergo self-renewal in the cortex or the corpus callosum

To determine whether reference memory influenced self-renewal, we compared the proportion of GFP+/PDGFRα+ cells that were also BrdU+ in trained and untrained animals (Figure 3D). Reference memory training did not influence self-renewal since no significant differences were found in the proportion of GFP+/PDGFRα+/BrdU+ cells between groups in both the corpus callosum (control group: n = 3, mean = 5.56±5.56%, experimental group: n = 5, mean = 6.88±2.73%, two-tailed t(6) = .242, p = .817) and cortex (control group: n = 3, mean = 2.41±2.21%, experimental group: n = 5, mean = 3.08±1.12%, two-tailed t(6) = .303, p = .772).

GFP+ cells can differentiate into an oligodendrocyte phenotype

To determine whether GFP+ cells could differentiate into oligodendrocytes, we calculated the proportion of GFP+ cells that co-expressed GST-pi, a marker of oligodendrocytes (Figure 6A and Figure 7). Approximately 39.72±4.11% of the total population of GFP+ cells in the corpus callosum were GST-pi+ (n = 8). In the cortex, this proportion was much lower, with only about 12.65±4.75% of GFP+ cells co-expressing GST-pi+ (n = 8). This difference was statistically significant (two-tailed $t(14) = 4.313$, $p = .001$) and is in line with previous evidence showing that OPCs located in white and grey matter have different properties, with white matter OPCs being more likely to differentiate into mature oligodendrocytes than grey matter OPCs (Dawson et al., 2003; Dimou, Simon, Kirchhoff, Takebayashi, & Gotz, 2008; Kang et al., 2010; Rivers et al., 2008). We also determined that GFP+/GST-pi+ cells represent 6.57±1.02% of the total estimated number of GST-pi+ cells located in the corpus callosum and 1.74±0.15% of GST-pi+ cells located in the cortex. The difference between the regions was statistically significant (two-tailed $t(14) = 4.692$, $p = .001$), lending further support to the idea that white- and grey matter OPCs have different properties, with OPCs located in myelin-rich areas being more likely to differentiate into oligodendrocytes than OPCs located in grey matter areas.

Finally, we show that not all GFP+/GST-pi+ cells are BrdU+. Only 12.15±6.24% of GFP+/GST-pi+ cells in the corpus callosum and 7.42±3.60% GFP+/GST-pi+ cells in the cortex co-expressed BrdU+ (n = 15). The difference in the proportion of GFP+/GST-pi+/BrdU+ cells between the two regions was not statistically significant (two tailed $t(14) = .655$, $p = .523$). This result supports the findings of Hughes, Kang, Fukaya, and Bergles (2013), who, using live imaging, were able to show that OPCs can differentiate into oligodendrocytes directly or without a prior cell division.

Reference memory training does not modify the number and proportion of GFP+ cells that differentiate into an oligodendrocyte phenotype

Environmental enrichment, physical exercise, and motor learning have all been shown to increase the differentiation of OPCs along the oligodendrocyte lineage (McKenzie et al., 2014; Simon et al., 2011; Xiao et al., 2016; Zhao et al., 2011). To determine whether reference memory training could do the same, we compared the proportion of GFP+ cells that were also GST-pi+ in the corpus callosum and cortex of trained and untrained animals (Figure 6B). No significant differences in the proportion of GST-pi+ cells were found in either one of the regions examined (corpus callosum – control group: n = 3, mean = 43.98±11.31%, experimental group: n = 5, mean = 37.17±2.13%, two-tailed t(6) = .779, p = .466; cortex – control group: n = 3, mean = 19.29±11.95%, experimental group: n = 5, mean = 8.66±3.10%, two-tailed t(6) = 1.102, p = .313). Similarly, the difference in the proportion of GFP+/GST-pi+ cells that incorporated BrdU was not statistically significant in both regions analyzed (corpus callosum – control group: n = 3, mean = 24.74±14.86%, experimental group: n = 5, mean = 4.59±2.21%, two-tailed t(6) = 1.792, p = .123; cortex – control group: n = 3, mean = 0.58±0.58%, experimental group: n = 5, mean = 11.52±5.00%, two-tailed t(6) = 1.636, p = .153).

A small proportion of GFP+ cells can differentiate into neurons

The potential for OPCs to differentiate into a neuronal phenotype in the mature brain is still under debate (Belachew et al., 2003; Nishiyama et al., 2016; Nishiyama, Suzuki, & Zhu, 2014). To determine whether NG2+ cells can differentiate into neurons in the adult, we counted the number of GFP+/NeuN+ cells located in the cortex of NG2-CreER^{TM}:EYFP reporter mice (Figure 8). In all animals studied, the stereological analysis estimated that 3,317±755 cells were GFP+/NeuN+ in the cortical volume sampled. This corresponds to approximately 4.25±1.00% of the total population of GFP+ cells in that volume (n = 8). It must be noted that these cells

exhibited typical neuron morphology (e.g. large cell body with few processes). Among the GFP+/NeuN+ cell population, only a small fraction was BrdU+, suggesting that cell division is not necessary for OPCs to differentiate into a neuronal phenotype (11.70±5.08% of GFP+/NeuN+ co-expressed BrdU).

Reference memory training does not alter the number of GFP+ cells that differentiate into neurons

Some reports have suggested that activity-dependent neural network activity can promote the differentiation of OPCs along the neuronal lineage (Belachew & Gallo, 2004). To evaluate whether reference memory training promotes the differentiation of OPCs into neurons, we compared the proportion of GFP+ cells that were co-localized with NeuN between groups (Figure 9). No significant difference in the proportion of GFP+/NeuN+ cells was found between the control (n = 3, mean = 4.02±1.76%) and the experimental group (n =5, mean = 4.39±1.37%) in this study (two-tailed $t(6) = .163$, $p = .876$). The difference in the proportion of GFP+/NeuN+ cells that incorporated BrdU in each group is not statistically significant (control: n = 3, mean = 19.05±12.60%; experimental: n = 5, mean = 7.30±3.41%; two-tailed $t(6) = 1.145$, $p = .296$).

Performance on the Barnes maze is correlated with some aspects of OPC proliferation and differentiation

Since taking part in reference memory training did not affect the proliferation and fate of adult OPCs, we examined whether performance on the task had an impact on these measures. To do so, we looked at the correlation existing between the estimated proportion of GFP+/BrdU+, GFP+/GST-pi+, and GFP+/NeuN+ reporter cells in the corpus callosum and cortex and the two recorded markers of performance (i.e. number of errors and latency) on the short-term and long-term probe trials. One animal was removed from the long-term probe trial analysis because it remained stationary and did not solve the maze in the allotted time. In both probe trials, number

of errors and latency were positively correlated, suggesting that a low number of errors and a short latency are both indicators of a good performance (short-term probe trial: Pearson correlation $r(5) = .843$, $p = .073$; long-term probe trial: Pearson correlation $r(4) = .610$, $p = .390$).

Performance on the short-term probe trail was positively correlated with the proportion of GFP+/GST-pi+ reporter cells in the cortex (number of errors: Pearson correlation $r(5) = .746$, $p = .148$; latency: Pearson correlation $r(5) = .587$, $p = .298$). Latency on the short-term probe was also negatively correlated with the proportion of GFP+/NeuN+ reporter cells (Pearson correlation $r(5) = -.572$, $p = .314$).

Performance on the long-term probe trial was positively correlated with the proportion of GFP+/GST-pi+ reporter cells in the corpus callosum (number of errors: Pearson correlation $r(4) = .776$, $p = .222$; latency: Pearson correlation $r(4) = .908$, $p = .092$). Latency on the long-term probe trial was also positively correlated with the proportion of GFP+/BrdU+ reporter cells in the corpus callosum (Pearson correlation $r(4) = .781$, $p = .219$), while the number of errors on the long-term probe trial was negatively correlated with the proportion of GFP+/NeuN+ reporter cells (Pearson correlation $r(4) = -.908$, $p = .020$). The overall pattern of correlations suggests that a good performance on a reference memory task such as the Barnes maze is associated with lower rates of proliferation in the corpus callosum, lower rates of differentiation along the oligodendrocyte lineage, and higher rates of differentiation along the neuronal lineage.

Discussion

The present experiment used stereology to estimate the overall number of OPCs in the cortex and the corpus callosum, examine the relative frequency of three possible fates of OPCs

and determine if a reference memory task had an impact on these values. Our study suggests that an estimated 80,000 OPCs are located in the corpus callosum and 375,000 in the cortex of the adult mouse. Using the estimated number of neurons obtained in this study, we propose that the ratio of OPCs to neurons in the cortex is 1 : 10. During the period that BrdU was administered, about 5% of OPCs in the corpus callosum and 8% of those in the cortex had undergone division. We found that OPCs are more likely to differentiate into oligodendrocytes in the corpus callosum compared to the cortex. Finally, it appears that a significant number of OPCs differentiate into neurons. Reference memory training had few significant effects on the proliferation and fate of adult OPCs. These results raise several questions that are addressed in the following sections.

Estimated number of OPCs in the corpus callosum and cerebral cortex of the adult mouse

Stereology consists of a systematic and random sampling strategy that allows for cell numbers to be counted from small fractions of tissue (Herculano-Houzel, von Bartheld, Miller, & Kaas, 2015). Using stereology, we were able to provide the first estimate of the total number of OPCs in the dorsal cerebral cortex and corpus callosum of the adult mouse brain. Since the density of OPCs in the healthy adult brain has been shown to remain stable across the lifetime, we believe that these estimates apply to mice of varying ages (Dawson, Levine, & Reynolds, 2000; Rivers et al., 2008).

Evidence to support the estimates is two-fold. First, Dawson et al. (2003) reported that the ratio of oligodendrocytes to OPCs is approximately 1 : 1 in the grey matter and 4-5 : 1 in the white matter. We quantified oligodendrocytes with an antibody for the isoenzyme GST-pi, which is found in the cytoplasm and nucleus of pre-myelinating and myelinating oligodendrocytes (Tamura et al., 2007a; Tansey & Cammer, 1991). Based on our estimates, we also find a 1 : 1

ratio of oligodendrocytes to OPCs in the cortex (43,032±6,825 GST-pi+ cells : 43,983±8,403 GFP+ cells, n = 8). However, the ratio of oligodendrocytes to OPCs that we obtained in the corpus callosum was approximately 7 : 1 (52,856±12,434 GST-pi+ cells : 7,208±869 GFP+ cells). This can be explained in a few ways. First, tamoxifen-induced Cre recombination is not always complete, and some NG2+ cells at the time of tamoxifen administration may not have been identifiable with the GFP antibody used in this study (Hayashi & McMahon, 2002; Zhu et al., 2011). Second, the GST-pi antibody labels a portion of NG2+ cells (Tamura et al., 2007a). More specifically, GST-pi translocation from the cytoplasm to the nucleus is required for cells to acquire a mature myelinating oligodendrocyte phenotype and begin to express CNPase, the oligodendrocyte marker used by Dawson et al. (2003). The GST-pi antibody used in this study most likely labeled some OPCs, thereby inflating the number of oligodendrocytes established by the stereological counts. This inflation would be more prominent in the corpus callosum because of the higher density of oligodendrocytes found in white matter tissue.

Second, the isotropic fractionator, a new and highly efficient method of counting cells, has determined that approximately 40% of cells in the adult rodent cortex are neurons (Herculano-Houzel & Lent, 2005). Assuming that this estimate is close to reality, based on the number of neurons that we counted, GFP+ cells would represent approximately 4.2% of the total cell population of the cerebral cortex. This suggests a ratio of OPC to neuron of approximately 1 :10 in the cerebral cortex, and supports the importance of this type of glial cell in the mammalian brain. This estimate is close, albeit slightly higher, than the number reported by Dawson et al. (2003), who proposed that OPCs represent 2-3% of the total cell population of the cortex. Differences in the methodology employed to count the density of OPCs relative to other cell types may account for these diverging results. First, Dawson et al. (2003) based their estimates

on the proportion of DAPI-labelled cell nuclei they themselves counted, which makes for a more precise and less theoretical estimate. However, they only counted cells in a small, discrete portion of the cortex. By using the optical fractionator stereological method, we were able to get an estimate as to the number of OPCs in the entire cortex, therefore controlling for the potential heterogeneity of the density of OPCs within that structure.

Proliferation of OPCs

In both the corpus callosum and cortex, few GFP+ cells incorporated BrdU. Since adult OPCs are the most mitotically active population of cycling cells outside of neurogenic zones, the low quantity of GFP+/BrdU+ cells may appear surprising (Dawson et al., 2003). However, these results are comparable to those obtained by Psachoulia et al. (2009), although their animals were slightly older than ours (i.e. 8 months of age at the time of BrdU administration). Through cumulative BrdU labelling and animal euthanasia at various time points, Psachoulia et al. (2009) were able to show that BrdU incorporation by OPCs is a function of age. More specifically, they showed that the length of the OPC cell cycle increases as animals get older, thereby limiting the number of OPCs that go through the S phase at the time when BrdU is available. Based on their results, we can estimate that the cell cycle of the cortical OPCs analyzed in this study was of 45 days, and that the cell cycle of OPCs located in the corpus callosum is shorter than 45 days. Since BrdU was only administered for 8 days, it is logical to assume that only a small proportion of GFP+ cells incorporated BrdU.

It must also be noted that BrdU systematically underestimates the fraction of proliferating OPCs. Indeed, while the growth fraction (i.e. fraction of the cell population that is actively cycling) of OPCs using BrdU labelling has been estimated to be between 50% (Psachoulia et al., 2009; Rivers et al., 2008) and 80% (Simon et al., 2011), labelling with 5-ethynyl-2'-

deoxyuridine (EdU) has revealed that it is near 100% (Clarke et al., 2012; Young et al., 2013), meaning that all OPCs are engaged in the cell cycle at any given time. Like BrdU, EdU is also a thymidine analog incorporated during the S phase of the cell cycle. Contrary to BrdU, EdU does not require DNA denaturation to be visualized during immunohistochemistry. DNA denaturation is not always successful and so BrdU is not as reliable a marker of cellular proliferation as EdU (Zeng et al., 2010), which may explain why the number of GFP+/BrdU+ cells counted in this study was relatively small.

Differentiation of OPCs into oligodendrocytes

To study the fate of adult OPCs, we made use of the NG2 CreER^{TM}:EYFP reporter mouse, where tamoxifen induces Cre recombination at the NG2 locus, rendering NG2+ cells EYFP+, independent of their future phenotype. By looking at the proportion of reporter cells that co-expressed PDGFRα, we determined that approximately 80% of reporter cells in the corpus callosum and cortex remained OPCs 75 days after tamoxifen induction. To establish whether a proportion of reporter cells differentiated into an oligodendrocyte phenotype, we also looked at the proportion of GFP+ cells that co-expressed GST-pi. The proportion of differentiated cells was significantly different between the corpus callosum and cortex, with approximately 40% of reporter cells being GST-pi+ in the corpus callosum and 13% in the cortex. The proportion of grey matter reporter cells that have and have not differentiated into oligodendrocytes corroborates the results reported by other groups using similar reporter mouse models (Dimou et al., 2008; Rivers et al., 2008; Zhu et al., 2011). However, while the proportion of white matter reporter cells that have differentiated into oligodendrocytes (i.e. GFP+/GST-pi+ cells) is similar to what other groups have reported (Dimou et al., 2008; Psachoulia et al., 2009; Rivers et al., 2008; Zhu et al., 2011), the proportion of reporter cells that have remained undifferentiated in the

corpus callosum is substantially higher than what has been established in the literature. This is most likely due to the fact that the number of reporter cells used to establish the proportion of undifferentiated and differentiated OPCs was different. To avoid antibody conflicts (e.g. PDGFRα and GST-pi antibodies have the same host) and because the microscope attached to the stereology software was equipped with three filter cubes (i.e. Y3, K3, and Y5), to view GFP, BrdU and a phenotypic marker together, PDGFRα, GST-pi, and NeuN immunostaining had to be done on different sets of slides. Immunostaining appears to have been more successful in the PDGFRα set for the corpus callosum (i.e. estimated number of GFP+ larger than in the GST-pi set), thereby inflating the proportion of undifferentiated cells counted in the corpus callosum.

We also showed that only a small proportion of OPCs incorporate BrdU prior to differentiating into an oligodendrocyte. It appears that OPCs can differentiate directly into an oligodendrocyte, that is, without undergoing cell division. In vivo two-photon microscopy imaging of adult OPCs has corroborated this result (Hughes et al., 2013).

Differentiation of OPCs into neurons

We observed a small proportion of GFP+ cells that co-expressed the neuronal marker NeuN in the cortex of adult mice. We are not the first group to provide evidence for the differentiation of OPCs into a neuronal phenotype. For instance, adult OPCs express transcriptional markers traditionally associated with neurons (Lau et al., 2008; Wang, O'Bara, Pol, & Sim, 2013; Zhang et al., 2014). A few groups have also suggested that OPCs can give rise to GABAergic interneurons in the adult neocortex (Dayer, Cleaver, Abouantoun, & Cameron, 2005; Tamura et al., 2007b). Using fate mapping studies with transgenic mouse models similar to our own, others have demonstrated that OPCs can differentiate into neurons in two distinct regions of the brain not analyzed in this study: the hypothalamus (Robins et al., 2013a) and the

piriform cortex (Guo et al., 2010; Rivers et al., 2008). Finally and still using fate mapping, a few more groups have observed a small number of reporter cells expressing neuronal markers in the adult forebrain (Clarke et al., 2012; Huang et al., 2014; Kang et al., 2010). However, not all groups using fate mapping mouse models have reported observing such cells (Dimou et al., 2008; Rivers et al., 2008; Zhu et al., 2011), keeping the debate alive.

Detection and/or the higher estimate of OPCs having differentiated into neurons observed in this study could be due to the unbiased systematic sampling provided by stereology. Whereas other groups typically looked only at a small portion of the cortex, by using stereology, we were able to sample the entire dorsal portion of the cerebrum. So, if OPCs capable of generating neurons are not homogenously distributed throughout the cortex, we were still able to capture them using systematic sampling. The proportion of GFP+/NeuN+ cells is extremely small, with only about 0.37% of NeuN+ cells co-expressing GFP. This makes them extremely difficult to observe. Even with the systematic sampling provided by stereology, only 5.88±1.53 GFP+/NeuN+ cells per animal were actually counted in this study (a number that is then multiplied by stereological variables established by the StereoInvestigator software). The stereological estimates are therefore based on a very small number of GFP+/NeuN+ cells, leaving open the question of artifactual observations.

It should also be noted that some of the GFP+/NeuN+ cells observed in this study were BrdU+. Since neural stem cells located in recognized adult neurogenic zones (i.e. the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampus) give rise to neurons through asymmetric cell division, it is hypothesized that, should OPCs also have this ability, they too would need to undergo cell division prior to differentiating into a neuronal phenotype (Gould, 2007; Kang et al., 2010; Zhao, Deng, & Gage, 2008). While Huang et al.

(2014) and Kang et al. (2010) did not find evidence for BrdU+/NeuN+ reporter cells, we did, lending support to the idea that adult OPCs are multipotent and can give rise to neurons.

However, we cannot discount problems associated with the Cre-loxP technology as a possible explanation for this controversial result (Nishiyama et al., 2016; Nishiyama et al., 2014). It has been hypothesized that when recombination strategies are non-homologous such as the one used here, physiological aging and stress may cause transient and/or ectopic Cre or NG2 expression in neurons. Upon tamoxifen administration, these cells would undergo recombination and express EYFP, even if they do not stem from the OPC lineage (Nishiyama et al., 2014). To circumvent this problem, Huang et al. (2014) generated a mouse model in which Cre recombinase was inserted into the NG2 locus by homologous recombination. However, they too observed a small population of reporter-positive neurons. Future fate mapping studies using alternatives to Cre technology will be needed to clearly determine if OPCs can in fact differentiate into neurons.

Reference memory training and OPC cell dynamics

OPC proliferation and differentiation appear subject to external modulation through environmental enrichment, physical exercise, and motor learning (Ehninger et al., 2011; McKenzie et al., 2014; Simon et al., 2011; Xiao et al., 2016). To determine whether similar effects could be caused by other external factors, we used stereological methods to study the proliferation and differentiation of reporter cells in the NG2-CreER^{TM}:EYFP mouse exposed to repeated trials on the Barnes Maze.

There were no statistically significant differences in the proportion of proliferated or differentiated OPCs in response to reference memory training. Part of this could be explained by the small sample size. However, it must also be said that the sample size is comparable to other

studies using stereological analysis. The goal of the random unbiased sampling at the core of the technique is to control for variability and so large samples are not typically used. Furthermore, our coefficient of error was within the acceptable range, therefore lending support to the parameters used in this study (Coggeshall & Lekan, 1996; Gundersen, 1986; Gundersen et al., 1999).

The lack of statistically significant results could also be explained by our approach in that we looked at the global effects of reference memory training on the entire dorsal cortex and corpus callosum. Reference memories are first processed by the hippocampus and then sent to the cortex for permanent storage. Cholinergic projections to the frontal and parietal cortices are thought to play a crucial role in that process (Kesner, DiMattia, & Crutcher, 1987; Maviel, Durkin, Menzaghi, & Bontempi, 2004). As such, focusing our analysis on more localized areas of the cortex may have been preferable since the effects of reference memory training on OPC proliferation and differentiation may not be homogenously distributed throughout the brain. Support for this hypothesis comes from Xiao et al. (2016), who demonstrated that learning a complex motor skill like running on a wheel with unevenly spaced rungs promotes the differentiation of OPCs into myelinating oligodendrocytes in the motor cortex only and not also in the visual cortex which, it could be argued, is also recruited by the task.

Concluding remarks

We have used an unbiased systematic stereological approach to demonstrate that OPCs are still present in large numbers in the corpus callosum and cortex of 4-5-month-old NG2-CreER^{TM}:EYFP reporter mice. We show that OPCs retain the ability to proliferate and to differentiate into oligodendrocytes at later stages of adulthood, with white matter OPCs being

more likely to differentiate into oligodendrocytes than grey matter OPCs. The differentiation of OPCs into an oligodendrocyte phenotype can occur directly or with a prior cell division. Oligodendrocyte differentiation represents an important aspect of neural plasticity called white matter plasticity. White matter plasticity contributes to cognitive functioning by increasing the speed and the synchronicity of neural inputs (Fields, 2005, 2008; Wang & Young, 2014). We also show evidence of neuronal differentiation by adult OPCs. Neuronal differentiation is rare, however, and some of the positive observations could be the result of problems with the Cre technology used to track the fate of OPCs (Nishiyama et al., 2014).

Activity-dependent neural network activation has been hypothesized to serve as a modulator of OPC proliferation and differentiation (Boda & Buffo, 2014; Tomlinson et al., 2015). For instance, physical exercise, environmental enrichment, and motor learning have all been shown to affect OPC proliferation and/or differentiation in specific regions of the brain (Ehninger et al., 2011; McKenzie et al., 2014; Simon et al., 2011; Xiao et al., 2016; Yang et al., 2013; Zhao et al., 2011). We looked at the effects of reference memory training on overall OPC proliferation and differentiation in the corpus callosum and neocortex of the adult NG2-CreER^{TM}:EYFP reporter mouse. While the effects of reference memory on OPC proliferation and differentiation were not conclusive, we believe that a more robust form of training or, alternatively, a more targeted stereological analysis (e.g. focused on the hippocampus, frontal cortex, or parietal cortex) may yield more significant results and further insights into the effects of learning and activity-dependent network activation on the modulation of white matter plasticity in the adult brain.

Tables

Table 1. List of antibodies used.

Host	Target	Concentration	Company
Primary Antibodies			
Rabbit	GFP	1/1000	Abcam (ab290)
Mouse	GFP	1/500	Abcam (ab1218)
Rabbit	$PDGFR_{\alpha}$	1/250	Santa Cruz (sc-338)
Rabbit	GST-pi	1/500	MBL (311-h)
Mouse	NeuN (Rbfox3)	1/500	Millipore (MAB377)
Rat	BrdU	1/500	Abcam (ab6326)
Secondary Antibodies			
Donkey	Anti-Rabbit Alexa 488	1/1000	Invitrogen
Donkey	Anti-Mouse Alexa 488	1/1000	Invitrogen
Donkey	Anti-Rabbit Alexa 594	1/1000	Invitrogen
Donkey	Anti-Mouse Alexa 594	1/1000	Invitrogen
Donkey	Anti-Rat Alexa 680	1/500	Abcam

Figure legends

Fig. 1. Experimental protocol. (A) Experimental timeline. 4-5 month-old NG2-CreER^{TM}:EYFP mice (n=8) received i.p. injections of tamoxifen once a day for 5 days (days 1-5). On day 10, drinking water was replaced with sweetened water. On day 12, sweetened water was replaced with BrdU sweetened water. On days 13-16, mice in the experimental group had three trials a day on the Barnes maze. On day 17, mice in the experimental group underwent the short-term reference memory probe on the Barnes maze. On day 19, BrdU sweetened water was replaced with regular drinking water. On day 26, mice in the experimental group underwent the long-term reference memory probe on the Barnes maze. Mice were euthanized on day 80, 8 weeks after the end of BrdU exposure. (B) Immunohistochemistry. To avoid antibody conflict and to allow for the visualization of reporter cells, BrdU, and different fates for GFP+ cells, immunostaining had to be done on different sets of slides. Each set consisted of 5 slides. For an OPC fate, immunochemistry was done with GFP, BrdU, and PDGFRα. For an oligodendrocyte fate, immunochemistry was done with GFP, BrdU, and GST-pi. For a neuronal fate, GFP, BrdU, and NeuN. (C) Stereology. The surface area of the corpus callosum (light grey) and dorsal cortex (dark grey) was manually traced at 10X. A sampling grid was placed onto of the traced areas by the StereoInvestigator software. Grid boxes were 900µm x 315µm for the corpus callosum and 1475µm x 382µm for the cortex. The StereoInvestigator software randomly selected equally spaced grid boxes and placed a 200µm x 200µm counting frame in their top left corner. All cells within the counting frames were imaged at 40X and counted using the software.

Fig 2. (A) Total estimated number of OPCs in the cortex and corpus callosum. Numbers were extrapolated from the cells counted between lateral 0.36mm and lateral 2.52mm using the stereological software. Totals were calculated using the immunostaining in the GFP/BrdU/PDGFRα set. The total estimated number of GFP+ cells (dark grey) represents the total number of NG2+ cells that underwent recombination upon tamoxifen administration. The total estimated number of PDGFRα (light grey) represents the total number of OPCs at the end of the experiment (animals aged between 6-7 months). (n=8; *error bars* SEM). (B) Mean proportion of GFP+ cells that entered the cell cycle during the 8-day BrdU pulse period in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm (n=8; *error bars* SEM). (C) Mean proportion of GFP+ cells that entered the cell cycle during the 8-day BrdU pulse period in the cortex and corpus callosum of animals exposed to the Barnes maze (Exp; dark grey; n=5) and control animals (Ctrl; light grey; n=3) between lateral 0.36mm and lateral 2.52mm. (*error bars* SEM).

Fig 3. (A) Mean proportion of GFP+ cells that remained PDGFRα+. These are cells that were NG2+ at the time of tamoxifen administration and remained OPCs or undifferentiated in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm (n=8; *error bars* SEM). (B) Mean proportion of GFP+ cells that remained PDGFRα+ in animals exposed to the Barnes maze (Exp; dark grey; n=5) and control animals (Ctrl; light grey; n=3). These are cells that were

NG2+ at the time of tamoxifen administration and remained OPCs or undifferentiated in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm (*error bars* SEM). (C) Mean proportion of GFP+/PDGFRα+ cells that incorporated BrdU, otherwise known as cells that underwent self-renewal in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm. These are cells that were NG2+ at the time of tamoxifen administration and that entered the cell cycle during the 8-day pulse period (n=8; *error bars* SEM). (D) Mean proportion of GFP+/PDGFRα+ cells that incorporated BrdU, otherwise known as cells that underwent self-renewal in the cortex and corpus callosum of animals exposed to the Barnes maze (Exp; dark grey; n=5) and control animals (Ctrl; light grey; n=3) between lateral 0.36mm and lateral 2.52mm. These are cells that were NG2+ at the time of tamoxifen administration and that entered the cell cycle during the 8-day pulse period (*error bars* SEM).

Fig. 4. Undifferentiated OPC. Pictures taken in the cortex at 40X using a laser scan confocal microscope. NG2+ cells at the time of tamoxifen administration (A; GFP) remained undifferentiated (B; PDGFRα) after the end of the experiment. GFP is therefore co-localized with PDGFRα (C; merged).

Fig. 5. Self-renewed OPCs. Pictures taken in the cortex (A-D) and corpus callosum (E-H) at 40X using a laser scan microscope. NG2+ cells at the time of tamoxifen administration (A, E; GFP) entered the cell cycle BrdU during the 8-day pulse period (C, G; BrdU) and remained OPCs at the end of the experiment (B, F; PDGFRα), making it so that GFP, PDGFRα, and BrdU are co-localized (D, H; merged).

Fig. 6. Oligodendrocyte differentiation. (A) Mean proportion of GFP+ cells that co-express GST-pi. These are cells that were NG2+ at the time of tamoxifen administration and that differentiated into oligodendrocytes by the end of the study in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm (n=8; *error bars* SEM; $*p < .05$). (B) Mean proportion of GFP+ cells that co-express GST-pi in the cortex and corpus callosum of animals exposed to the Barnes maze (Exp; dark grey; n=5) and control animals (Ctrl; light grey; n=3). These are cells located between lateral 0.36mm and lateral 2.52mm that were NG2+ at the time of tamoxifen administration and that differentiated into oligodendrocytes by the end of the study (*error bars* SEM).

Fig. 7. Oligodendrocyte differentiation. Pictures taken in the cortex at 40X using a laser scan microscope. These are cells that were NG2+ at the time of tamoxifen administration (A, E; GFP) and that differentiated into oligodendrocytes by the end of the study (B, F; GST-pi). Some of these cells differentiate directly, that is, without a prior cell division (C; BrdU). Other cells enter the cell cycle first and incorporated BrdU during the 8-day pulse period (G; BrdU). Cells that

differentiate directly are GFP+/GST-pi+/BrdU- (D; merged) and cells that go through the cell cycle first are GFP+/GST-pi+/BrdU+ (H; merged).

Fig. 8. Neuronal differentiation. Pictures taken at 40X in the cortex using a laser scan microscope. These are cells that were NG2+ at the time of tamoxifen administration (A; GFP) and that differentiated into neurons (B; NeuN) by the end of the study, making them GFP+/NeuN+ (C; merged).

Fig. 9. Neuronal differentiation. Mean proportion of GFP+ cells that co-express NeuN in the cortex of animals exposed to the Barnes maze (Exp; dark grey; n=5) and control animals (Ctrl; light grey; n=3). These are cells located between lateral 0.36mm and lateral 2.52mm that were NG2+ at the time of tamoxifen administration and that differentiated into neurons by the end of the study (*error bars* SEM).

Figures

Figure 1.

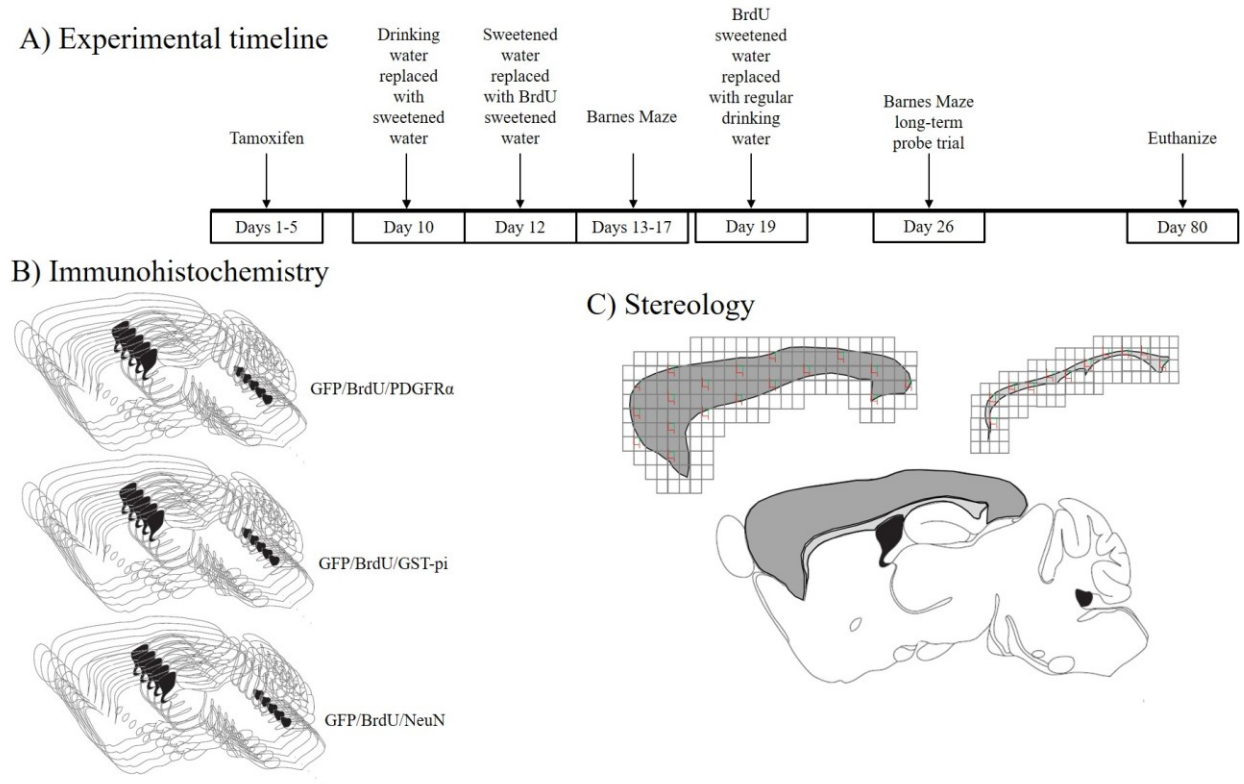
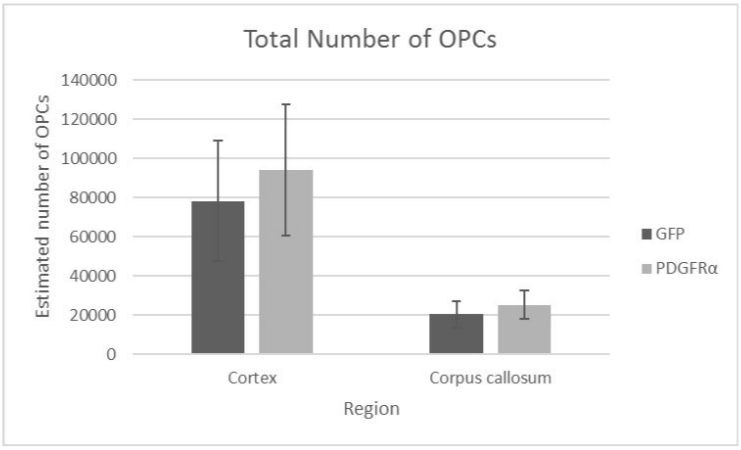
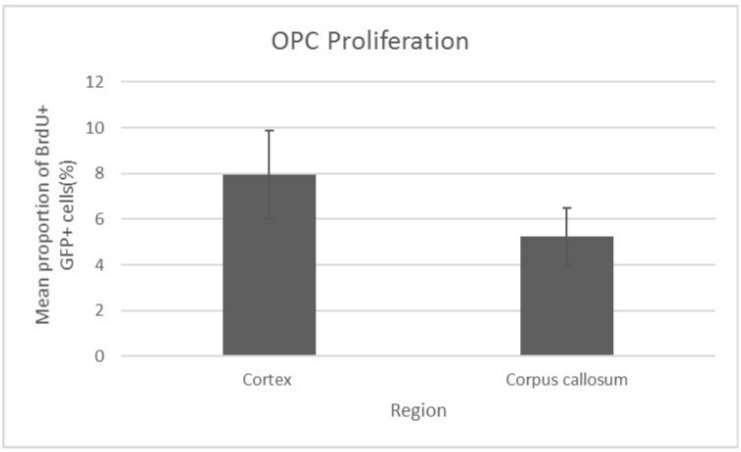


Figure 2.

A


Total Number of OPCs
Estimated number of OPCs
140000
120000
100000
80000
60000
40000
20000
0
Cortex
Corpus callosum
Region
GFP
PDGFRα

B


OPC Proliferation
Mean proportion of BrdU+ GFP+ cells(%)
12
10
8
6
4
2
0
Cortex
Corpus callosum
Region

C

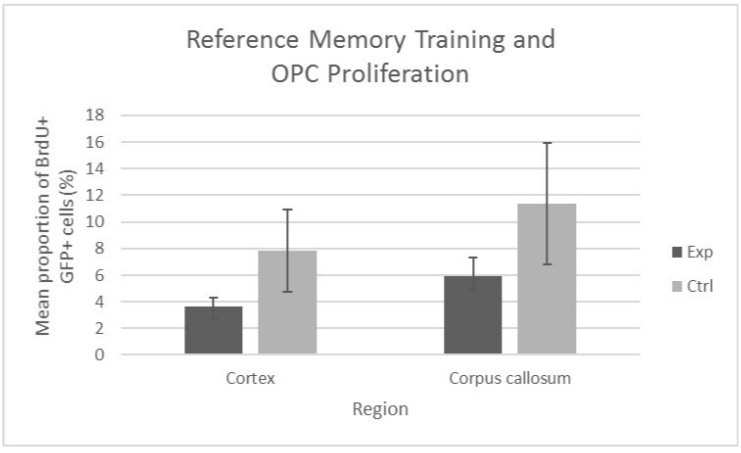

Reference Memory Training and OPC Proliferation
Mean proportion of BrdU+ GFP+ cells (%)
18
16
14
12
10
8
6
4
2
0
Cortex
Corpus callosum
Region
Exp
Ctrl

Figure 3.

