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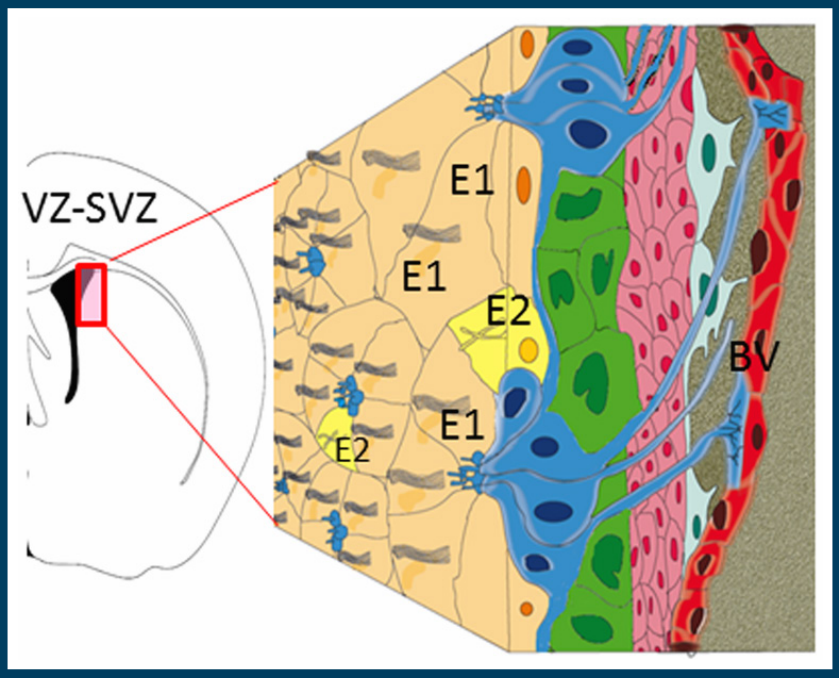

THE VENTRICULAR-SUBVENTRICULAR ZONE: A SOURCE OF OLIGODENDROCYTES IN THE ADULT BRAIN

Topic Editors
Oscar Gonzalez-Perez,
Jose Manuel Garcia-Verdugo and Adan Aguirre

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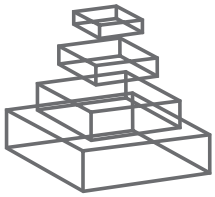

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THE VENTRICULAR-SUBVENTRICULAR ZONE: A SOURCE OF OLIGODENDROCYTES IN THE ADULT BRAIN

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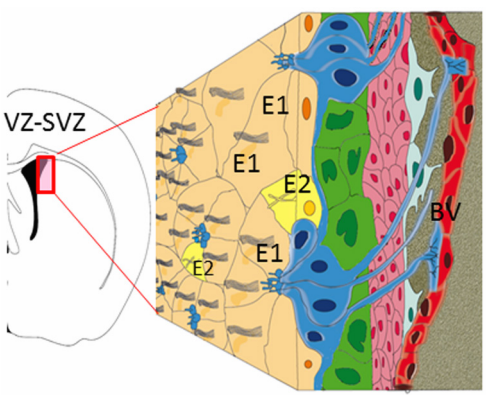

Schematic 3D-reconstruction of the ventricular-subventricular zone (V-SVZ), the niche of adult neural stem cells in the adult mouse brain
Figure taken from: Galvez-Contreras AY, Quiñones-Hinojosa A and Gonzalez-Perez O (2013) The role of EGFR and ErbB family related proteins in the oligodendrocyte specification in germinal niches of the adult mammalian brain. Front. *Cell. Neurosci.* 7:258. doi: 10.3389/fncel.2013.00258

Demyelinating diseases are characterized by an extensive loss of oligodendrocytes and myelin sheaths from axolemma, which commonly result in disability in young adults. To date, there is no effective treatment against these neurological disorders. In the adult brain, there are neural stem cells (NSCs) that reside within a niche denominated ventricular-subventricular zone (V-SVZ) in the lateral wall of the cerebral ventricles. NSCs give rise to neurons and oligodendrocytes that help preserve cellular homeostasis. Growing evidence indicates that V-SVZ progenitor cells may represent an endogenous source of oligodendrocytes that can be useful to treat demyelinating diseases.

This e-Book "The ventricular-subventricular zone as a source of oligodendrocytes in the adult brain" collected the most recent evidence regarding the mechanisms that modulate the proliferation, migration, quiescence, cell-fate choices and survival of oligodendrocyte precursors generated in the V-SVZ. Herein, we compiled information about the role of Sonic hedgehog, NMDA receptors, ErbB proteins, hemopressin, erythropoietin, osmolarity and microglia in the oligodendrocyte production. Some chapters also describe the role of oligodendrocyte precursors in the preservation of cellular homeostasis, aging and white matter repair. All these information is presented as novel research findings, short communications, and review articles, which were written by experts in the field of oligodendrocyte generation, myelin production and white matter re-myelination.

Acknowledgements

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EDITORIAL
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The ventricular-subventricular zone: a source of oligodendrocytes in the adult brain

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Keywords: neural stem cells, ventricular-subventricular zone, oligodendrocyte, myelin, white matter, oligodendrocyte precursor cells, remyelination, demyelinating disease

Demyelinating diseases are characterized by an extensive loss of oligodendrocytes and myelin sheaths from axolemma, which commonly result in disability in young adults. To date, there is no effective treatment against these neurological disorders. In the adult brain, there are neural stem cells (NSCs) that reside within a niche denominated ventricular-subventricular zone (V-SVZ) in the lateral wall of the cerebral ventricles. These NSCs give rise to neurons and oligodendrocytes that help preserve cellular homeostasis (Kriegstein and Alvarez-Buylla, 2009). Growing evidence indicates that V-SVZ progenitor cells may represent an endogenous source of oligodendrocytes that can be useful to treat demyelinating diseases.

The e-Book "*The ventricular-subventricular zone as a source of oligodendrocytes in the adult brain*" has collected the most recent evidence regarding the mechanisms that modulate the proliferation, migration, quiescence, cell-fate choices and survival of oligodendrocyte precursors generated in the V-SVZ. This e-Book begins with an excellent study performed by the members of the Nada Zecevic's laboratory. They analyzed the effects of Sonic hedgehog (Shh) signaling on proliferation and specification of human cortical Olig2+ progenitors *in vitro*. They demonstrated that Shh increased the number of oligodendrocyte progenitors (OPCs). However, inhibition of endogenous Shh did not reduce the density of Olig2+ cells, which suggest an additional Shh-independent mechanism for oligodendrocyte generation (Ortega et al., 2013).

In the adult V-SVZ, the activation of NMDA receptors (NMDAR) increases the oligodendrocyte differentiation. Using the reporter gene luciferase, Carlos Matute et al. (Chapter 2) found that the activation of NMDR stimulates PKC, which precedes the activation of NADPH oxidase (NOX) (Cavaliere et al., 2013). Hence, the authors propose that NOX2 is involved in the transduction of the signal from NMDAR through PKC activation. This work also suggests that signaling through the cascade NMDAR–PKC-NOX2 generates ROS that, in turn, activate the PI3/mTOR pathway and drives oligodendrogenesis.

Tyrosine kinase (Trk) receptors play a key role in regulating the function of V-SVZ NSCs. Particularly, V-SVZ neural progenitor cells express the epidermal growth factor receptor (EGFR). EGF strongly stimulates proliferation, survival, migration and differentiation into the oligodendrocyte lineage (Gonzalez-Perez and Alvarez-Buylla, 2011). In the chapter 3, Galvez-Contreras and colleagues compiled the current data regarding the role of EGFR and ErbB family signaling on V-SVZ NSCs and explained the downstream cascades involved in oligodendrogenesis (Galvez-Contreras et al., 2013). The authors also proposed a hypothetical model to support the EGF-induced oligodendrogenesis, which involves homodimerization between ErbB1 and ErbB3, which probably activate the PI3K or the STAT pathways. Inconsequence AKT is activated and the expression of Olig-2 is induced, which determines oligodendroglial specification.

Endocannabinoids have been involved in oligodendrogenesis. As reported by the Dr. Malva's group (chapter 4), hemopressin, a modulator of cannabinoid receptor-1, can increase the oligodendroglial differentiation in V-SVZ progenitor cells (Xapelli et al., 2014). Their results suggest that hemopressin may be of potential interest to treat demyelinating diseases.

Erythropoietin (EPO) promotes the V-SVZ-derived neurogenesis and oligodendrogenesis. Sawamoto and coworkers recently demonstrated that the EPO derivative asialo-EPO promotes the differentiation of V-SVZ-derived OPCs into myelin-forming mature oligodendrocytes in the injured white matter of neonatal mice without causing erythropoiesis (Kaneko et al., 2013). These findings and the therapeutic proposal are discussed in the chapter 5 of this book.

In the chapter 6, the group of Prof. Dietzel found that the addition of equiosmolar supplements of mannitol or NaCl associated with microglia cells can influence the proliferation of OPCs (Kleinsimlinghaus et al., 2013). Interestingly, a maximal yield of OPCs is obtained by combining a cell culture medium with osmolarity >280 mOsm and low density of microglia cells.

Aging progressively decline in the proliferative capacity of V-SVZ progenitor cells that subsequently affects the incorporation of new cells in the olfactory bulb. In the chapter 7, Quiñones-Hinojosa's group presents the effects of aging across the V-SVZ-OB system. They surprisingly found that neurogenesis decline with aging, but oligodendrogenesis in the rostral migratory stream is not compromised (Capilla-Gonzalez et al., 2013).

In the chapter 8, Ken Arai and colleagues (Maki et al., 2013) summarized the recent studies on extrinsic (extracellular matrix, cerebrospinal fluid, vasculature) and intrinsic (transcription factors or epigenetic modifiers) factors, which mediate oligodendrocyte generation from the V-SVZ progenitor cells. These factors appear to decrease by aging and compromise the efficiency of remyelination into the brain. In the chapter 9, significant questions and hypotheses regarding this topic are analyzed by Agathou et al. (2013). In the last chapter, Zhang et al. compiled evidence

that indicates that a pathological condition as stroke can increase oligodendrogenesis in the white matter and V-SVZ during brain repair (Zhang et al., 2013), which support the notion that many inflammatory cytokines regulate the V-SVZ cells (Gonzalez-Perez et al., 2012).

In summary, growing evidence indicates that V-SVZ NSCs are good candidates to establish stem cell-based therapies in demyelinating disorders. Nevertheless, chemical mediators and signaling pathways involved in all these processes are to be completely elucidated. Understanding the mechanisms that regulate the V-SVZ progenitor cells may help develop therapeutic approaches to treat demyelinating diseases.

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Sonic hedgehog promotes generation and maintenance of human forebrain Olig2 progenitors

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Function of oligodendrocytes (OLs), myelin forming cells in the CNS, is disrupted in demyelinating diseases such as periventricular leukomalacia or multiple sclerosis. It is, thus, important to better understand factors that can affect generation or differentiation of human OLs. In rodents, Sonic hedgehog (Shh) is influencing expression of Olig2, a helix-loop-helix transcription factor required for development of OLs. In humans, Olig2 is present in cortical progenitors at midgestation, however the role of Shh in the specification of human OLs, including Olig2 positive (Olig2$^+$) progenitors, is not fully understood. Here we studied *in vitro* effects of Shh signaling on proliferation and specification of human cortical Olig2$^+$ progenitors at midgestation. First, we established that the spatial pattern of Olig2 expression in the human developing CNS, described on cryosections, was preserved in mixed and enriched radial glia cell (RGC) cultures. Next, we demonstrated that *in vitro* treatment with Shh induced an increase in the number of Olig2$^+$ progenitors. Shh treatment increased the density of early oligodendrocyte progenitors (OPCs) at the expense of RGC, while the number of late OPCs, did not change. However, inhibition of endogenous Shh with cyclopamine did not reduce the density of Olig2$^+$ cells, implying the presence of an additional Shh-independent mechanism for OLs generation *in vitro*. These results suggest that the primary role of Shh signaling in the human dorsal oligodendrogenesis is the expansion and specification of multipotent radial glia progenitors into Olig2$^+$ early OPCs. These results obtained *in vitro* are relevant to understand primary myelination during CNS development, as well as remyelination in demyelinating diseases.

Keywords: glia, cortical development, cellular fate, myelination, transcription factors

INTRODUCTION

The origin and differentiation of oligodendrocytes (OLs) have been extensively studied in animal models, and are especially well documented in rodents, mainly due to advances in genetic mapping of cell lineages (He et al., 2001; Marshall and Goldman, 2002; Fogarty et al., 2005; Kessaris et al., 2006). In the rodent telencephalon, the majority of embryonic OLs are generated in the ventral forebrain (ganglionic eminence, GE), but a subpopulation of OLs is also generated postnatally in the cortical subventricular zone (SVZ) (Levison and Goldman, 1993; Gorski et al., 2002; Kessaris et al., 2006; Richardson et al., 2006; Kessaris et al., 2008). Notably, it has been suggested that only dorsally generated OLs remain in the adult mouse brain (Kessaris et al., 2006). In the human developing brain, OLs differentiation proceeds through similar stages described in rodents (Rivkin et al., 1995; Back et al., 2001; Ulfig et al., 2002; Wilson et al., 2003; Jakovcevski and Zecevic, 2005; Jakovcevski et al., 2009). At midgestation, human oligodendrocyte progenitor cells (OPCs) originate both in the ventral and dorsal telencephalon (Rakic and Zecevic, 2003; Jakovcevski et al., 2009). Cells generated from these two sources could potentially differ in vulnerability or response to environmental factors. This might be clinically important in various diseases, from the ones that present with demyelination, such as multiple sclerosis (MS) or hereditary leukodistrophy, to complex neurological disorders such as Alzheimer's disease and schizophrenia (Goldman et al., 2012).