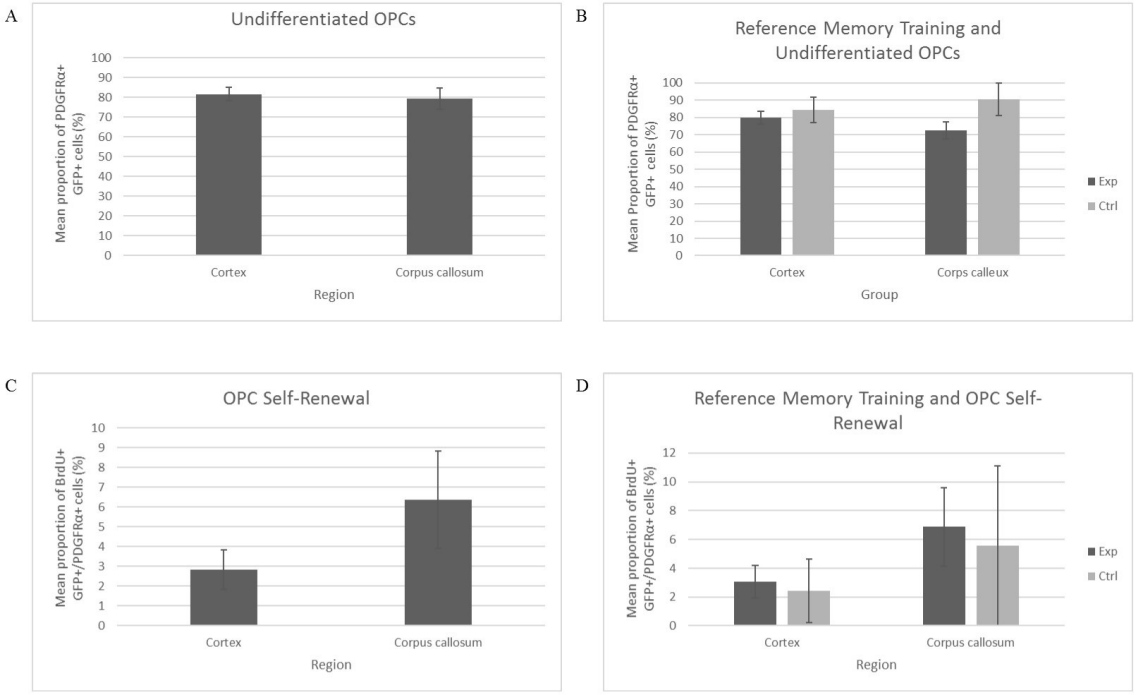

A
Undifferentiated OPCs
Mean proportion of PDGFRα+ GFP+ cells (%)
100
90
80
70
60
50
40
30
20
10
0
Cortex
Corpus callosum
Region
B
Reference Memory Training and Undifferentiated OPCs
Mean Proportion of PDGFRα+ GFP+ cells (%)
100
90
80
70
60
50
40
30
20
10
0
Cortex
Corps calleux
Group
Exp
Ctrl
C
OPC Self-Renewal
Mean proportion of BrdU+ GFP+/PDGFRα+ cells (%)
10
9
8
7
6
5
4
3
2
1
0
Cortex
Corpus callosum
Region
D
Reference Memory Training and OPC Self-Renewal
Mean proportion of BrdU+ GFP+/PDGFRα+ cells (%)
12
10
8
6
4
2
0
Cortex
Corpus callosum
Region
Exp
Ctrl

Figure 4.

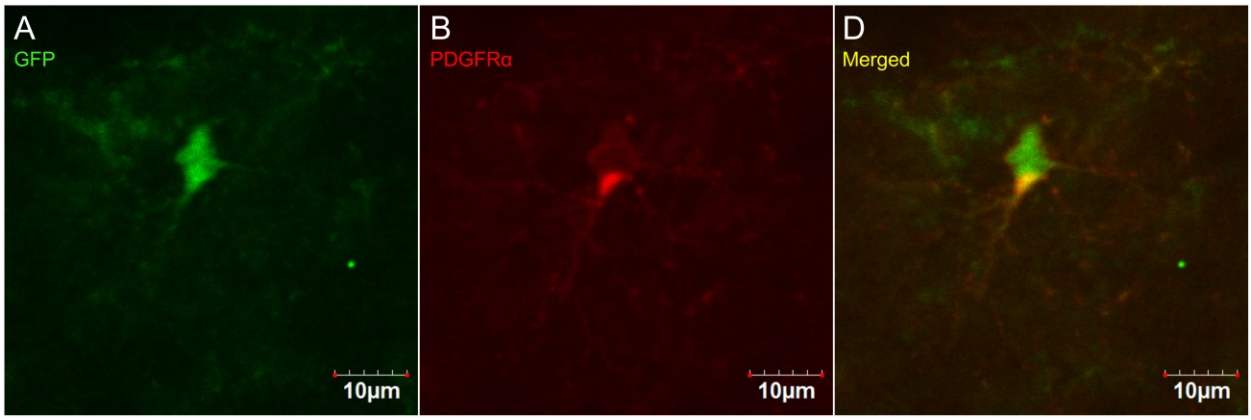


Figure 5.

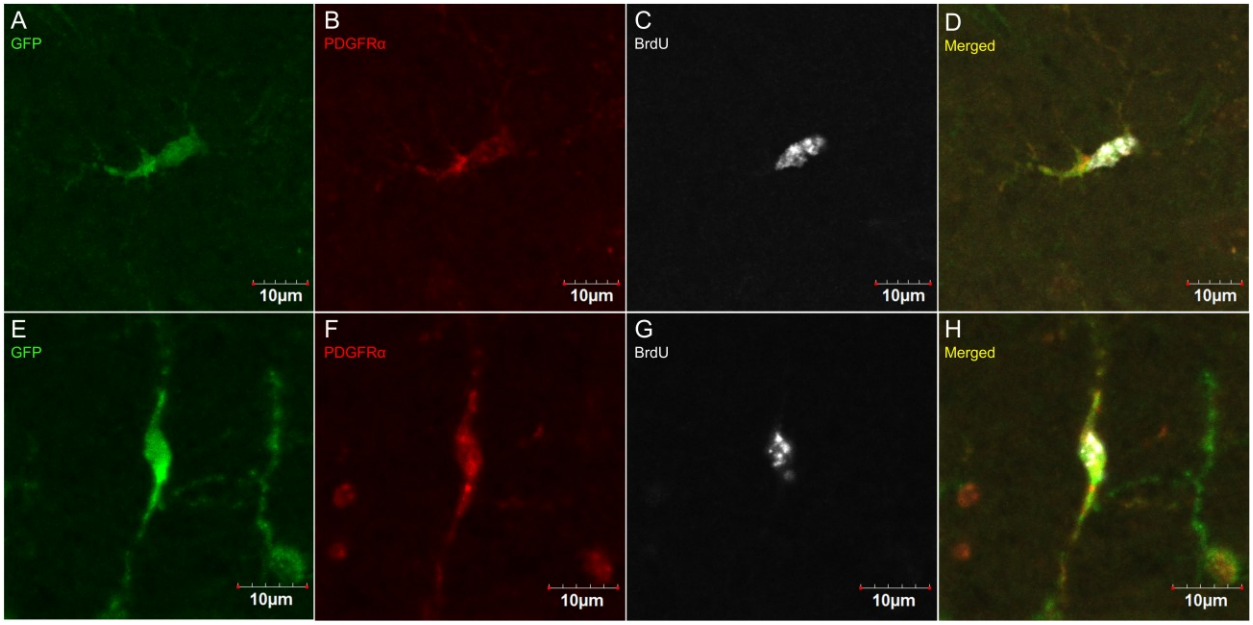
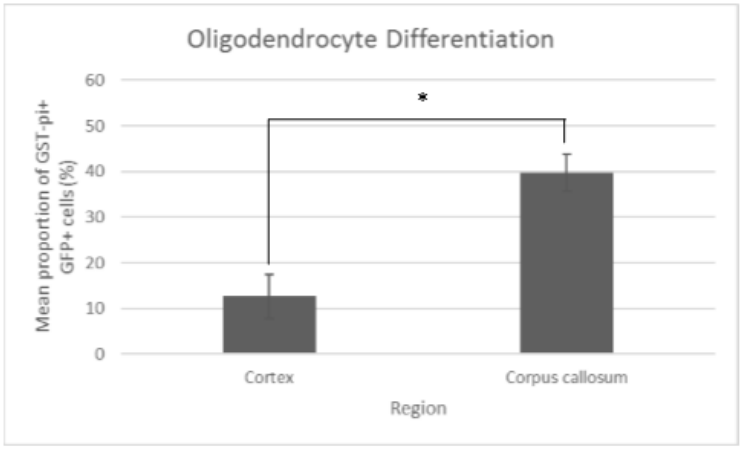

A
GFP
10μm
B
PDGFRα
10μm
C
BrdU
10μm
D
Merged
10μm
E
GFP
10μm
F
PDGFRα
10μm
G
BrdU
10μm
H
Merged
10μm

Figure 6.

A


Oligodendrocyte Differentiation
Mean proportion of GST-pi+ GFP+ cells (%)
60
50
40
30
20
10
0
*
Cortex
Corpus callosum
Region

B

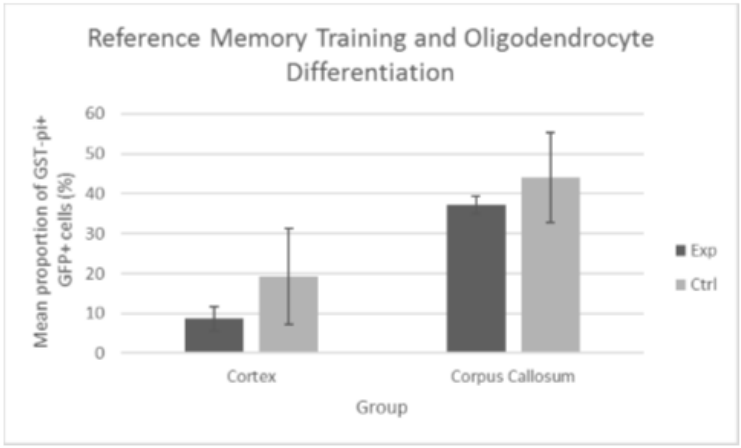

Reference Memory Training and Oligodendrocyte Differentiation
Mean proportion of GST-pi+ GFP+ cells (%)
60
50
40
30
20
10
0
Exp
Ctrl
Cortex
Corpus Callosum
Group

Figure 7.

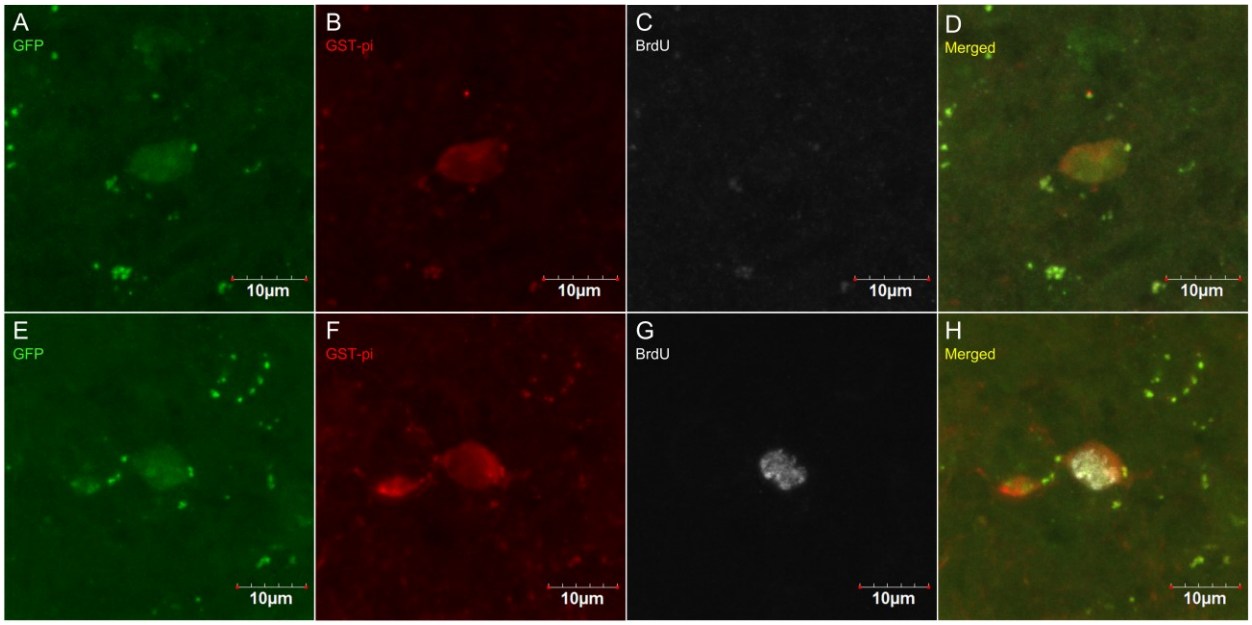

A
GFP
10μm
B
GST-pi
10μm
C
BrdU
10μm
D
Merged
10μm
E
GFP
10μm
F
GST-pi
10μm
G
BrdU
10μm
H
Merged
10μm

Figure 8.

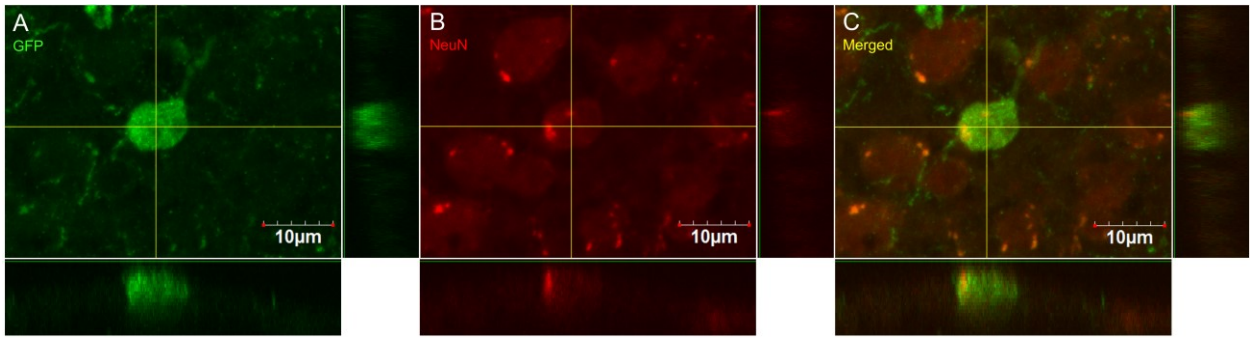
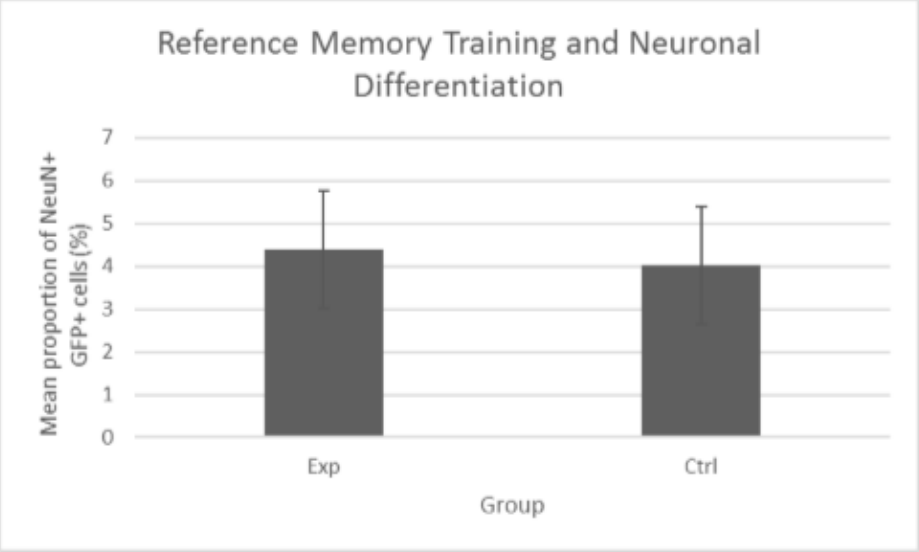


Figure 9.


Reference Memory Training and Neuronal Differentiation
Mean proportion of NeuN+ GFP+ cells (%)
7
6
5
4
3
2
1
0
Exp
Ctrl
Group

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Running Head: Oligodendrocyte progenitor cells and GABA-receptor agonists and antagonists

EXPERIMENT 3: UNBIASED STEREOLOGICAL ANALYSIS OF THE FATE OF OLIGODENDROCYTE PROGENITOR CELLS IN THE ADULT MOUSE BRAIN AND EFFECT OF GABA-RECEPTOR AGONISTS AND ANTAGONISTS

Jenna J. BOULANGER and Claude MESSIER

School of Psychology, University of Ottawa

Correspondence to: C. Messier, School of Psychology, University of Ottawa, 136 Jean-Jacques Lussier Room 2076A, Ottawa, Ontario, K1N 6N5, CANADA. Tel (613) 562-5800 ext. 4562, Fax: (613) 562-5147; E-mail: cmessier@uottawa.ca

Abbreviations

In alphabetical order:

BrdU: 5-bromo-2’-deoxyuridine

EYFP: enhanced fluorescent yellow protein

GFP: green fluorescent protein

GST-pi: glutathione S-transferase pi

HuCD: HuC + HuD neuronal protein

NeuN: Neuronal nuclear protein, also known as Fox-3 or Rbfox3

NG2: chondroitin sulfate proteoglycan neuron-glia antigen 2

OPC: oligodendrocyte progenitor cell

PDGF: platelet-derived growth factor

PDGFRα: platelet-derived growth factor receptor alpha

Sox10: SRY-related HMG-box transcription factor 10

Abstract

Oligodendrocyte progenitor cells (OPC) are glial cells that differentiate into myelinating oligodendrocytes during early stages of post-natal life. However, OPCs persist beyond developmental myelination and represent an important population of cycling cells in the grey and white matter of the adult brain. Here, we used unbiased systematic stereological analysis of the NG2-CreER^{TM}:EYFP reporter mouse to evaluate the proliferation and fate of OPCs. We show that white matter OPCs are more likely to proliferate and to differentiate into an oligodendrocyte phenotype than grey matter OPCs. We also establish that OPCs can differentiate into an oligodendrocyte phenotype either directly from spontaneous differentiation or following OPC cell division. We also provide evidence for the neuronal differentiation of adult OPCs in the cortical grey matter. Since OPCs have receptors for neurotransmitters such as GABA and because receptor activation has been hypothesized to influence OPC proliferation and differentiation, we also examined the effects of $GABA_A$- and $GABA_B$-receptor agonists and antagonists on these physiological processes. While none of the agents analyzed yielded a significant effect, $GABA_A$-receptor agonism does appear to play a modulatory role on adult OPC proliferation and differentiation.

Key Words: myelin, adult neurogenesis, myelin remodelling, oligodendrocyte, plasticity, gliogenesis, oligodendrocyte progenitor cells, stereology, $GABA_A$-receptor, $GABA_B$-receptor

Introduction

Oligodendrocyte progenitor cells (OPCs; also known as NG2-glial cells, synantocytes, or polydendrocytes) are a type of glial cell that give rise, as their name suggests, to myelinating oligodendrocytes during early stages of post-natal life (Kang, Fukaya, Yang, Rothstein, & Bergles, 2010; Nishiyama, Watanabe, Yang, & Bu, 2002). OPCs specifically express the platelet-derived growth factor receptor alpha (PDGFRα) and the chondroitin sulfate proteoglycan neuron-glia antigen 2 (NG2), both of which are downregulated in favour of O4 and myelin-specific antigens as the cell transitions from an OPC to an oligodendrocyte phenotype (Butt et al., 1997; Levine, Stincone, & Lee, 1993; Nishiyama, Lin, Giese, Heldin, & Stallcup, 1996; Stallcup, 2002).

Interestingly, a large number of OPCs remain undifferentiated after developmental myelination is completed, and OPCs correspond to 2-9% of the total cell population of the adult brain (Dawson, Polito, Levine, & Reynolds, 2003; Ffrench-Constant & Raff, 1986; Nishiyama, Chang, & Trapp, 1999; Pringle, Mudhar, Collarini, & Richardson, 1992; Wolswijk & Noble, 1989). Although neonatal and adult OPCs are very similar, the latter have a more mature transcriptome that resembles that of an oligodendrocyte more so than their neonatal counterpart (Moyon et al., 2015). Adult OPCs are uniformly distributed between the grey and white matter of the central nervous system (CNS) and, while their proliferative activity does decline with age due to a slowing down of the cell cycle, they continue to undergo cell division in the adult, representing the mature brain's most active population of cycling cells (Dawson et al., 2003; Psachoulia, Jamen, Young, & Richardson, 2009; Simon, Gotz, & Dimou, 2011). The primary fates of daughter cells of adult OPCs are self-renewal and oligodendrocyte differentiation (Nishiyama, Boshans, Goncalves, Wegrzyn, & Patel, 2016). However, there is a debate as to

their ability to also differentiate into astrocytes and neurons (Nishiyama et al., 2016; Nishiyama, Komitova, Suzuki, & Zhu, 2009; Vigano & Dimou, 2016; Wigley & Butt, 2009). If these fates are possible, they are classified as minor compared to self-renewal and differentiation into oligodendrocytes (Nishiyama et al., 2016; Robins et al., 2013b).

The production of new oligodendrocytes after developmental myelination is completed corresponds to a form of neural plasticity known as white matter plasticity (reviewed in (Wang & Young, 2014)). White matter plasticity encompasses two mechanisms: the *de novo* myelination of previously unmyelinated axons and myelin remodeling, where new oligodendrocytes replace those that have been lost and/or insert themselves into pre-exiting myelin sheaths (Young et al., 2013). White matter plasticity increases the speed of transmission and/or the synchronicity of inputs, which ultimately serves to optimize neural communication (Lillard & Erisir, 2011; Pajevic, Basser, & Fields, 2014; Young et al., 2013).

Since adult OPCs give rise to functional oligodendrocytes, research has primarily examined the proliferation and fate of OPCs following demyelination, as it could have important implications for neurodegenerative diseases such as multiple sclerosis (reviewed in (Boulanger & Messier, 2014)). However, much less is known about the dynamics of OPC proliferation and differentiation under normal conditions (Boda & Buffo, 2014). Recent reports have pointed to experience-dependent neural network activity, with neurotransmitters specifically identified as potential modulators of OPC proliferation and differentiation (Boda & Buffo, 2014; Fields, 2008, 2015; Gallo, Mangin, Kukley, & Dietrich, 2008; Gibson et al., 2014; Karadottir & Attwell, 2007; Purger, Gibson, & Monje, 2015; Tomlinson, Leiton, & Colognato, 2015; Wake, Lee, & Fields, 2011). OPCs are equipped with functional glutamatergic (kainate, AMPA and NMDA) and GABAergic ($GABA_A$ and $GABA_B$) receptors (Belachew & Gallo, 2004; Chittajallu, Aguirre, &

Gallo, 2004; Gallo & Ghiani, 2000; Gallo et al., 2008; Hill & Nishiyama, 2014; Karadottir, Cavelier, Bergersen, & Attwell, 2005; Lin & Bergles, 2004a, 2004b; Luyt et al., 2007; Maldonado & Angulo, 2015; Maldonado, Velez-Fort, & Angulo, 2011; Patneau, Wright, Winters, Mayer, & Gallo, 1994), and receptor activation has been shown to occur through synaptic or non-synaptic actions (Maldonado & Angulo, 2015; Maldonado et al., 2011; Velez-Fort, Maldonado, Butt, Audinat, & Angulo, 2010). Indeed, OPCs establish synapses directly with neurons (Bergles, Roberts, Somogyi, & Jahr, 2000; Lin & Bergles, 2004a; Ong & Levine, 1999; Velez-Fort et al., 2010). They also extend processes equipped with glutamatergic and GABAergic receptors to nodes of Ranvier and neuron-to-neuron synapses where neurotransmitters are released in the extracellular environment (Butt et al., 1999; Kukley, Capetillo-Zarate, & Dietrich, 2007; Velez-Fort et al., 2010; Ziskin, Nishiyama, Rubio, Fukaya, & Bergles, 2007).

Activation of both glutamate and $GABA_A$ receptors induces the depolarization of OPCs (Bakiri, Attwell, & Karadottir, 2009; Boda & Buffo, 2014; Gallo et al., 2008; Lin & Bergles, 2004a; Maldonado & Angulo, 2015; Tanaka et al., 2009). $GABA_A$ receptor activation induces depolarization because of the highly negative intracellular resting potential of adult OPCs. When the concentration of Cl^- is high within a cell, as it is in OPCs, the opening of GABA receptor channels results in Cl^- efflux and depolarization (Lin & Bergles, 2004a; Tanaka et al., 2009). Whether depolarization is caused by an influx of Na^+ (i.e. activation of glutamate receptors) or by an efflux of Cl^- (i.e. activation of GABA receptors), the resulting depolarisation blocks outward rectifying K^+ channels to prevent repolarization (Berger, Schnitzer, & Kettenmann, 1991; Borges & Kettenmann, 1995; Borges, Ohlemeyer, Trotter, & Kettenmann, 1994; Gallo et al., 1996; Pastor, Chvatal, Sykova, & Kettenmann, 1995).

In cultured OPCs and organotypic brain slices, K^+ channel blockade appears to inhibit OPC proliferation (Karadottir & Attwell, 2007; Knutson, Ghiani, Zhou, Gallo, & McBain, 1997; Pastor et al., 1995; Yuan, Eisen, McBain, & Gallo, 1998). Conversely, mitogens such as platelet-derived growth factor (PDGF) increase the expression of K^+ channels and, consequently, the proliferation of cultured OPCs (Chittajallu, Aguirre, & Gallo, 2005; Vautier, Belachew, Chittajallu, & Gallo, 2004). Thus, potassium channel blockade occurring in response to GABA or glutamate receptor activation and the concomitant OPC depolarization appears to be a negative regulator of OPC proliferation.

In addition to K^+ channel blockade, glutamate and $GABA_A$ receptor activation also increase the concentration of intracellular Ca^{2+} by opening voltage-gated calcium channels (Bernstein, Lyons, Moller, & Kettenmann, 1996; Karadottir & Attwell, 2007; Kirchhoff & Kettenmann, 1992; Kirischuk, Scherer, Moller, Verkhratsky, & Kettenmann, 1995; Tanaka et al., 2009; Verkhratsky, Trotter, & Kettenmann, 1990). Calcium signalling is involved in many cellular processes, including the production of transcription factors responsible for cellular differentiation (Bakiri et al., 2009; Kirischuk et al., 1995; Velez-Fort, Audinat, & Angulo, 2012). Interestingly however, calcium signalling has been hypothesized to hinder differentiation along the oligodendrocyte lineage, perhaps maintaining OPCs in an undifferentiated state so that they can take on roles other than the generation of new myelinating oligodendrocytes in the healthy adult brain (Gallo et al., 2008; Gallo et al., 1996; Karadottir & Attwell, 2007; Velez-Fort et al., 2012). Of note, calcium-regulated protein kinase C expression has been shown to downregulate the production of myelin-associated proteins in cultured OPCs (Baron, de Jonge, de Vries, & Hoekstra, 1998). Since OPC daughter cells are born with functional neuron-OPC synapses inherited from their parent cell, and because receptor activation appears to interfere with OPC

proliferation and differentiation, glutamatergic and GABAergic neurotransmission has been hypothesized to act as an anti-myelination signal to OPCs of the healthy and mature brain (Gallo et al., 2008; Kukley et al., 2008).

There are, however, other groups of research reports that suggest that experience-dependent neural network activity and neurotransmission may instead promote the proliferation of OPCs and/or their differentiation along the oligodendrocyte lineage. For instance, $GABA_B$ receptor activation has specifically been shown to increase the proliferation of cultured OPCs (Luyt et al., 2007). Moreover, the vesicular release of glutamate by neurons has been shown to favour the terminal differentiation of cultured OPCs into myelin-producing oligodendrocytes (Wake et al., 2011). Similarly, OPCs derived from the subventricular zone and recruited to the corpus callosum following a demyelinating injury receive transient glutamatergic inputs from neurons, and these inputs have been hypothesized to promote their differentiation into myelinating oligodendrocytes (Etxeberria, Mangin, Aguirre, & Gallo, 2010). Conversely, loss of GABAergic synaptic input in response to hypoxia has been shown to decrease the differentiation of OPCs into oligodendrocytes *in vivo* (Zonouzi et al., 2015). Finally, independent of neurotransmission, electrical activity in neurons appears to promote OPC proliferation, the differentiation of OPCs along the oligodendrocyte lineage, and myelination in both cell cultures and *in vivo* (Barres & Raff, 1993; Demerens et al., 1996; Gibson et al., 2014; Hines, Ravanelli, Schwindt, Scott, & Appel, 2015; Li, Brus-Ramer, Martin, & McDonald, 2010). So, the findings presented in this paragraph indicate that neurotransmitter activation of OPCs would favour the proliferation and/or differentiation of OPCs into myelinating oligodendrocytes instead of downregulating these processes.

We decided to study *in vivo* the effects of systemic administration of GABAergic agonists and antagonists on the proliferation and fate of OPCs. More specifically, we used stereology to evaluate the effects of a $GABA_A$-receptor agonist (i.e. muscimol), a $GABA_B$-receptor agonist (i.e. baclofen), a $GABA_A$-receptor antagonist (i.e. picrotoxin), and a $GABA_B$-receptor antagonist (i.e. saclofen) on the proliferation and differentiation of OPCs in the adult NG2-CreER^{TM}:EYFP reporter mouse, which allows for genetic fate mapping of OPCs. We show that OPCs are numerous in the corpus callosum and cortex of the adult brain and retain the ability to proliferate and to differentiate into oligodendrocytes. We also show that OPCs located in the corpus callosum are more likely to proliferate and differentiate into an oligodendrocyte phenotype than cortical OPCs. Finally, while $GABA_A$- and $GABA_B$-receptor agonists and antagonists did not have a significant effect on the proliferation and differentiation of adult OPCs, our results suggest that $GABA_A$-receptor activation modulates some aspects of OPC proliferation and differentiation.

Methods

Animals

NG2CreER^{TM} BAC transgenic mice (Jackson Laboratory, Bar Harbour, Maine, USA; described in (Zhu et al., 2011)) were bred in house with gtROSA26-EYFP transgenic mice on a C57BL/6J background (Jackson Laboratory, Bar Harbour, Maine, USA) to generate the NG2-CreER^{TM}:EYFP reporter mouse. The NG2-CreER^{TM}:EYFP reporter mouse allows for genetic fate mapping of OPCs through tamoxifen-induced Cre-recombination in the NG2 locus, which activates EYFP expression in NG2+ cells. These EYFP+ cells continue to express EYFP+ even if they are no longer NG2+, therefore enabling lineage tracing of labelled cells. All the animals

used in this study were individually housed in a 21+/- 1ºC vivarium, maintained on a reverse 12-hour light/dark cycle and had *ad libitum* access to food and water. All animal procedures were done in accordance with the recommendations of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Procedure

Figure 1 presents the timeline of the experimental procedure.

Tamoxifen. Tamoxifen was dissolved in corn oil at a concentration of 10 mg/ml**.** Offspring resulting from the crossbreeding aged 4-5 months were given a 0.4 ml i.p. injection of tamoxifen once a day for 5 days (n=30).

BrdU. Five days following the last tamoxifen injection and three days prior to the first day of GABAergic agent administration, animals' drinking water was replaced with sweetened water (100 ml water + 0.125 g saccharine + 3 g dextrose) to habituate them to the new taste and reduce neophobia. The sweetened water was replaced with sweetened water containing BrdU (100 ml water + 0.125 g saccharine + 3 g dextrose + 0.1 g BrdU) 7 days following the last tamoxifen injection and 24 hours prior to the beginning of the GABAergic drug administration period. Forty-eight hours following the end of the GABAergic drug administration period, the BrdU sweetened water was replaced with regular drinking water.

GABAergic drug administration. Twenty-four hours following the introduction of BrdU sweetened water and 8 days following the last injection of tamoxifen, animals were split into 5 groups. Once a day and for a period of 5 days, mice received an injection of either saline (n=7), muscimol ($GABA_A$ receptor agonist; 1.0 mg/kg, n=5), picrotoxin ($GABA_A$ receptor antagonist; 1.0 mg/kg; n=8), baclofen ($GABA_B$ receptor agonist; 7.5 mg/kg; n=6), or saclofen

($GABA_B$ receptor antagonist; 5.0 mg/kg; n=4). A first group of mice were euthanized 2 weeks following the end of BrdU exposure (n=14) and a second group (n=16) was euthanized 8 weeks following the end of BrdU exposure.

Tissue Processing

Anesthetized mice were transcardially perfused with saline followed by Lana's fixative (4% paraformaldehyde-picric acid; modified from (Zamboni & Demartin, 1967)). Brains were post-fixed in this fixative for one hour before being incubated in 10% sucrose in sodium phosphate buffer (0.1 M, pH 7.2) overnight at 4°C. Brains were then frozen using CO_2 and cut in 15 μm sagittal sections using a cryostat.

Immunochemistry

Antibodies used and dilutions are presented in Table 1.

The sections in the two-week survival group were stained for the following combinations of primary antibodies: GFP/BrdU/PDGFRα to evaluate OPC proliferation, OPC self-renewal and overall number of OPCs with transgene expression; GFP/BrdU/Sox10 to evaluate OPC proliferation and OPCs committed to the oligodendrocyte lineage; and GFP/BrdU/HuCD to evaluate OPC proliferation and OPCs committed to the neuronal lineage.

The sections in the eight-week survival group were stained for the following combinations of primary antibodies: GFP/BrdU/GST-pi to evaluate the terminal differentiation of OPCs into oligodendrocytes; and GFP/BrdU/NeuN to examine the terminal differentiation of OPCs into neurons. NeuN counts were also used to evaluate the total number of neurons to validate the stereological method utilized in this study.

Primary Antibodies. Primary antibody solutions were diluted in 0.3% Triton in 10X PBS. Tissue sections were covered with 50 µl of the primary antibody solution and parafilm was placed on top to prevent evaporation. Slides were incubated at room temperature in a humidified chamber for three hours. Following incubation, slides were washed successively three times for 5 minutes in 10X PBS. An anti-GFP antibody that also recognizes EYFP was used to maximize the visualization of EYFP+ NG2 reporter cells. As such, EYFP+ cells will henceforth be referred to as GFP+ cells. Pericytes also express NG2. Therefore, the NG2-CreER^{TM}:EYFP reporter mouse allows for the visualization of both OPCs and pericytes. To ensure that the stereological estimates only included OPCs, we looked at the morphology of the GFP-labelled cells. While OPCs have a bipolar or multipolar shape with multiple processes, pericytes have a flat, slightly elongated soma, no processes and are associated with blood vessels. Only cells that exhibited the typical morphology of OPCs were counted.

Secondary antibodies. Secondary antibody solutions were diluted in 0.3% Triton in 10X PBS. Tissue sections were covered with 50µl of the secondary antibody solution and parafilm was placed on top to prevent evaporation. Slides were incubated in a humidified chamber for 30 minutes at 37ºC. Following incubation, slides were washed successively three times for 5 minutes in 10X PBS.

BrdU immunostaining. To preserve immunostaining during the acid/heat denaturation step required for BrdU visualization, a previously described protocol was used (Boulanger et al., 2016). Briefly, immunohistochemistry for GFP and PDGFRα, Sox10, HuCD, GST-pi, or NeuN was conducted first (primary and secondary antibodies). This was followed by an overnight post-fixation step where slides were incubated in a humid chamber with Lana's fixative overnight at 4

ºC. Slides were then rinsed in 10X PBS 3 times for 5 minutes. This protects the first immunochemistry from the DNA denaturation step, during which slides were incubated in HCl 2N for 30 minutes at 37 ºC. Slides were then rinsed 3 times for 5 minutes in a 0.1M borate buffer pH 8 and 3 times in 10X PBS for 5 minutes. For BrdU immunohistochemistry, slides were incubated in a humid chamber with a rat anti-BrdU antibody dissolved in PBS with 0.3% Triton-X in the dark for 3 hours at room temperature. Finally, slides were incubated in a humid chamber with a donkey anti-rat secondary antibody dissolved in PBS with 0.3% Triton-X for 30 minutes at 37ºC. Slides were then rinsed in PBS 3 times for 5 minutes, treated with custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

Microscopy

Microscopy observations were made in a small group of NG2-CreER^{TM}:EYFP reporter mice. To establish that OPC/neurons couple were not specific to this transgenic mouse, casual observations in outbred CD-1 mice were also made (but not shown here) to confirm the existence the couples and the identity of the neurons. Immunofluorescence results were visualized using an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) attached to a ProgRes MF Scan camera (Jenoptik, Jena, Thuringe, Germany). Digital images were captured using the ProgRes CapturePro 2.5 software (Jenoptik, Jena, Thuringe, Germany). Objectives were Olympus 20X UPlanSApo (0.7n.a.) and Olympus 40X UPlanSApo (0.9n.a.). High-resolution observations were carried out on an Olympus FV1000 BX61 laser scan confocal microscope (Olympus Corporation, Tokyo, Japan) and images were captured using the Olympus Fluoview software (Olympus Corporation, Tokyo, Japan). The objective for this microscope was an Olympus 60X PlanApo oil SC (1.4n.a.). Filters for both microscopes were Semrock Brightline TXRed-4040A; FITC-3540B; and DAPI. A Chroma 41023 was used for Alexa680.

Stereology

Stereological counts were made in one cortical hemisphere and in the corpus callosum of that same hemisphere using the optical fractionator method of the StereoInvestigator software (StereoInvestigator v.11; MBF Bioscience, VT, USA) on a Leica DMR microscope (Leica Microsystems, Germany). The filters were K3, (Leica #513809), Y3 (Leica #513837), Y5 (Leica #513844). The objectives were 10X (Leica#506505, HC PL Fluotar, 0.30n.a.) and 40X (Leica #506144, HCX PL Fluotar, 0.75n.a.).

To determine the appropriate number of sections that should be evaluated in the present study, the sample over-sample method was first carried out on seventeen sections separated by 112µm and originating from one hemisphere of a given animal (starting approximately at lateral 0.36mm and ending approximately at lateral 2.52mm). The data suggested that the estimated number of GFP+, GFP+/BrdU+, GFP+/PDGFRα+ and GFP+/PDGFRα+/BrdU+ cells would show similar values had they been counted at intervals of 336µm instead of 112µm (data not shown). Therefore, in this study, the section evaluation interval was 336µm, with five sections being evaluated for each animal. The starting section of each series was randomly selected to ensure that all areas between lateral 0.36mm and lateral 2.52mm had an equal chance of being examined.

The cross-sectional area of the dorsal portion of the cortex and the corpus callosum was manually traced at a low magnification using a 10X objective lens. The limits of the corpus callosum are imprecise at the junction between the tightly bundled callosal fibres and the cortical areas where the callosal fibres spread into the cortex. In the present experiment, we traced the limits of the corpus callosum where it starts to lose its solid appearance. The optical fractionator method created an equally spaced sampling grid that was superimposed onto the traced areas.