In rodents, radial glial cells (RGCs) and several intermediate precursor cells, presumably generated from RGCs, give rise to OLs in the developing CNS (Raff et al., 1998; Rao et al., 1998; Fogarty et al., 2005; Casper and Mccarthy, 2006; Kriegstein and Alvarez-Buylla, 2009). Along oligodendroglial lineage, cells change their morphologies and expression pattern of OLs- specific proteins. Early OPCs are bipolar migratory cells that express chondroitin-sulfate proteoglycan (NG2) and platelet-derived growth factor receptor alpha (PDGFRα). They differentiate into late OPCs which can be labeled with anti-O4 antibody, and finally into premyelinating and myelinating OLs recognized by anti-O1 antibody and antibodies to myelin basic protein (MBP) and proteolipid protein (PLP), respectively; these cells do not proliferate or migrate (Pfeiffer et al., 1993; De Castro and Bribián, 2005; Meijer et al., 2012).

In addition to similar sequential expression of immunohistochemical markers during the progression of OLs lineage in humans, we have reported that *in vitro* human fetal RGCs can generate OLs and that this process is enhanced by Sonic hedgehog (Shh) (Jakovcevski et al., 2009; Mo and Zecevic, 2009). Shh

is an essential morphogen critical for normal development of the brain, especially for its ventral patterning (Gritli-Linde et al., 2001; Marti and Bovolenta, 2002; Ruiz I Altaba et al., 2002). Moreover, Shh promotes OPCs specification from both ventral and dorsal sources (Kessaris et al., 2004). This effect of Shh is particularly important after brain injury, since it might contribute to remyelination (Amankulor et al., 2009; Ferent et al., 2013). Shh exerts its function through spatiotemporal interaction with a variety of transcription factors such as Pax6, Dlx2, Nkx2.1, Nkx2.2, Olig1, and Olig2 (Nery et al., 2001; Fuccillo et al., 2006; Xu et al., 2010; Balaskas et al., 2012). A dynamic combination of these transcription factors plays an important role in OLs differentiation (Nery et al., 2001; Nicolay et al., 2007).

Olig 1 and 2 (Oligodendrocyte Lineage Genes) are basic helix-loop-helix (bHLH) transcription factors expressed in response to ventrally secreted Shh in rodents. Gain-and-loss-of-function studies confirmed that Olig genes are necessary and sufficient for generation of OLs (Lu et al., 2000; Zhou et al., 2000; Tekki-Kessaris et al., 2001). Since the human brain is much larger, has a prolonged time of differentiation and myelination, and numerous evolutionary adaptations, the findings on OL lineage cells and Olig genes obtained in animal models cannot be directly extrapolated to the human brain. Olig genes have a great clinical relevance since they might be involved in demyelination/remyelination pathologies in MS patients and in brain lesions (Arnett et al., 2004; Fancy et al., 2004; Meijer et al., 2012). Moreover, Olig2 is selectively up-regulated after brain injury (Buffo et al., 2005), in diffuse gliomas (Ligon et al., 2004), and is linked to schizophrenia and Alzheimer's disease (Georgieva et al., 2006). Furthermore, this transcription factor has an unusual dual role in development; it first promotes expansion of the neural progenitor pool, and later affects specification and differentiation of OL lineage (reviewed in Meijer et al., 2012). Olig2 has been reported by us and others to be more involved in OL specification than Olig1 (Jakovcevski and Zecevic, 2005; Meijer et al., 2012; Mei et al., 2013). This is why in this study we focused on Olig2 progenitors, and on the effect of Shh signaling on their proliferation and differentiation in human fetal telencephalon at midgestation (ages ranging from 14 to 19 gestational weeks, gw).

We demonstrate that Shh signaling promotes Olig2$^+$ cell proliferation and influences specification of RGCs into early OPCs (PDGFRα^+), but its role in further differentiation into late OPCs is still not clear.

MATERIALS AND METHODS

HUMAN FETAL BRAIN TISSUE AND CELL CULTURES

Tissues for cell cultures were dissected from human fetal forebrains ($n = 8$, **Table 1**) ranging in age from 14 to 19 gw, obtained from Advanced Bioscience Resources (ABR, Alameda, CA) and StemEx (Diamond Springs, CA) with proper parental consent and the approval of the Ethics Committees. No evidence of disease or abnormalities was observed after ultrasound and neuropathological examination of fetal brains. The stage of development was estimated as gw after conception and ultrasound findings. Brain tissue was collected in oxygenized Hank's balanced salt solution (Invitrogen, Carlsbad, CA) and transported on ice. Tissue from the cerebral cortex and ganglionic eminence was dissected out and dissociated mechanically and enzymatically (0.025% trypsin-EDTA, Invitrogen, Carlsbad, CA; DNAse I, 2 mg/ml, Sigma, Saint Louis, MO). Cells were seeded in expansion medium: DMEM/F12 Medium (Invitrogen, Carlsbad, CA) supplemented with B27 conditional medium (Invitrogen, Carlsbad, CA), basic fibroblast growth factor (bFGF) (10 ng/ml, Peprotech, Rocky Hill, NJ), epidermal growth factor (EGF) (10 ng/ml, Millipore, Billerica, MA) and Penicillin/Streptomycin antibiotics (Invitrogen, Carlsbad, CA) on Poly-D-Lysine (Sigma, Saint Louis, MO) coated flasks. RGC were isolated using MACS immunomagnetic sorting protocol with CD15 microbeads (Miltenyi Biotech, Auburn, CA). To isolate PDGFRα^+ OPCs, cells were first immunolabeled with mouse anti-human CD140a antibody (1:25, BD Pharmingen, San Jose, CA) and then magnetically immunosorted by rat anti-mouse IgG2a+b secondary antibody conjugated with magnetic beads (Miltenyi Biotech, Auburn, CA).

Table 1 | Fetal human brain tissue used in this study.

Case	Gestational week (gw)	Gender	Direct tissue application	Cell culture application
1	14	NP	–	ICC, RT-PCR, WB
2	15	♀	IHC	–
3	16	♂	RT-PCR	–
4	17	♂	–	ICC, RT-PCR, WB
5	17	♂	–	RT-PCR
6	17	NP	–	ICC
7	18	NP	RT-PCR	ICC, RT-PCR
8	19	♂	–	ICC

♀, female; ♂, male; ICC, immunocytochemistry; IHC, immunohistochemistry; NP, not provided; RT-PCR, real-time PCR; WB, western blot.

For pharmacological treatments, 2×10^6 and 25×10^4 cells were seeded in Poly-D-lysine coated 6-well and 24-well plates respectively. Cells were cultured for 1–3 days in expansion medium and then treated with Shh (200 ng/ml, R&D systems, Minneapolis, MN) and/or cyclopamine (2.5 μM, EnzoLife Sciences, NY) in differentiation medium (expansion medium without bFGF and EGF) applied to cells every 3 days for 14 days *in vitro* (DIV).

Three distinct differentiation media were used to promote OLs generation and maturation from RGC and OPC cultures. Apart from control medium (DMEM/F12, B27), we used OPCs expansion medium to potentiate differentiation of OPCs. This medium contained DMEM/F12, N2 conditional medium (Invitrogen, Carlsbad, CA) and PDGFaa (10 ng/ml, Peprotech, Rocky Hill, NJ). In order to get more mature OLs, we used a OLs differentiation medium and a protocol where cells were cultured first for 2 days in DMEM/F12, N2 and PDGFaa and then for an additional 14 DIV in DMEM/F12, N2, NT3 (10 ng/ml, Peprotech, Rocky Hill, NJ) and T3 (30 ng/ml, Sigma, Saint Louis, MO) (Dugas et al., 2006).

For immunohistochemistry, an additional 10 specimens were available, ranging in age from 5 gw to newborn. These cases were subject of previous reports (Jakovcevski and Zecevic, 2005; Zecevic et al., 2005; Jakovcevski et al., 2009).

REAL-TIME PCR

A Real-time PCR was used to determine the expression of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), Olig2, and PDGFRα. Total RNA was extracted from cells using TRIZOL® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Approximately 1 μg of RNA was used in the reverse transcription reaction using M-MuLV reverse transcriptase with random hexamers (Fermentas, Vilnus, Lithuania) according to the manufacturer's instructions. Real-time PCR was performed in a Realplex2 Mastercycler (Eppendorf, Hamburg, Germany) using 96-well reaction plates (Eppendorf, Hamburg, Germany). The reactions were prepared according to the standard protocol for one-step QuantiTect SYBR Green RT-PCR (Applied Biosystems, Cheshire, UK). The sequences $5' \rightarrow 3'$ of the forward (F) and reverse (R) primers were as follows:

GAPDH:(F)ACCACCATGGAGAAGGC/(R)GGCATGGACTG TGGTCATGA
Olig2:(F)AGTCATCCTCGTCCAGCACC/(R)TCCATGGCGAT GTTGAGGT
PDGFRα:(F)AAATCTATGTTAGACTCAGAAGTC/(R) AGTAGAATCCACCATCATGCC

The thermal cycle conditions were 95°C for 2 min followed by 40 cycles of 15 s at 95°C, 15 s at 55°C and 20 s at 68°C. All assays were performed in triplicates. Averaged cycle of threshold (Ct) values of GAPDH triplicates were subtracted from Ct values of target genes to obtain ΔCt, and then relative gene expression was determined as $2-\Delta Ct$. The results were presented relative to the control value, which was arbitrarily set to 1.

WESTERN BLOT ANALYSIS

Cells were homogenized in lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride, and protease inhibitor cocktail] on ice for 30 min, centrifuged at 14,000 g for 15 min at 4°C, and the supernatants were collected as the cell lysates. Protein extracts obtained from cultures were separated by SDS-PAGE and electro-transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked and incubated first with primary antibodies anti-Olig2 (1:500, Millipore) and anti-GAPDH (1:3000, Millipore) overnight at 4°C, and then with their corresponding secondary HRP-conjugated antibodies (1:15000, Thermo Fisher Scientific, Temecula, CA). Protein signal was detected using SuperSignal West Pico Chemiluminescent system (Thermo Fisher Scientific, Temecula, CA).

IMMUNOCYTOCHEMISTRY AND IMAGE ANALYSIS

Cell cultures were fixed with 4% paraformaldehyde/0.2% glutaraldehyde, blocked 1 h in PBS with 0.2% bovine serum albumin (BSA) and subsequently incubated with primary antibodies diluted in phosphate buffer solution (PBS) with 0.5% BSA, 5% normal goat serum (NGS) and 0.5% Tween 20® at 4°C overnight. Primary antibodies against the following proteins were used: rabbit anti-brain lipid binding protein (BLBP; 1:1000, Abcam, UK), mouse anti-Ki67 (1:50, Dako, Denmark), mouse anti-MAP2 (1:200, Sigma, Saint Louis, MO), mouse anti-NG2 (1:100, Chemicon), mouse anti-PDGFRα (1:25, Pharmingen, San Diego, CA), rabbit anti-Olig2 (1:500, Millipore), mouse anti-O4 (1:50, generous gift from Dr. Bansal), mouse anti-vimentin (1:100, Sigma, Saint Louis, MO). Primary antibodies were followed by the appropriate secondary antibodies conjugated with Alexa488 or Alexa555 fluorophores (1:500, Molecular Probes, Eugene, Oregon) for 1 h and a short incubation in bisbenzimide (Sigma) for nuclear staining.

Immunohistochemistry on frontally cut cryosections (15 μm) of fetal brains was performed as previously described (Jakovcevski and Zecevic, 2005). Tissue sections and cell cultures were visualized with the Axioskop microscope (Zeiss, Germany) using Axiovision software and photographed with a digital camera. Images were assembled in Adobe Photoshop (v. 7.0), with adjustments for contrast, brightness and color balance to obtain optimum visual reproduction of data. Immunolabeled cells from nine predesigned adjacent optical fields were analyzed by Image J software (National Institutes of Health, Bethesda, Maryland). T-test was used to discriminate between the means, with significance levels set at $p < 0.05$ and $p < 0.01$.