The size of the grid boxes was 1475µm x 382µm for the cortex and 900µm x 315µm for the corpus callosum. Based on the size of the traced area, the StereoInvestigator software randomly selected equally spaces grid boxes inside of which a 200µm x 200µm counting frame was placed. All cells within the counting frames were imaged using a 40X objective lens. The software selected on average 52 sampling sites in the corpus callosum and 91 sampling sites in the cortex. The optical disector height (thickness) was 16µm with a 1µm top guard zone. The average section thickness was 14.99µm. Therefore, for each sampling site, the volume examined was 200µm x 200µm x 15µm.

The analysed region (between lateral 0.36 mm and lateral 2.52 mm of one hemisphere) ranged roughly from the rhinal fissure anteriorly to the post-subiculum posteriorly. The regions included were the insular, cingulate, frontal association infralimbic, orbital, primary and secondary motor, parietal association, prelimbic, retrosplenial, primary somatosensory, primary and secondary visual cortex. The more lateral part of the cortex that was not sampled comprises extensions of the same structures and we assumed similar cellular distributions. All stained cell bodies found within the randomly chosen sampling sites were manually counted with the help of the software. The estimated number of each cell type within the analyzed region (between lateral 0.36mm and lateral 2.52mm of one hemisphere) was calculated using the total number of cells counted at the sampling sites, the section sampling fraction (number of sections/total area), the area sampling fraction (area of section sampled/total area), and the thickness sampling fraction (mean section thickness measured at each disector site/disector height).

Data analysis

Statistical analyses were performed using SPSS (v.24; IBM Corporation, USA). To compare the properties of white matter- and grey matter OPCs, independent samples two-tailed t-

tests were conducted. To evaluate the effects of GABAergic agonists and antagonists on OPC proliferation and differentiation, one-way ANOVAs were conducted for the white and grey matter regions separately. For all statistical analyses, significance was accepted at *p* value of 0.05. Values are expressed as mean ± standard error of the mean (SEM).

Results

Validation of the stereological protocol

Validation of our stereological protocol is provided by the coefficient of error (CE), a parameter of within sample variation generated by the StereoInvestigator software. A CE below 10% is considered to be sufficient for valid stereological analysis and suggests that enough cells per probe were counted in the study (Coggeshall & Lekan, 1996; Gundersen, 1986; Pakkenberg & Gundersen, 1988). For HuCD and associated markers, the CE was 2.57 (range 2-4%); for Sox10 and associated markers in the corpus callosum, the CE was 3.67 (range 3-6%); for Sox10 and associated markers in the cortex, the CE was 3.87 (range 3-4%); for PDGFRα and associated markers in the corpus callosum, the CE was 7.67 (range 7-10%); for PDGFRα and associated markers in the cortex, the CE was 5.93 (range 5-7%); for NeuN and associated markers, the CE was 2.93 (range 2-4%); for GSTpi and associated markers in the corpus callosum, the CE was 5.53 (range 3-9%); for GSTpi and associated markers in the cortex, the CE was 5.20 (range 4-8%). These estimates suggest that the stereological parameters chosen were appropriate (Gundersen, Jensen, Kieu, & Nielsen, 1999).

Neuronal estimates

We compared the estimated total number of cortical NeuN+ cells to previously published results. The average number of NeuN+ cells found in all mice tested was 1,183,571±122,495 for a 2.16mm-thick portion of the cerebral cortex obtained between lateral 0.36mm and lateral 2.52mm of one hemisphere (n = 16). This portion of the cortex represents roughly 50.4% of the total cortical tissue of one cerebral hemisphere (as estimated using anatomical landmarks from (Paxinos & Franklin, 2004)). Similarly, we estimated that our 2.16mm-thick sample contained 63.8% of the corpus callosum found in one hemisphere. Based on the stereological estimate obtained from that portion of the cortex, there would be approximately 2,348,355±243,045 NeuN+ cells per hemisphere, or 4,696,710±486,090 NeuN+ cells in the entire cerebral cortex of the mouse. This is comparable to previously obtained estimates in mice, stating that the cerebral cortex is comprised of approximately 4,000,000 neurons (Haug, 1987; Roth & Dicke, 2005). The concordance of these two estimates validates our stereological methodology and the estimates that we will present concerning OPCs.

Total number of OPCs in the cerebral cortex and corpus callosum and validation of the NG2-CreER^{TM}:EYFP reporter mouse model

PDGFRα is selectively expressed by OPCs (Butt et al., 1997; Nishiyama et al., 1996). To determine the total number of OPCs in the dorsal portion of the cerebral cortex and corpus callosum of adult mice, we looked at the estimated total number of PDGFRα+ cells located between lateral 0.36mm and lateral 2.52mm in those regions. We determined that an average of 20,962±1,092 PDGFRα+ cells can be found between those coordinates in the corpus callosum, while 92,206±4,975 are located in the cortex (n = 14) two weeks after the administration of GABAergic agents. Assuming our sample represents roughly 50.4% of the total cortical and 63.8% of the corpus callosum tissue of one cerebral hemisphere, this would correspond to

approximately 65,712±3,423 PDGFRα+ cells in the corpus callosum and 365,897±19,742 PDGFRα+ cells in the cerebral cortex.

Like PDGFRα, NG2 is also a selective OPC marker (Nishiyama et al., 1996; Stallcup, 2002). To study the fate of adult OPCs, we made use of the NG2-CreER^{TM}:EYFP reporter mouse, in which NG2+ cells come to express EYFP upon tamoxifen-induced Cre recombination at the gene locus (Zhu et al., 2011). To optimize the visualization of reporter cells, we used a GFP antibody that recognizes EYFP. Henceforth, NG2+ reporter cells will be referred to as GFP+ cells. To verify the effectiveness of the NG2-CreER^{TM}:EYFP reporter mouse at identifying OPCs, we compared the estimated number of GFP+ cells to the estimated number of PDGFRα+ cells in the portion of the cortex and corpus callosum analyzed in this study. The estimated number of GFP+ cells in the areas studied was similar to the estimated total number of PDGFRα+ cells, with an average of 24,839±2,009 GFP+ cells in the corpus callosum and 104,993±5,824 in the dorsal portion of the cerebral cortex (n = 14). Since the estimated number of GFP+ cells is slightly higher than the estimated number of PDGFRα+ cells, it implies that a proportion of GFP+ reporter cells differentiated into a phenotype other than that of an OPC during the two weeks following GABAergic agent administration.

Although the estimated number of GFP+ cells in the neocortex and corpus callosum reflect those provided by other reports (Dawson et al., 2003), we used relative values (i.e. proportion of cells of a particular phenotype relative to the total estimated number of GFP+ cells) to evaluate the remaining questions. This controls for variability between animals caused by the efficiency of Cre recombination, the relative success of immunostaining, and the relative impact of GABAergic agents.

The proportion of mitotically active GFP+ cells is significantly larger in the corpus callosum than in the cerebral cortex

To examine the proliferative activity of OPCs in 4-5-month-old animals, we estimated the proportion of GFP+ cells that were also BrdU+. Since GFP and BrdU immunostaining were present in all combinations of antibodies used in both the two-week and the eight-week survival groups and because the proportion of BrdU+ reporter cells was similar at both timepoints, we combined the results of all of these analyses in an effort to increase statistical power. Results show that 23.16±1.91% of GFP+ cells in the corpus callosum and 11.15±0.79% of GFP+ cells in the dorsal portion of the cerebral cortex were also BrdU+. The difference in the proliferative activity of GFP+ cells between regions was statistically significant (two sample $t(146) = 5.818$, $p = .001$ Fig. 2A). These results are comparable with what has been observed for an eight-day BrdU labelling period (Simon et al., 2011). They also agree with other results showing that white- and grey matter OPCs have different levels of proliferative activity (Kang et al., 2010; Psachoulia et al., 2009; Rivers et al., 2008).

GABAergic agents do not alter the proportion of proliferated GFP+ in the corpus callosum and cortex

There are conflicting reports concerning the effect of GABA receptor activation on OPC cycling activity. By preventing outward currents of potassium, *in vitro* and organotypic studies have shown that $GABA_A$ receptor activation blocks OPC proliferation (Bernstein et al., 1996; Kirchhoff & Kettenmann, 1992; Lin & Bergles, 2004b). Conversely, loss of $GABA_A$ receptor-mediated synaptic input following hypoxia has been shown to increase OPC proliferation *in vivo* (Zonouzi et al., 2015). However, Luyt et al. (2007) have shown that baclofen-induced $GABA_B$ receptor activation promotes OPC proliferation *in vitro*. To determine whether GABAergic

agents could modify the cycling activity of OPCs *in vivo*, we compared the proportion of BrdU+ reporter cells between groups after a two-week BrdU chase period. There was no significant difference in the proportion of GFP+/BrdU+ cells between groups in both the corpus callosum ($F(4,69) = 1.349$, $p = .261$) and the dorsal portion of the cerebral cortex ($F(4,69) = 1.189$, $p = .324$). Graphical representation of this result is shown in Figure 2B, results for each group are presented in Table 2.

The proportion of cells that remain OPCs is significantly higher in the cortex than it is in the corpus callosum

To determine the number of reporter cells that had an OPC phenotype two weeks after the end of the study, we estimated the proportion of GFP+ cells that co-expressed PDGFRα in the corpus callosum and in the dorsal portion of the cerebral cortex. In the corpus callosum, 55.36±2.95% of all GFP+ cells were PDGFRα+, while 64.12±2.47% of GFP+ cells were PDGFRα+ in the cortex (n = 14). The difference in the proportion of PDGFRα+ reporter cells between the two regions is statistically significant (two tailed $t(14) = 2.280$, $p = .031$). Graphical representation of this result is shown in Figure 2C.

GABAergic agents do not alter the proportion of GFP+ that have an OPC phenotype in the corpus callosum and cortex

It has been hypothesized that extra-synaptic GABAergic input maintains OPCs in an undifferentiated state, allowing them to undertake functions other than the formation of new myelinating oligodendrocytes (Velez-Fort et al., 2012). To determine whether GABAergic agents do in fact maintain OPCs in an undifferentiated state, we calculated the proportion of reporter cells that were PDGFRα two weeks after GABAergic agent administration had been

terminated in both the corpus callosum and in the dorsal portion of the cerebral cortex. There was no significant difference in the proportion of GFP+/PDGFRα cells between groups in both the corpus callosum ($F(4,9) = 1.927$, $p = .190$) and cortex ($F(4,9) = .182$, $p = .942$). Graphical representation is shown in Figure 2D and results for each group are presented in Table 2.

The proportion of cells that undergo self-renewal is significantly higher in the corpus callosum than in the cortex

Self-renewal refers to OPCs that have divided but remain in an undifferentiated state following cell cycle exit (i.e. keep an OPC phenotype). To determine the amount of reporter cells that underwent self-renewal, we estimated the proportion of GFP+/PDGFRα+ cells that were co-localized with BrdU in the two-week survival group. In the corpus callosum, 43.85±5.13% (n=14) of all GFP+/PDGFRα+ cells were BrdU+, while only 13.75±1.84% (n=14) of GFP+/PDGFRα+ cells were BrdU+ in the cortex. This difference in the proportion of self-renewed OPCs between regions is statistically significant (two tailed $t(26) = 5.518$, $p = .001$). Graphical representation of this result is shown in Figure 2E. GFP+/PDGFRα+ cells that did and that did not undergo self-renewal are shown in Figure 3.

GABAergic agents do not influence the proportion of GFP+ cells that undergo self-renewal in the corpus callosum and cortex

It was demonstrated that GABAergic interneurons in the hippocampus inhibit the self-renewal of neural stem cells through $GABA_A$ receptor activation in organotypic slices and *in vivo* (Song et al., 2012). To determine whether GABAergic agents could influence self-renewal in OPCs, we calculated the proportion of GFP+/PDGFRα+ cells that were co-localized with BrdU in the corpus callosum and cortex two weeks after GABAergic had been administered.

There were no statistically significant differences in the proportion of GFP+/PDGFRα+/BrdU+ cells between groups in the corpus callosum ($F(4,9) = .396$, $p = .807$) and cortex ($F(4,9) = .497$, $p = .739$). Graphical representation is shown in Figure 2F and results for each group are presented in Table 2.

A large proportion of GFP+ are committed to the oligodendrocyte lineage in both the corpus callosum and cerebral cortex

Sox10 is an oligodendrocyte lineage-specific transcription factor required for the terminal differentiation of cells into myelin-forming oligodendrocytes. Sox10 is selectively expressed by cells that have committed to the oligodendrocyte lineage but that have not yet reached a mature oligodendrocyte phenotype (Li, Lu, Smith, & Richardson, 2007; Stolt et al., 2002). To determine the proportion of GFP+ cells committed to the oligodendrocyte lineage, we counted the number of GFP+ cells that co-expressed Sox10 two weeks after GABAergic agent administration. Approximately 76.47±2.17% of the GFP+ population of the corpus callosum and 73.22±2.91% of the GFP+ population of the cortex was Sox10+ (n = 14). This difference in proportion between regions was not statistically significant (two-tailed $t(26) = .896$, $p = .378$). Graphical representation of this result is shown in Figure 4A.

We also found that not all GFP+/Sox10+ cells are BrdU+. Approximately 33.71±3.99% of GFP+/Sox10+ cells in the corpus callosum and 14.13±2.31% GFP+/Sox10+ cells in the cortex were localized with BrdU (n = 14). The difference in the proportion of GFP+/Sox10+/BrdU+ cells between the two regions is statistically significant (two tailed $t(26) = 4.243$, $p = .001$). These results support findings suggesting that OPCs can differentiate along the oligodendrocyte lineage either directly or without a prior cell division (Hughes, Kang, Fukaya, & Bergles, 2013). It also supports the notion that OPCs located in the white and grey matter have different

properties, with white matter OPCs being more likely to differentiate into oligodendrocytes than grey matter OPCs (Dawson et al., 2003; Dimou, Simon, Kirchhoff, Takebayashi, & Gotz, 2008; Huang et al., 2014; Kang et al., 2010; Rivers et al., 2008). Figure 5 shows GFP+/Sox10+ cells that did or that did not incorporate BrdU.

While Sox10 is a marker of pre-oligodendrocytes, GST-pi is a marker of pre-myelinating and myelinating oligodendrocytes (Tamura et al., 2007a; Tansey & Cammer, 1991). To determine whether GFP+ cells committed to the oligodendrocyte lineage can terminally differentiate into mature oligodendrocytes, we estimated the proportion of GFP+/GST-pi+ reporter cells 8 weeks after the end of the experimental period. At this time point, 41.58±5.02% of GFP+ cells of the corpus callosum and 21.62±3.50% of GFP+ cells of the cortex were GST-pi+. The difference in the proportion of GFP+/GST-pi+ cells across regions is statistically significant (two-tailed $t(30) = 3.261$, $p = .003$). This result is shown in Figure 4C and is consistent with reports showing that white matter OPCs are more likely to differentiate into oligodendrocytes than grey matter OPCs (Dawson et al., 2003; Dimou et al., 2008; Kang et al., 2010; Rivers et al., 2008).

The proportion of BrdU+ GFP+/GST-pi+ cells is smaller than what was observed with Sox10, with only about 18.76±3.66% in the corpus callosum and 10.63±3.01% of GFP+/GST-pi+ cells incorporating BrdU. This difference is not statistically significant (two-tailed $t(30) = 1.716$, $p = .096$) and supports the notion proposed by Hughes et al. (2013) that OPCs can terminally differentiate into an oligodendrocyte phenotype directly, that is, without going through a cell cycle first. Examples of GFP+/GST-pi+ cells that did not and that did incorporate BrdU are shown in Figure 5.

GABAergic agents do not influence the proportion of GFP+ cells that differentiate along the oligodendrocyte lineage in the corpus callosum and cortex

Receptor activation-mediated increases in intracellular Ca^{2+} is thought to regulate the differentiation of OPCs (Bakiri et al., 2009; Gallo et al., 2008). However, whether GABA promotes the differentiation of OPCs along the oligodendrocyte lineage or prevents it is unclear. For instance, loss of $GABA_A$ receptor-mediated synaptic input due to diffuse white matter injury in neonatal mice has been shown to promote the differentiation of OPCs into myelinating oligodendrocytes *in vivo* (Zonouzi et al., 2015). Conversely, endogenously released GABA that binds to the $GABA_A$ receptor reduces the number of oligodendrocyte lineage cells in organotypic slices of the cerebral cortex of perinatal mice (Hamilton et al., 2017). To determine the effects of $GABA_A$ and $GABA_B$ agonists and antagonists on OPC differentiation along the oligodendrocyte lineage, we compared the proportion of GFP+ cells that were Sox10+ across groups in the corpus callosum and dorsal portion of the cerebral cortex two weeks after the agents were administered. There was no significant difference in the proportion of GFP+/Sox10+ cells between groups, both in the corpus callosum ($F(4, 9) = .498$, $p = .738$) and cortex ($F(4, 9) = .310$, $p = .864$). There was also no significant difference across groups in the proportion of GFP+/Sox10+ cells that incorporated BrdU (corpus callosum: $F(4,9) = .838$, $p = .534$; cortex: $F(4,9) = .571$, $p = .691$). Graphical representation is shown in Figure 4B and results for each group are presented in Table 2.

To determine whether GABAergic agents affected the terminal differentiation of GFP+ cells into an oligodendrocyte phenotype, we compared the proportion of GFP+/GST-pi+ cells across groups. There was no significant difference in the proportion of GFP+/GST-pi between experimental groups in the corpus callosum ($F(4,11) = .323$, $p = .857$) or the cortex ($F(4,11) =$

1.353, p = .312) 8 weeks after GABAergic agent administration. Similarly, there was no significant difference in the proportion of GFP+/GST-pi+ cells that incorporated BrdU between groups in the corpus callosum (F(4,11) = 2.177, p = .139) and cortex (F(4.11) = 1.787, p = .202). Graphical representation is shown in Figure 4D and results for each group are presented in Table 2.

A small proportion of GFP+ cells are committed to the neuronal lineage in the cortex

$GABA_A$ receptor activation has been shown to promote the differentiation of hippocampal neural progenitors along the neuronal lineage in organotypic slices of adult mice (Tozuka, Fukuda, Namba, Seki, & Hisatsune, 2005). HuCD is a marker expressed by post-mitotic cells committed to the neuronal lineage, making it a marker of both young and mature neurons (Fornaro, Geuna, Fasolo, & Giacobini-Robecchi, 2003; Okano & Darnell, 1997). To determine whether OPCs could become committed to the neuronal lineage, we calculated the proportion of GFP+/HuCD+ reporter cells in the dorsal portion of the cerebral cortex two weeks after GABAergic agent administration. Approximately 5.83±1.63% of reporter cells located in the cerebral cortex were GFP+/HuCD+ (n = 14). Of the GFP+/HuCD+ cells, only 15.63±7.19% had incorporated BrdU, suggesting that OPCs can differentiate along the neuronal lineage directly or without a prior cell division (n = 14).

NeuN or Rbfox3 is a neuronal specific nuclear protein that appears when cells undergo terminal differentiation into a neuronal phenotype (Mullen, Buck, & Smith, 1992). It is expressed later than HuCD as cells progress along the neuronal lineage. In the 8-week survival group, approximately 2.74±0.79% of reporter cells were NeuN+ (n =16), and of these cells, only 19.72±4.84% were BrdU+. GFP+/NeuN+ cells are shown in Figure 7. This suggests OPCs that enter a neuronal fate do it mostly without a prior cell division.

GABAergic agents do not influence the proportion of GFP+ cells that differentiate along the neuronal lineage in the cortex

To determine whether GABAergic agents could promote the differentiation of OPCs along the neuronal lineage, we compared the proportion of GFP+ that were also HuCD+ across groups. There was no significant difference in the proportion of GFP+/HuCD+ across groups ($F(4,9) = 0.967$, $p = .471$). There also was not a significant difference between groups in the proportion of GFP+/HuCD+ cells that incorporated BrdU ($F(4,9) = .966$, $p = .471$). Graphical representation is shown in Figure 6A and results for each group are presented in Table 2.

To determine whether GABAergic agents could affect the terminal differentiation of GFP+ into neurons, we estimated the proportion of GFP+ that were NeuN+. There was no significant difference in the proportion of GFP+/NeuN+ cells between groups ($F(4,11) = .824$, $p = .537$). There also was no significant difference in the proportion of BrdU+ GFP+/NeuN+ cells between groups ($F(4,11) = .442$, $p = .776$). Graphical representation is shown in Figure 6B and results for each group are presented in Table 2.

Discussion

The present experiment used stereology to estimate the overall number of OPCs in the corpus callosum and cortex, examined the relative frequency of three possible fates of OPCs and determined if GABAergic agonists and antagonists influence the probability of each fate. Our study suggests that approximately 65,000 PDGFRα-positive cells are located in the corpus callosum and 365,000 are found in the cortex of the adult mouse. During the period that BrdU was administered, about 25% of OPCs in the corpus callosum and 11% of those in the cortex had

undergone cell division. We found that OPCs are more likely to proliferate in the corpus callosum than in the cortex. They are also more likely to differentiate into oligodendrocytes in the corpus callosum than in the cortex. OPCs can differentiate into oligodendrocytes directly or by going through the cell cycle first. Finally, it appears as though a significant number of OPCs differentiate into neurons. GABAergic agent administration had no significant effect on the proliferation and fate of adult OPCs. These results raise several questions that are addressed in the following sections.

Estimated number of OPCs in the corpus callosum and cerebral cortex of the adult mouse

Stereology consists of a systematic and random sampling strategy that allows for cell numbers to be counted from small volumes of tissue (Herculano-Houzel, von Bartheld, Miller, & Kaas, 2015). Using stereology, we were able to provide the first estimates of the total number of OPCs in the dorsal cerebral cortex and corpus callosum of the adult mouse brain. Two estimates of the total number of OPCs in the corpus callosum and cortex can be derived from this study: one based on the estimated total number of PDGFRα-positive cells, and another from the estimated total number of GFP-positive reporter cells. GFP-positive reporter cells are derived from the transgenic mouse model used in this study, the NG2-CreER^{TM}:EYFP reporter mouse, in which tamoxifen-induced Cre-recombination at the NG2 locus activates EYFP expression in NG2-positive cells. A GFP antibody was used to enhance the visualization of EYFP-positive cells, which is why we refer to reporter cells as GFP-positive cells. Since EYFP expression relies on effective Cre recombination, and because recombination is typically not complete, the estimated total number of GFP (EYFP)-positive cells was expected to be lower than the estimated total number of PDGFRα-positive cells (Hayashi & McMahon, 2002; Zhu et al., 2011). However, this was not the case. In the corpus callosum, the estimated total number of

PDGFRα-positive cells was 65,712, while the estimated total number of GFP-positive cells was of 77,865. Similarly, in the cortex, the estimated total number of PDGFRα-positive cells was of 365,897, while the estimated total number of GFP-positive cells was of 416,639. It must first be noted that the tamoxifen-administration protocol used in this study was quite robust, which may account for the extensive recombination and large number of GFP-positive cells. However, this cannot explain why the estimated total number of GFP-positive cells is greater than the estimated total number of PDGFRα-positive cells. The discrepancy between the two estimated totals may be due to the relative strength of the immunochemistry with the antibodies we used to detect PDGFRα and GFP. Also, it is possible that the PDGFRα antibody was more sensitive to the DNA-denaturation protocol required for BrdU visualization and/or was less protected by the post-fixation step we added to protect antigens against heat and acid (Boulanger et al., 2016). Still, both results are similar to those obtained in the previous study and are therefore believed to be an adequate estimate of the total number of OPCs in the corpus callosum and cortex of the adult mouse. Evidence to support the estimates is two-fold.

First, Dawson et al. (2003) reported that the ratio of oligodendrocytes to OPCs is approximately 1 : 1 in the grey matter and 4-5 : 1 in the white matter. We quantified oligodendrocytes with an antibody directed at the isoenzyme GST-pi, which is found in the cytoplasm and nucleus of pre-myelinating and myelinating oligodendrocytes (Tamura et al., 2007a; Tansey & Cammer, 1991). Based on our estimates, the ratio of oligodendrocytes to OPCs is of 2 : 1 in the cortex (171,926 GST-pi+ cells : 78,239 GFP+ cells, n=16) and 3 : 1 in the corpus callosum (94,404 GST-pi+ cells : 32,133 GFP+ cells). These proportion are similar but not identical to those obtained by Dawson et al. (2003). The discrepancies may be due to a number of factors. First, tamoxifen-induced Cre recombination is not always complete, and some

NG2+ cells at the time of tamoxifen administration may have not been labeled with the GFP antibody used in this study (Hayashi & McMahon, 2002; Zhu et al., 2011). Second, Dawson et al. (2003) did not use a transgenic mouse model in their study, which may partly explain why our results are slightly different to theirs. Third, the GST-pi antibody labels a portion of NG2+ cells (Tamura et al., 2007a). More specifically, GST-pi translocation from the cytoplasm to the nucleus is required for cells to acquire a mature myelinating oligodendrocyte phenotype and begin to express CNPase, which was the oligodendrocyte marker used by Dawson et al. (2003). The GST-pi antibody used in this study most likely labeled some OPCs, thereby inflating the number of oligodendrocytes established by the stereological counts using that antibody. Finally, the relative success of the immunochemistry with antibodies for GFP and GST-pi may have been different across animals and regions, thereby explaining why our results have a relatively high rate of variability and why our ratios are slightly different than those established by Dawson et al. (2003).

Second, the isotropic fractionator, a new and highly efficient method of counting cells, has determined that approximately 40% of cells in the adult rat cortex are neurons (Herculano-Houzel & Lent, 2005). Assuming that this estimate is close to reality, based on the number of neurons that we counted, GFP+ cells would represent approximately 3.5% of the total cell population of the cerebral cortex. This estimate is close, albeit slightly higher, than the number reported by Dawson et al. (2003), who proposed that OPCs represent 2-3% of the total cell population of the cortex. Differences in the methodology employed to count the density of OPCs relative to other cell types may account for these diverging results. First, Dawson et al. (2003) based their estimates on the proportion of DAPI-labelled cell nuclei they themselves counted, which makes for a more precise and less theoretical estimate. However, they only counted cells

in a small, discrete portion of the cortex. By using the optical fractionator stereological method, we were able to get an estimate as to the number of OPCs in the entire cortex, therefore controlling for the potential heterogeneity of the density of OPCs within that structure.

Proliferation of OPCs

We determined that approximately 23% of GFP+ cells in the corpus callosum and 11% of those located in the cortex incorporated BrdU during the course of the study in 4-5-month old mice. These results are lower than those established by Simon et al. (2011) and Rivers et al. (2008), who used animals aged 2-3 months, but higher than those obtained by Psachoulia et al. (2009) in animals 8 months of age. Through cumulative BrdU labelling and the euthanasia of animals of various ages at different time points, Psachoulia et al. (2009) demonstrated that BrdU incorporation by OPCs is a function of age. More specifically, they showed that the length of the cell cycle increases as animals get older, thereby limiting the number of OPCs that went through the S phase at the time when BrdU is available. As such, the age of the animals used in this study can explain the level of proliferative activity identified in this study.

It must also be noted that BrdU appears to systematically underestimate the fraction of proliferating OPCs. Indeed, while the growth fraction (i.e. fraction of the cell population that is actively cycling) of OPCs using BrdU labelling has been estimated to be between 50% (Psachoulia et al., 2009; Rivers et al., 2008) and 80% (Simon et al., 2011), labelling with 5-ethynyl-2'-deoxyuridine (EdU) has revealed that it is instead closer to 100% (Clarke et al., 2012; Young et al., 2013), suggesting that all OPCs are engaged in the cell cycle at any given time. Like BrdU, EdU is also a thymidine analog incorporated during the S phase of the cell cycle. Contrary to BrdU, however, EdU does not require DNA denaturation to be visualized during immunohistochemistry. DNA denaturation is not always successful and so BrdU is not regarded

as reliable a marker of cellular proliferation as EdU (Zeng et al., 2010), which may explain why the number of GFP+/BrdU+ cells counted in this study was smaller.

Grey and white matter OPCs have different properties

OPCs are distributed evenly throughout the grey and white matter of the adult brain, where they establish unique territories and continue to proliferate (Hughes et al., 2013). However, we observed that the mitotic activity of grey and white matter OPCs is different, with white matter OPCs being more likely to enter the S phase of the cell cycle during an eight-day BrdU administration period than grey matter OPCs. Similar observations have previously been made using different methodologies (Dawson et al., 2003; Kang et al., 2010; Psachoulia et al., 2009; Rivers et al., 2008).

In addition to estimating the proliferative activity of OPCs, the NG2-CreER^{TM}:EYFP reporter mouse used in this study allowed us to track the fate of adult OPCs. We show that a larger proportion of proliferated cells undergo self-renewal, or go through a cell division and remain OPCs, in the corpus callosum than in the cortex (i.e. significantly more GFP+/BrdU+/PDGFRα+ cells in the corpus callosum than in the cortex). Lastly, whether they have divided or not, we show that a larger proportion of reporter cells remain OPCs two-weeks after the end of the study in the cortex than in the corpus callosum (i.e. larger proportion of GFP+/PDGFRα+ cells in the cortex than in the corpus callosum). We also found a larger proportion of reporter cells with an oligodendrocyte phenotype in the eight-week survival group (as discussed in the next section). Together, these results suggest that grey and white matter adult OPCs have different properties (Dawson et al., 2003; Dimou et al., 2008; Kang et al., 2010; Psachoulia et al., 2009; Rivers et al., 2008).

Differentiation of OPCs into oligodendrocytes

The primary fates of adult OPCs are self-renewal (i.e. an OPC phenotype) and oligodendrocyte differentiation (Nishiyama et al., 2016). To determine whether OPCs can differentiate into oligodendrocytes in adulthood, we calculated the proportion of GFP+ cells that co-expressed Sox10 in the two-week survival group and GST-pi in the eight-week survival group. Sox10 is a transcription factor that can be expressed by both OPCs and oligodendrocytes and that is required for cells to begin expressing myelin-related proteins (Li et al., 2007; Rivers et al., 2008; Stolt et al., 2002). It is an early marker of an oligodendrocyte fate in cells that still express OPC-specific markers such as NG2 and PDGFRα. We found a similarly high proportion of Sox10-positive reporter cells in the corpus callosum and cortex (i.e. approximately 76% of GFP+ cells in the corpus callosum and 73% of those in the cortex were Sox10+).

To determine whether a similar proportion of reporter cells terminally differentiate into an oligodendrocyte phenotype, we estimated the proportion of GST-pi+ reporter cells in the eight-week survival group. At this time point, we found that approximately 42% of reporter cells in the corpus callosum and 22% of those in the cortex were GST-pi-positive. This significant difference shows that white matter OPCs are more likely to differentiate into an oligodendrocyte phenotype than grey matter OPCs. This result is similar to what has been reported by other groups (Dimou et al., 2008; Psachoulia et al., 2009; Rivers et al., 2008; Zhu et al., 2011). Furthermore, the smaller proportion of GST-pi-positive reporter cells compared to the proportion of Sox10-positive reporter cells suggests that OPCs committed to the oligodendrocyte lineage either take longer than 8 weeks to complete differentiation, or never fully differentiate into an oligodendrocyte phenotype. The difference in the proportion of GST-pi-positive reporter cells in

the corpus callosum and cortex also points to a role of myelin-rich environments in promoting the differentiation of OPCs into mature oligodendrocytes (Vigano et al., 2016).

Finally, we also show that only a small proportion of reporter cells incorporate BrdU prior to differentiating along the oligodendrocyte lineage. We must point out that, based on the results obtained in the two-week survival group, OPCs located in the corpus callosum are significantly more likely to go through the cell cycle prior to differentiating along the oligodendrocyte lineage. Still, it appears as though OPCs can differentiate into an oligodendrocyte phenotype directly, that is, without undergoing cell division first. In vivo two-photon microscopy imaging of adult OPCs has corroborated this finding (Hughes et al., 2013).

Differentiation of OPCs into neurons

We observed a small proportion of reporter cells that co-expressed the neuronal marker HuCD in the two-week survival group and NeuN in the eight-week survival group in the dorsal portion of the cortex analyzed in this study. HuCD was used in the two-week survival group because it is expressed earlier than NeuN in newly generated neurons (Fornaro et al., 2003; Okano & Darnell, 1997). The reporter cells that expressed either HuCD or NeuN exhibited typical neuron morphology (e.g. large cell body with few processes), suggesting that these reporter cells were in fact neurons. Since the proportion of GFP+/NeuN+ cells was smaller than the proportion of GFP+/HuCD+ cells, it appears as though OPCs take longer than 8 weeks to terminally differentiate into neurons or, alternatively, fail to do so entirely.

We are not the first group to provide evidence for the differentiation of OPCs into a neuronal phenotype. For instance, adult OPCs have been shown to express transcriptional markers traditionally associated with neurons (Lau et al., 2008; Wang, O'Bara, Pol, & Sim, 2013;

Zhang et al., 2014). A few groups have also suggested that OPCs can give rise to GABAergic interneurons in the adult neocortex (Dayer, Cleaver, Abouantoun, & Cameron, 2005; Tamura et al., 2007b). Using fate mapping studies with transgenic mouse models similar to our own, others have demonstrated that OPCs can differentiate into neurons in two distinct regions of the brain not analyzed in this study: the hypothalamus (Robins et al., 2013a) and the piriform cortex (Guo et al., 2010; Rivers et al., 2008). Finally using fate mapping, a few more groups have observed a small number of reporter cells expressing neuronal markers in the adult forebrain parenchyma (Clarke et al., 2012; Huang et al., 2014; Kang et al., 2010). However, not all groups using fate mapping mouse models have reported observing such cells (Dimou et al., 2008; Rivers et al., 2008; Zhu et al., 2011), keeping the debate alive.

Detection and/or the higher estimate of OPCs having differentiated into neurons observed in this study could be due to the unbiased systematic sampling provided by stereology. Whereas other groups typically looked at only a small portion of the cortex, by using stereology, we were able to sample the entire dorsal portion of the cerebral cortex. So, if OPCs capable of generating neurons are not homogenously distributed throughout the cortex, we were still able to capture them. The proportion of reporter cells that co-expressed neuronal markers is also extremely small, with only about 0.25% of HuCD-positive cells and 0.31% of NeuN-positive cells co-expressing GFP. This makes them extremely difficult to observe. Even with the systematic sampling provided by stereology, only 14.50±4.08 GFP+/HuCD+ and 7.63±2.83 GFP+/NeuN+ cells per animal were actually counted in this study (a number that is then multiplied by stereological variables by the StereoInvestigator software). The stereological estimates are therefore based on a very small number of GFP+/HuCD+ and GFP+/NeuN+ observed cells.

While our observations suggest that OPCs can in fact differentiate into neurons in cortical grey matter, we cannot discount problems associated with the Cre-loxP technology used in this study as a possible explanation for this controversial result (Nishiyama et al., 2016; Nishiyama, Suzuki, & Zhu, 2014). It has been hypothesized that when recombination strategies are non-homologous such as the one used here, physiological aging and stress may cause transient and/or ectopic Cre or NG2 expression in neurons. Upon tamoxifen administration, these cells would undergo recombination and express EYFP, even if they do not stem from the OPC lineage (Nishiyama et al., 2014). To circumvent this problem, Huang et al. (2014) generated a mouse model in which Cre recombinase was inserted into the NG2 locus by homologous recombination. However, they too observed a small population of reporter-positive neurons in the forebrain. Future fate mapping studies using alternatives to Cre technology will be needed to clearly determine if OPCs can in fact differentiate into neurons.

GABAergic agents and OPC dynamics

OPCs possess receptors for neurotransmitters such as GABA, and receptor-activation has been hypothesized to modulate OPC proliferation and differentiation (Boda & Buffo, 2014; Fields, 2008, 2015; Gallo et al., 2008; Gibson et al., 2014; Karadottir & Attwell, 2007; Nishiyama et al., 2009; Purger et al., 2015; Tomlinson et al., 2015; Wake et al., 2011). However, evidence as to the effects of GABA on OPC proliferation and differentiation *in vivo* in the adult brain is lacking. Furthermore, the effects of GABA receptor-activation on OPC dynamics are at times contradictory and not entirely resolved. For instance, while $GABA_A$-receptor activation has been shown to decrease the proliferation of neonatal OPCs in culture and in organotypic slices (Karadottir & Attwell, 2007; Knutson et al., 1997; Pastor et al., 1995; Yuan et al., 1998), $GABA_B$-receptor activation was shown to increase the proliferation of cultured neonatal OPCs

(Luyt et al., 2007). Furthermore, while $GABA_A$-receptor activation is believed to decrease the differentiation of OPCs along the oligodendrocyte lineage (Baron et al., 1998; Gallo et al., 2008; Gallo et al., 1996; Karadottir & Attwell, 2007), the effects of $GABA_B$-receptor activation on OPC differentiation have yet to be studied. We therefore wished to examine the effects of $GABA_A$- and $GABA_B$-receptor agonists and antagonists on OPC proliferation and differentiation *in vivo* in the adult mouse. Since a similar study determined that GABAergic agonists promote the differentiation of neural progenitors of the hippocampus along the neuronal lineage (Tozuka et al., 2005), and because of the conflicting evidence as to the ability of adult OPCs to differentiate into neurons (Clarke et al., 2012; Dayer et al., 2005; Guo et al., 2010; Huang et al., 2014; Kang et al., 2010; Rivers et al., 2008; Robins et al., 2013a; Tamura et al., 2007b), we looked at three possible fates for labeled reporter cells: OPC, oligodendrocyte, or neuron.

However, we did not find any significant differences in the proliferation and differentiation of OPCs across groups in both regions analyzed. There are a few possible explanations for this result. First, the lack of findings in the corpus callosum may be explained by the fact that almost, if not all, axons within that structure are excitatory in nature (Bakiri et al., 2009; Velez-Fort et al., 2012). Some reports have even suggested that OPCs located within the corpus callosum respond only to glutamatergic currents (Kukley et al., 2007; Velez-Fort et al., 2012; Ziskin et al., 2007). As such, even if the GABAergic agents were able to make their way to the corpus callosum, the OPCs located within that structure may not have been able to respond to them.

A second possibility is related to the concentration of the agents that were administered. To prevent sedation in the case of GABAergic agonists and seizures in the case of GABAergic antagonists, lower concentrations of the agents were administered only once a day for five days

to the animals. Since the effects of glutamatergic neurotransmission on OPC proliferation and differentiation have specifically been shown to be concentration-dependent, it is very likely that the effects of GABA are concentration-dependent as well and that the administered doses were simply too low to evoke any changes in OPC dynamics *in vivo* (Mangin & Gallo, 2011).