RESULTS AND DISCUSSION

Olig2$^+$ CELLS IN HUMAN TELENCEPHALON AT MIDGESTATION

We first assessed distribution of Olig2 immunolabeled cells through increasing developmental stages, from 5–19 gw and in neonatal forebrain (**Figure 1A**). A considerable difference in the number of Olig2$^+$ cells was observed throughout the forebrain, with higher density seen in the ventral telencephalon, in the GE, whereas in the cerebral cortex, Olig2$^+$ cells were observed mainly in the subplate layer and in the ventricular/subventricular zone (VZ/SVZ), but not in the cortical plate. Example of Olig2$^+$ cells distribution is shown in the 15 gw forebrain (**Figure 1B**). Real-time PCR analysis from human fetal brain tissues (16 and 18 gw) supported these findings and demonstrated that the levels of Olig2 mRNA were significantly higher in the GE compared to the cortex (CX) (**Figure 1C**). Taken together, these results are in accord with the finding that during midgestation, human OPCs are more numerous in the GE than in the cortex, suggesting their initial generation in the ventral telencephalon (Rakic and Zecevic, 2003; Jakovcevski and Zecevic, 2005; Jakovcevski et al., 2009). Scattered Olig2$^+$ cells observed in the subplate layer probably represent the first cortical OPCs, as both O4$^+$ and PDGFRα$^+$cells have been described in the same region and at the same developmental stages (Rakic and Zecevic, 2003; Jakovcevski et al., 2009). It is possible that the transient subplate layer, which hosts numerous afferent and efferent fibers (McConnell et al., 1989; Kostovic and Rakic, 1990; Jacobs et al., 2007), supports OPCs differentiation (Jakovcevski et al., 2009). As cortical development proceeds, enriched Olig2 expression is observed in the expanded human outer SVZ (Back et al., 2001; Rakic and Zecevic, 2003; Jakovcevski and Zecevic, 2005; Jakovcevski et al., 2009). In rodents, similar expression of Olig2 is reported in the intermediate progenitors of the SVZ and along white matter tracts, such as the corpus callosum, during perinatal development and even in the gray matter where it remains in quiescent progenitors for mature OL turnover (Levison and Goldman, 1993;

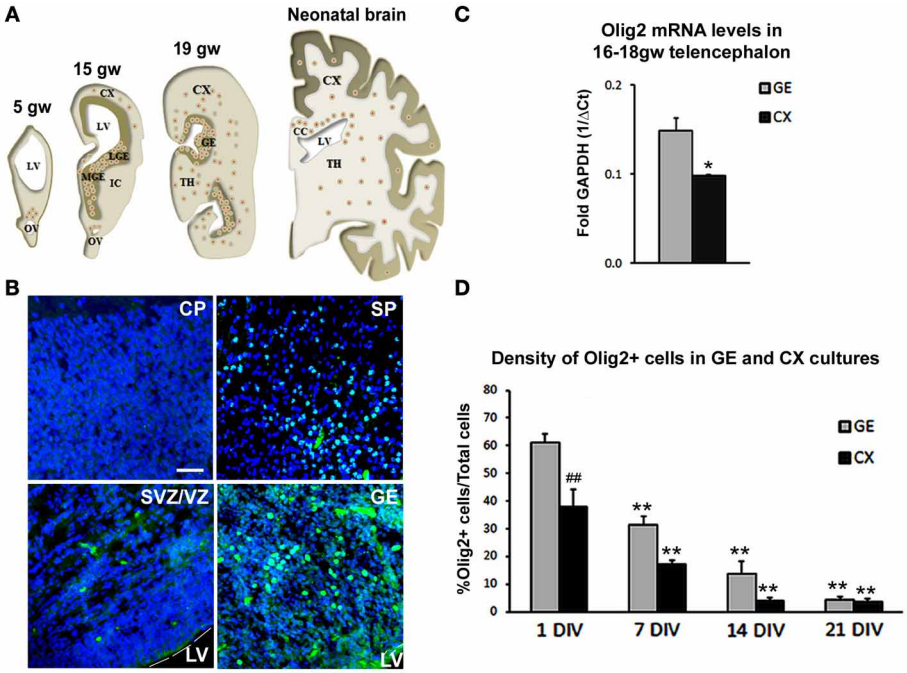

FIGURE 1 | Olig2 expression in the human fetal forebrain. (A) Schematic representation of the distribution of Olig2$^+$ cells (red dots) in frontal sections of the fetal (5–19 gw) and neonatal human telencephalon. **(B)** Representative Olig2 (green) immunostaining in 15 gw fetal forebrain. Nuclear staining with bisbenzimide (BB) in blue. **(C)** RT-PCR levels for mRNA Olig2 in CX and GE in 16–18 gw human brains. Data are presented as mean ± standard deviation. **(D)** Percentage of Olig2$^+$ cells in ventral (GE) and cortical (CX) cultures. Data are presented as mean ± s.e.m. (9 optical fields quantified in each culture). $^*p < 0.05$, $^{**}p < 0.01$ vs. control; $^{\#\#}p < 0.01$- GE vs. CX. Scale bar: 20 μm. CC, Corpus Callosum; CP, Cortical Plate; IC, Internal Capsule; IZ, Intermediate Zone; LGE, Lateral Ganglionic Eminence; LV, Lateral Ventricle; MGE, Medial Ganglionic Eminence; OV, Optical Vesicle; SP, Subplate layer; TH, Thalamus; VZ/SVZ, Ventricular/Subventricular Zone.

Nait-Oumesmar et al., 1999; Ligon et al., 2006; Menn et al., 2006).

We were interested whether Shh regulates the expression of Olig2 during human OLs development. For that purpose, we established mixed cell cultures of dorsal (CX) and ventral (GE) regions of 17 gw forebrain. As expected from the results of immunostained cryosections, the density of Olig2$^+$ cells was significantly higher in the ventral cultures compared to the dorsal (**Figure 1D**). Olig2$^+$ cells represented 61% of total cells in ventral cultures compared to 38% in dorsal cultures 24 h post-isolation (**Figure 1D**). The maintenance of Olig2 in cultured cells is in line with Olig2 expression reported in the majority of progenitor cells kept in defined medium with bFGF and EGF (Hack et al., 2004). As it was observed with other neural progenitors, the number of Olig2$^+$ cells decreased progressively with the withdrawal of these growth factors, which led to cell differentiation (Gabay et al., 2003; Mo et al., 2007). At 21 days *in vitro* (DIV), density of Olig2$^+$ cells was only 4% in both ventral and dorsal cell cultures (**Figure 1D**), which is a 15 and 10 times reduction respectively from their initial numbers.

Based on the observed pattern of expression and developmental changes of Olig2$^+$ cells in tissue and *in vitro*, we concluded that dissociated human cell cultures represent a reliable model for the study of human OLs development.

NEURAL CELL TYPES THAT EXPRESS Olig2 IN HUMAN CORTICAL CELL CULTURES

In rodents, even though Olig2 is mainly expressed in OLs progenitors, its presence has also been reported in astrocyte precursors in the SVZ at early postnatal stages and in reactive astrocytes after injury (Marshall and Goldman, 2002; Cahoy et al., 2008). Moreover, several studies pointed out the importance of Olig2 in the generation of neurons in the ventral telencephalon (Petryniak et al., 2007) and in the spinal cord (Lu et al., 2002; Zhou and Anderson, 2002). Indeed, progress in genetic mapping studies in rodents indicates that during CNS development, the origin of oligodendroglial lineage is more closely associated to the neurons than to astrocytes (reviewed by Meijer et al., 2012). We reported previously that Olig2 was expressed not only in human fetal OPCs but also in MAP2$^+$ neurons (Jakovcevski and Zecevic, 2005). In the present study, we explored which cell types expressed Olig2 in the human fetal cortical cultures at midgestation. The expression of Olig2 was examined in cortical mixed cell cultures at 17 gw according to the experimental design presented in **Figure 2A**. One day after seeding cortical cells, Olig2 was observed in early OPCs labeled with NG2 (**Figure 2B**). After 14 DIV Olig2 was present in early PDGFRα$^+$OPCs (**Figure 2C**), in late OPCs marked with O4$^+$ (**Figure 2D**), MAP2$^+$ neurons (**Figure 2E**) and vimentin$^+$ RGCs (**Figure 2F**). The same pattern of Olig2 distribution was

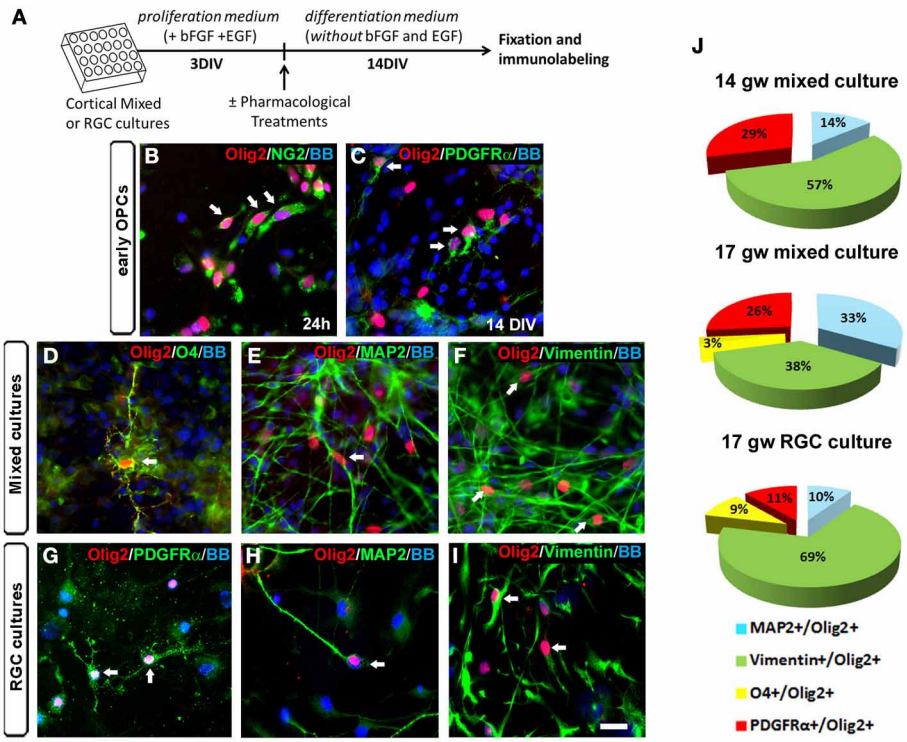

FIGURE 2 | Olig2 expression in human mixed and radial glia cell (RGC) cultures. (A) Time-line of the cell cultures protocol used. **(B–I)** Olig2 (red) expression in mixed cell cultures in NG2$^+$ cells at 24 h **(B)** and in PDGFRα$^+$ early OPCs at 14 DIV **(C)**. Olig2 expression in mixed cell cultures at 14 DIV was observed in O4$^+$ late OPCs **(D)**, in MAP2$^+$ neurons (**E**) and in vimentin$^+$ RGCs (**F**). Similar double-labeling results were demonstrated in RGC cultures after 14 DIV differentiation **(G–I)**. Arrows show double-stained cells. **(J)** The percentages of neuronal (MAP2$^+$), RGC (vimentin$^+$) and OPCs (PDGFRα$^+$, O4$^+$) cells from total Olig2$^+$ cells in control mixed cell cultures (14 and 17 gw), and in 17 gw RGC culture after 14 DIV. Scale bar: 20 μm.

observed in enriched RGC cultures from the same case (17 gw) (**Figures 2G–I**).

We next analyzed differentiation of Olig2$^+$ progenitors in cultures from two fetal brains, aged 14 and 17 gw. At 14 gw, in mixed cortical cultures after 14 DIV, 57% of Olig2$^+$ cells were co-labeled with RGC marker vimentin, 14% with neuronal marker MAP2$^+$, 29% with early OPCs marker PDGFRα$^+$ and no cells were labeled with late OPC marker O4 (**Figure 2J**). In slightly older case (17 gw), the percentage of vimentin$^+$/Olig2$^+$ cells was reduced to 38%; 33% were MAP2$^+$, 26% were PDGFRα$^+$ and 3% were O4$^+$ (**Figure 2J**). These results, although obtained on a small number of cases, indicate that with increased fetal age from 14 to 17 gw, there is a decrease in vimentin$^+$ and an increase in density of MAP2$^+$cells within Olig2$^+$ population of cells. Late O4$^+$ OPCs although still sparse, were first demonstrated in 17 gw mixed cultures. Obtained *in vitro* results suggest that there is a gradual differentiation of Olig2$^+$ cells from multipotent RGC progenitors to neurons (19% more at 17 than at 14 gw cultures) and from early OPCs to late OPCs. Age-dependent potential of O4$^+$ pre-OLs production has also been recently reported by (Cui et al., 2012). These authors observed that sorted OPCs from older fetal tissue have increased myelination potential, which argues for the existence of intrinsic mechanisms controlling OLs differentiation and myelination (Cui et al., 2012). On the other hand, comparison between mixed cultures and RGC cultures at 17 gw showed that the sum of Olig2$^+$ early and late OPCs was reduced in RGC cultures (11% of PDGFRα$^+$ and 9% of O4$^+$). As expected, the percentage of Olig2$^+$/RGCs was higher in RGC cultures (69%) compared to the mixed cultures (38%) (**Figure 2J**). In contrast, the percentage of MAP2$^+$ neurons within Olig2$^+$ population of cells was higher in mixed cell cultures (33%) compared to the RGC cultures (10%). These differences highlight the importance of distinct cellular support needed for OLs specification and differentiation that is present in mixed cell cultures and absent in enriched RGC culture. Indeed, the requirement of neurons and astrocytes to preserve OPCs proliferation and avoid their rapid loss in culture, as well as promote subsequent differentiation has been previously shown in multiple studies (Crang and Blakemore, 1997; Zhang et al., 2000; Filipovic and Zecevic, 2008; Emery, 2010; Monaco et al., 2012).