Beyond the issue of concentration, the cellular depolarization and calcium currents that accompany GABA receptor-activation have been shown to be very localized events that take place mostly in OPC cell processes. The effects of receptor-activation have therefore been hypothesized to be spatially restricted and limited to the local synthesis of proteins involved in cellular motility and/or secretions, without much of an influence on whole-cell physiological processes such as proliferation and differentiation (Boda & Buffo, 2014; Haberlandt et al., 2011; Hughes et al., 2013; Lin et al., 2005; Wake et al., 2011). This appears to be especially true in the adult because OPC-neuron synapses are lost once developmental myelination is completed. As a result, GABAergic neurotransmission in OPCs achieved through the spillover of neurotransmitters released in neuron-to-neuron synapses rather than from neuron-to-OPC synaptic contacts (Velez-Fort et al., 2010). Spillover transmission is associated with transient responses with slow kinetics, and rarely with the evoked miniature events triggered by the synaptic transmission observed during development (Boda & Buffo, 2014; Velez-Fort et al., 2010). The animals used in our study likely had few neuron to OPC synapses because of their age: this may explain the lack of effects of GABA drugs in our experiment. Lending further support to this hypothesis are reports suggesting that action potentials in axons to which OPCs extend processes modulate OPC proliferation and distribution only during a narrow window of postnatal development (Demerens et al., 1996; Mangin, Li, Scafidi, & Gallo, 2012).

We were able to identify a few trends concerning the effects of GABAergic agents on OPC dynamics. First, much like it was established in cultured OPCs and in organotypic slices (Karadottir & Attwell, 2007; Knutson et al., 1997; Pastor et al., 1995; Yuan et al., 1998), $GABA_A$-receptor agonism appears to decrease the proliferation of adult OPCs *in vivo*. This trend was observed in both the cortex and the corpus callosum. The assumed mechanism for this action is K+ channel blockage in response to $GABA_A$-receptor induced cellular depolarization (Karadottir & Attwell, 2007; Knutson et al., 1997; Pastor et al., 1995; Yuan et al., 1998). Contrary to what has been hypothesized (Gallo et al., 2008; Gallo et al., 1996; Karadottir & Attwell, 2007; Velez-Fort et al., 2012), $GABA_A$-receptor agonism appears to favour differentiation in a phenotype other than that of an OPC in the corpus callosum. This result is therefore more in line with the Wake et al. (2011) study that looked at the effects of vesicular neurotransmitter release on OPC differentiation, where it was found that receptor-activation increases oligodendrocyte differentiation in OPCs. However, whether reporter cells of the corpus callosum are more likely to differentiate into an oligodendrocyte phenotype is difficult to determine. Indeed, in the two-week survival group, analysis of Sox10 expression yielded similar results across groups. In the eight-week survival group, there was a high degree of variability in the muscimol group for reporter cell co-localization with GST-pi. When it comes to GST-pi co-localization, it's also interesting to note that $GABA_B$-receptor agonism and antagonism appears to decrease the levels of oligodendrocyte differentiation, and that is especially true in the cortex. A more thorough study of the effects of $GABA_B$-receptor activation on OPC proliferation, perhaps in culture or in organotypic slices, could be helpful. Finally, in terms of neuronal differentiation, the $GABA_A$-receptor agonist muscimol appears to have had opposing effects in the two-week survival group and in the eight-week survival group. If $GABA_A$-receptor activation

does in fact promote the differentiation of OPCs into neurons, as it does in neural progenitors of the hippocampus (Tozuka et al., 2005), then perhaps two weeks is too short a timeframe for differentiated cells to begin expressing neuronal markers, thereby explaining the discrepancy in the results. Together, these trends suggest that $GABA_A$-receptor activation plays at least a modulatory role in OPC proliferation and differentiation in the adult brain.

Concluding remarks

We have used an unbiased systematic stereological approach to demonstrate that OPCs are still present in large numbers in the corpus callosum and cortex of 4-5-month-old NG2-CreER^{TM}:EYFP reporter mice, where they retain the ability to proliferate and to differentiate into oligodendrocytes. We also show that white- and grey matter OPCs have different properties, with white matter OPCs being more likely to proliferate and to adopt a fate other than that of an OPC, including terminal differentiation into an oligodendrocyte phenotype. While $GABA_A$- and $GABA_B$-receptor agonists and antagonists did not yield a significant effect on the proliferation and differentiation of OPCs, $GABA_A$-receptor agonism does appear to play a regulatory role these physiological processes. Alternatively, the lack of results could serve in support of the hypothesis that GABAergic neurotransmission in adulthood maintains OPCs in an undifferentiated state to accomplish a yet-to-be-identified function other than the generation of myelinating oligodendrocytes (Velez-Fort et al., 2012).

Tables

Table 1. List of antibodies used in the study.

Host	Target	Concentration	Company
Primary Antibodies			
Rabbit	GFP	1/1000	Abcam (ab290)
Mouse	GFP	1/500	Abcam (ab1218)
Goat	Sox10	1/500	Santa Cruz (sc-17342)
Mouse	HuCD	1/2000	Thermo Scientific (A-21271)
Rabbit	PDGFR$_\alpha$	1/250	Santa Cruz (sc-338)
Rabbit	GST-pi	1/500	MBL (311-h)
Mouse	NeuN (Rbfox3)	1/500	Millipore (MAB377)
Rat	BrdU	1/500	Abcam (ab6326)
Secondary Antibodies			
Donkey	Anti-Rabbit Alexa 488	1/1000	Invitrogen
Donkey	Anti-Mouse Alexa 488	1/1000	Invitrogen
Donkey	Anti-Rabbit Alexa 594	1/1000	Invitrogen
Donkey	Anti-Mouse Alexa 594	1/1000	Invitrogen
Donkey	Anti-Goat Alexa 594	1/1000	Invitrogen
Donkey	Anti-Rat Alexa 680	1/500	Abcam

OPC proliferation (Mean proportion of BrdU+ reporter cells - %)			
Corpus callosum		Cortex	
Saline	19.20±3.70	Saline	11.73±1.83
Muscimol	17.11±4.72	Muscimol	7.73±1.33
Picrotoxin	28.94±2.98	Picrotoxin	12.72±0.99
Baclofen	23.29±4.53	Baclofen	11.93±2.47
Saclofen	25.43±6.56	Saclofen	9.95±2.04

Undifferentiated OPCs (Mean proportion of PDGFRα+ reporter cells - %)				Self-renewed OPCs (Mean proportion of PDGFRα+/BrdU+ reporter cells - %)			
Corpus callosum		Cortex		Corpus callosum		Cortex	
Saline	50.23±4.91	Saline	67.84±4.13	Saline	48.60±5.13	Saline	17.38±2.26
Muscimol	40.76±7.02	Muscimol	64.47±10.13	Muscimol	32.37±23.44	Muscimol	8.12±1.60
Picrotoxin	60.91±6.24	Picrotoxin	62.41±5.55	Picrotoxin	43.60±4.08	Picrotoxin	12.69±1.46
Baclofen	59.60±4.53	Baclofen	64.94±7.47	Baclofen	38.80±19.72	Baclofen	14.91±8.38
Saclofen	60.16±2.91	Saclofen	60.40±2.90	Saclofen	56.30±12.00	Saclofen	14.31±3.44

OPCs committed to the oligodendrocyte lineage (Mean proportion of Sox10+ reporter cells - %)				OPC differentiation into oligodendrocytes (Mean proportion of GST-pi+ reporter cells - %)			
Corpus callosum		Cortex		Corpus callosum		Cortex	
Saline	71.42±3.21	Saline	73.62±2.23	Saline	37.98±10.00	Saline	24.82±10.09
Muscimol	78.51±4.59	Muscimol	77.21±2.58	Muscimol	46.77±21.52	Muscimol	32.59±6.81
Picrotoxin	79.37±3.81	Picrotoxin	74.86±2.36	Picrotoxin	49.65±8.26	Picrotoxin	23.16±5.83
Baclofen	74.13±8.37	Baclofen	66.53±14.33	Baclofen	36.58±10.01	Baclofen	12.88±3.17
Saclofen	79.69±1.33	Saclofen	75.35±2.20	Saclofen	32.39±3.36	Saclofen	8.80±0.29

OPCs committed to the neuronal lineage (Mean proportion of HuCD+ reporter cells - %)		OPC differentiation into neurons (Mean proportion of NeuN+ reporter cells - %)	
Saline	8.24±3.04	Saline	3.27±1.46
Muscimol	0.18±0.18	Muscimol	5.20±3.86
Picrotoxin	3.89±3.19	Picrotoxin	1.46±0.14
Baclofen	6.49±4.17	Baclofen	1.03±0.43
Saclofen	10.77±5.40	Saclofen	3.08±0.61

Table 2. Effects of GABAergic agent administration on OPC proliferation and differentiation (mean ± SEM).

Figure legends

Fig. 1. Experimental protocol. (A) Experimental timeline. 4-5 month-old NG2-CreER^{TM}:EYFP mice (n=30) received i.p. injections of tamoxifen once a day for 5 days (days 1-5). On day 10, drinking water was replaced with sweetened water. On day 12, sweetened water was replaced with BrdU sweetened water. On days 13-17, mice received one injection per day of either saline (n=7), muscimol ($GABA_A$ receptor agonist; 1.0 mg/kg, n=5), picrotoxin ($GABA_A$ receptor antagonist; 1.0 mg/kg; n=8), baclofen ($GABA_B$ receptor agonist; 7.5 mg/kg; n=6), or saclofen ($GABA_B$ receptor antagonist; 5.0 mg/kg; n=4). A first group of mice were euthanized on day 33, 2 weeks following the end of BrdU exposure (n=14) and a second group (n=16) was euthanized on day 80, 8 weeks following the end of BrdU exposure. (B) Immunohistochemistry. To avoid antibody conflict and to allow for the visualization of reporter cells, BrdU, and different fates for GFP+ cells, immunostaining had to be done on different sets of slides. Each set consisted of 5 slides. For an OPC fate, immunochemistry on slides from the 2-week group was done with GFP, BrdU, and PDGFRα. For an oligodendrocyte fate, immunochemistry was done with GFP, BrdU, and Sox10 on slides from the 2-week group and with GFP, BrdU, and GST-pi on slides from the 8-week group. For a neuronal fate, immunochemistry was done with GFP, BrdU, and HuCD on slides from the 2-week group and with GFP, BrdU, and NeuN. On slides from the 8-week group. (C) Stereology. The surface area of the corpus callosum (light grey) and dorsal cortex (dark grey) was manually traced at 10X. A sampling grid was placed onto of the traced areas by the StereoInvestigator software. Grid boxes were 900µm x 315µm for the corpus callosum and 1475µm x 382µm for the cortex. The StereoInvestigator software randomly selected equally spaced grid boxes and placed a 200µm x 200µm counting frame in their top left corner. All cells within the counting frames were imaged at 40X and counted using the software.

Fig. 2. (a) Mean proportion of GFP+ cells that entered the cell cycle during the 8-day BrdU pulse period (i.e. proliferated cells) in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm (n=30; *error bars* SEM; *$p < .05$). (B) Mean proportion of GFP+ cells that entered the cell cycle during the 8-day BrdU pulse period (i.e. proliferated cells) in the cortex and corpus callosum of animals that received either saline (n=7, darkest grey), muscimol (n=5, light grey), picrotoxin (n=8, dark grey), baclofen (n=6, black), or saclofen (n=4, lightest grey) between lateral 0.36mm and lateral 2.52mm (*error bars* SEM). (C) Mean proportion of GFP+ cells that remained PDGFRα+ (i.e. undifferentiated cells) in the two-week survival group in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm (n=14; *error bars* SEM; *$p < .05$). (D) Mean proportion of GFP+ cells that remained PDGFRα+ (i.e. undifferentiated cells) in animals that received either saline (n=3, darkest grey), muscimol (n=2, light grey), picrotoxin (n=4, dark grey), baclofen (n=3, black), or saclofen (n=2, lightest grey) in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm (*error bars* SEM). (E) Mean proportion of GFP+/PDGFRα+ cells that incorporated BrdU (i.e. self-renewed cells) in the cortex and corpus callosum between lateral 0.36mm and lateral 2.52mm in the two-week-survival group (n=14; *error bars* SEM; *$p < .05$). (F) Mean proportion of GFP+/PDGFRα+ cells

that incorporated BrdU (i.e. self-renewed cells) in the cortex and corpus callosum of animals that received either saline (n=3, darkest grey), muscimol (n=2, light grey), picrotoxin (n=4, dark grey), baclofen (n=3, black), or saclofen (n=2, lightest grey) between lateral 0.36mm and lateral 2.52mm in the two-week survival group (*error bars* SEM).

Fig. 3. Undifferentiated OPC (A-D). Pictures taken in the cortex at 40X using a laser scan confocal microscope. NG2+ cell at the time of tamoxifen administration (A; GFP) did not incorporate BrdU (B; BrdU) and remained undifferentiated (C; PDGFRα) after the end of the experiment. GFP is therefore co-localized only with PDGFRα (D; merged). Self-renewed OPC (E-H). Pictures taken in the cortex at 40X using a laser scan microscope. NG2+ cells at the time of tamoxifen administration (E; GFP) entered the cell cycle BrdU during the 8-day pulse period (F; BrdU) and remained OPCs at the end of the experiment (G; PDGFRα), making it so that GFP, BrdU, and PDGFRα are co-localized (H; merged).

Fig. 4. Oligodendrocyte differentiation. (A) Mean proportion of GFP+ cells that co-express Sox10 between lateral 0.36mm and lateral 2.52mm in the two-week survival group. These are cells that were NG2+ at the time of tamoxifen administration and that committed to the oligodendrocyte lineage in the cortex and corpus callosum (n=14; *error bars* SEM). (B) Mean proportion of GFP+ cells that co-express Sox10 in the cortex and corpus callosum of animals in the two-week survival group that received either saline (n=3, darkest grey), muscimol (n=2, light grey), picrotoxin (n=4, dark grey), baclofen (n=3, black), or saclofen (n=2, lightest grey). These are cells located between lateral 0.36mm and lateral 2.52mm that were NG2+ at the time of tamoxifen administration and that committed to the oligodendrocyte lineage by the end of the study (*error bars* SEM). (C) Mean proportion of GFP+ cells that co-express GST-pi in the eight-week survival group between lateral 0.36mm and lateral 2.52mm. These are cells that were NG2+ at the time of tamoxifen administration and that differentiated into oligodendrocytes in the cortex and corpus callosum by the end of the study (n=16; *error bars* SEM; $*p < .05$). (D) Mean proportion of GFP+ cells that co-express GST-pi in the cortex and corpus callosum of animals from the eight-week survival group that received either saline (n=4, darkest grey), muscimol (n=3, light grey), picrotoxin (n=4, dark grey), baclofen (n=3, black), or saclofen (n=2, lightest grey). These are cells located between lateral 0.36mm and lateral 2.52mm that were NG2+ at the time of tamoxifen administration and that differentiated into oligodendrocytes by the end of the study (*error bars* SEM).

Fig. 5. GFP+ cells committed to the oligodendrocyte lineage in the two-week survival group (A-H). Pictures taken in the cortex at 40X using a laser scan microscope. These are cells that were NG2+ at the time of tamoxifen administration (A, E; GFP) and that committed to the oligodendrocyte lineage by the end of the study (B, F; Sox10). Some of these cells differentiate directly, that is, without a prior cell division (C; BrdU). Other cells entered the cell cycle first and incorporated BrdU during the 8-day pulse period (G; BrdU). Cells that differentiated directly

are GFP+/GST-pi+/BrdU- (D; merged) and cells that went through the cell cycle first are GFP+/GST-pi+/BrdU+ (H; merged). GFP+ cells that differentiated into oligodendrocytes in the eight-week survival group (I-P). Pictures taken in the cortex at 40X using a laser scan microscope. These are cells that were NG2+ at the time of tamoxifen administration (I, M; GFP) and that differentiated into oligodendrocytes by the end of the study (J, N; GST-pi). Some of these cells differentiate directly, that is, without a prior cell division (K; BrdU). Other cells entered the cell cycle first and incorporated BrdU during the 8-day pulse period (O; BrdU). Cells that differentiated directly are GFP+/GST-pi+/BrdU- (L; merged) and cells that went through the cell cycle first are GFP+/GST-pi+/BrdU+ (P; merged).

Fig. 6. Neuronal differentiation. (A) Mean proportion of GFP+ cells that co-express HuCD in the cortex of animals in the two-week survival group that received either saline (n=3, darkest grey), muscimol (n=2, light grey), picrotoxin (n=4, dark grey), baclofen (n=3, black), or saclofen (n=2, lightest grey). These are cells located between lateral 0.36mm and lateral 2.52mm that were NG2+ at the time of tamoxifen administration and that committed to the neuronal lineage by the end of the study (*error bars* SEM). (B) Mean proportion of GFP+ cells that co-express NeuN in the cortex of animals in the eight-week survival group that received either saline (n=4, darkest grey), muscimol (n=3, light grey), picrotoxin (n=4, dark grey), baclofen (n=3, black), or saclofen (n=2, lightest grey). These are cells located between lateral 0.36mm and lateral 2.52mm that were NG2+ at the time of tamoxifen administration and that differentiate into neurons by the end of the study (*error bars* SEM).

Fig. 7. Neuronal differentiation. Pictures taken at 40X in the cortex using a laser scan microscope. These are cells that were NG2+ at the time of tamoxifen administration (A; GFP) and that differentiated into neurons (B; NeuN) by the end of the study, making them GFP+/NeuN+ (C; merged).

Figures

Figure 1.

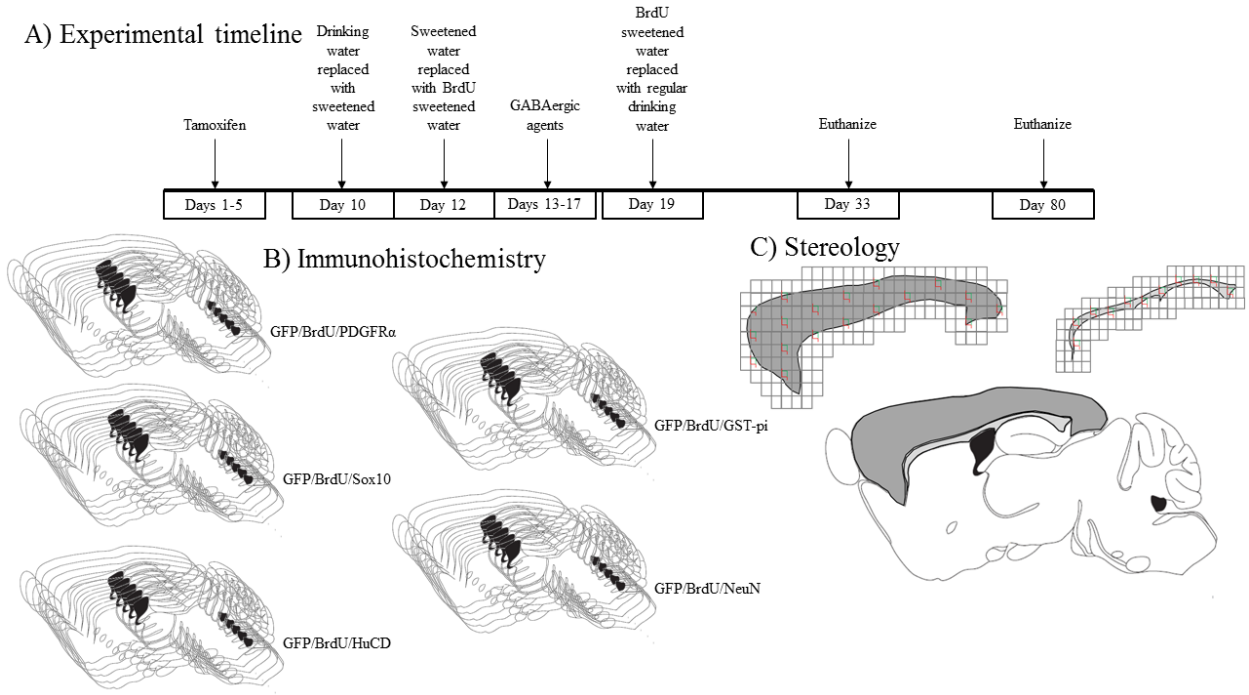


Figure 2.

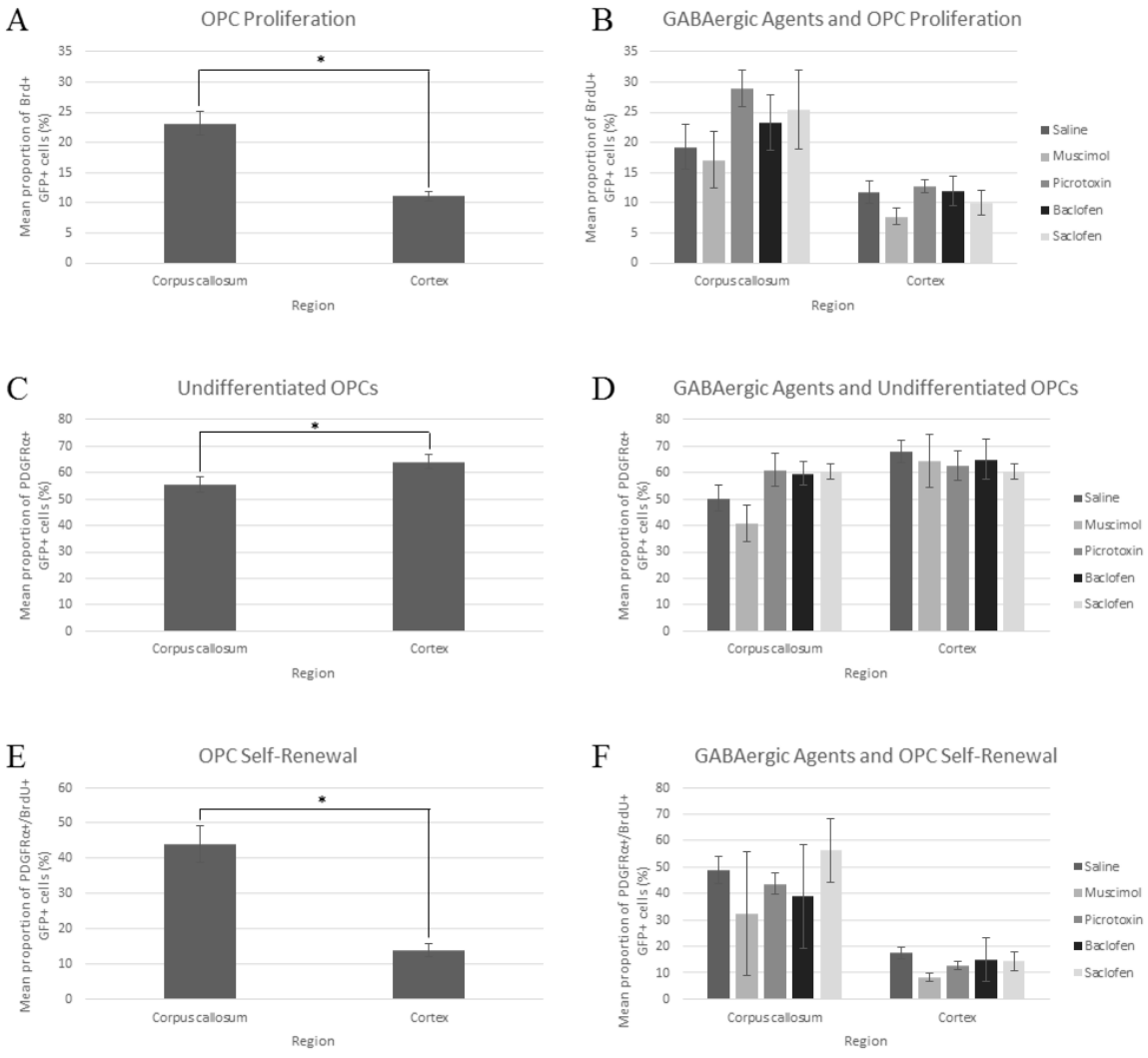
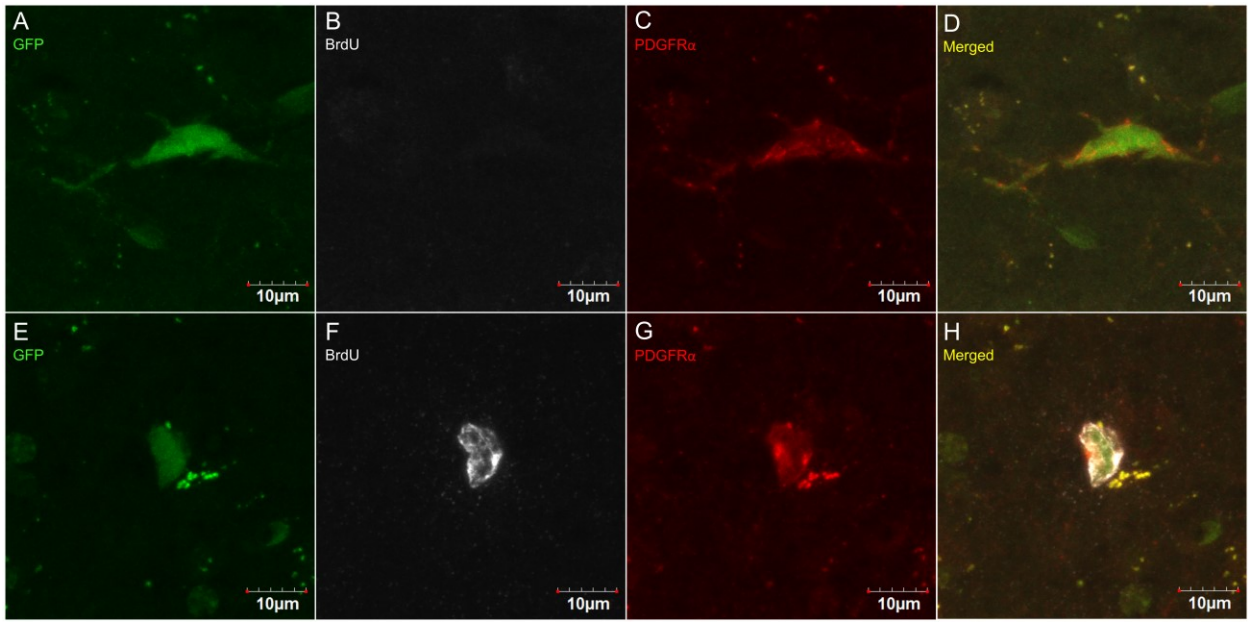

A
OPC Proliferation
Mean proportion of Brd+ GFP+ cells (%)
Corpus callosum
Cortex
Region
B
GABAergic Agents and OPC Proliferation
Mean proportion of BrdU+ GFP+ cells (%)
Saline
Muscimol
Picrotoxin
Baclofen
Saclofen
C
Undifferentiated OPCs
Mean proportion of PDGFRα+ GFP+ cells (%)
D
GABAergic Agents and Undifferentiated OPCs
E
OPC Self-Renewal
Mean proportion of PDGFRα+/BrdU+ GFP+ cells (%)
F
GABAergic Agents and OPC Self-Renewal

Figure 3.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GFP
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BrdU
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C
PDGFRα
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D
Merged
10μm
E
GFP
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F
BrdU
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G
PDGFRα
10μm
H
Merged
10μm

Figure 4.

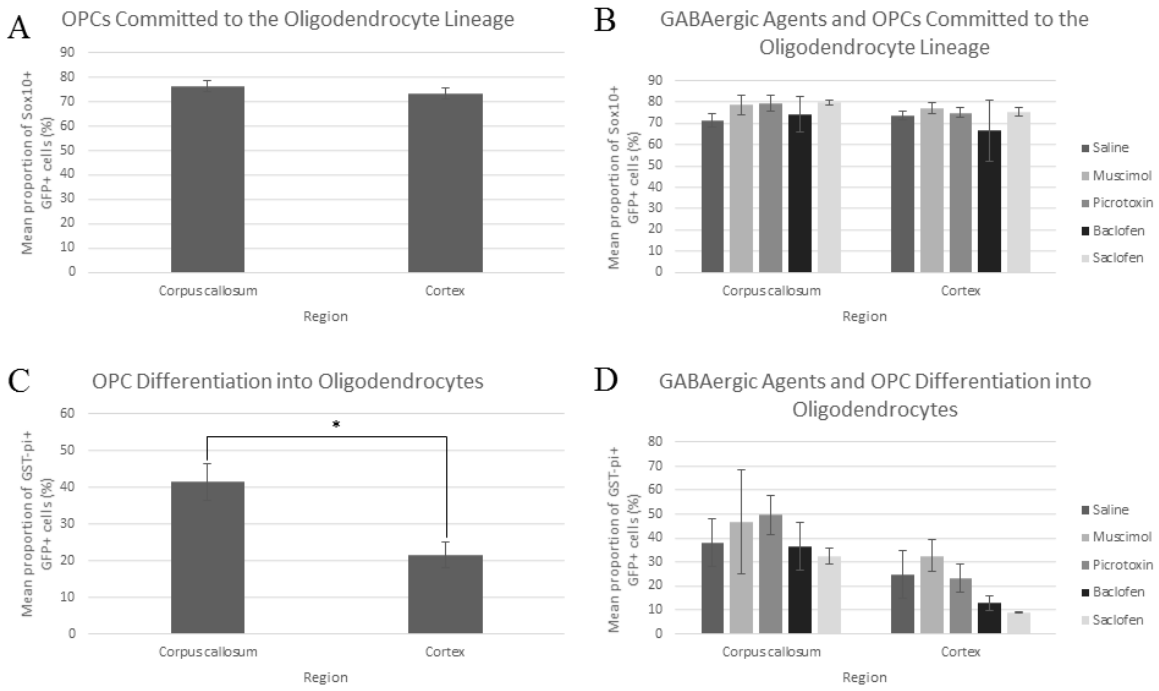
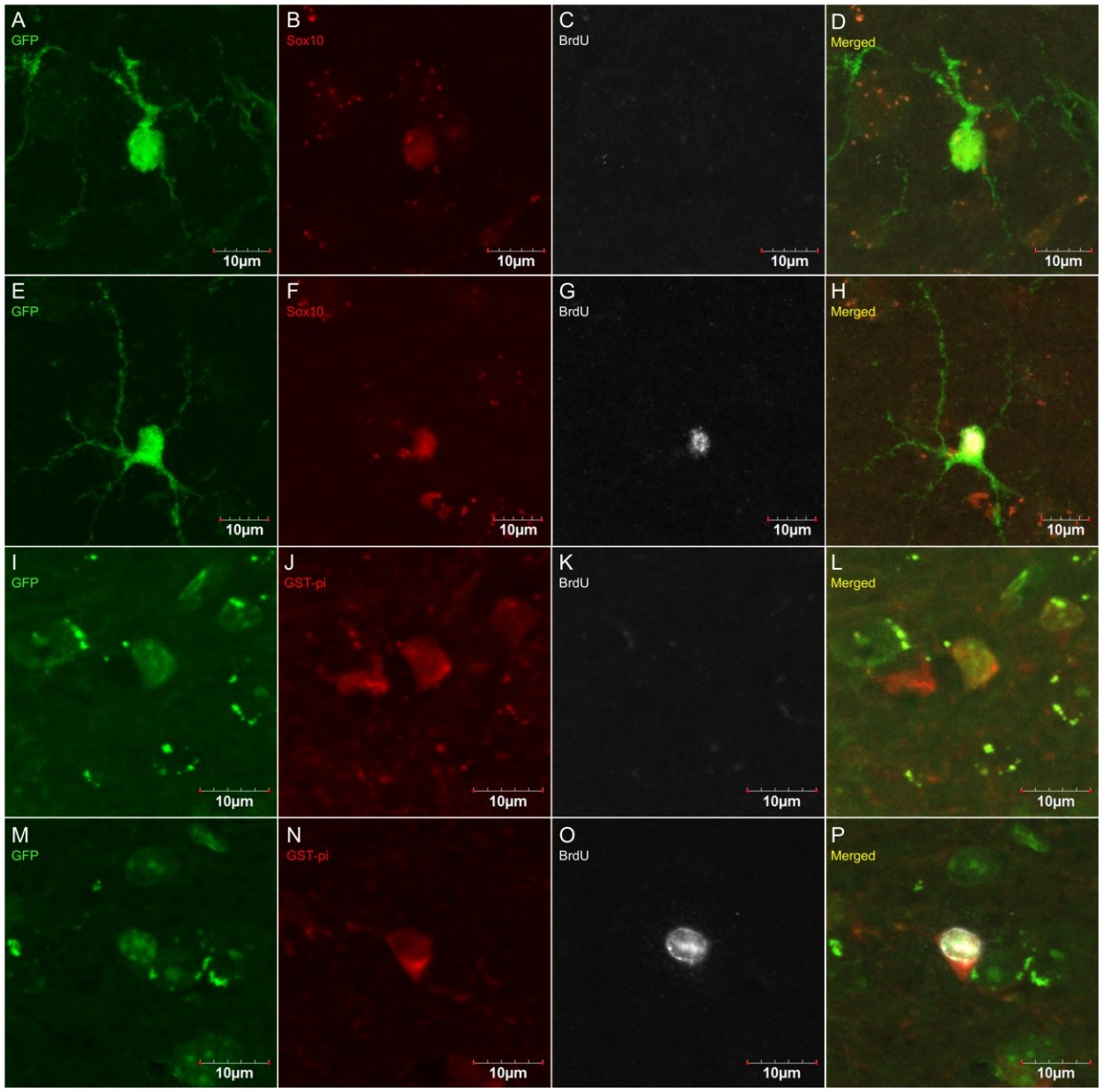

A
OPCs Committed to the Oligodendrocyte Lineage
Mean proportion of Sox10+ GFP+ cells (%)
Corpus callosum
Cortex
Region
B
GABAergic Agents and OPCs Committed to the Oligodendrocyte Lineage
Saline
Muscimol
Picrotoxin
Baclofen
Saclofen
C
OPC Differentiation into Oligodendrocytes
Mean proportion of GST-pi+ GFP+ cells (%)
*
D
GABAergic Agents and OPC Differentiation into Oligodendrocytes

Figure 5.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GFP
10μm
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Figure 6.

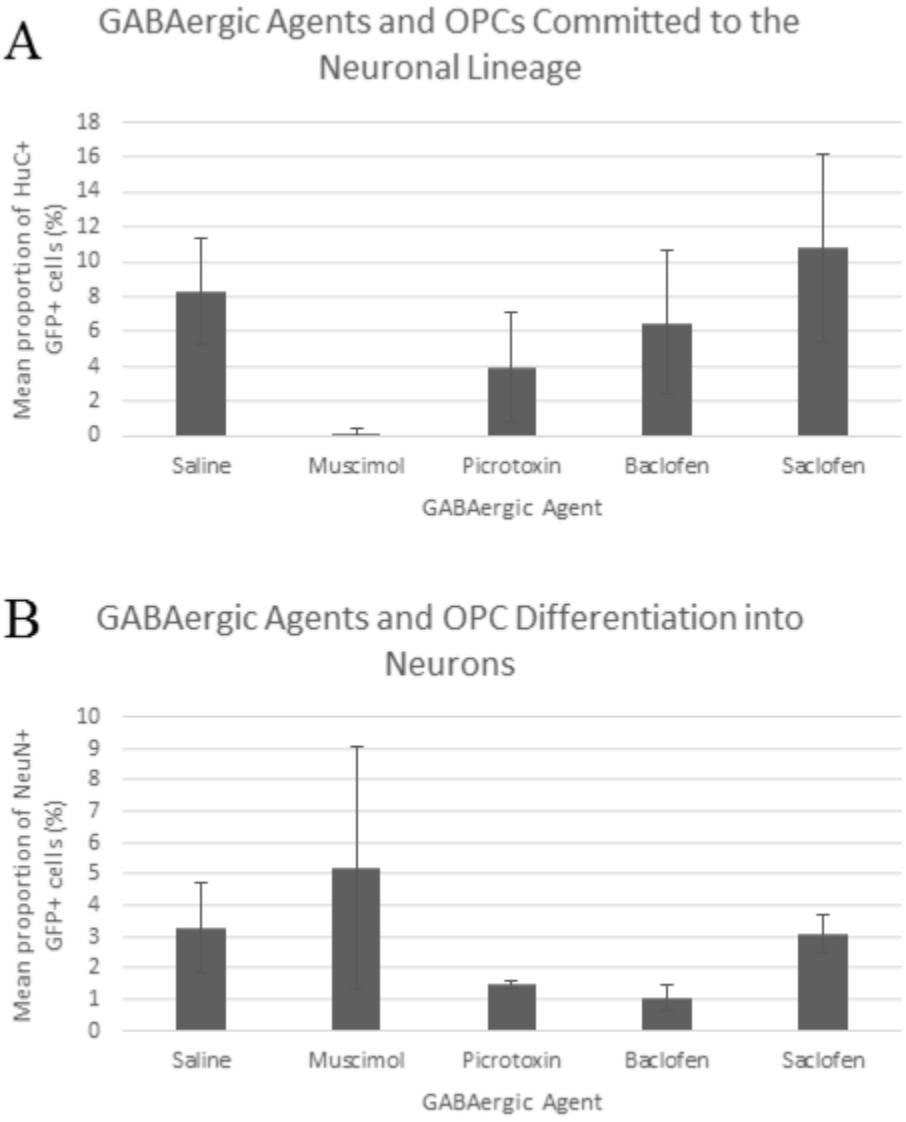
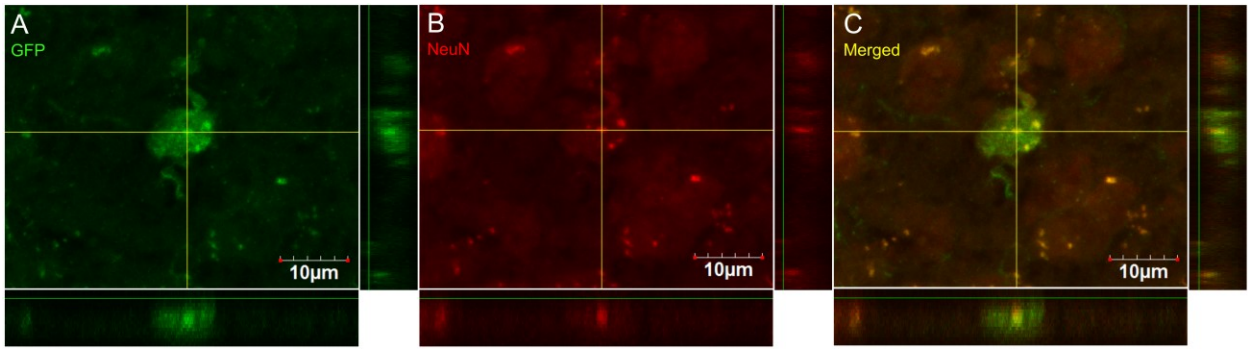

A
GABAergic Agents and OPCs Committed to the Neuronal Lineage
Mean proportion of HuC+ GFP+ cells (%)
18
16
14
12
10
8
6
4
2
0
Saline
Muscimol
Picrotoxin
Baclofen
Saclofen
GABAergic Agent
B
GABAergic Agents and OPC Differentiation into Neurons
Mean proportion of NeuN+ GFP+ cells (%)
10
9
8
7
6
5
4
3
2
1
0
Saline
Muscimol
Picrotoxin
Baclofen
Saclofen
GABAergic Agent

Figure 7.