EFFECT OF SHH ON Olig2 EXPRESSION

OLs specification and differentiation can be modulated by intrinsic factors, such as Olig2, but also by extrinsic cues. One of these extrinsic factors is Shh, a potent morphogen secreted early during development by the notochord and floor plate and later on by progenitor cells in ventral (Pringle et al., 1996; Orentas et al., 1999) and dorsal telencephalic regions both in rodents (Gulacsi and Lillien, 2003; Komada et al., 2008) and in humans (Mo and Zecevic, 2009). Modulation of transcription factors expression,

such as Olig1 and Olig2, by Shh is both necessary and sufficient to regulate initial oligodendrogenesis at the ventral telencephalon (Lu et al., 2000; Nery et al., 2001; Cai et al., 2005; Ligon et al., 2006; Petryniak et al., 2007). Later in development, OLs are generated in dorsal cortical regions in a process often considered to be Shh-independent (Nery et al., 2001; Cai et al., 2005; Vallstedt et al., 2005; Richardson et al., 2006). However, several studies in mice reported that Shh receptors, Patched1 and Smoothened, are also expressed in dorsal cortical progenitor cells, and respond to Shh treatment by adopting OLs fate (Nery et al., 2001; Murray et al., 2002). Moreover, loss of function models of Shh and Olig1 and 2 demonstrated the importance of these extrinsic and intrinsic factors in regulation of ventrally and dorsally OLs derived populations, both in spinal cord and in forebrain (Lu et al., 2000; Kessaris et al., 2001, 2006; Fuccillo et al., 2006)

In contrast to the numerous reports on animal models, the effect of Shh signaling on OLs genesis in the human fetal brain remains elusive. Expression of Shh has been reported in human embryonic ventral spinal cord and mesencephalon (Hajihosseini et al., 1996; Orentas et al., 1999), and Shh mutation is related to brain defects such as holoprosencephaly or cyclopia (Schell-Apacik et al., 2003). Experimentally, these defects can be induced by the selective Shh inhibitor cyclopamine, a steroidal alkaloid known to interrupt Shh signaling by binding to its co-receptor Smoothened (Incardona et al., 1998). Previously, we reported that RGCs, isolated from human fetal forebrain at mid-term (20 gw), contain Shh and its receptors, Patched1 and Smoothened (Mo and Zecevic, 2009). Additionally, our unpublished results also demonstrate elements of a Gli signaling pathway in the human fetal cortical cultures (Radonjić et al., under revision). Notably, the capability of human RGC *in vitro* to generate cells of OL lineage can be modulated by Shh treatment (Mo and Zecevic, 2009). It would be particularly important to better understand the effect of Shh on dorsal oligodendrogenesis which probably plays a more significant role in the large human forebrain than in much smaller rodent brains.

We studied the effect of Shh treatment on Olig2 expression in mixed dissociated cultures obtained from human fetal cerebral cortex in four fetal brains (14, 17, 18, and 19 gw) (**Figure 3A**). Although we observed a trend of Shh-induced increase in the number of Olig2$^+$ cells with progressively older cases, these values did not reach significance level. We, thus, combined four specimens together and compared them with controls (non Shh treated cultures). Treatment of cortical mixed cultures with Shh resulted in a significant increase in the number of Olig2$^+$ cells, whereas treatment with Shh inhibitor, cyclopamine, did not affect the percentage of Olig2$^+$ cells (**Figure 3A**). In order to demonstrate that the effect of Shh on expression of Olig2 is specific, we also treated mixed cultures with both Shh and cyclopamine. This combined treatment did not change the number of Olig2$^+$ cells compared to the control, and it inhibited the increase of Olig2$^+$ cells produced by the treatment with Shh alone (**Figure 3A**).

Similar results were observed in RGC cultures by Real-time PCR ($n = 4$; 14, 17, 17, and 18 gw). Levels of Olig2 mRNA were higher in Shh-treated cultures but not in cyclopamine-treated cultures compared to control (**Figure 3B**). The finding that blocking endogenous Shh did not produce an effect, suggests

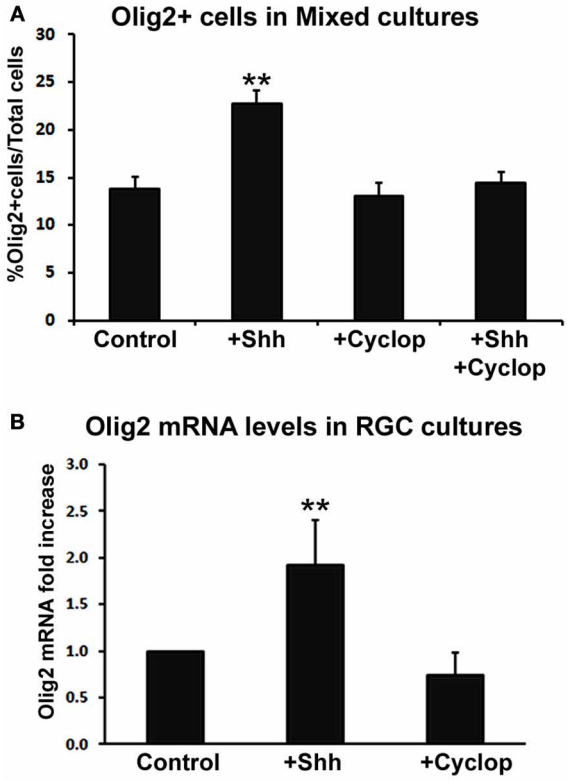

FIGURE 3 | The effect of Shh signaling on Olig2 expression *in vitro*. (A) Treatment with exogenous Shh significantly increased the percentage of Olig2$^+$ cells from total cells in human cortical mixed cell cultures, whereas either cyclopamine or cyclopamine/Shh treatment did not show any effect ($n = 4$; 14, 17, 18, and 19 gw mixed cultures). Data are presented as mean ± s.e.m. $^{**}p < 0.01$ vs. control. **(B)** Shh signaling modulated Olig2 mRNA levels in RGC cultures ($n = 4$; 14, two cases of 17 and 18 gw); cyclopamine treatment did not affect Olig2 mRNA levels. Data are presented as mean ± standard deviation. $^{**}p < 0.01$ vs. control.