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Running Head: Oligodendrocyte progenitor cells and neurons

EXPERIMENT 4: OLIGODENDROCYTE PROGENITOR CELLS ARE PAIRED WITH GABA NEURONS IN THE MOUSE CORTEX: UNBIASED STEREOLOGICAL ANALYSIS

Jenna J. BOULANGER and Claude MESSIER

School of Psychology, University of Ottawa

Correspondence to: C. Messier, School of Psychology, University of Ottawa, 136 Jean-Jacques Lussier Room 2076A, Ottawa, Ontario, K1N 6N5, CANADA. Tel (613) 562-5800 ext. 4562, Fax: (613) 562-5147; E-mail: cmessier@uottawa.ca.

Abbreviations

In alphabetical order:

BrdU: 5-bromo-2’-deoxyuridine

EYFP: enhanced fluorescent yellow protein

GFP: green fluorescent protein

NeuN: Neuronal nuclear protein, also known as Fox-3 or Rbfox3

NG2: chondroitin sulfate proteoglycan neuron-glia antigen 2

OPC: oligodendrocyte progenitor cell

PDGF: platelet-derived growth factor

PDGFRα: platelet-derived growth factor receptor alpha

Abstract

Oligodendrocyte progenitor cells (OPC) are glial cells that differentiate into myelinating oligodendrocytes during early stages of post-natal life. However, OPCs persist beyond developmental myelination and represent an important population of cycling cells in the grey and white matter of the adult brain. While adult OPCs form unique territories that are maintained through self-avoidance, some cortical OPCs appear to position their cell body very close to that of a neuron, forming what are known as OPC-neuron pairs. We used unbiased systematic stereological analysis of the NG2-CreER^{TM}:EYFP reporter mouse to determine that close to 170,000 OPC-neuron pairs can be found in the dorsal portion of the adult neocortex, with approximately 40% of OPCs and 4% of neurons in pairs. Through stereological analysis, we also determined that reference memory training does not change the prevalence of OPC-neuron pairs or the proportion of OPCs and neurons that form them. GABAergic agent administration did not affect the proportion of OPCs and neurons that can be found in pairs. However, the $GABA_B$-receptor agonist baclofen and the $GABA_A$ receptor antagonist picrotoxin significantly increased the estimated number of pairs when compared to the control group and the $GABA_B$-receptor antagonist (i.e. saclofen) group. Density of OPC-neuron pairs were increased by the $GABA_A$ receptor antagonist picrotoxin. Finally, histological analysis of OPC-neuron pairs suggested that in the dorsal portion of the cortex, GABAergic interneurons represent the most common neuronal component of the pairs, and that calbindin, calretinin and parvalbumin GABAergic interneurons found in the cortex take part in these pairs. Using previous estimates of the number of GABAergic neurons in the rodent cortex, we estimate that roughly one in four GABAergic neurons are paired with an OPC.

Key Words: myelin, oligodendrocyte, plasticity, gliogenesis, oligodendrocyte progenitor cells, stereology, GABA, calbindin, calretinin, parvalbumin

Introduction

Oligodendrocyte progenitor cells (OPCs; also known as NG2-glial cells, synantocytes, or polydendrocytes) are a type of glial cell that give rise, as their name suggests, to myelinating oligodendrocytes during early stages of post-natal life (Kang, et al., 2010; Nishiyama, et al., 2002). OPCs specifically express the platelet-derived growth factor receptor alpha (PDGFRα) and the chondroitin sulfate proteoglycan neuron-glia antigen 2 (NG2), both of which are downregulated in favour of O4 and myelin-specific antigens as the cell transitions from an OPC to an oligodendrocyte phenotype (Levine, et al., 1993; Nishiyama, et al., 1996; Stallcup, 2002). Interestingly, a large number of OPCs remain undifferentiated after developmental myelination is completed, and OPCs correspond to approximately 2-9% of the total cell population of the adult brain (Butt, et al., 2005; Dawson, et al., 2003a; Ffrench-Constant and Raff, 1986; Nishiyama, et al., 1999; Pringle, et al., 1992; Wolswijk and Noble, 1989).

Adult OPCs have a few characteristics that distinguish them from other types of glial cells. Firstly, they are homogenously distributed between the grey and white matter of the central nervous system (CNS), where they maintain unique territories through self-avoidance (Hughes, et al., 2013) . However, it is interesting to note that some grey matter OPCs establish extremely close anatomical contacts with neurons, their soma and processes being adjoined to and/or intertwined with that of a neuron (Butt, et al., 2005; Boda and Buffo, 2014; Gallo, et al., 2008) . In fact, spatial analysis has revealed that the cell bodies of OPC-neuron pairs are statistically closer to one another than what is to be expected from random distribution, leading some researchers to consider OPCs as satellite cells of the closely apposed neurons (Gallo, et al., 2008; Dayer, et al., 2005; Mangin, et al., 2008).

Secondly, adult OPCs possess functional glutamatergic (kainate, AMPA and NMDA) and GABAergic ($GABA_A$ and $GABA_B$) receptors that induce depolarization, K^+ channel blockage, and an intracellular influx of Ca^{2+} upon activation (Berger, et al., 1991; Bernstein, et al., 1996; Borges and Kettenmann, 1995; Borges, et al., 1994; Gallo, et al., 1996; Káradóttir and Attwell, 2007; Kirchhoff and Kettenmann, 1992; Kirischuk, et al., 1995; Luyt, et al., 2004; Pastor, et al., 1995; Tanaka, et al., 2009; Verkhratsky, et al., 1990). GABA-receptor activation induces depolarization because of the highly negative intracellular charge of adult OPCs. When the concentration of Cl^- is high within a cell, the opening of GABA receptor channels results in Cl^- efflux and depolarization (Tanaka, et al., 2009; Lin and Bergles, 2004a). Glutamate- and GABA-receptor activation can occur through both synaptic and non-synaptic means: OPCs form synapses directly with neurons and extend processes to nodes of Ranvier and neuron-to-neuron synapses where spill over neurotransmitters may be released into the extracellular environment (Bergles, et al., 2000; Butt, et al., 1999; Kukley, et al., 2007; Lin and Bergles, 2004b; Ong and Levine, 1999; Vélez-Fort, et al., 2010; Ziskin, et al., 2007). While it could be assumed that receptor-activation would come from a closely apposed neuron, Mangin, et al. (2008) have demonstrated that there is no neurotransmitter-based connectivity between the somata of OPC-neuron pairs. Instead, the OPC appears to monitor the electrical activity occurring within the neuron, displaying synchronized bursts of activity upon its activation (Butt, et al., 2005; Mangin, et al., 2008; Maldonado, et al., 2013). Since grey matter OPCs contact many different neurons through their extensive arborisation, OPCs in OPC-neuron pairs (i.e. somata of the OPC and the neuron are closely apposed to one another) have been hypothesized to be play a neuromodulatory role in neural network activity, integrating the synaptic activity of its paired neuron with that of neighbouring neurons (Butt, et al., 2005; Boda and Buffo, 2014; Gallo, et al., 2008).

Thirdly, adult OPCs maintain the ability to proliferate into adulthood and represent the mature brain's most active population of cycling cells (Dawson, et al., 2003b; Psachoulia, et al., 2009; Simon, et al., 2011). The primary fates of daughter cells of adult OPCs are self-renewal and oligodendrocyte differentiation, and daughter cells have been shown to migrate away from one another to maintain those unique territories that were referenced previously (Hughes, et al., 2013; Nishiyama, et al., 2016; Zhu, et al., 2011). It must also be noted that there is a debate as to adult OPCs' ability to differentiate into astrocytes and neurons (Nishiyama, et al., 2016; Nishiyama, et al., 2009; Viganò and Dimou, 2016; Wigley and Butt, 2009). If these fates are possible however, they are usually classified as minor compared to self-renewal and differentiation into oligodendrocytes (Nishiyama, et al., 2016; Robins, et al., 2013).

While the dynamics of OPC proliferation, migration, and differentiation in the healthy adult brain are not fully understood, recent reports have pointed to experience-dependent neural network activity as a potential modulator, with neurotransmitters specifically identified as key mediators in this relationship (Boda and Buffo, 2014; Gallo, et al., 2008; Fields, 2008; Fields, 2015; Gibson, et al., 2014; Purger, et al., 2016; Tomlinson, et al., 2016; Wake, et al., 2011). For instance, factors such as physical exercise and environmental enrichment have been shown to promote OPC proliferation and differentiation along the oligodendrocyte lineage (Simon, et al., 2011; Ehninger, et al., 2011; McKenzie, et al., 2014; Xiao, et al., 2016; Zhao, et al., 2010). On the other hand, $GABA_B$-receptor activation has been shown to increase the proliferation of cultured OPCs (Luyt, et al., 2007), while glutamate- and $GABA_A$-receptor activation have been shown to inhibit OPC proliferation and differentiation along the oligodendrocyte lineage, thereby enabling OPCs to take on roles other than the generation of oligodendrocytes after developmental myelination is completed (Gallo, et al., 2008; Gallo, et al., 1996; Káradóttir and

Attwell, 2007; Pastor, et al., 1995; Baron, et al., 1998; Knutson, et al., 1997; Vélez-Fort, et al., 2012; Yuan, et al., 1998).

Since one of these roles appears to be the monitoring of neural network activity by way of OPC-neuron pairs, we tested whether experience-dependent neural network activation and neurotransmission could influence that relationship as well. More specifically, we used stereology to measure the effects of reference memory training and $GABA_A$- and $GABA_B$-receptor agonists and antagonists on the number of closely apposed OPC- and neuron cell bodies in the dorsal portion of the adult mouse neocortex. We also provide a histological analysis of OPC-neuron pairs in the dorsal portion of the cortex.

Methods

Animals

NG2CreER^{TM} BAC transgenic mice (Jackson Laboratory, Bar Harbour, Maine, USA; described in (Zhu, et al., 2011)) were bred in house with gtROSA26-EYFP transgenic mice on a C57BL/6J background (Jackson Laboratory, Bar Harbour, Maine, USA) to generate the NG2-CreER^{TM}:EYFP reporter mouse. The NG2-CreER^{TM}:EYFP reporter mouse allows for genetic fate mapping of OPCs through tamoxifen-induced Cre-recombination in the NG2 locus, which activates EYFP expression in NG2+ cells. These EYFP+ cells continue to express EYFP+ even if they are no longer NG2+, therefore enabling lineage tracing of labelled cells. All the animals (n=24) used in this study were individually housed in a 21+/- 1ºC vivarium, maintained on a reverse 12-hour light/dark cycle and had *ad libitum* access to food and water. All animal

procedures were done in accordance with the recommendations of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Procedure

Figure 1 presents the timeline of the experimental procedures.

Tamoxifen. Tamoxifen was dissolved in corn oil at a concentration of 10 mg/ml**.** Offspring resulting from the crossbreeding aged 4-5 months were given a 0.4 ml i.p. injection of tamoxifen once a day for 5 days.

BrdU. Five days following the last tamoxifen injection and three days prior to the first day of the experimental procedure, animals' drinking water was replaced with sweetened water (100 ml water + 0.125 g saccharine + 3 g dextrose) to habituate them to the new taste and reduce neophobia. The sweetened water was replaced with sweetened water containing BrdU (100 ml water + 0.125 g saccharine + 3 g dextrose + 0.1 g BrdU) 7 days following the last tamoxifen injection and 24 hours prior to the beginning of the experimental procedure. Forty-eight hours following the end of the experimental procedure, the BrdU sweetened water was replaced with regular drinking water.

Reference memory training. Figure 1A illustrates the procedure. Twenty-four hours following the introduction of BrdU sweetened water and 8 days following the last injection of tamoxifen, animals were split into 2 groups. In the control group (n=3), mice stayed in their cages throughout the entire length of the study. The animals in the experimental group (n=5) were trained in a Barnes Maze task. The Barnes maze protocol was adapted from (Sunyer, et al., 2007). The Barnes Maze consisted in a circular platform with 18 equally spaced holes elevated 105 cm above the floor. A bright spotlight was placed above the maze so that mice would be

motivated to escape into a dark chamber (escape box) that could be accessed through one of the holes (target hole). A curtain onto which visual cues were hanged surrounded the maze.

Mice were exposed to the maze three times a day for 4 days. Each trial of a specific day was separated from the next trial by 15-20 minutes. The maze was cleaned with 70% ethanol after each animal. At the beginning of each trial, the lights in the room were switched off and the mouse was placed inside a cylindrical black start chamber at the center of the maze. The floodlight was then turned on and the animal remained in the start chamber for 10 seconds. The first trial of the first day consisted in an adaptation period where the animal was guided to the target hole and encouraged to enter the escape box. Once the mouse was inside the escape box, the floodlight was turned off and the mouse remained in the escape box for 1 minutes. If the mouse tried to escape the box, it was gently guided back inside.

In the spatial acquisition trials that followed, the animals were allowed to explore the maze for three minutes. If three minutes had elapsed and the mouse had not yet entered the target hole, it was gently guided to it. Mice were allowed to stay in the escape box for 30 seconds before being returned to their cage. The fifth day consisted in a probe trial evaluating short-term memory where the escape box was removed from the maze. A similar probe trial to evaluate long-term learning was conducted on day 26 (7 days following the short-term probe). For each probe session, the total number of errors (nose pokes inside a hole other than the target hole) and escape latency (time elapsed before the mouse entered the escape box) were recorded.

GABAergic drug administration. Figure 1B illustrates the procedure. Twenty-four hours following the introduction of BrdU sweetened water and 8 days following the last injection of tamoxifen, animals were split into 5 groups. Once a day and for a period of 5 days, mice

received an injection of either saline (n=4), muscimol ($GABA_A$ receptor agonist; 1.0 mg/kg, n=3), picrotoxin ($GABA_A$ receptor antagonist; 1.0 mg/kg; n=4), baclofen ($GABA_B$ receptor agonist; 7.5 mg/kg; n=3), or saclofen ($GABA_B$ receptor antagonist; 5.0 mg/kg; n=2). Mice were euthanized 8 weeks following the end of BrdU exposure.

Tissue Processing

Anesthetized mice were transcardially perfused with saline followed by Lana's fixative (4% paraformaldehyde-picric acid; modified from (Zamboni & Demartin, 1967)). Brains were post-fixed in this fixative for one hour before being incubated in 10% sucrose in sodium phosphate buffer (0.1 M, pH 7.2) overnight at 4ºC. Brains were then frozen using CO_2 and cut in 15 µm sagittal sections using a cryostat.

Immunochemistry

Antibodies used and dilutions are presented in Table 1. For the stereological analysis, all sections were stained for GFP/BrdU/NeuN.

Primary Antibodies. Primary antibody solutions were diluted in 0.3% Triton in 10X PBS. Tissue sections were covered with 50 µl of the primary antibody solution and parafilm was placed on top to prevent evaporation. Slides were incubated at room temperature in a humidified chamber for three hours. Following incubation, slides were washed successively three times for 5 minutes in 10X PBS. An anti-GFP antibody that also recognizes EYFP was used to maximize the visualization of EYFP+ NG2 reporter cells. As such, EYFP+ cells will henceforth be referred to as GFP+ cells. Pericytes also express NG2. Therefore, the NG2-CreER^{TM}:EYFP reporter mouse allows for the visualization of both OPCs and pericytes. To ensure that the stereological estimates only included OPCs, we examined the morphology of the GFP-labelled cells. OPCs

have a bipolar or multipolar shape with multiple processes. Pericytes have a flat, slightly elongated soma, no processes and are associated with blood vessels. Only cells that exhibited the typical morphology of OPCs were counted.

Secondary antibodies. Secondary antibody solutions were diluted in 0.3% Triton in 10X PBS. Tissue sections were covered with 50µl of the secondary antibody solution and parafilm was placed on top to prevent evaporation. Slides were incubated in a humidified chamber for 30 minutes at 37ºC. Following incubation, slides were washed successively three times for 5 minutes in 10X PBS.

BrdU immunostaining. To preserve immunostaining during the acid/heat denaturation step required for BrdU labeling, a previously described protocol was used (Boulanger, et al., 2016). Briefly, immunohistochemistry for GFP and PDGFRα, Sox10, HuCD, GST-pi, or NeuN was conducted first (primary and secondary antibodies). This was followed by an overnight post-fixation step where slides were incubated in a humid chamber with Lana's fixative overnight at 4 ºC. Slides were then rinsed in 10X PBS 3 times for 5 minutes. This protects the first immunochemistry from the DNA denaturation step, during which slides were incubated in HCl 2N for 30 minutes at 37 ºC. Slides were then rinsed 3 times for 5 minutes in a 0.1 M borate buffer pH 8 and 3 times in 10X PBS for 5 minutes. For BrdU immunohistochemistry, slides were incubated in a humid chamber with a rat anti-BrdU antibody dissolved in PBS with 0.3% Triton-X in the dark for 3 hours at room temperature. Finally, slides were incubated in a humid chamber with a donkey anti-rat secondary antibody dissolved in PBS with 0.3% Triton-X for 30 minutes at 37 ºC. Slides were then rinsed in PBS 3 times for 5 minutes, treated with custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

Microscopy

Microscopy observations were made in a small group of NG2-CreER^{TM}:EYFP reporter mice (n=4). To establish that OPC/neurons couple were not only specific to this transgenic mouse, casual observations in outbred CD-1 mice (n=4) were also made (but not shown here) to confirm the existence the couples and the identity of the neurons in couples with OPC. Immunofluorescence results were visualized using an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) attached to a ProgRes MF Scan camera (Jenoptik, Jena, Thuringe, Germany). Digital images were captured using the ProgRes CapturePro 2.5 software (Jenoptik, Jena, Thuringe, Germany). Objectives were Olympus 20X UPlanSApo (0.7 n.a.) and Olympus 40X UPlanSApo (0.9 n.a.). High-resolution observations were carried out on an Olympus FV1000 BX61 laser scan confocal microscope (Olympus Corporation, Tokyo, Japan) and images were captured using the Olympus Fluoview software (Olympus Corporation, Tokyo, Japan). The objective for this microscope was an Olympus 60X PlanApo oil SC (1.4 n.a.). Filters for both microscopes were Semrock Brightline TXRed-4040A, FITC-3540B and DAPI. A Chroma 41023 filter was used for Alexa680.

Stereology

Stereological counts were made in one cortical hemisphere using the optical fractionator method of the StereoInvestigator software (StereoInvestigator v.11; MBF Bioscience, VT, USA) on a Leica DMR microscope (Leica Microsystems, Germany). The filters were K3, (Leica #513809), Y3 (Leica #513837), Y5 (Leica #513844). The objectives were 10X (Leica#506505, HC PL Fluotar, 0.30 n.a.) and 40X (Leica #506144, HCX PL Fluotar, 0.75 n.a.).

To determine the appropriate number of sections that should be evaluated in the present study, the sample over-sample method was first carried out on seventeen sections separated by 112 μm and originating from one hemisphere of a given animal (starting approximately at lateral 0.36 mm and ending approximately at lateral 2.52 mm). The data suggested that the estimated number of cells would show similar values had they been counted at intervals of 336 μm instead of 112 μm (data not shown). Therefore, in this study, the section evaluation interval was 336μm, with five sections being evaluated for each animal. The starting section of each series was randomly selected to ensure that all areas between lateral 0.36 mm and lateral 2.52 mm had an equal chance of being examined.

The cross-sectional area of the dorsal portion of the cortex was manually traced at a low magnification using a 10X objective lens. The limits of the corpus callosum are imprecise at the junction between the tightly bundled callosal fibres and the cortical areas where the callosal fibres spread into the cortex. In the present experiment, we traced the limits of the corpus callosum where it starts to lose its solid appearance. A sampling grid with 1475 μm x 382 μm spacing was randomly placed on the traced areas by the StereoInvestigator software, and a single 200 μm x 200 μm counting frame was placed systematically at each sampling spot. All cells within the counting frames were imaged using a 40X objective lens. The software selected on average 87 sampling sites. The optical disector height (thickness) was 16 μm with a 1-μm top guard zone. The average section thickness was 16.08 μm ± 1.03 μm. Therefore, for each sampling site, the volume examined was 200 μm x 200 μm x 15 μm.

The analysed region (between lateral 0.36 mm and lateral 2.52 mm of one hemisphere) ranged roughly from the rhinal fissure anteriorly to the post-subiculum posteriorly. The regions included were the insular, cingulate, frontal association infralimbic, orbital, primary and

secondary motor, parietal association, prelimbic, retrosplenial, primary somatosensory, primary and secondary visual cortex. The more lateral part of the cortex that was not sampled comprises extensions of the same cortical regions and we assumed similar cellular distributions. All stained cell bodies found within the randomly chosen sampling sites were manually counted with the help of the software. The estimated number of each cell type within the analyzed region (between lateral 0.36 mm and lateral 2.52 mm of one hemisphere) was calculated using the total number of cells counted at the sampling sites, the section sampling fraction (number of sections/total area), the area sampling fraction (area of section sampled/total area), and the thickness sampling fraction (mean section thickness measured at each disector site/disector height). In addition to looking at the total number of OPC-neuron pairs, we also estimated the density of these pairs in the dorsal portion of the adult mouse cortex by dividing the estimated total number of OPC-neuron pairs by the estimated total volume of tissue in each animal of our sample.

Data analysis

For each animal and each measure, the average of the stereological estimate found for all counting frames was used. Statistical analyses were performed using SPSS (v.24; IBM Corporation, USA). To compare the properties of cortical OPCs, independent samples two-tailed t-tests were conducted. To determine how reference memory training affected OPC-neuron pairs, differences between the experimental group trained in the Barnes maze and the control group were evaluated using independent samples two-tailed t-tests. Degrees of freedom for t-tests were adjusted when unequal variance was found. To evaluate the effects of GABAergic agonists and antagonists on OPC-neuron pairs, one-way ANOVAs were conducted followed as necessary by Bonferroni pairwise tests. Homogeneity of variance hypothesis was tested with Levene's test.

When the hypothesis was rejected, the non-parametric test Kruskal-Wallis was used followed by Dunn's test for pairwise comparisons as necessary. For all statistical analyses, significance was accepted at *p* value of 0.05. Values are expressed as mean ± standard error of the mean (SEM). All results are presented in Table 2.

Results

Validation of the stereological method

The precision of our stereological protocol is provided by the coefficient of error (CE), a parameter of within sample variation generated by the StereoInvestigator software. A CE below 10% is considered sufficient for a valid stereological analysis and suggests that enough cells per probe were counted in the study (Coggeshall and Lekan, 1996; Gundersen, 1986; Pakkenberg and Gundersen, 1988). For NeuN and associated markers, the CE was 3.15 (range 2-5%); for GSTpi and associated markers in the cortex, the CE was 5.35 (range 4-8%). These estimates suggest that the stereological parameters chosen were appropriate (Gundersen, et al., 1999).

Overall neuron estimates

We compared the estimated total number of cortical NeuN+ cells to previously published results. The average number of NeuN+ cells found in all mice tested was 1,079,619±89,281 for a 2.16 mm-thick portion of the cerebral cortex obtained between lateral 0.36mm and lateral 2.52 mm of one hemisphere (n = 24). This portion of the cortex represents roughly 50.4% of the total cortical tissue of one cerebral hemisphere (estimated using coordinates from Franklin and Paxinos (2008)). Based on the stereological estimate obtained from that portion of the cortex, there would be approximately 2,142,101±177,145 NeuN+ cells per cerebral hemisphere, or

4,284,202±354,290 NeuN+ cells in the entire cerebral cortex of the mouse. This is comparable to previously obtained estimates in mice, stating that the mouse cerebral cortex contains approximately 4,000,000 neurons (Haug, 1987; Roth and Dicke, 2005).

Validation of the NG2-CreER^{TM}:EYFP reporter mouse model and total number of OPCs in the cerebral cortex and corpus callosum

PDGFRα is selectively expressed by OPCs (Nishiyama, et al., 1996; Butt, et al., 1997). To determine the total number of OPCs in the dorsal portion of the cerebral cortex of adult mice, we looked at the estimated total number of PDGFRα+ cells located between lateral 0.36 mm and lateral 2.52 mm in those regions. We determined that an average of 92,934±12,081 are located in the dorsal portion of one hemisphere of the cortex (n = 24). Assuming our sample represents roughly 50.4% of the total cortical of one cerebral hemisphere, this would correspond to approximately 368,786±47,940 PDGFRα+ cells in the cerebral cortex.

Like PDGFRα, NG2 is also a selective OPC marker (Nishiyama, et al., 1996; Butt, et al., 1997). To study the fate of adult OPCs, we made use of the NG2-CreER^{TM}:EYFP reporter mouse, in which NG2+ cells come to express EYFP upon tamoxifen-induced Cre recombination at the gene locus (Zhu, et al., 2011). To optimize the visualization of reporter cells, we used a GFP antibody that recognizes EYFP. Henceforth, NG2+ reporter cells will be referred to as GFP+ cells. To verify the effectiveness of the NG2-CreER^{TM}:EYFP reporter mouse at identifying OPCs, we compared the estimated number of GFP+ cells to the estimated number of PDGFRα+ cells in the dorsal portion of the cortex analyzed in this study. The estimated number of GFP+ cells in the areas studied was similar to the estimated total number of PDGFRα+ cells, with an average of 95,986±5,398 in the dorsal portion of the cerebral cortex (n = 24). Since the estimated number of GFP+ cells is slightly higher than the estimated number of PDGFRα+ cells,

it implies that a proportion of GFP+ reporter cells differentiated into a phenotype other than that of an OPC during the two weeks following GABAergic agent administration. Alternatively, immunohistochemistry with PDGFRα may not have completely successful, failing to label some PDGFRα+ cells.

Total number of OPC-neuron pairs in the cortex of the NG2-CreER^{TM}:EYFP reporter mouse and effect of reference memory training and GABAergic agent administration

To study OPC-neuron pairs, we used the NG2-CreER^{TM}:EYFP reporter mouse, in which NG2+ cells come to express EYFP upon tamoxifen-induced Cre recombination (Zhu, et al., 2011). To optimize the visualization of reporter cells, we used a GFP antibody that recognizes EYFP. Henceforth, NG2+ reporter cells will be referred to as GFP+ cells. Our stereological analysis estimated that an average of 42,410±3,511 OPC-neuron pairs are located between lateral 0.36 mm and lateral 2.52 mm of the dorsal portion of one hemisphere of the cortex of adult mice (n=24). Using the approximation presented in the previous section, (i.e. that our sample corresponds to 50.4% of the cortex in one hemisphere), we propose that approximately 168,294±13,933 OPC-neuron pairs are present in the adult mouse cerebral cortex. Examples of OPC-neuron pairs can be seen in Figures 2, 3, and 4.

We also looked at the effects of reference memory training and GABAergic agent administration on the estimated number of OPC-neuron pairs. Reference memory training did not significantly affect the estimated number of OPC-neuron pairs located in the dorsal portion of the cortex of experimental animals between lateral 0.36 mm and lateral 2.52 mm ($F(1,6) = 0.48$).

However, GABAergic agent administration did have a significant effect on the estimated number of OPC-neuron pairs (Kruskal-Wallis exact probability = 0.026). Post hoc comparisons using Dunn's test indicated that the mean score for the saline group (32,979±2,280) was different from the baclofen group (60,036±5,583) and the picrotoxin group (53,643 ± 6,289).

Density of OPC-neuron pairs in the cortex of the NG2-CreER^{TM}:EYFP reporter mouse and effect of reference memory training and GABAergic agent administration

We estimated that there are, on average, 3470±273 OPC-neuron pairs per mm^3 in the dorsal portion of the cortex of the adult mouse. In contrast, we estimated that there are, on average, 8580 ± 530 OPCs per mm^3 and 88400 ± 5960 neurons per mm^3 of the dorsal portion of the cortex.

We also looked at the effects of reference memory training and GABAergic agent administration on the estimated density of OPC-neuron pairs. Reference memory training did not significantly affect the estimated density of OPC-neuron pairs located in the dorsal portion of the cortex of experimental animals (Kruskal-Wallis exact probability = 0.88).

However, GABAergic agent administration did have an overall significant effect on the estimated number of OPC-neuron pairs (Kruskal-Wallis exact probability = 0.026). Post hoc comparisons using Dunn's test indicated that the picrotoxin (4546 ± 550) group had significantly more OPC-neuron pairs per mm^3 than the control group (2617 ± 180). Furthermore, the picrotoxin and baclofen (4260 ± 620) groups had significantly more OPC-neuron pairs per μm^3 than the saclofen group (2338 ± 67).

Proportion of OPCs in OPC-neuron pairs

Based on the total number of GFP+ cells estimated in the stereological analysis, we were able to determine that approximately 40.44 ± 1.78% of all GFP+ cells (i.e. OPCs) are closely apposed to NeuN+ somata (i.e. neurons). While animals in the experimental group of the reference memory training cohort had a larger proportion of GFP+ cells in OPC-neuron pairs (Experimental group: 48.80 ± 5.22%; Control group: 38.71 ± 2.56), this difference was not statistically significant ($F(1,6) = 1.96$, $p = .21$). Similarly, the difference between groups in the proportion of GFP+ cells in OPC-neuron pairs in the GABAergic agent cohort was not statistically significant ($F(4,11) = 2.16$, $p = .14$).

A small proportion of OPCs in OPC-neuron pairs are mitotically active

Of the OPC-neuron pairs identified in this study, 7.20±1.67% were composed of an OPC that had incorporated BrdU (i.e. GFP+/BrdU+ cells) during the 8-day period when it was available (n=24). Figure 2 presents an example BrdU-positive OPC associated in an OPC-neuron pair. As to the effects of reference memory training on the number of GFP+/BrdU+ cells in OPC-neuron pairs, only one animal in the cohort had such cells. However, most of the animals in the GABAergic agent cohort had GFP+/BrdU+ cells in OPC-neuron pairs. The difference between the groups in the proportion of these cells was not statistically significant ($F(4,11) = .681$, $p = .62$).

Proportion of neurons in OPC-neuron pairs

Based on the total number of NeuN+ cells estimated in the stereological analysis, we were able to determine that approximately 4.14±0.27% of all neurons are closely apposed to an OPC somata. There was no significant difference in the proportion of NeuN+ cells in OPC-neuron pairs between groups in the reference memory cohort ($F(1,6) = 0.1$, $p = 0.77$). Similarly,

there was no significant difference in the proportion of NeuN+ cells in OPC-neuron pairs between groups in the GABAergic agents cohort ($F(4,11) = .36$, $p = .83$).

Histological analysis of OPC-neuron pairs

Finally, we did a preliminary attempt at identifying the types of neurons in OPC-neuron pairs in the dorsal portion of the cortex. Figures 3 and 4 presents examples of OPC-neuron pairs in the dorsal portion of the cortex. As can be seen in Figure 4 and 5, the two cells are closely apposed to one another. We also used immunohistochemistry to show that all three classical subgroups of cortical interneurons form OPC-neuron pairs (Figures 6A-C, 7, and 9 A-D : somatostatin identified with calbindin; Figures 6D-F: parvalbumin; Figures 6G-I, 8, and 9 E-H,: calretinin (Kawaguchi and Kubota, 1997; Wonders and Anderson, 2006)).

Discussion

In the present experiment, we used stereology and histological analysis to study OPC-neuron pairs in the dorsal neocortex of the adult mouse. OPC-neuron pairs correspond to an OPC cell body that is closely apposed that of a neuron, forming pairs or possibly even satellite cells (Gallo, et al., 2008; Dayer, et al., 2005; Mangin, et al., 2008). Our results suggest that there are close to 170,000 OPC-neuron couples in the adult mouse dorsal neocortex. This corresponds to approximately 40% of all cortical OPCs and 4% of all cortical neurons. We also established that reference memory training does not affect the prevalence of OPC-neuron pairs or the number of OPCs and neurons in these pairs. We also determined that pharmacologically-induced GABAergic neurotransmission does not affect the proportion of OPCs and neurons in pairs. However, the $GABA_B$-receptor agonist baclofen and the $GABA_A$ receptor antagonist picrotoxin

significantly increased the estimated number of pairs when compared to the control group and the $GABA_B$-receptor antagonist (i.e. saclofen) group. Density of OPC-neuron pairs were increased by the $GABA_A$ receptor antagonist picrotoxin. Finally, we provided histological evidence to suggest that many of the neurons in OPC-neuron pairs are GABAergic interneurons.

Stereological analysis of OPC-neuron pairs

Stereology allows for cell numbers to be counted from tissue in an unbiased manner (Herculano-Houzel, et al., 2015). Using stereology, we were able to provide the first estimates of the total number of OPC-neuron pairs in the dorsal portion of the cerebral cortex of the adult mouse brain. We determined that approximately 170,000 OPC-neuron pairs are found in the adult mouse dorsal cerebral cortex or 3470 OPC-neuron pairs per mm^3 of tissue in the dorsal portion of the adult mouse cerebral cortex. We also estimated that about 40% of OPCs and 4% of neurons form OPC-neuron pairs.

We used the NG2-CreER^{TM}:EYFP reporter mouse to establish these numbers. NG2 is a selective marker of OPCs (Nishiyama, et al., 1996; Stallcup, 2002) and in the NG2-CreER^{TM}:EYFP reporter mouse, NG2+ cells come to express EYFP upon tamoxifen-induced Cre recombination at the gene locus (Zhu, et al., 2011). Although NG2 is also expressed in pericytes, these cells are easily distinguished from OPC. Immunohistochemistry with a GFP+ antibody that also recognizes EYFP allows for the visualization of cells that were OPCs at the time of tamoxifen administration. We recognize that using a mouse model that makes use of Cre technology to identify OPCs may have resulted in an underestimation of the prevalence of OPC-neuron pairs since Cre recombination is rarely complete. In other words, not all NG2+ cells at the time of tamoxifen administration undergo Cre recombination and come to express EYFP (Zhu, et al., 2011; Hayashi and McMahon, 2002). However, it must be noted that the Cre

recombination achieved in the reference memory and GABAergic agents experiments was high, which suggests that our estimates probably only slightly underestimated the actual number of OPC-neuron pairs found in the dorsal portion of the cortex of the adult mouse.

Reference memory training and OPC-neuron pairs

It has been suggested that OPCs in OPC-neuron pairs play a modulatory role in neural network activity, integrating the synaptic activity of the neuron it is associated to with that of neighbouring cells (Butt, et al., 2005; Boda and Buffo, 2014; Gallo, et al., 2008). Conversely, experience-dependent neural network activity has been shown to influence OPCs, affecting their rates of proliferation and differentiation along the oligodendrocyte lineage (Boda and Buffo, 2014; Gallo, et al., 2008; Káradóttir and Attwell, 2007; Fields, 2008; Fields, 2015; Gibson, et al., 2014; Purger, et al., 2016; Tomlinson, et al., 2016; Wake, et al., 2011). We therefore tested whether training on a task that recruits a number of brain areas could affect the prevalence of OPC-neuron pairs. Reference memory training was not found to have an effect on the number of OPC-neuron pairs or on the proportion of OPCs and neurons that form these pairs.

The lack of statistically significant results could also be explained by our approach in that we looked at the global effects of reference memory training on the entire dorsal portion of the cortex. Reference memories are first processed by the hippocampus and then sent to the cortex for permanent storage. Cholinergic projections to the frontal and parietal cortices are thought to play a crucial role in that process (Kesner, et al., 1987; Maviel, et al., 2004). A more segmented analysis on smaller areas of the cortex may have been preferable since the effects of reference memory training on OPC proliferation and differentiation may not be homogenously distributed throughout the brain. Support for this hypothesis comes from (Xiao, et al., 2016), who demonstrated that learning a complex motor skill like running on a wheel with unevenly spaced

rungs promotes the differentiation of OPCs into myelinating oligodendrocytes in the motor cortex but not in the visual cortex.

However, the lack of significant results reported here does not exclude the possibility that experience-dependent neural network activity has a small but distributed effect on the prevalence of OPC-neuron pairs. Examples of global activities that increase the proliferation and differentiation of OPCs include voluntary physical exercise, environmental enrichment, behavioural stimulation, and motor learning (Simon, et al., 2011; Ehninger, et al., 2011; McKenzie, et al., 2014; Xiao, et al., 2016; Zhao, et al., 2010).

GABAergic agents and OPC-neuron pairs

OPCs have functional $GABA_A$ and $GABA_B$ receptors that can be stimulated through synaptic and extra-synaptic transmitter release (Lin and Bergles, 2004a; Kukley, et al., 2007; Lin and Bergles, 2004b; Vélez-Fort, et al., 2010; Ziskin, et al., 2007; Luyt, et al., 2007; Hill and Nishiyama, 2014; Maldonado and Angulo, 2015; Maldonado, et al., 2011). Much like experience-dependent neural network activity, GABAergic neurotransmission has been hypothesized to modulate OPC proliferation and differentiation (Gallo, et al., 2008; Káradóttir and Attwell, 2007; Pastor, et al., 1995; Luyt, et al., 2007; Baron, et al., 1998; Knutson, et al., 1997; Vélez-Fort, et al., 2012; Hill and Nishiyama, 2014; Bakiri, et al., 2009; Belachew and Gallo, 2004). We therefore tested whether the systemic administration of $GABA_A$ and $GABA_B$ receptor agonists and antagonists would have an effect on OPC-neuron pairs.

We were able to show that the $GABA_B$-receptor agonist baclofen and the $GABA_A$ antagonist picrotoxin significantly increased the number of OPC-neuron pairs *in vivo* when compared to the saline control group and the $GABA_B$-receptor agonist saclofen. Similarly, the

density of OPC-neuron pairs was significantly higher in animals exposed to picrotoxin than in controls and those exposed to saclofen. The $GABA_A$-receptor agonist muscimol, on the other hand, was not found to have an effect on the prevalence of OPC-neuron pairs.

Since we have no dose-response data for the GABA-modulating drugs we used, it is difficult to ascribe specific roles of the $GABA_A$ and $GABA_B$ receptors to the modulation of OPC-neuron couples. However GABA receptor modulation appears to be significant. In the next paragraph, we discuss possible interaction between GABAergic neurotransmission and OPC's

While $GABA_A$ is a ligand-gated ionotropic receptor that leads to fast GABAergic neurotransmission, $GABA_B$ is a G-coupled receptor that mediates slow GABAergic neurotransmission (Bowery, et al., 2002). Fast and slow GABAergic neurotransmission may therefore have different effects on the prevalence of OPC-neuron pairs. Similarly, fast and slow GABAergic neurotransmission have been shown to have different effects on OPC proliferation *in vitro* (Káradóttir and Attwell, 2007; Pastor, et al., 1995; Knutson, et al., 1997), with $GABA_A$-receptor activation inhibiting OPC proliferation and $GABA_B$-receptor activation promoting it (Luyt, et al., 2007). In addition to increasing the proliferation of cultured OPCs, the $GABA_B$-receptor agonist baclofen has also been shown to facilitate their migration (Luyt, et al., 2007). If baclofen had similar effects on the OPCs located in the brain of the animals analyzed in this study, it could have increased the prevalence of OPC-neuron pairs by facilitating the migration of OPCs toward neuronal cell bodies.

Histological analysis of OPC-neuron pairs

We provided a histological analysis of OPC-neuron pairs. More specifically, we showed that the three classical subtypes of cortical GABAergic interneurons take part in such pairs

(Kawaguchi and Kubota, 1997; Wonders and Anderson, 2006). While we don't exclude the possibility that OPCs form pairs with other types of neurons, it appears as though, based on the literature and observations done in our lab, that OPC-neuron pairs of the dorsal portion of the cortex are mostly restricted to GABAergic interneurons (Butt, et al., 2005).

GABAergic interneurons are the main inhibitory neurons of the central nervous system. In the cortex of the rat, GABAergic interneurons have been estimated to comprise 16% of all cortical neurons (Beaulieu, 1993). The combination of our observations with that estimate suggests that up to one in four GABAergic interneurons could be paired with an OPC in the mouse cortex. Cortical GABAergic interneurons are surrounded by perineuronal nets, and OPC-neuron pairs appear to be a critical component of these nets (Butt, et al., 2005; Gallo, et al., 2008; Dayer, et al., 2005; Mangin, et al., 2008; Butt, et al., 2002). The role of GABAergic interneurons is compatible with one of the hypothesized role of OPCs in the cortex, which is to monitor neural network activity (Butt, et al., 2005; Boda and Buffo, 2014; Gallo, et al., 2008). Since GABAergic interneurons are also involved in coordinating neural synchrony, it is possible that the close contacts between OPCs and GABAergic interneurons act in tandem to promote this function (Sohal, et al., 2009).

Concluding remarks

We have used an unbiased systematic stereological approach to determine the total number of OPC-neuron pairs in the dorsal portion of the adult neocortex. Stereological analysis also showed that approximately 40% of OPCs and 4% of neurons of the dorsal cerebral cortex take part in OPC-neuron pairs. Since OPC-neuron pairs are thought to assist OPCs in their role as monitors of neural network activity, we sought to determine whether two factors that may

influence neural networks, reference memory training and GABAergic neurotransmission, affected the prevalence of OPC-neuron pairs. While reference memory training did not appear to have an overall effect, GABA receptor modulation increased the number of OPC-neuron pairs. Finally, we found that the three subtypes of cortical GABAergic interneurons can form OPC-neuron pairs. Since GABAergic interneurons support circuit performance by ensuring the synchrony of inputs, OPC-neuron pairs appear to assist OPCs in their role as monitors of neural network activity.

Tables

Table 1. List of antibodies used in the study.

Host	Target	Concentration	Company
Primary Antibodies			
Rabbit	GFP	1/1000	Abcam
Mouse	GFP	1/500	Abcam
Goat	Sox10	1/500	Santa Cruz
Mouse	HuCD	1/2000	Thermo Scientific
Rabbit	PDGFR$_\alpha$	1/250	Santa Cruz
Rabbit	GST-pi	1/500	MBL
Mouse	NeuN (Rbfox3)	1/500	Millipore
Guinea-pig	NeuN (Rbfox3)	1/1000	Millipore
Sheep	Calbindin	1/200	Swant
Rabbit	Calbindin	1/300	C. Gerfen (private antibody)
Goat	Calretinin	1/500	Swant
Rat	Parvalbumin	1/500	Abcam
Rat	BrdU	1/500	Abcam
Secondary Antibodies			
Donkey	Anti-Rabbit Alexa 488	1/1000	Invitrogen
Donkey	Anti-Mouse Alexa 488	1/1000	Invitrogen
Donkey	Anti-Rabbit Alexa 594	1/1000	Invitrogen
Donkey	Anti-Mouse Alexa 594	1/1000	Invitrogen
Donkey	Anti-Goat Alexa 594	1/1000	Invitrogen
Donkey	Anti-Rat Alexa 680	1/500	Abcam

Table 2. Results of stereological analysis

Total estimated number of OPC-neuron pairs	
Global result	**42,410±3,511**
Reference memory training	**F(1,6) = 0,48 *p=0.51 (power=0.09)***
Control group	30,764 ± 768
Experimental group	41,984 ± 12,042
GABAergic agents	**Kruskal-Wallis prob = .026***
Saline	32,979 ± 2,280
Muscimol	39,374 ± 7,295
Picrotoxin	53,643 ± 6,289**
Baclofen	60,036 ± 5,583**
Saclofen	28,322 ± 856
Density of OPC-neuron pairs (number/mm^3)	
Global result	**3470 ± 273**
Reference memory training	**Kruskal-Wallis prob = .88**
Control group	2450 ± 91
Experimental group	3457 ± 816
GABAergic agents	**Kruskal-Wallis prob = .026***
Saline	2617 ± 180
Muscimol	3142 ± 380
Picrotoxin	4546 ± 550**
Baclofen	4260 ± 620
Saclofen	2338 ± 67.1
Mean proportion of GFP+ cells in OPC-neuron pairs (%)	
Global result	**40.44±1.78**
Reference memory training	**F(1,6)) = 1.96, *p=0.21 (power=0.22)***
Control group	38.71±2.56
Experimental group	48.80±5.22
GABAergic agents	**F(4,11) = 2.16, *p* = .14 *(power=0.45)***
Saline	40.55±2.58
Muscimol	27.49±4.70
Picrotoxin	41.24±3.13
Baclofen	39.09±1.54
Saclofen	34.96±6.16
Mean proportion of NeuN+ cells in OPC-neuron pairs (%)	
Global result	**4.14±0.27**
Reference memory training	**F(1,6) = 0.1, *p=0.77 (power=0.06)***
Control group	4.67±1.14
Experimental group	4.22±0.89
GABAergic agents	**F(4,11) = .36, *p* = .83 *(power=0.11)***
Saline	4.18±0.94
Muscimol	3.48±0.06

Picrotoxin	4.39±0.31
Baclofen	3.69±0.48
Saclofen	3.75±0.09

Figure legends

Fig. 1. Experimental protocol. (A) Experimental timeline for the reference memory training cohort. 4-5 month-old NG2-CreER^{TM}:EYFP mice (n=8) received i.p. injections of tamoxifen once a day for 5 days (days 1-5). On day 10, drinking water was replaced with sweetened water. On day 12, sweetened water was replaced with BrdU sweetened water. On days 13-16, mice in the experimental group had three trials a day on the Barnes maze. On day 17, mice in the experimental group underwent the short-term reference memory probe on the Barnes maze. On day 19, BrdU sweetened water was replaced with regular drinking water. On day 26, mice in the experimental group underwent the long-term reference memory probe on the Barnes maze. Mice were euthanized on day 80, 8 weeks following the end of BrdU exposure (B) Experimental timeline for the GABAergic agents cohort. 4-5 month-old NG2-CreER^{TM}:EYFP mice (n=16) received i.p. injections of tamoxifen once a day for 5 days (days 1-5). On day 10, drinking water was replaced with sweetened water. On day 12, sweetened water was replaced with BrdU sweetened water. On days 13-17, mice received one injection per day of either saline (n = 4), muscimol ($GABA_A$ receptor agonist; 1.0 mg/kg, n = 3), picrotoxin ($GABA_A$ receptor antagonist; 1.0 mg/kg; n = 4), baclofen ($GABA_B$ receptor agonist; 7.5 mg/kg; n = 3), or saclofen ($GABA_B$ receptor antagonist; 5.0 mg/kg; n = 2). Mice were euthanized on day 80, 8 weeks following the end of BrdU exposure. (C) Immunohistochemistry. Five slides per animal were stained for GFP, BrdU, and NeuN. (D) Stereology. The cross-sectional area of the dorsal cortex (dark grey) was manually traced at 10X. A sampling grid with 1475 μm x 382 μm spacing was randomly placed on the traced areas by the StereoInvestigator software, and a single 200 μm x 200 μm counting frame was placed systematically at each sampling spot in their top left corner. All cells within the counting frames were imaged at 40X and counted using the software.

Fig. 2. BrdU-positive OPC-neuron pair. Pictures taken in the cortex of 4-5 month-old NG2-CreER^{TM}:EYFP mice at 60X using a laser scan microscope. GFP (mouse anti-GFP) in green (A), neuronal marker HuCD in red (B), BrdU-positive OPC in grey (C), and merged (D).

Fig. 3. OPC-neuron pairs. Pictures taken in the cortex of 4-5 month-old NG2-CreER^{TM}:EYFP mice at 20X using a fluorescence microscope. GFP (mouse anti-GFP) in green (A and E), PDGFRα in red (B and F), NeuN in blue (C and G), and merged (D and H).

Fig. 4. OPC-neuron pairs. Pictures taken in the cortex of 4-5 month-old NG2-CreER^{TM}:EYFP mice at 60X using a laser scan microscope. GFP (mouse anti-GFP) in green (A and E), PDGFRα in red (B and F), NeuN in grey (C and G), and merged (D and H).

Fig. 5. OPCs form close contacts with neurons. Pictures taken in the cortex of 4-5 month-old NG2-CreER^{TM}:EYFP mice at 60X using a laser scan microscope. GFP (mouse anti-GFP) in green (A), PDGFRα in red (B), NeuN in grey (C), and merged (D).

Figure 6. Subtypes of GABAergic interneurons form OPC-neuron pairs. Pictures taken in the cortex of 4-5 month-old NG2-CreER^{TM}:EYFP mice at 60X using a laser scan microscope. GABAergic interneuron subtype in green (calbindin in A, parvalbumin in D, and calretinin in G), PDGFRα in red (B, E, and H), and merged (C, F, and I).

Figure 7. Calbindin-positive GABAergic interneurons form OPC-neuron pairs. Pictures taken in the cortex of 4-5 month-old NG2-CreER^{TM}:EYFP mice at 40X using a fluorescence microscope. Calbindin in green (A and D), PDGFRα in red (B and E), and merged (C and F).

Figure 8. Calretinin-positive GABAergic interneurons form OPC-neuron pairs. Pictures taken in the cortex of 4-5 month-old NG2-CreER^{TM}:EYFP mice at 40X using a fluorescence microscope. Calretinin in green (A and D), PDGFRα in red (B and E), and merged (C and F).

Fig. 9. OPC-neuron pairs. Pictures taken in the cortex of 4-5 month-old NG2-CreER^{TM}:EYFP mice at 60X using a laser scan microscope. GFP (mouse anti-GFP) in green (A and E), PDGFRα in red (B and F), GABAergic interneuron in grey (calbindin in C and calretinin in G), and merged (D and H).

Figures

Figure 1.

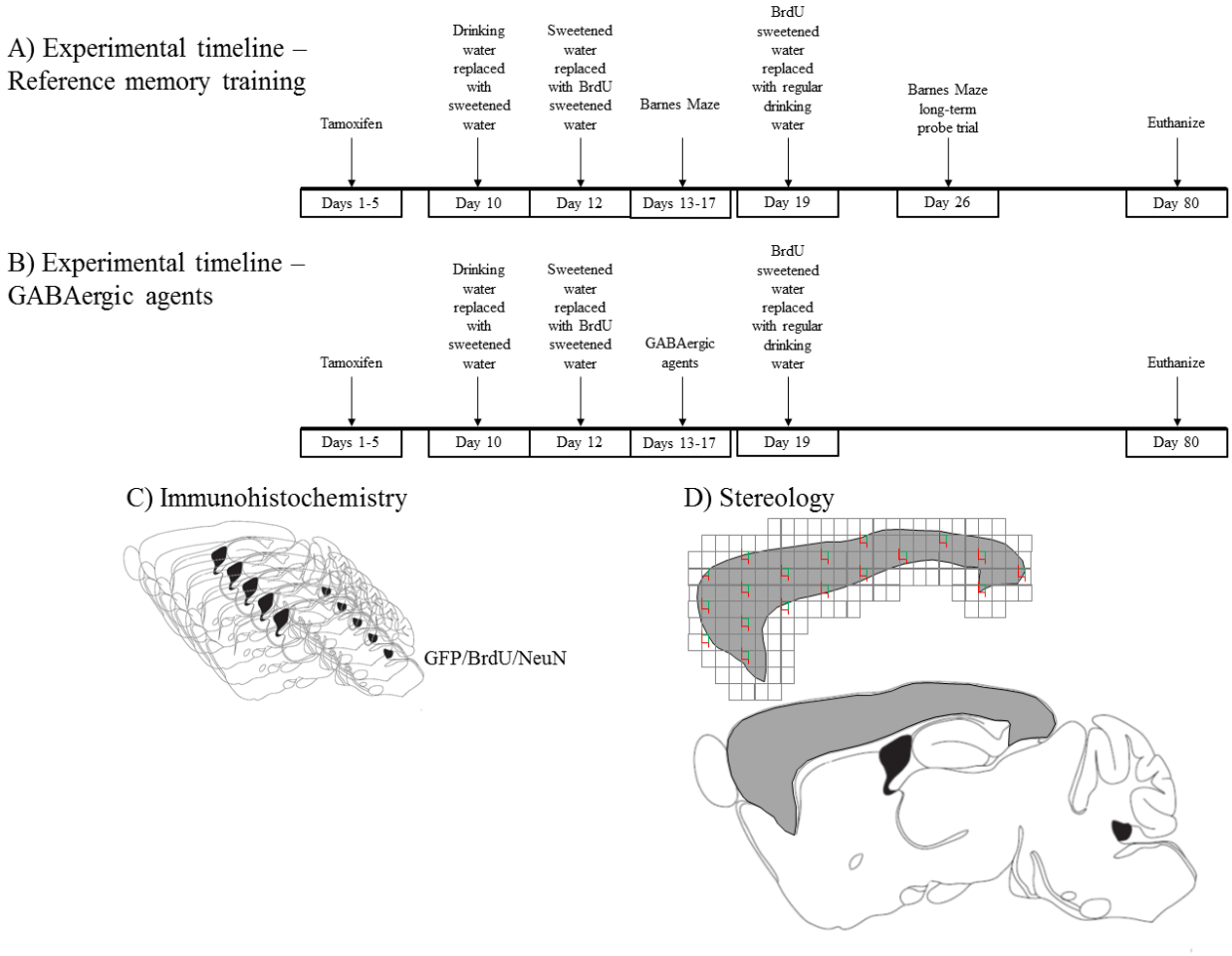
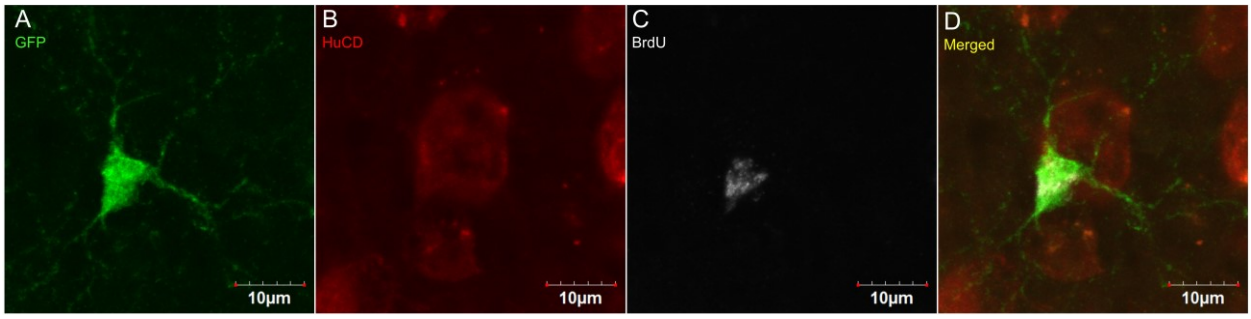


Figure 2.



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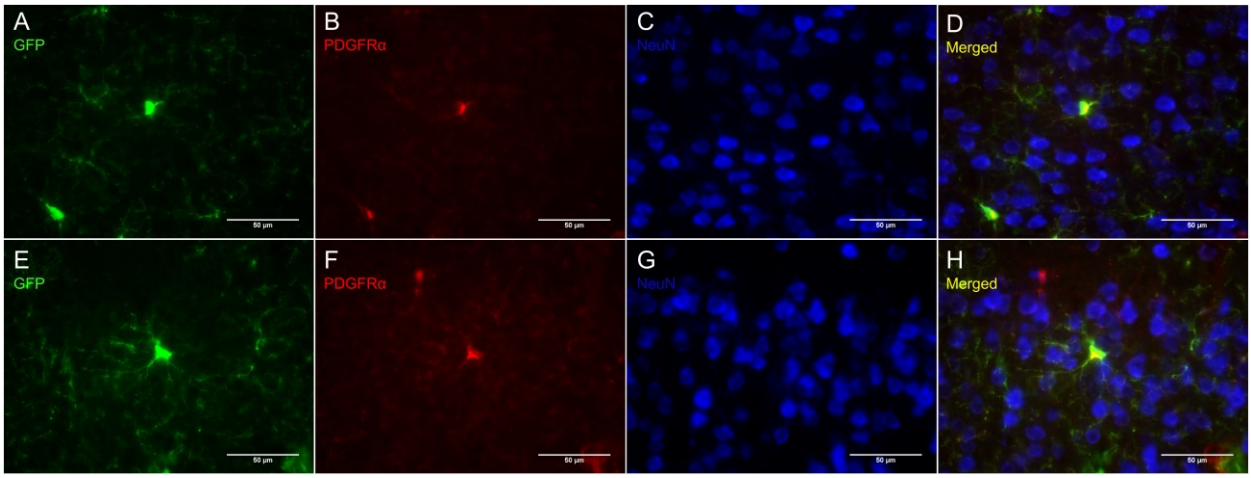

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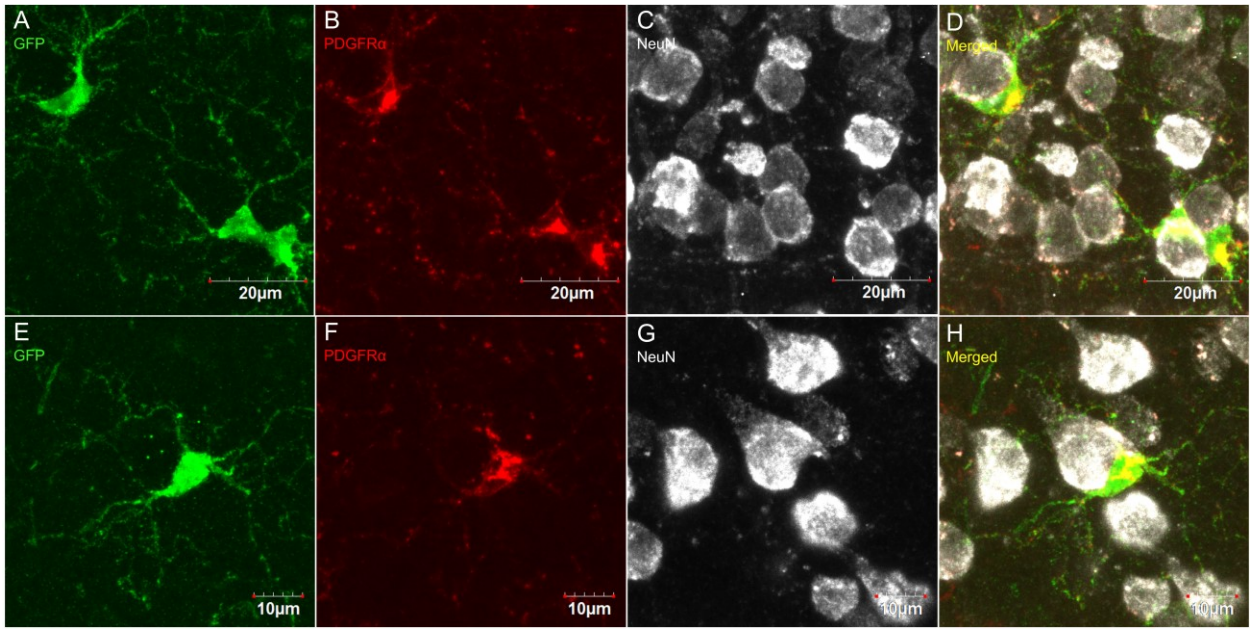
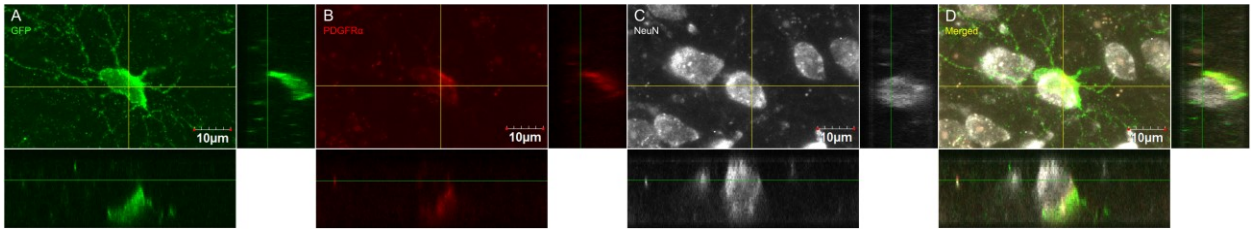

A
GFP
20μm
B
PDGFRα
20μm
C
NeuN
20μm
D
Merged
20μm
E
GFP
10μm
F
PDGFRα
10μm
G
NeuN
10μm
H
Merged
10μm

Figure 5.



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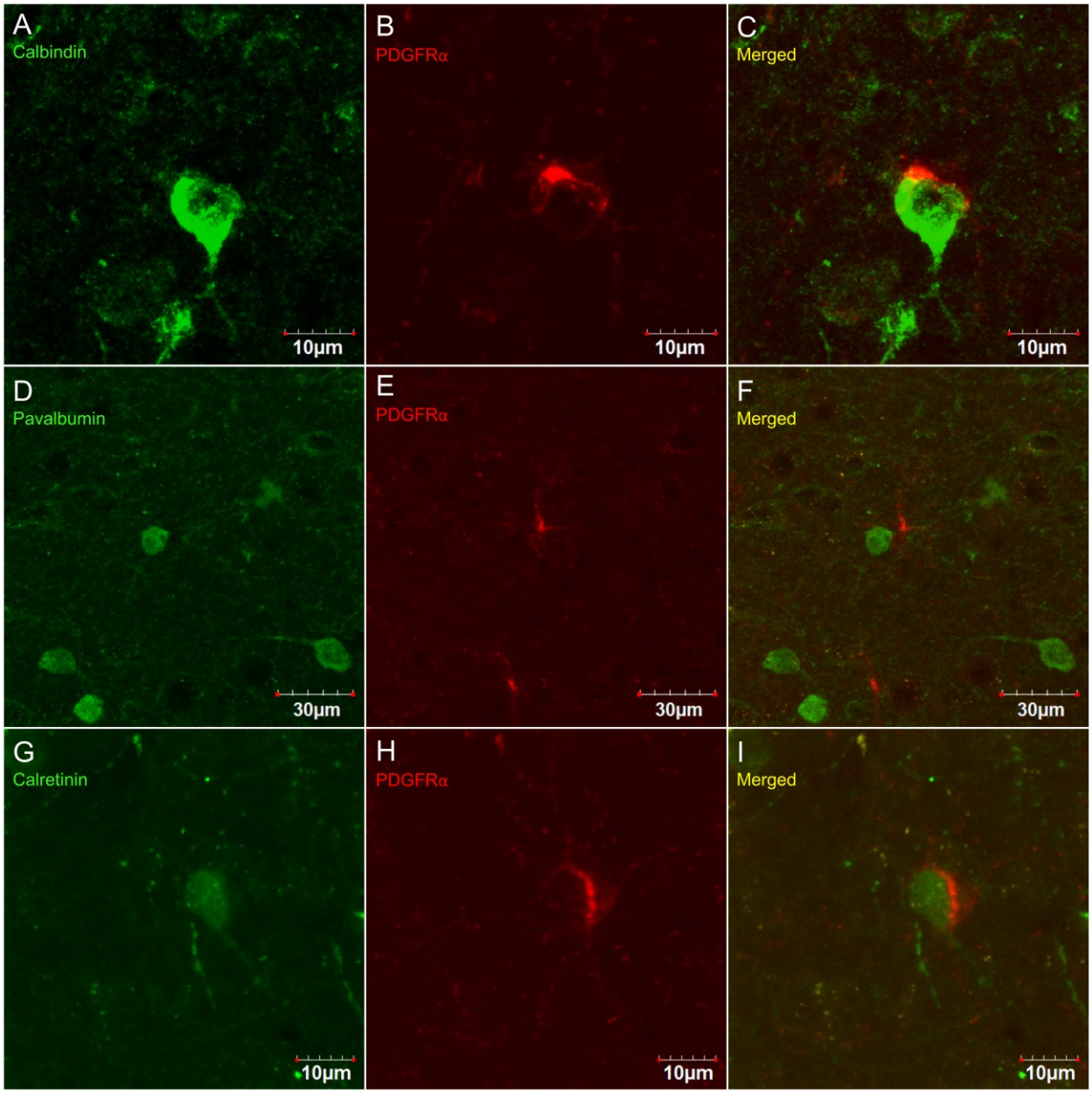
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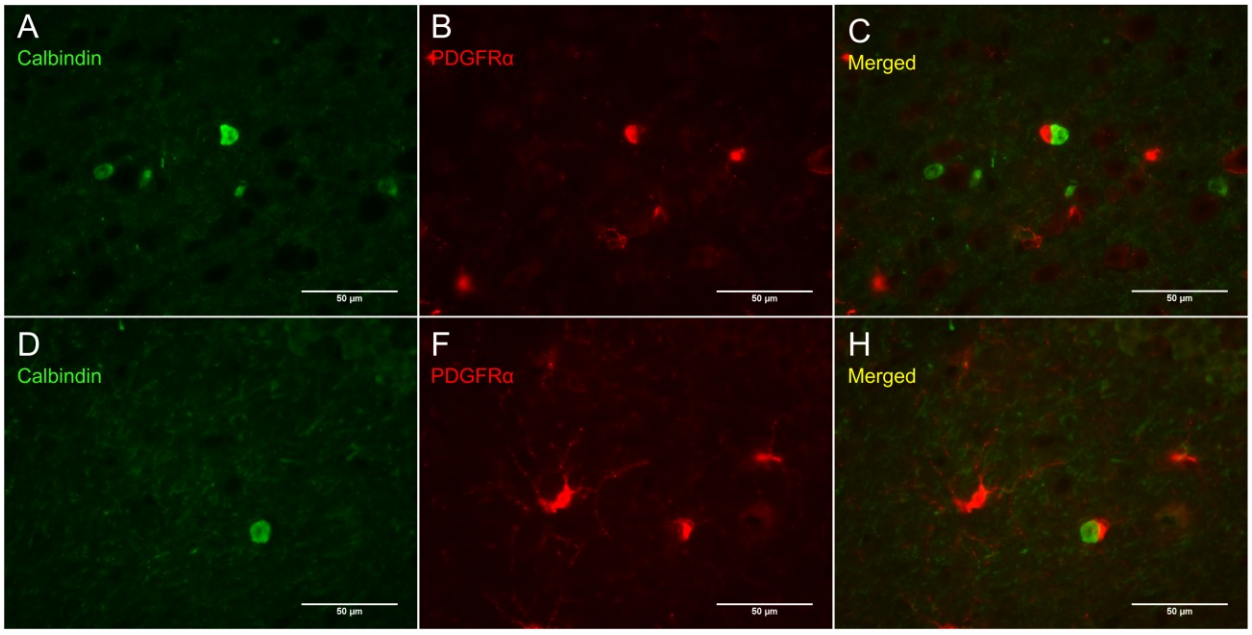
Calbindin
10μm
B
PDGFRα
10μm
C
Merged
10μm
D
Pavalbumin
30μm
E
PDGFRα
30μm
F
Merged
30μm
G
Calretinin
10μm
H
PDGFRα
10μm
I
Merged
10μm

Figure 7.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Calbindin
B
PDGFRα
C
Merged
D
Calbindin
F
PDGFRα
H
Merged
50 µm

Figure 8.

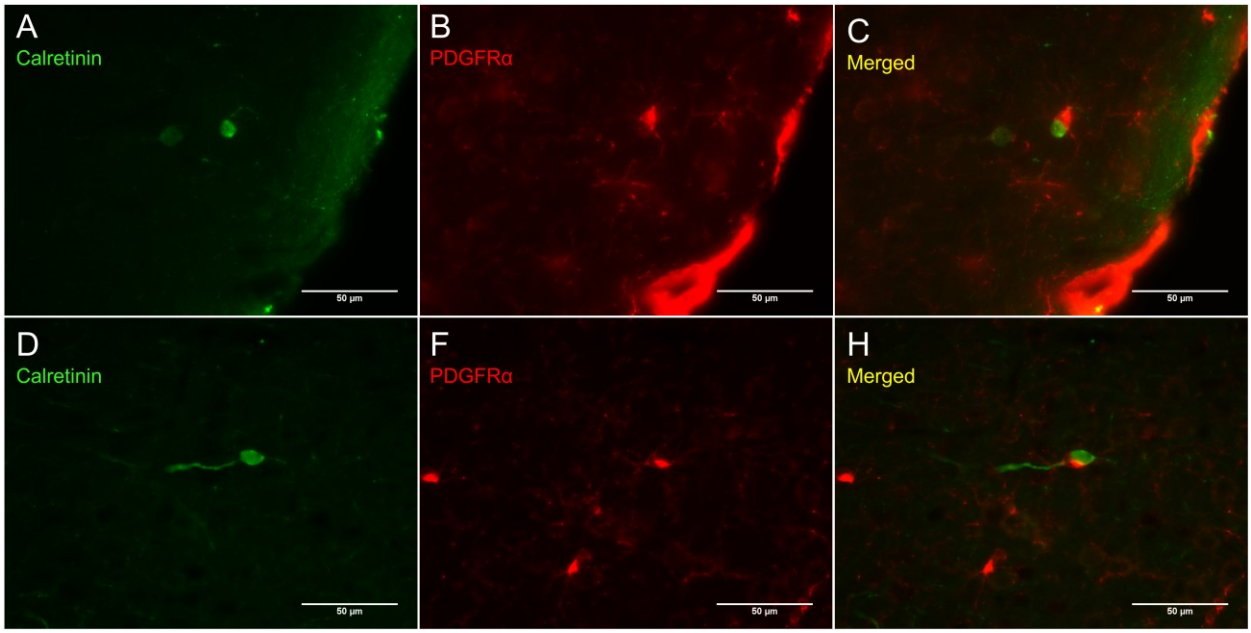
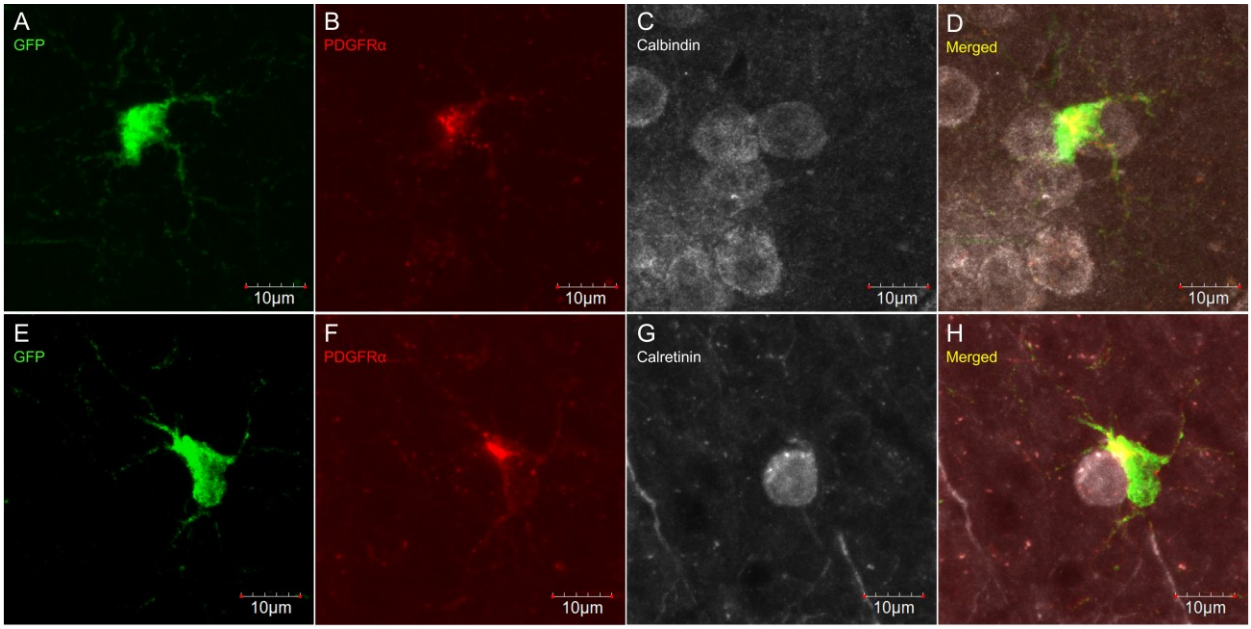

A
Calretinin
B
PDGFRα
C
Merged
D
Calretinin
F
PDGFRα
H
Merged
50 µm

Figure 9.


A
GFP
10μm
B
PDGFRα
10μm
C
Calbindin
10μm
D
Merged
10μm
E
GFP
10μm
F
PDGFRα
10μm
G
Calretinin
10μm
H
Merged
10μm

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Running Head: Doublecortin in oligodendrocyte precursor cells

EXPERIMENT 5: DOUBLECORTIN IN OLIGODENDROCYTE PROGENITOR CELLS IN THE ADULT MOUSE BRAIN

Jenna J. BOULANGER and Claude MESSIER

School of Psychology, University of Ottawa

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Correspondence to: C. Messier, School of Psychology, University of Ottawa, 136 Jean-Jacques Lussier Room 2076A, Ottawa, Ontario, K1N 6N5, CANADA. Tel (613) 562-5800 ext. 4562, Fax: (613) 562-5147; E-mail: cmessier@uottawa.ca.

Abbreviations

In alphabetical order:

DCX: Doublecortin

GSTπ: glutathione S-transferase pi

NG2: chondroitin sulfate proteoglycan neuron-glia antigen 2

Olig1: bHLH transcription factor 1

OPC: oligodendrocyte precursor cell

PDGFRα: platelet-derived growth factor receptor alpha

Sox10: SRY-related HMG-box transcription factor 10

Abstract

Key Points

- Oligodendrocyte precursor cells express doublecortin, a microtubule-associated protein.
- Oligodendrocyte precursor cells express doublecortin, but at a lower level of expression than in neuronal precursor.
- Doublecortin is not associated with a potential immature neuronal phenotype in Oligodendrocyte precursor cells.

Oligodendrocyte precursor cells (OPC) are glial cells that differentiate into myelinating oligodendrocytes during embryogenesis and early stages of post-natal life. OPCs continue to divide throughout adulthood and some eventually differentiate into oligodendrocytes in response to demyelinating lesions. There is growing evidence that OPCs are also involved in activity-driven *de novo* myelination of previously unmyelinated axons and myelin remodeling in adulthood. Considering these roles in the adult brain, OPCs are likely mobile cells that can migrate on some distances before they differentiate into myelinating oligodendrocytes. A number of studies have noted that OPCs express doublecortin (DCX), a microtubule-associated protein expressed in neural precursor cells and in migrating immature neurons. Here we describe the distribution of DCX in OPCs. We found that almost all OPCs express DCX, but the level of expression appears to be much lower than what is found in neural precursor. We found that DCX is downregulated when OPCs start expressing mature oligodendrocyte markers and is absent in myelinating oligodendrocytes. DCX does not appear to signal an immature neuronal phenotype in OPCs in the adult mouse brain. Rather, it could be involved either in cell migration, or as a marker of an immature oligodendroglial cell phenotype.

Keywords: myelin, adult neurogenesis, myelin remodeling, oligodendrocyte, plasticity, gliogenesis, cell migration

Introduction

Although traditionally perceived as non-regenerative tissue, the adult brain does retain the ability to generate new neurons, as first described by Messier (Messier et al., 1958) and confirmed by Altman and Das (1964). More specifically, studies have determined that neurogenesis in the adult rodent brain takes place in two distinct regions: the subgranular layer of the dentate gyrus of the hippocampus, with newly generated neurons migrating to the granular layer of the dentate gyrus (Kaplan and Bell, 1984; Stanfield and Trice, 1988; Cameron et al., 1993; Kuhn et al., 1996) and in the subventricular zone of the lateral ventricles, from where the newly generated neurons migrate to the olfactory bulbs using a pathway known as the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Alvarez-Buylla and Garcıa-Verdugo, 2002).

The migration of these newly generated neuroblasts to their final destination is thought to be facilitated by the expression of doublecortin (DCX), a protein that participates in the polymerization of microtubules (Francis et al., 1999; Gleeson et al., 1999). DCX is only transiently expressed in proliferating progenitor cells and in newly generated neuroblasts and its expression is downregulated as the cells begin to express markers of a mature neuronal state. This has led to the use of DCX as a selective marker of adult neurogenesis (Brown et al., 2003). However, DCX expression is not restricted to the dentate gyrus or areas involved in the addition of new neurons to the olfactory bulbs (Nacher et al., 2001; Dayer et al., 2005; Luzzati et al., 2006; Xiong et al., 2008; Cai et al., 2009; Klempin et al., 2011; Werner et al., 2012; Saul et al., 2014). Some authors have also observed DCX in cells expressing immature neurons markers located in the layer III of the piriform cortex and endopiriform nucleus of adult rodents which do not appear to be migrating neurons (Rivers et al., 2008; Guo F. et al., 2010; Klempin et al., 2011; Clarke et al., 2012). Finally, as we show here, DCX is widely expressed in oligodendrocyte

precursor cells (OPCs; also known as NG2-glial cells, synantocytes, or polydendrocytes; Tamura et al., 2007a; Ehninger et al., 2011).

OPCs are a type of glial cell that give rise, as their name suggests, to myelinating oligodendrocytes during embryogenesis and early stages of post-natal life (Nishiyama et al., 2002). However, a large number of OPCs maintain their undifferentiated state after these initial developmental stages and OPCs are thus abundant in the adult brain, corresponding to ~5–8% of the total cell population (Dawson et al., 2003; Gallo et al., 2008). Adult OPCs form non-overlapping fields that are uniformly distributed between the gray and white matter of the central nervous system (Dawson et al., 2003; De Biase et al., 2010; Kukley et al., 2010; Hughes et al., 2013). While their proliferative activity does decline with age, they continue to undergo cell division throughout adulthood, representing the most active population of cycling cells within the adult brain (Dawson et al., 2003; Psachoulia et al., 2009). The fate of these adult-generated OPCs has not been clearly established (Boulanger and Messier, 2014). While most daughter cells appear to maintain an OPC phenotype, it has been demonstrated that a subset of these cells differentiates into a mature myelinating oligodendroglial phenotype (Dimou et al., 2008; Kang et al., 2010; Clarke et al., 2012). Furthermore, some researchers have reported that postnatal OPCs are multipotent and have the capacity to differentiate into astrocytes and neurons in multiple regions of the adult CNS (Belachew et al., 2003; Aguirre and Gallo, 2004; Aguirre et al., 2004; Dayer et al., 2005; Tamura et al., 2007a; Rivers et al., 2008; Zhu et al., 2008; Guo F. Z. et al., 2009, 2010; Robins et al., 2013). However, this ability to differentiate into neurons is not supported by other studies (Dimou et al., 2008; Komitova et al., 2009; Kang et al., 2010; Richardson et al., 2011; Zhu et al., 2011; Nishiyama et al., 2016). This remains a debated point at this time (Larson et al., 2016; Nishiyama et al., 2016; Viganò and Dimou, 2016).

Here, we show that despite evidence for multipotency in OPCs, DCX is not associated with an immature neuronal phenotype in these cells. Rather, it could be involved either in cell migration, or as a marker of an immature oligodendroglial cell phenotype.

Materials and Methods

Animals

Animals were 4–5 month old Long-Evans rats (Charles River, St-Constant, Qc, Canada), 5–10 weeks old CD-1 mice (Charles River, St-Constant, Qc, Canada), and 3–5 month-old C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, USA) All the animals used in this study were individually housed in a 21 ± 1°C vivarium, maintained on a 12-h light/dark cycle, and had *ad libitum* access to food and water. All animal procedures were done in accordance with the recommendations of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Ottawa.

Transgenic animals

NG2CreER BAC transgenic mice (Jackson Laboratory, Bar Harbor, Maine, USA; described in Zhu et al., 2011) were bred in house with R26-stop-EYFP transgenic mice on a C57BL/6J background (Jackson Laboratory, Bar Harbor, Maine, USA) to generate the NG2-CreER:EYFP reporter mouse. Three to five-month old animals (n = 8) received i.p injections of 6 mg of tamoxifen per day, over a period of 5 days. Six days after the last tamoxifen injection, the animals' drinking water was replaced with 5-bromo-2′-deoxyuridine (BrdU) in sweetened water

(100 ml water + 0.125 g saccharine + 3 g dextrose + 0.1 g BrdU) for a total of 8 days. Mice were perfused 8 weeks following the end of BrdU exposure.

Tissue processing

Anesthetized rats or mice were transcardially perfused with saline followed by Lana's fixative (4% paraformaldehyde-picric acid; modified from Zamboni and Demartin, 1967). Brains were post-fixed in this fixative for 1 h before being incubated in 10% sucrose in sodium phosphate buffer (0.1 M, pH 7.2) overnight at 4°C. Brains were then frozen using CO_2 and cut in 14 µm sagittal sections using a cryostat.

Immunochemistry

Peptide competition assay

To determine the specificity of the goat anti-DCX antibody (Santa Cruz, sc-8066) and the guinea-pig anti-DCX antibody (Chemicon, AB5910) a peptide competition assay was performed. The DCX peptide was no longer available at Chemicon. As such, both antibodies were submitted to a competition assay with the DCX peptide provided by Santa Cruz Biotechnology (sc-8066). A 1:5 solution of anti-DCX antibody and anti-DCX peptide (sc-8066P, Santa Cruz) was incubated at room temperature for 1 h. The preparation was then diluted in 0.3% Triton and each section was covered with 50 µl and incubated at room temperature for 3 h. Following incubation, slides were washed successively three times for 5 min in PBS (10 mM). Each section was then incubated for 30 min at 37°C with 50 µl of the secondary antibody Alexa488 donkey anti-Goat (1:1,000, Invitrogen) or Alexa488 anti-guinea pig (1:500, Jackson Immuno Research) diluted in 0.3% Triton in 10X PBS. After incubation, slides were washed successively three times for 5

min in 10X PBS. Sections were imbedded in custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped with micro cover glasses (VWR Scientific).

Primary antibodies

Antibodies used and dilutions are presented in Table Table1.1. Primary antibody solutions were diluted in 0.3% Triton in 10X PBS. Tissue sections were covered with 50 µl of the primary antibody solution and parafilm was placed on top to prevent evaporation. Slides were incubated at room temperature in a humidified chamber for 3 h. Following incubation, slides were washed successively three times for 5 min in 10X PBS. An anti-GFP antibody that also recognize EYFP was used to label the NG2-CreER:EYFP-positive OPCs because it improved visualization of the EYFP NG2 reporter.

Secondary antibodies

Antibodies used and dilutions are presented in Table Table1.1. Secondary antibody solutions were diluted in 0.3% Triton in 10X PBS. Tissue sections were covered with 50 µl of the secondary antibody solution and parafilm was placed on top to prevent evaporation. Slides were incubated in a humidified chamber for 30 min at 37°C. Following incubation, slides were washed successively three times for 5 min in 10X PBS.

Cell nuclei staining

Cell nuclei were stained using the DNA stain Hoechst 33342 (Invitrogen). The Hoechst dye was diluted in 0.3% Triton in 10X PBS to yield a final concentration of 1:20,000. Each section was covered with 100 µl of this solution and was left to incubate for 10 min in a humidified chamber at room temperature. Slides were washed successively three times for 5 min in 10X PBS.

Sections were imbedded in custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

BrdU immunostaining

To preserve DCX and other immunostaining during the acid/heat denaturation step required for BrdU labeling, a previously described protocol was used (Boulanger et al., 2016). Immunohistochemistry for DCX and GFP were conducted first. This was followed by an overnight post-fixation step where slides were incubated in a humid chamber with Lana's fixative overnight at 4°C. Slides were then rinsed in 10X PBS 3 times for 5 min. This post-fixation step allowed the preservation of the DCX and GFP immunohistochemistry during the acid/heat denaturation step required for BrdU labeling. This was followed by DNA denaturation, where slides were incubated in HCl 2N for 30 min at 37°C. Slides were then rinsed 3 times for 5 min in a 0.1M borate buffer pH 8 and 3 times in 10X PBS for 5 min. For BrdU immunohistochemistry, slides were incubated in a humid chamber with a rat anti-BrdU antibody dissolved in PBS with 0.3% Triton-X in the dark for 3 h at room temperature. Finally, slides were incubated in a humid chamber with a donkey anti-rat secondary antibody dissolved in PBS with 0.3% Triton-X for 30 min at 37°C. Slides were then rinsed in PBS 3 times for 5 min, treated with custom-made anti-fade solution (p-Phenylenediamine and glycerol in PBS solution) and cover-slipped.

Microscopy

Immunofluorescence results were visualized using an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) attached to a ProgRes MF Scan camera (Jenoptik, Jena, Thuringe, Germany). Digital images were captured using the ProgRes CapturePro 2.5 software (Jenoptik, Jena, Thuringe, Germany). High-resolution observations were carried out on an

Olympus FV1000 BX61 laser scan confocal microscope (Olympus Corporation, Tokyo, Japan) and images were captured using the Olympus Fluoview software (Olympus Corporation, Tokyo, Japan).

Results

Specificity of the weak DCX staining in OPC

Figure 1 shows the difference in relative intensity of staining of cells in neurogenic areas (Figures 1A–C) and non-neurogenic areas (Figures 1D–F). All pictures were taken with the same objectives, the same gain but exposure times were generally twice as long for non-neurogenic DCX labeling. Although, we have observed this weak DCX staining with different immunohistochemistry protocol variants in rat and mouse tissue, the best results appear to be dependent on a short 1-h post-fixation period.

Figure 2A shows DCX immunostaining using a goat anti-DCX from Santa Cruz (sc-8066) used by many researchers. Figures 2B, C show DCX immunostaining using respectively a guinea pig anti-DCX from Chemicon (Ab5910) and a rabbit anti-DCX from Abcam (Ab18723). These observations suggest that light DCX cell labeling outside of neurogenic zones is not specific to one primary antibody. In general, the original guinea pig anti-DCX from Chemicon (Ab5910) produced brighter labeling but with slightly increased background. Because others and we typically use the Santa Cruz DCX primary antibodies, we also confirmed its specificity through the absence of staining after blocking the DCX primary antibody with the corresponding DCX immunizing peptide (Santa Cruz sc-8066P) that we also used to block the guinea pig anti-DCX (Figure 3). The absence of labeling observed in the presence of the immunizing peptide

indicated that the primary anti-DCX antibody recognizes DCX protein. Figure Figure33 also shows the relative intensity of DCX in OPCs compared to the cells in the subgranular layer of the dentate gyrus. Together, these observations confirm that the weak DCX staining observed in OPCs throughout the mouse and rat brain is a specific and reproducible finding.

In DCX lightly stained cells, there is a characteristic higher intensity staining of one pole of the cell: this is very typical of the DCX in OPCs. The rest of the cell body is weakly stained while the membrane appears slightly more stained (Figure 4). The most intense staining is found at the hillock of the main branch of the cell processes. Most of the cell processes also appear to contain DCX with weaker staining being found in the finer processes. Most cells that are weakly stained for DCX are multipolar with a minority of cells that are bipolar or, more rarely, unipolar. In DCX-labeled cell with a bipolar form, only one of the poles had intense DCX labeling, usually the one with the most processes. In instances where OPCs were newly divided, as demonstrated by PDGFR$_\alpha$ labeling coupled with BrdU immunostaining, we observed early DCX labeling (Figure 5).

Identity of cells labeled with DCX outside neurogenic areas

Virtually all DCX weakly stained cells were also labeled with PDGFR$_\alpha$, an OPC marker (Figure (Figure6).6). These double-labeled cells are observed throughout the brain and follow the usual distribution of OPCs. DCX lightly-labeled OPC-like cells outside of neurogenic zones are co-localized with GFP in NG2-CreER:EYFP reporter mice (Figure 7). When tamoxifen is intraperitoneally injected into these mice, it induces a Cre-mediated recombination of the floxed sequences and EYFP expression is thus observed in NG2-positive cells. Since NG2 is also a marker of OPCs, this confirms that OPCs express DCX at least at some point in time, including when they have proliferated, as demonstrated by BrdU immunostaining (Figure 7).

Figure Figure88 shows that PDGFR$_\alpha$-positive OPC immunostaining are sometimes co-localized with the immature oligodendrocyte marker Sox10 (Stolt et al., 2002), but never with the marker of mature oligodendrocytes GST$_\pi$ (Deloulme et al., 2004; Polito and Reynolds, 2005; Nishiyama, 2007; Taupin, 2010). Similarly, DCX immunostaining outside of neurogenic zones are sometimes co-localized with Sox10 (Figure 9) but not with GST$_\pi$ (Figure 10). Sox10 regulates myelin gene expression in oligodendrocytes and is therefore expressed by OPCs that are transitioning to a mature, myelinating oligodendroglial phenotype (Stolt et al., 2002; Liu et al., 2007). During that transition, some, but not all, adult-brain OPCs express Sox10 and conversely, some, but not all oligodendrocytes, express Sox10.

Some researchers have reported DCX labeling outside of neurogenic zones together with labeling for neuronal markers (for example NeuN; Nacher et al., 2001; Dayer et al., 2005; Luzzati et al., 2006; Xiong et al., 2008; Cai et al., 2009; Klempin et al., 2011; Werner et al., 2012; Saul et al., 2014). We have observed numerous instances of closely apposed OPC-neuron pairs (Figures 11C,F,G) that can appear in some instances as double-labeled cells (Figure 11H). These were found in outbred mice (CD-1; Figures 11A–F) as well as in the NG2-CreER:EYFP reporter mice (Figures 11F,G). The occurrence of OPC-neuron pairs has been mentioned previously (Butt et al., 2002; Sakry et al., 2011).

Discussion

In the present report, we found that most OPCs express low levels of DCX in all parts of the brain where these cells are found. Low levels of DCX protein in OPCs is better visualized in lightly fixed brain tissue. The intensity of labeling also varies between commercially available

DCX antibodies. Although, we did not determine when exactly DCX starts being expressed in OPCs after division, the observation that virtually all OPCs appear to express DCX suggest that, in the adult rodent brain, OPCs express DCX sometime after division and continue to express DCX until they differentiate into myelinating oligodendrocytes. This conclusion is supported by the sparse number of OPCs that express both DCX and the transcription factor Sox10, which is crucial for the final transformation of OPCs into myelinating oligodendrocytes (Stolt et al., 2002), and the absence of co-labeling of DCX together with GST_{π}, a marker of mature myelinating oligodendrocytes (Tansey and Cammer, 1991; Tamura et al., 2007b; Simon et al., 2011).

Classically, DCX has been described as a selective marker of adult neurogenesis (Brown et al., 2003). As such, the observation of DCX-positive cells in various parts of the mammalian CNS previously led to the suggestion that new neurons are produced outside of the dentate gyrus and the subventricular zone (Dayer et al., 2005; Tamura et al., 2007a). In support of this hypothesis came reports that NG2-positive precursors are multipotent and can generate functional neurons (Belachew et al., 2003; Aguirre and Gallo, 2004; Aguirre et al., 2004; Dayer et al., 2005; Tamura et al., 2007a; Rivers et al., 2008; Zhu et al., 2008; Guo F. Z. et al., 2009, 2010; Robins et al., 2013).

Because of these reports, we examined if DCX-positive found outside of neurogenic zones also express mature or immature neuronal markers. We examined patterns of labeling using antibodies against Pax6—a paired box gene which is expressed by immature glutamatergic neurons (Bayatti et al., 2008), Pax2—a paired box gene expressed in GABAergic neurons (Batista and Lewis, 2008), and HuCD and Rbfox3 (NeuN)—markers of mature neurons. We found some very rare examples of co-labeling of lightly stained DCX cells either with Pax2,

Rbfox3, or HuCD but that did not express OPC markers such as PDGFR$_{\alpha}$. Sometimes the closely apposed OPC-neuron pairs could be interpreted as double-labeled cells. In these instances, an OPC and a neuron could be superimposed in the field of view, as shown in Figure Figure11.11. While these observations do not rule out the possibility that OPCs can generate mature neurons, it may help explain the conclusion drawn by others that all DCX-positive cells, including those located outside of the dentate gyrus and subventricular zone, have neuronal attributes. These observations do not rule out the possibility of OPC-derived neurogenesis but they suggest additional caution to exclude other possibilities (see further discussion of this issue in Dimou and Gallo, 2015; Feliciano et al., 2015).

Finally, the question remains as to the role of doublecortin in OPCs. Since DCX is a microtubule-associated protein and because DCX-positive cells outside of neurogenic zones do not co-express mature neuronal markers, it is unlikely to be associated with a potential for OPCs to differentiate into neurons. It may, however, be involved in their migration over small distances as they monitor a unique territory that is not shared by other OPCs (Hughes et al., 2013). Furthermore, recent studies in missense DCX gene expression suggest a role in tubulin organization that could be associated with migration but also with process extension to establish and remodel the synaptic connections between neurons and OPCs (Tsai et al., 2016). This is significant since OPCs are known to form glutamatergic and GABAergic synapses with neurons (Bergles et al., 2000; Lin and Bergles, 2004). Therefore, this report as well as others suggest that it is time to stop viewing DCX as a marker of newly generated neurons but, rather, as a marker of cells that are undergoing migration or other forms of process reorganization.

Tables

Table 1. List of antibodies used.

Host	Target	Concentration	Company
Rabbit	GFP (EYFP)	1/1000	Abcam (ab290)
Rabbit	$PDGFR_{\alpha}$	1/250	Santa Cruz (sc-338)
Rat	$PDGFR_{\alpha}$	1/500	Abcam (ab93531)
Rabbit	GST_{π}	1/500	MBL (311-h)
Goat	DCX	1/100	Santa Cruz (sc-8066)
Guinea Pig	DCX	1/500	Chemicon (AB5910)
Rabbit	DCX	1/1000	Abcam (Ab18723)
Mouse	Rbfox3 (NeuN)	1/500	Millipore (MAB377)
Goat	Sox10	1/500	Santa Cruz (sc-17342)
Rat	BrdU	1/500	Abcam (ab6326)
Mouse	OLIG1	1/1000	Millipore (MAB5540)
Donkey	Anti-Rabbit Alexa 488	1/1000	Invitrogen
Donkey	Anti-Goat Alexa 488	1/1000	Invitrogen
Donkey	Anti-Guinea Pig Alexa 488	1/500	Jackson Immuno Research
Donkey	Anti-Rabbit Alexa 594	1/1000	Invitrogen
Donkey	Anti-Mouse Alexa 594	1/1000	Invitrogen
Donkey	Anti-Goat Alexa 594	1/1000	Invitrogen
Donkey	Anti-Rat Alexa 594	1/1000	Invitrogen
Donkey	Anti-Rabbit Alexa 680	1/500	Invitrogen
Donkey	Anti-Mouse Alexa 680	1/500	Invitrogen
Donkey	Anti-Guinea Pig Alexa 680	1/500	Jackson Immuno Research
Donkey	Anti-Rat Alexa 680	1/500	Abcam

Figures legends

Fig. 1. Intensity of DCX immunostaining in neurogenic (A–C) and in non-neurogenic zones (D–F) using guinea pig anti-DCX and Alexa 488 anti-guinea pig antibodies. Exposure times for DCX labeling in non-neurogenic areas were in general double those of neurogenic areas. Pictures taken with a fluorescence microscope in the subventricular zone (A, 20X), rostral migratory stream (B, 40X), subgranular zone of the dentate gyrus (C, 40X), cortex (D, 20X), cortex (E, 40X), and striatum (F, 40X).

Fig. 2. DCX immunostaining in corpus callosum and hippocampus of rat (A) and mouse brain (B,C) at 10X with a fluorescence microscope using a variety of anti-DCX antibodies. Goat anti-DCX from Santa Cruz (A, 1/100 in PBS-Triton), guinea pig anti-DCX from Chemicon (now Millipore; B, 1/500 in PBS-Triton), and rabbit anti-DCX from Abcam (C, 1/1000 in PBS-Triton).

Fig. 3. DCX immunostaining in hippocampus of mouse brain with (B,D) and without anti-DCX peptide (A,C). Pictures were taken at 10X using a fluorescence microscope. Goat anti-DCX from Santa Cruz Biotechnology was used in images (A,B) and guinea pig anti-DCX from Chemicon (now Millipore) was used in images (C,D). Arrows show DCX staining in OPCs located outside of the known neurogenic zone that is the dentate gyrus of the hippocampus.

Fig. 4. (A,B) Two examples of DCX-positive cells outside of neurogenic zones: DCX immunostaining (guinea pig anti-DCX) is concentrated in one pole of the cell. Pictures were taken in the cortex at 40X with a fluorescence microscope.

Fig. 5. DCX immunostaining appears after division of an OPC. Pictures taken in the cortex at 60X with a laser scan confocal microscope. DCX in green (A), $PDGFR_{\alpha}$ in red (B), BrdU in blue (C), and merged (D).

Fig. 6. DCX immunostaining (guinea pig anti-DCX) outside of neurogenic zones is co-localized with PDGFR$_\alpha$ (rat anti-PDGFR$_\alpha$), an OPC-marker. Pictures taken at 40x with a fluorescence microscope in the cortex (A–C) and in the cerebellum (D–F) and at 40x with a laser scan confocal microscope in the cortex (G–I). DCX in green (A,D,G), PDGFR$_\alpha$ in red (B,E,H), and merged (C,F,I).

Fig. 7. DCX immunostaining (goat anti-DCX) in OPC-like cells outside of neurogenic zones is co-localized with GFP in NG2-CreER:EYFP reporter mice. Pictures taken in the cortex at 40X with a laser scan confocal microscope (A–D). Pictures taken in the cortex at 60X with a laser scan confocal microscope (E–H). GFP in green (A,E), DCX in red (B,F), BrdU in blue (C,G), and merged (D,H).

Fig. 8. PDGFR$_\alpha$-positive OPC immunostaining (rat anti-PDGFR$_\alpha$) are sometimes co-localized with the immature oligodendrocyte marker Sox10, but never with the marker of mature oligodendrocytes GST$_\pi$. Pictures taken in the cortex at 40X using a laser scan confocal microscope. Sox10 in green (A), GST$_\pi$ in red (B), PDGFR$_\alpha$ in blue (C), and merged (D). Sox10-positive/GST$_\pi$-negative/PDGFR$_\alpha$-negative cell (long yellow arrow), Sox10-negative/GST$_\pi$-positive/PDGFR$_\alpha$-negative cell (short and narrow orange arrow), Sox10-negative/GST$_\pi$-negative/PDGFR$_\alpha$-positive cell (short and thick aqua arrow), Sox10-positive/GST$_\pi$-positive/PDGFR$_\alpha$-negative cell (long and narrow white arrow), and Sox10-positive/GST$_\pi$-negative/PDGFR$_\alpha$-positive cell (pink arrow head).

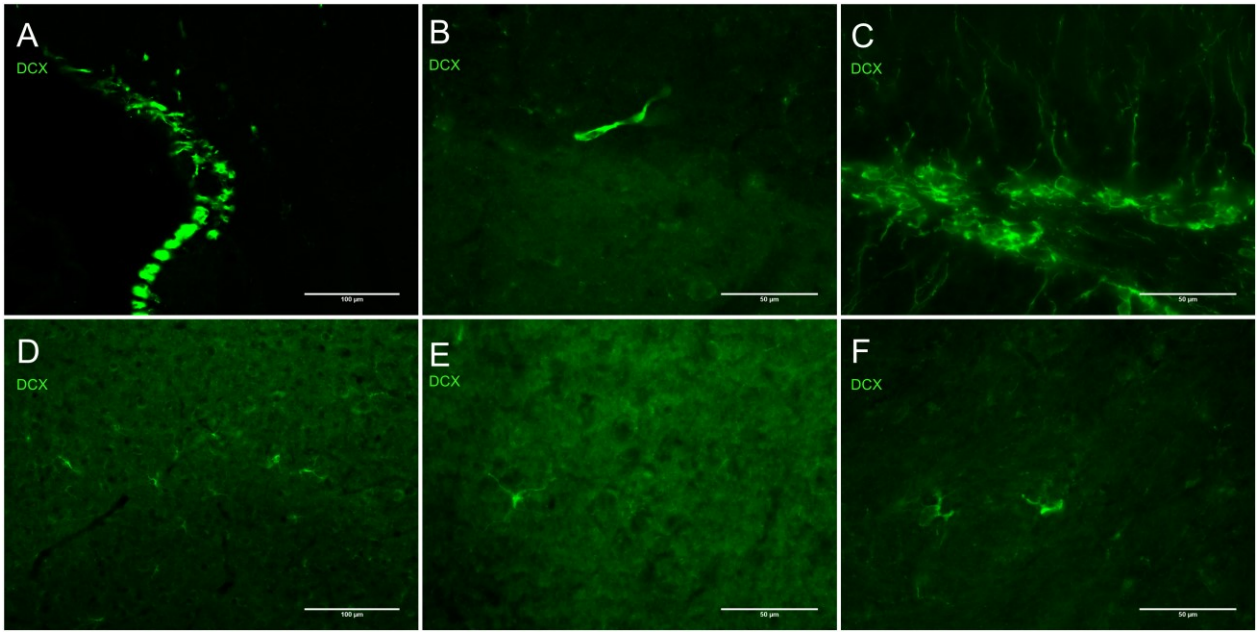
Fig. 9. DCX immunostaining (guinea pig anti-DCX) outside of neurogenic zones is sometimes co-localized with Sox10, a marker of immature oligodendrocytes. Pictures were taken at 40X using a fluorescence microscope with a fluorescence microscope in the cerebellum (A–C) and corpus callosum (D–F). Pictures were taken at 40X using a laser scan confocal microscope in the cortex (G–I). DCX in green (A,D,G), Sox10 in red (B,E,H), and merged (C,F,I).

Fig. 10. DCX immunostaining (guinea pig anti-DCX) outside of neurogenic zones is not co-localized with GST_{π}, a marker of mature oligodendrocytes. Pictures were taken in the cortex at 20X with a fluorescence microscope. DCX in green (A), GST_{π} in red (B), and merged (C).

Fig. 11. OPCs form close contacts with neurons, but these cells are not co-localized. Pictures taken in the cortex of CD-1 mice at 40X using a fluorescence microscope (A–C) and a laser scan confocal microscope (D–F). DCX (guinea pig anti-DCX) in green (A,D), NeuN in red (B,E), and merged (C,F). Pictures taken in the cortex of NG2-CreER:EYFP reporter mice at 60X using a laser scan confocal microscope with GFP in green and NeuN in blue (G,H).

Figures

Figure 1.



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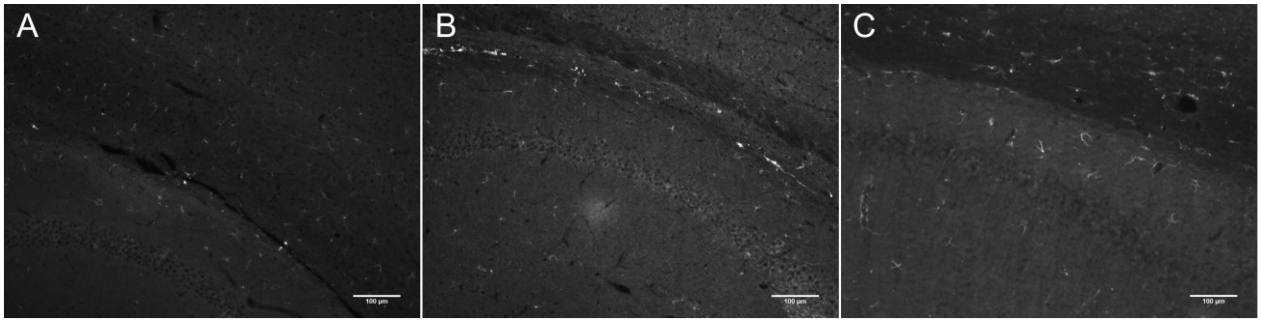

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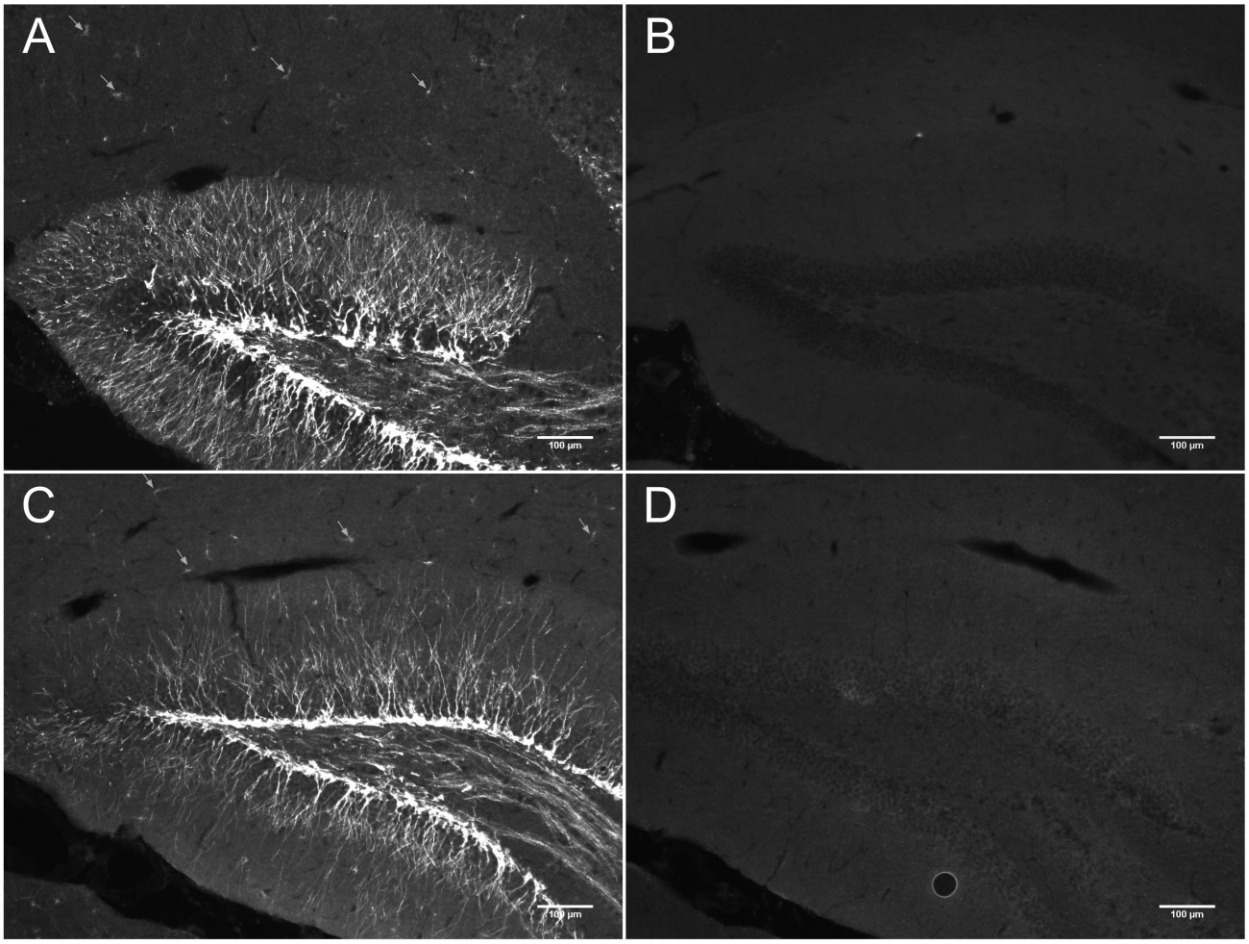

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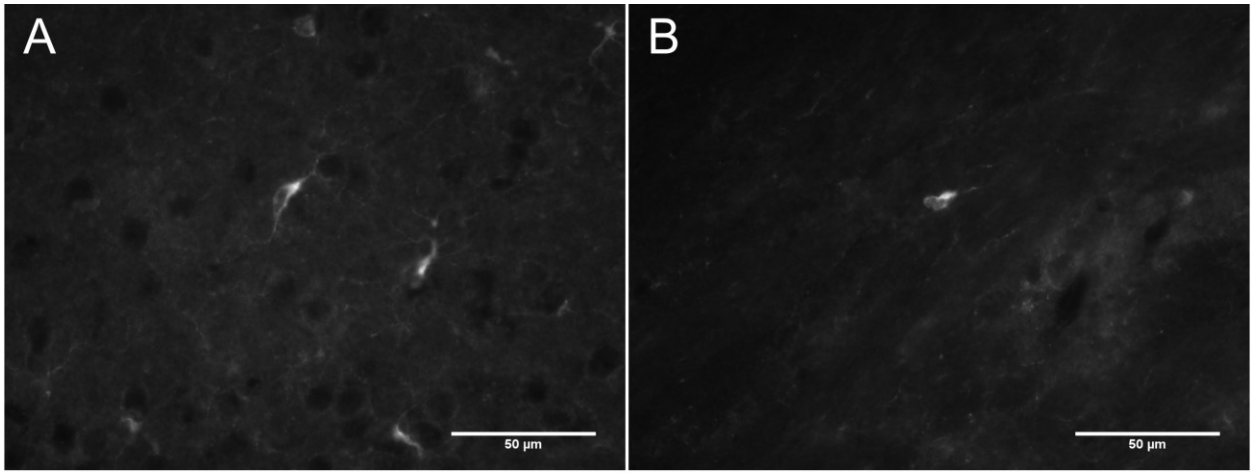

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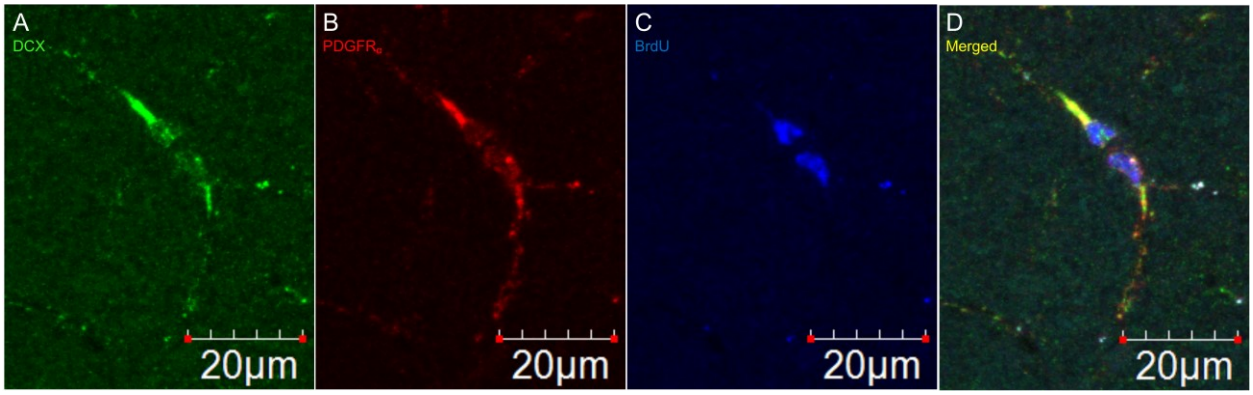

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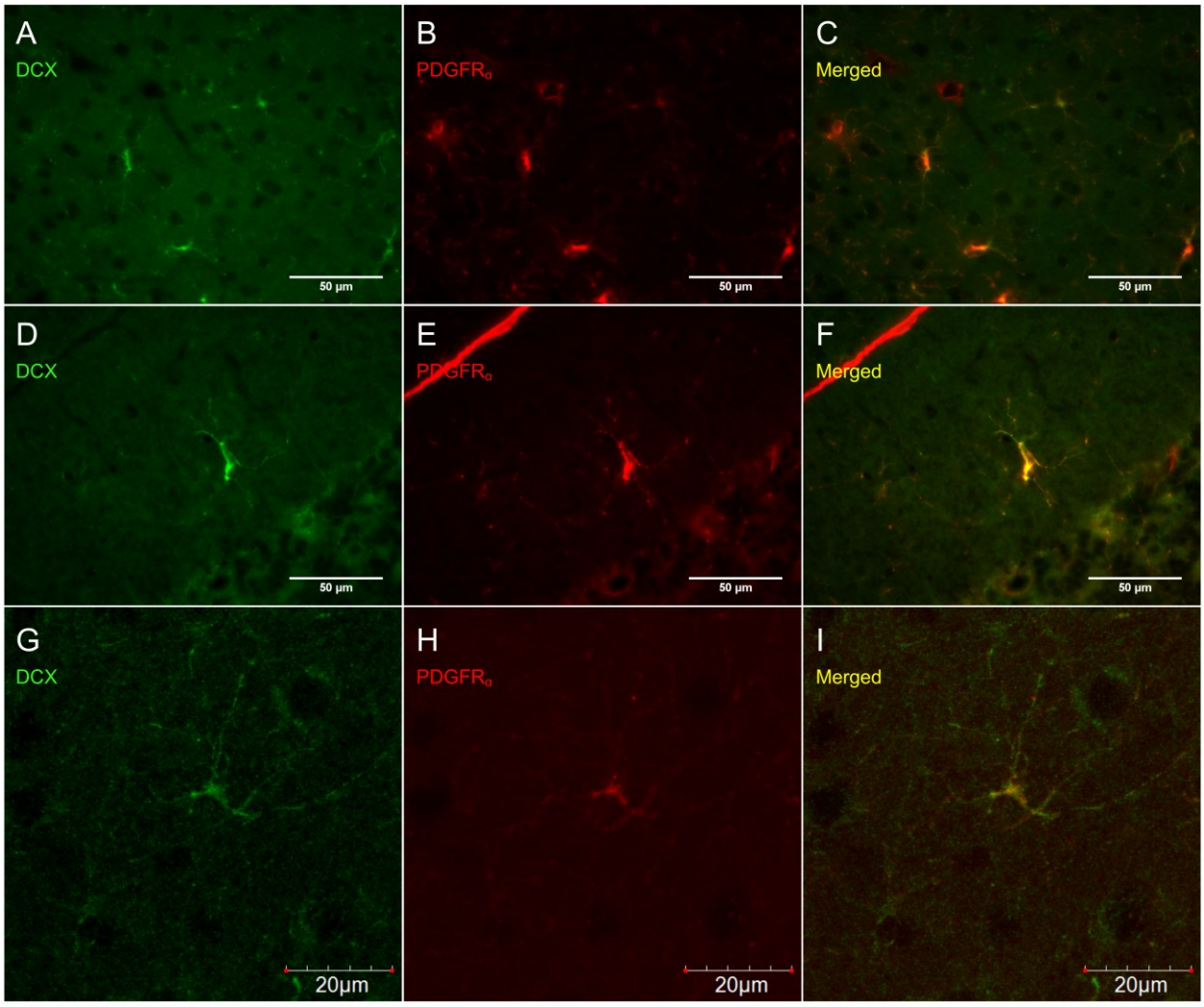
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Figure 7.

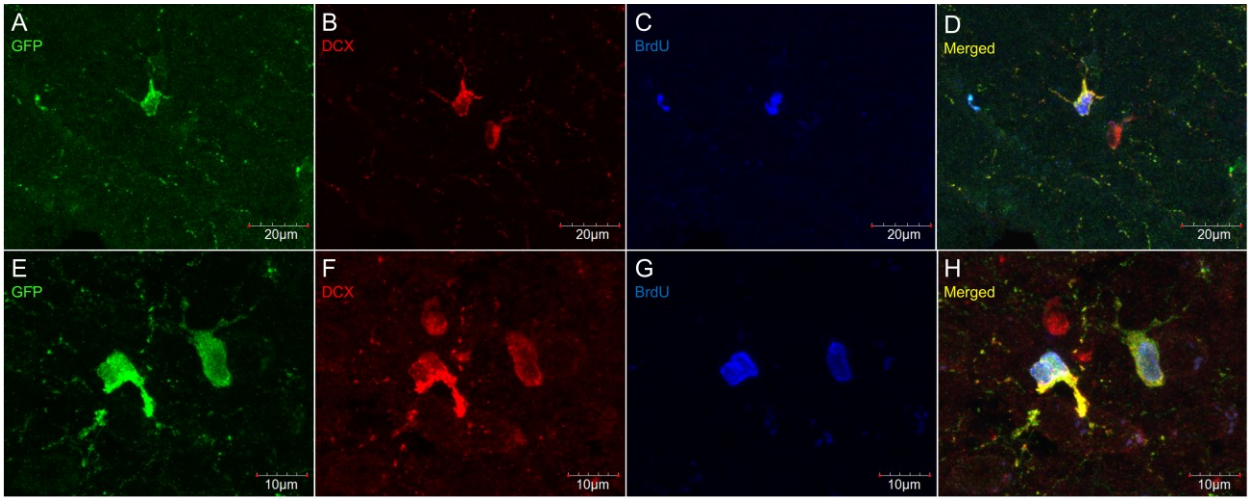
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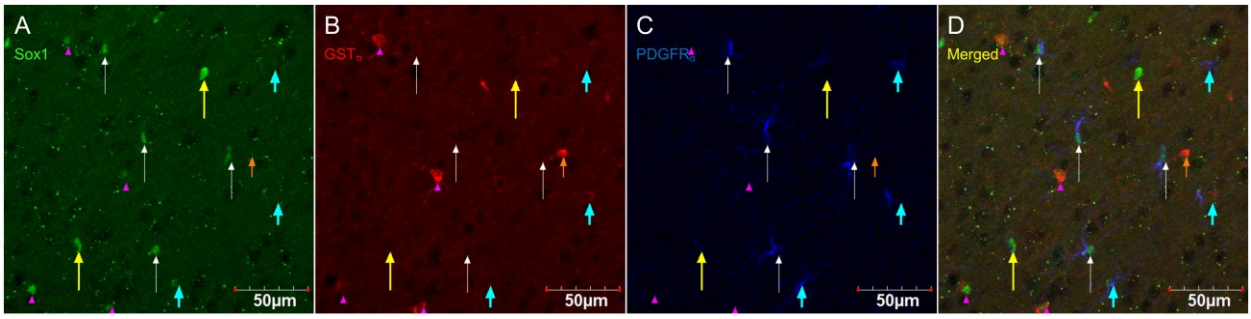
Figure 8.



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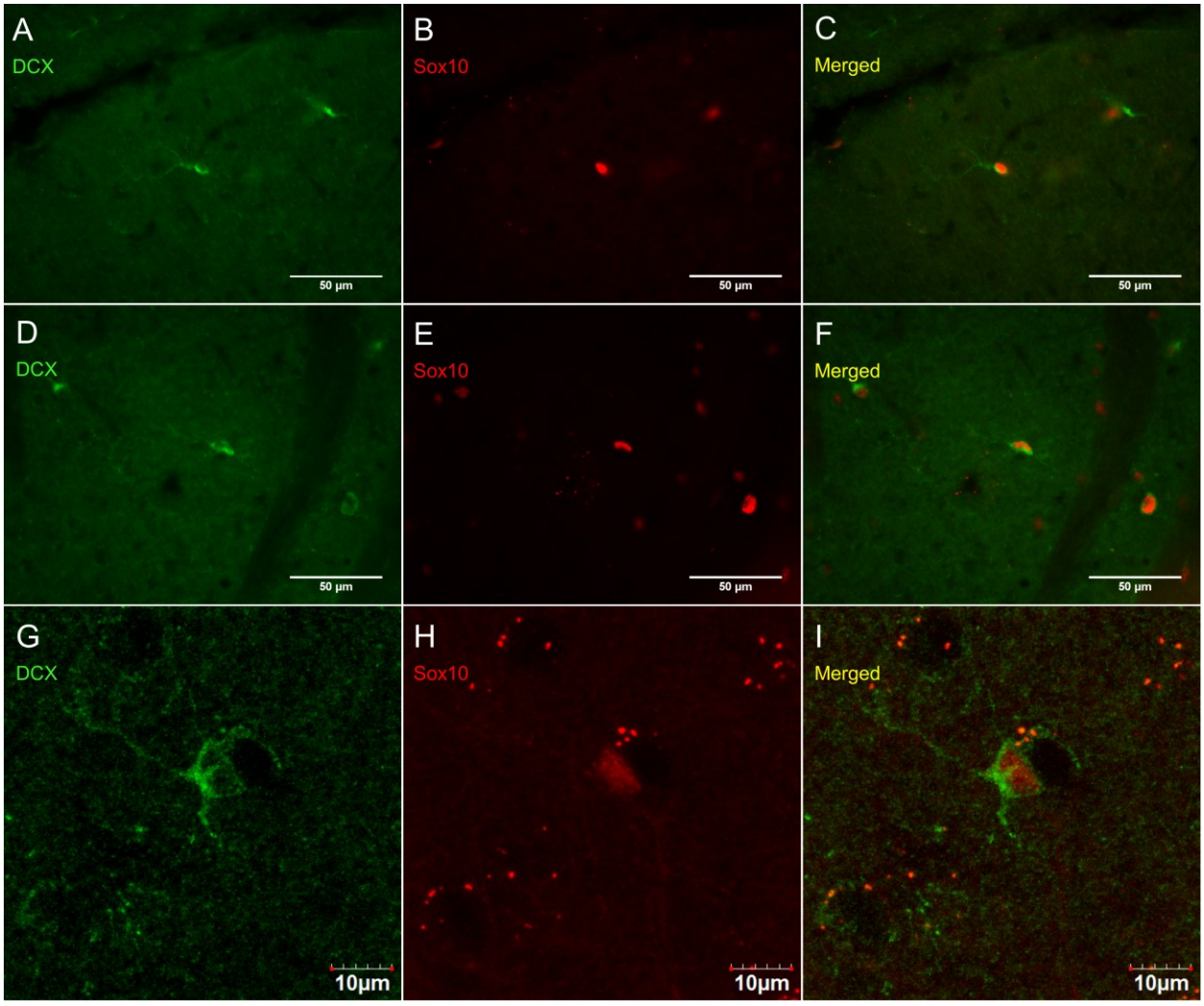
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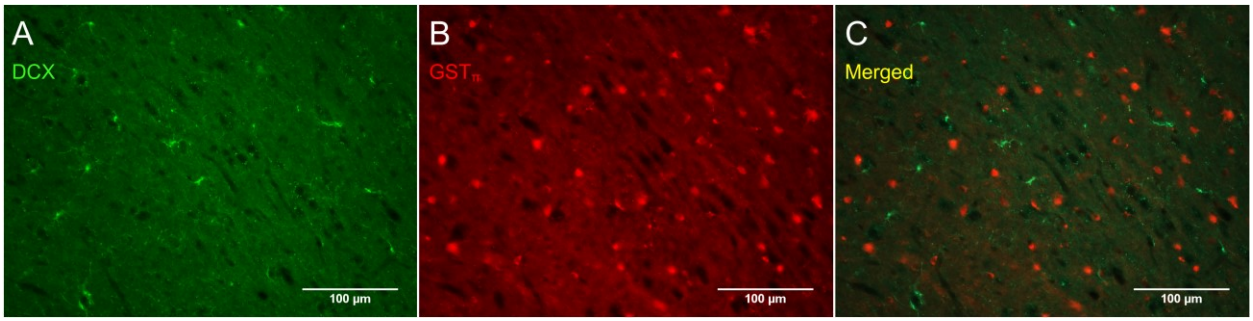
Figure 10.



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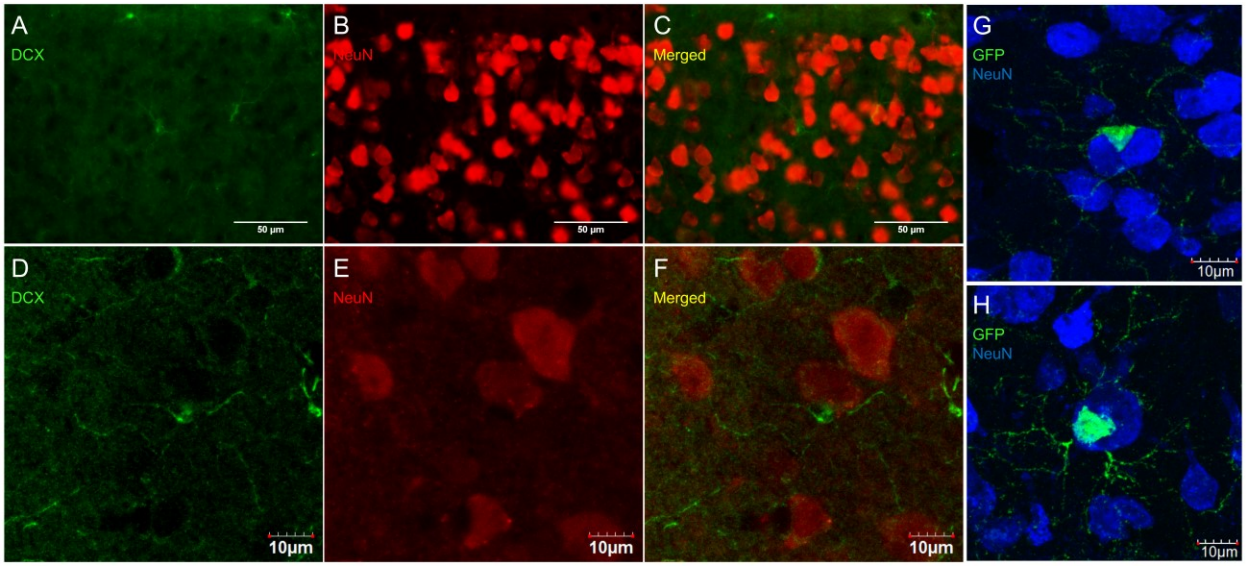
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GENERAL DISCUSSION

The goal of this thesis was to learn more about OPCs in the intact adult brain, with a special focus on the factors that affect their proliferation and differentiation, as it may impact white matter plasticity and, consequently, learning and memory. To this end, we performed a stereological analysis of the corpus callosum and cortex of the adult NG2-CreER^{TM}:EYFP reporter mouse exposed to BrdU. We also looked at the impact that an experience-related factor, reference memory training, and GABAergic neurotransmission have on the proliferation and differentiation of adult OPCs, as well as on the prevalence of OPCs as satellite cells of neurons. Finally, we examined the question of neuronal differentiation of adult OPCs by analysing doublecortin (DCX) expression in these cells.

Summary of findings

In experiment 1, we demonstrated that adding a post-fixation step prevents fading and false negatives when trying to visualize BrdU with other antigens of interest thereby increasing the range of proteins that can be detected in combination with BrdU. More specifically, we showed that it is best to do the immunochemistry with the antigens of interest first. Before proceeding to the second immunochemistry with BrdU, we introduced an overnight post-fixation step that protected immunostaining of the other proteins from the heat and acid used to denature the DNA during the BrdU procedure.

In experiments 2 and 3, we used the technique presented in experiment 1, along with a stereological analysis of adult NG2CreER:EYFP reporter mice, to estimate the total number of OPCs in the corpus callosum and cortex and to study OPC proliferation and differentiation in

these structures. We present for the first time stereological estimates of the number of OPCs in the cortex and corpus callosum of the mouse. We established that adult OPCs maintain the ability to proliferate and to differentiate into oligodendrocytes in adulthood, and that differentiation can occur both spontaneously and following OPC cell division. We also showed that OPCs located in the white and grey matter have different properties, with OPCs located in the white matter being more likely to proliferate and to differentiate into oligodendrocytes than grey matter OPCs. Finally, we provided evidence for neuronal differentiation of OPCs in the mouse cerebral cortex.

In experiment 2, we measured the effects of reference memory training on OPC proliferation and differentiation. Although activity-dependent neural network activity has been hypothesized to serve as a modulator of OPC proliferation and differentiation, we found no evidence to show that reference memory training affects these factors in the adult mouse brain. However, the pattern of correlations between performance on the reference memory task and OPC proliferation and differentiation suggested that learning a reference memory task is associated with lower rates of proliferation in the corpus callosum, lower rates of differentiation along the oligodendrocyte lineage, and higher rates of differentiation along the neuronal lineage.

In experiment 3, we evaluated the effects of systematic GABAergic agent administration on adult OPC proliferation and differentiation *in vivo*. More specifically, we measured the effects of a $GABA_A$-receptor agonist, a $GABA_A$-receptor antagonist, a $GABA_B$-receptor agonist, and a $GABA_B$-receptor antagonist on OPC proliferation and differentiation in the adult NG2-CreER^{TM}:EYFP reporter mouse. While none of the agents analyzed yielded a significant effect, the $GABA_A$-receptor agonist did appear to play a modulatory role on adult OPC proliferation and

differentiation. Indeed, the $GABA_A$-receptor agonist appeared to decrease the proliferative activity of OPCs and to favour differentiation into a phenotype other than that of an OPC.

In experiment 4, we provided a stereological estimate of the total number of OPC-neuron pairs in the cortex of the adult mouse and determined that approximately 40% of OPCs and 4% of neurons form such pairs. Through histological analysis, we showed that the neurons in these pairs are GABAergic interneurons. GABA interneurons are the main inhibitory neurons of the brain and have been estimated to comprise 16% of cortical neurons (Beaulieu, 1993). The combination of our observations with that estimate yields the conclusion that one in four GABA neurons is paired with an OPC. Finally, we also looked at the effects of reference memory training and GABAergic neurotransmission on the prevalence of these pairs. While reference memory training did not have an effect, we determined that the $GABA_B$-receptor agonist baclofen significantly increased the number of OPC-neuron pairs when compared to the saline-injected control group and the $GABA_B$-receptor antagonist saclofen.

In experiment 5, we looked at DCX expression in adult OPCs. We found that all OPCs express low levels of DCX, and that DCX expression is downregulated as OPCs differentiate along the oligodendrocyte lineage so that DCX is absent in mature oligodendrocytes. Despite evidence for neuronal differentiation in OPCs, DCX expression did not appear to be associated with an immature neuronal phenotype in OPCs. Rather, we hypothesize that DCX is involved with the migration of these cells along short distances as they travel away from sister cells and survey their local environment, as described by Hughes, Kang, Fukaya, and Bergles (2013). The following sections of this thesis will discuss some overarching issues not already addressed including the similarities and differences across experiments.

Properties of white and grey matter OPCs in the adult brain: comparing the results of experiments 2 and 3

We examined the general characteristics of white and grey matter adult OPCs in experiments 2 and 3. While some of the analyses yielded comparable results, others were less consistent. The first factor we measured was the estimated total number of OPCs in the adult corpus callosum and cortex. While both PDGFRα and GFP can be used to estimate the total number of OPCs, PDGFRα is preferable since GFP staining relies on Cre recombination, which is not always complete and therefore likely underestimates total number of OPCs present in a sample. The total number of PDGFRα+ cells resulting from the stereological analyses in experiments 2 and 3 yielded very similar results. Approximately 25,000 OPCs were estimated to be located between lateral 0.36mm and lateral 2.52mm of the corpus callosum in experiment 2 and 21,000 were estimated to be present in experiment 3. In the cortex, approximately 94,000 OPCs were estimated to be located between these coordinates in experiment 2 and 92,000 in experiment 3. These estimates demonstrate that the total number of OPCs estimated in each experiment was reliable. When we combined the results of both experiments and translated them to the entire volume of the adult corpus callosum and cortex, we estimated that approximately 70,000 OPCs are located in the corpus callosum and 370,000 in the cerebral cortex.

Our second analysis examined the proliferative activity of OPCs in the adult corpus callosum and cortex. More specifically, it looked at the survival of cells that were in the S-phase of the cell cycle during the period when BrdU was administered. Experiments 2 and 3 yielded different results, with experiment 2 estimating that approximately 8% of OPCs in the corpus callosum and 5% of those in the cortex incorporated BrdU and experiment 3 showing that approximately 23% of OPCs in the corpus callosum and 11% of those in the cortex incorporated

the thymidine-analog. In fact, any analysis that looked at BrdU incorporation yielded very different results, with experiment 2 consistently giving much lower levels of BrdU incorporation than experiment 3. BrdU was administered for 8 days in both studies but survival times were different between experiments, with euthanasia occurring 8-weeks post BrdU removal in experiment 2 and 2- and 8-weeks post-BrdU in experiment 3. While 8 weeks gives OPCs more time to undergo multiple cell divisions, which dilutes the BrdU and makes it harder to detect, results for the 2- and 8-week timepoints were similar in experiment 3, with approximately 25% of reporter cells in the corpus callosum and 11% of those in the cortex having incorporated BrdU in the 2-week survival group and approximately 21% of OPCs in the corpus callosum and 12% of those in the cortex having incorporated BrdU in the 8-week survival group. BrdU dilution due to cell division does not appear to be the reason why results for experiments 2 and 3 are different. The differing experimental protocols that the animals were exposed also does not appear to be a valid explanation since there were no significant differences between the control and experimental groups in either study. Instead, the DNA denaturation required for BrdU visualization may have been less successful in experiment 2 as compared to experiment 3. It must be noted that the results obtained in experiment 3 concerning the fraction of reporter cells having incorporated BrdU were more consistent with the literature than those obtained in experiment 2 (Psachoulia, Jamen, Young, & Richardson, 2009; Rivers et al., 2008; Simon, Gotz, & Dimou, 2011). Thus, the results obtained in experiment 3 are most likely more indicative of the proliferative activity of OPCs in 4-5-month old animals.

The third factor we looked at was adult OPC differentiation in the corpus callosum and cortex. More specifically, we looked at three possible fates for adult OPCs: OPC, oligodendrocyte, and neuron. While results for oligodendrocyte and neuronal differentiation

were similar in both experiments, those for undifferentiated OPCs were quite different, with experiment 2 estimating that approximately 79% of reporter cells in the corpus callosum and 82% of those in the cortex remained OPCs and experiment 3 estimating that approximately 55% of reporter cells in the corpus callosum and 64% of those in the cortex kept an OPC phenotype. Since Cre recombination is never complete, the number of GFP+ cells should be lower than the number of PDGFRα+ cells (Hayashi & McMahon, 2002; Zhu et al., 2011). This was the case in experiment 2. However, in experiment 3, the estimated number of GFP+ cells was higher than the number of PDGFRα+ cells. Again, differing experimental protocols do not appear to be the reason for the conflicting result since no significant differences were found between the control and experimental groups in either study. There are thus two possible explanations for this difference. First, PDGFRα immunostaining success in experiment 3 might have been worse than experiment 2. Second, higher rates of differentiation into a phenotype other than OPC in experiment 3. Since results for oligodendrocyte differentiation and neuronal differentiation were similar in both experiments, a less successful PDGFRα immunostaining is the most likely explanation for differences between the proportion of undifferentiated OPCs in experiments 2 and 3. After using all the original order of the PDGFRα antibody from our commercial supplier, a new sample was ordered. This new sample had a different lot, and immunostaining with that lot was not as successful as immunostaining with the original lot used in experiment 2.

So, based on our evaluation of the technical limitations of each experiment, as well as previously published results, we think experiment 2 provided a better estimate of the proportion of OPCs that remain undifferentiated, while experiment 3 provided a better estimate of OPC proliferation. Taking into account the potential biases listed above, we think that using the most

reliable results of each study provides a better estimate of the total number of OPCs in the corpus callosum and cortex and OPC differentiation into oligodendrocytes and neurons.

DCX expression in OPCs: Neuronal differentiation or substrate used for migration

We found evidence for the neuronal differentiation of OPCs in experiments 2 and 3. In those experiments, reporter cells that co-expressed neuronal markers displayed typical neuron morphology, having a large cell body and few processes. Since signs of neuronal differentiation have been linked to problems with the Cre-loxP technology used to study the fate of OPCs (Nishiyama, Boshans, Goncalves, Wegrzyn, & Patel, 2016; Nishiyama, Suzuki, & Zhu, 2014), we looked to another marker traditionally associated with adult neurogenesis, DCX, for answers.

Analysis of DCX expression suggests that DCX is not a selective marker of adult neurogenesis but, rather, a marker of migrating cells. A number of observations support this hypothesis. First, DCX is a protein that participates in the polymerisation of microtubules (Francis et al., 1999; Gleeson, Lin, Flanagan, & Walsh, 1999), and microtubules play a role in cellular migration (Watanabe, Noritake, & Kaibuchi, 2005). Consequently, DCX is only transiently expressed by newly generated neurons as they migrate toward their final destination, and downregulated as they adopt a mature neuronal phenotype and begin to functionally integrate into neural networks (Brown et al., 2003). DCX expression in neuroblasts could be related to the fact that they are migrating cells rather than a unique clue to their eventual neuronal phenotype.

Secondly, we found low-intensity DCX-staining outside of known neurogenic zones (i.e. dentate gyrus of the hippocampus, subventricular zone of the lateral ventricles and its associated rostral migratory stream, piriform cortex, and hypothalamus). Double-labeling with PDGFRα identified these cells as OPCs. OPCs have been shown to survey small, unique territories through

migration and other processes. It is therefore plausible that OPCs would require low levels of DCX expression to migrate and/or develop processes within their local environment.

Third, none of the low-intensity DCX+ cells found outside of neurogenic zones had the typical neuronal morphology observed in the GFP+/NeuN+ cells of experiments 2 and 3 and GFP+/HuCD+ cells of experiment 3. However, it must be noted that OPCs that appear to have differentiated into neurons are not numerous and systematic analysis of DCX was not conducted. We cannot completely exclude the possibility that OPCs that are in the process of differentiating into neurons are also DCX+, and that DCX expression would be linked to their neuronal fate. However, the fact that very few OPCs differentiate into neurons and that all OPCs have low-intensity DCX labeling suggests that this is very unlikely.

Finally, some DCX+/PDGFRα+ cells observed outside of neurogenic zones were also Sox10+. Sox10 is selectively expressed by cells that have committed to the oligodendrocyte lineage but that have not yet reached a mature oligodendrocyte phenotype (Li, Lu, Smith, & Richardson, 2007; Stolt et al., 2002). DCX-labelling thus appears to be associated with oligodendrocyte differentiation by OPCs, which is in contradiction with neuronal differentiation. Together, these observations point to DCX expression in OPCs being related to something other than neuronal differentiation, with migration being a likely candidate.

Roles of OPCs in adulthood

As their name suggests, OPCs are responsible for generating myelinating oligodendrocytes during the peri-natal period (Zhu, Bergles, & Nishiyama, 2008). However and as we show here, OPCs are still present in high number in the adult brain and they are distributed

rather evenly throughout the grey and white matter. Beyond developmental oligodendrocyte production, what are the other potential roles of OPCs once myelination is completed?

One role is the continued generation of oligodendrocytes, as demonstrated in experiments 2 and 3. This role appears to serve two purposes. First, oligodendrocytes are extremely vulnerable to damage or death under pathological conditions (Bradl & Lassmann, 2010). The prevalence of adult OPCs, as well as their ability to generate new oligodendrocytes, could serve to restore myelination after white matter damage. Factors involved in the OPC response to white matter injury were discussed in the first chapter of this thesis and that role is predominantly studied because of the devastating nature of demyelinating diseases. However, the overall low prevalence of white matter pathology in the mammalian brain does not seem to justify the widespread distribution of OPCs and their continued ability to generate oligodendrocytes throughout life, nor does it explain the diminishing abilities of OPCs to proliferate in the older brain.

Rather it appears to subserve a form of plasticity known as white matter plasticity (reviewed in (Purger, Gibson, & Monje, 2015; Wang & Young, 2013)). Until recently, the turnover rate of oligodendrocytes was thought to be relatively low in the healthy adult brain (Mccarthy & Leblond, 1988). However, it has now been shown that white matter is subject to plastic changes well into adulthood. These changes include the myelination of previously unmyelinated axons, as well as the remodelling of pre-existing myelin sheaths (Young et al., 2013). White matter plasticity appears to be modulated by neural activity and be associated with increased cognitive functioning (Fields, 2015; Nunez, Nelson, Pych, Kim, & Juraska, 2000; Stevens, Porta, Haak, Gallo, & Fields, 2002; Tomlinson, Leiton, & Colognato, 2015). That is partly why we decided to measure the effects of reference memory training (experiment 2) and

GABAergic neurotransmission (experiment 3) on OPC proliferation and differentiation. While no overall significant effects were found, these two factors did appear to be somewhat related to OPC proliferation and differentiation along the oligodendrocyte lineage.

Many researchers now believe that the role of adult OPCs goes beyond the generation of new oligodendrocytes. More specifically, it has been hypothesized that OPCs are involved in monitoring, and possibly even modulating, neural activity (Boda & Buffo, 2014; Gallo, Mangin, Kukley, & Dietrich, 2008; Mangin & Gallo, 2011; Velez-Fort, Audinat, & Angulo, 2012). Our experiments touched on this issue in a few ways. First, learning a reference memory task relies on the creation of a new neural network. Second, GABAergic neurotransmission, the effects of which were explored in experiment 3, may be a mechanism by which OPCs can sense neural activity. Indeed, OPCs have functional GABAergic receptors that can be activated through synaptic innervation with neurons, or through GABA spillover at neuron-to-neuron synapses (Bernstein, Lyons, Moller, & Kettenmann, 1996; Kirchhoff & Kettenmann, 1992; Luyt et al., 2007; Maldonado, Velez-Fort, & Angulo, 2011; Pastor, Chvatal, Sykova, & Kettenmann, 1995; Velez-Fort, Maldonado, Butt, Audinat, & Angulo, 2010). Spillover neurotransmission is thought to be the primary mode of GABAergic communication for OPCs in the adult brain and has specifically been hypothesized to allow OPCs to integrate neural activity patterns that correspond to *in vivo* stimulation (Velez-Fort et al., 2010). Third, we showed in experiment 5 that 40% of OPCs position their cell body close to that of a neuron, creating OPC-neuron pairs where the OPC is thought of as a satellite cell to the neuron (Dayer, Cleaver, Abouantoun, & Cameron, 2005; Gallo et al., 2008; Mangin, Kunze, Chittajallu, & Gallo, 2008). OPC-neuron pairs are believed to facilitate neural network monitoring and signal integration by OPCs because the OPC displays synchronized bursts of activity upon activation of the closely apposed cell (Boda &

Buffo, 2014; Butt, Hamilton, Hubbard, Pugh, & Ibrahim, 2005; Gallo et al., 2008; Maldonado, Velez-Fort, Levavasseur, & Angulo, 2013; Mangin et al., 2008). Furthermore, the fact that GABAergic interneurons form OPC-neuron pairs lends support to the idea that OPCs are involved in neural network monitoring, as they are involved in regulating neural synchrony to ensure circuit performance (Sohal, Zhang, Yizhar, & Deisseroth, 2009). The results obtained in this thesis lend support to the dual potential roles of adult OPCs.

Limitations

The experiments in this thesis are based on histology. Even when it is paired with a quantitative analysis like stereology, histology remains qualitative and can be plagued with problems like fading, tissue damage, low intensity of staining and overall variability. The biggest obstacle to histological analysis in my thesis was the use of BrdU to study OPC proliferation. The DNA denaturation required to visualize BrdU can severely damage the tissue, as well as antigens of interest and the antibodies used to observe them. The technique developed and presented in experiment 1 mitigated the impact of a lot of these issues but, as was mentioned previously, we did experience some problems with PDGFRα staining in experiment 3. Furthermore, DNA denaturation itself is not always successful, and, consequently, BrdU appears to underestimate levels of cellular proliferation (Clarke et al., 2012; Young et al., 2013; Zeng et al., 2010). To prevent problems related to BrdU-immunohistochemistry, EdU, a thymidine-analog that does not require DNA denaturation to be visualized, has been proposed as a better alternative to BrdU (Zeng et al., 2010).

Another potential limitation is the use of the NG2-CreER^{TM}:EYFP reporter mouse. Cre recombinase is an enzyme that recognizes genetic sequences known as LoxP sites. Cre excises

the DNA sequence that lies between two LoxP sites and rejoins their exposed ends. When Cre is fused with the human estrogen receptor (ER), it creates an inducible recombination system reliant on tamoxifen, an estrogen receptor modulator, for recombination to take place. In the absence of tamoxifen, the CreER enzyme is kept in the cytoplasm. After the administration of tamoxifen, CreER undergoes a conformational change that allows for its translocation to the nucleus. Once in the nucleus, Cre can induce the recombination of LoxP sites. To create the NG2-CreER^{TM}:EYFP reporter mouse used in this study, a mouse with CreER inserted at the NG2 locus was bred with one with a LoxP-STOP-LoxP sequence and EYFP inserted at the *Rosa26* locus. Tamoxifen administration in adulthood brings on the translocation of Cre to the nucleus and activates the expression of EYFP in NG2-positive cells.

This reporter mouse model creates a few problems. First, NG2 is expressed not only by OPCs but also by pericytes. To ensure that our stereological counts only included OPCs, we had to rely on the morphology of labeled cells and only count those that exhibited a shape typical of OPCs. While the morphology of OPCs and pericytes is very different, with OPCs typically having highly arborized processes and pericytes having none, it is possible that certain pericytes were included in our stereological counts, thereby slightly inflating our results.

Second, the NG2-CreER^{TM}:EYFP reporter mouse makes it so that OPC visualization is reliant on the success of tamoxifen-induced Cre recombination. While we did reach extensive recombination in this study due to a robust tamoxifen-administration protocol (see experiments 2 and 3 for details), it is likely that not all NG2-positive cells at the time of tamoxifen administration underwent recombination. To control for efficiency of recombination, as well as success of immunostaining, we used ratios to address most of the questions addressed in this study. The Cre-loxP technology associated with the NG2-CreER^{TM}:EYFP reporter mouse may

also have be responsible for our observation of neuronal markers in GFP+ cells. It has been hypothesized that when recombination strategies are non-homologous such as the one used here, physiological aging and stress may cause transient and/or ectopic Cre or NG2 expression in neurons. Upon tamoxifen administration, these cells would undergo recombination and express EYFP, even if they do not stem from the OPC lineage (Nishiyama et al., 2014). Future fate mapping studies using alternatives to Cre technology will be needed to clearly determine if OPCs can in fact differentiate into neurons.

Third, the NG2-CreER^{TM}:EYFP reporter mouse requires tamoxifen for OPCs to be visualized. While tamoxifen is widely used in research and clinically in patients receiving cancer treatments, it can have toxic side-effects (Huh et al., 2012). Similarly, BrdU can also be toxic to animals (Taupin, 2007). Some NG2-CreER^{TM}:EYFP did not respond well to the combined administration of tamoxifen and BrdU. We therefore had a limited pool of animals to draw from, leading to the use of both male and female NG2-CreER^{TM}:EYFP mice in our studies. Since the proliferation and maturation of cultured neonatal OPCs is differentially regulated by male and female sex steroid hormones, and because proliferation and death of oligodendrocytes and myelin proteins are differentially regulated in male and female rodents, it is possible that the use of animals of both sexes has impacted our results (Marin-Husstege et al., 2004; Cerget et al., 2006).

The NG2-CreER^{TM}:EYFP reporter mouse was used to evaluate the fate of OPCs. Three possible fates were examined in this study: OPC, oligodendrocyte, and neuronal. However, it must be noted that OPCs may also have the ability to differentiate into astrocytes (Nishiyama et al., 2016; Nishiyama, Komitova, Suzuki, & Zhu, 2009; Vigano & Dimou, 2016). However, if this fate is possible, it is classified as minor compared to self-renewal and oligodendrocyte

differentiation (Nishiyama et al., 2016). Furthermore, we were mostly interested in adult OPCs role in white matter plasticity and neural networks (i.e. OPC-neuron pairs). Hence, we did not examine a potential astrocytic fate.

The stereological analyses were conducted in the entire corpus callosum and dorsal portion of the cortex. Furthermore, a core principle of stereology is homogenous distribution of features of interest. This should not have been a problem for the analysis of the general properties of white and grey matter OPCs since OPCs have been found to be evenly distributed in the adult brain (Dawson, Polito, Levine, & Reynolds, 2003). However, if reference memory training and GABAergic agent administration had regional impact (as opposed to a general one) on parts of the corpus callosum and cortex, our experiments were not designed to address this issue. Finally, the initial determination of the stereological parameters was based on a large number of sections but they all came from one animal stained for GFP/PDGFRα/BrdU. This could have led to biased parameters that were not optimal for the other combinations of antigen staining used in this study. However, for all combinations of stains studied, we obtained coefficients of error below 10%. These low coefficients are usually taken as a demonstration that the selected parameters are appropriate for stereological analysis (Gundersen, Jensen, Kieu, & Nielsen, 1999).

Finally, since electrophysiological analysis was not conducted, we cannot be certain that the GABAergic agents administered through i.p. injection reached the brain in sufficient quantities to act upon neurotransmission. Future studies may want to use intracerebral injections or infusion. However, we were interested in studying the effects of GABAergic neurotransmission *in vivo* in the healthy and intact adult brain, which would have made

electrophysiological analysis and the intracerebral administration of pharmacological agents problematic in that context.

Future directions

It has become increasingly clear that the function of adult OPCs goes beyond the generation of new oligodendrocytes in the face of white matter injury. Their continued ability to give rise to oligodendrocytes suggests that adult OPCs are key players in a form of neural plasticity known as white matter plasticity, where previously unmyelinated axons receive myelin sheaths and pre-existing sheaths are remodelled. White matter plasticity contributes to cognitive functioning by increasing the speed and the synchronicity of neural inputs (Fields, 2005, 2008; Wang & Young, 2014). While activities such as physical exercise, environmental enrichment, behavioural stimulation, and motor learning have been shown to participate in white matter plasticity by modulating OPC proliferation and differentiation (Ehninger et al., 2011; McKenzie et al., 2014; Simon et al., 2011; Xiao et al., 2016; Yang et al., 2013; Zhao et al., 2011), it remains to be seen whether cognitive learning can do the same. Future studies may examine behavioural training that has been shown to change white matter density locally and/or in specific areas of the cortex and focus stereological analysis on these rather than use an overall analysis (e.g. visual training / optic radiation / visual cortex). Skilled unilateral reach training where rats are trained to reach with one paw while the other remains untrained could also be a powerful approach to examine local OPC changes (Sampaio-Baptista et al., 2013). Similarly, behavioural training that use a specific sensory modality could be useful models.

The ability of behavioural factors to change OPC proliferation and differentiation seems to be linked to a second role of adult OPCs, which is the monitoring of neural network activity.

Based on *in vitro* evidence, neurotransmitters appear to be key players in OPCs' ability to sense and respond to experience-dependent neural activity, but the *in vivo* evidence needed to confirm this hypothesis is lacking. While the anatomical connections that OPCs form through their processes with multiple neurons are in line with the idea that they monitor neural network activity, the purpose of OPC-neuron pairs is not as clear. The sheer prevalence of these pairs, which has been established in experiment 5, should warrant further studies into their function. While it has already been shown that OPC-neuron pairs are not connected through synapses between their cell bodies, the results of experiment 5 suggest that GABAergic neurotransmission through the $GABA_B$-receptor could be a good starting point. In vivo studies using 2-photon microscopy and cranial windows in NG2-CreER^{TM}:EYFP reporter mice could be a possible avenue to study OPC-neuron interactions though calcium imaging and/or electrophysiological recording (Aharoni, 2013; Kherlopian et al., 2008; Zuo, Yu, Tennant, & Jones, 2013). Also, we performed a preliminary identification of paired neurons using classic protein labels (calbindin, calretinin, and parvalbumin). Additional experiments could specifically identify if precise GABA neurons subtypes are more likely to be found with OPC satellites (Tasic et al., 2016).

Finally, determining whether adult OPCs have the ability to generate new oligodendrocytes is of the upmost importance since it could have implications for various neurodegenerative diseases. The generation of a new reporter mouse model that allows for the fate mapping of OPCs but that does not make use of Cre-loxP technology appears to be a promising path. Also, a closer investigation as to the role of DCX, both in OPCs and in newly generated neurons, could help elucidate whether its expression by adult OPCs is linked to multipotency or migration in these cells.

Concluding remarks

We have used an unbiased systematic stereological approach to examine the general characteristics of OPCs located in the corpus callosum and cortex of 4-5-month-old NG2-CreER^{TM}:EYFP reporter mice. We show that OPCs retain the ability to proliferate and to differentiate into oligodendrocytes at later stages of adulthood, with white matter OPCs being more likely to differentiate into oligodendrocytes than grey matter OPCs. The differentiation of OPCs into an oligodendrocyte phenotype can occur directly or with a prior cell division. Oligodendrocyte differentiation represents an important aspect of neural plasticity called white matter plasticity. White matter plasticity contributes to cognitive functioning by increasing the speed and the synchronicity of neural inputs (Fields, 2005, 2008; Wang & Young, 2014). We also show evidence of neuronal differentiation by adult OPCs. Neuronal differentiation is rare, however, and some of the positive observations could be the result of problems with the Cre technology used to track the fate of OPCs (Nishiyama et al., 2014). Furthermore, DCX expression by OPCs appears to be linked to migration over short distances and not to a neuronal fate. Finally, we examined anatomical connections between neurons and OPCs and showed that almost half of OPCs take part in OPC-neuron pairs, where the cell body of the neuron and the OPC are closely apposed to one another.

Experience-dependent neural network activity has been shown to serve as a modulator of OPC proliferation and differentiation (Boda & Buffo, 2014; Tomlinson et al., 2015). We looked at the effects of reference memory training on overall OPC proliferation and differentiation in the corpus callosum and neocortex of the adult NG2-CreER^{TM}:EYFP reporter mouse. However, the effects of reference memory on OPC proliferation and differentiation were not conclusive. Neurotransmitters have been identified as potential mediators in the relationship between

experience-dependent neural network activity and OPCs. There is also conflicting evidence as to the effects of neurotransmitter-receptor activation on OPC proliferation and differentiation. While $GABA_A$- and $GABA_B$-receptor agonists and antagonists did not yield a significant effect on the proliferation and differentiation of OPCs, $GABA_A$-receptor agonism does appear to play a regulatory role these physiological processes. Finally, we looked and the effects of reference memory and GABAergic agents on OPC-neuron pairs and determined that GABAergic neurotransmission through the $GABA_B$-receptor can modulate the prevalence of these pairs.

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