an additional Shh-independent regulation of Olig2 expression *in vitro*. This is consistent with a previous report on different *in vitro* and *in vivo* requirements for Shh in oligodendrogenesis (Nery et al., 2001). A possible explanation is that *in vivo* Shh is necessary to overcome a constitutive inhibition for oligodendrogenesis that does not exist *in vitro* (Nery et al., 2001; Jakovcevski et al., 2009). Besides Shh, other factors such as bFGF (basic fibroblast growth factor), are implicated in OLs development (Kessaris et al., 2004; Furusho et al., 2011). bFGF and its receptor FGFR1 are constitutively expressed in astroglia and neurons in the cortex (Leadbeater et al., 2006) and bFGF acts synergistically with Shh inducing the expression of Olig2 and the generation of OLs from cortical progenitors *in vitro* (Kessaris et al., 2004). Hence, the difference in response to pharmacological treatments observed here may be due to the different endogenous expression levels of these molecules depending on the age and the brain region studied.

SHH PROMOTES THE GENERATION OF OPC FROM RGC AND PRESERVES THEM IN UNDIFFERENTIATED STATE

Animal studies showed that Shh increases the number of Olig2 progenitors probably by its well established function as

a promoter of progenitor proliferation (Rowitch et al., 1999; Amankulor et al., 2009; Ferent et al., 2013). We analyzed the effect of Shh treatment on proliferation of Olig2 progenitors in mixed cortical cultures using proliferation marker Ki67 (**Figure 4A**). After 14 DIV, Shh treatment (**Figure 2A**) increased the number of cortical proliferative Olig2$^+$cells (**Figure 4B**). In mixed cultures treated with cyclopamine, or with combined Shh and cyclopamine, there was no change in the number of proliferating Olig2$^+$ cells compared to the control (**Figure 4B**).

Next, we assessed the effect of Shh on early specification of cortical Olig2$^+$ progenitors analyzing the percentages of neurons (Olig2$^+$/MAP2$^+$), RGCs (Olig2$^+$/vimentin$^+$), early OPCs (Olig2$^+$/PDGFRα$^+$) and late OPCs (Olig2$^+$/O4$^+$) within the Olig2$^+$ cell population (normalized to 100% in the chart, **Figure 4C**). We observed that exogenous Shh induced an increase of the number of early OPCs (Olig2$^+$/PDGFRα$^+$) by 9% (control, 26% ± 3; Shh, 35% ± 2) at the expense of RGC (Olig2$^+$/vimentin$^+$) which decreased by 10% (control, 38% ± 4; Shh, 28% ± 2) (**Figure 4C**). Treatment with cyclopamine or combined cyclopamine and Shh did not show significant difference in the number of Olig2$^+$/ PDGFRα$^+$ cells and Olig2$^+$/vimentin$^+$ compared to control. The percentages of Olig2 expression in either MAP2$^+$ neurons or O4$^+$ late OPCs did not change among the treatments in 17 gw cortical mixed cultures, indicating that Shh might specifically target early OPCs (**Figure 4C**).

Comparable results were obtained by Real-Time PCR analysis from 14, 17, and 18 gw RGC cultures. Parallel to the increase in Olig2 mRNA levels (Control, 1 ± 0; Shh, 1.7 ± 0.3; Fold increase mean ± standard deviation), we observed that Shh treatment also enhances mRNA levels of PDGFRα (Control, 1 ± 0; Shh, 1.3 ± 0.01). On the contrary, cyclopamine treatment resulted in a significant decrease in mRNA levels of PDGFRα (0.5 ± 0.2) as well as in the mRNA levels of Olig2 (0.7 ± 0.3) in 2 of the 3 cases analyzed.

Thus, we showed here that Shh induced an increase in the number of proliferating Olig2$^+$ cells as well as an increase in the total number of PDGFRα$^+$/Olig2$^+$early OPCs, most likely at the expense of multipotent RG progenitors. Taking into consideration the low number of Olig2$^+$/O4$^+$ cells obtained in our cultures, it is difficult to assess the effect of Shh on differentiation of Olig2$^+$ progenitors further into oligodendroglial lineage at the stages studied here.

WHICH CELLS IN OL LINEAGE ARE THE MOST RESPONSIVE TO SHH?

To further clarify which progenitor cell type contributes the most to Shh response, we did a pilot study sorting two distinct cell populations from two fetal brains (14 and 17 gw): enriched RGC cultures (CD15 immunosorted cells) and OPC cultures (CD140a immunosorted cells). To confirm the identity of isolated cells, we performed immunostaining 24 h after isolation. For RGC that are CD15$^+$, we used anti-BLBP antibody and for CD140a$^+$ cells we used anti-PDGFRα antibody (**Figure 5A**). In order to also assess a possible differential induction of Olig2 along OL lineage, both populations of sorted cells were then cultured for 18DIV with three distinct media that promote differentiation into different maturation stages of OLs lineage (**Figure 5A**): (a) control medium supplemented with B27 (B27), (b) medium supplemented with N2 and PDGFaa, a potent promotor of OPC proliferation (PDGF) (Hu et al., 2012), and (c) medium initially supplemented with N2 and PDGF and afterwards with NT3 and T3 (NT3+T3) to obtain late OPCs/early OLs (Dietrich et al., 2002; Dugas et al., 2006; Neri et al., 2010; Cui et al., 2012). We tested the effect of the three different media in RGC cultures, and as expected, PDGF medium produced the highest number of PDGFRα$^+$ cells (**Figure 5B**). In the NT3+T3 medium, where cells were exposed to PDGFaa for 2 days, we observed a significant increase in the number of PDGFRα$^+$ cells compared to the control, B27 medium. The highest levels of O4$^+$ cells were observed in NT3+T3 maturation medium (**Figure 5B**). These results confirmed the effects of PDGFaa and NT3+T3 on OPC proliferation and maturation respectively.

We next showed that in RGC cultures Olig2 protein levels increased after the Shh treatment regardless of the culture medium, while no changes were observed in OPC cultures (**Figure 5C**). This difference suggests that RGC progenitors are more susceptible to Shh and increase their Olig2 content in response to Shh treatment. The highest level of Olig2 after Shh treatment was demonstrated in PDGF medium (**Figure 5C**). Under these conditions the number of early OPCs (PDGFRα$^+$) was also the highest (**Figure 5B**). This result indicates that Shh and PDGF might synergistically promote the differentiation of RGCs into OPCs, through the increase in the expression of Olig2. The lack of induction in OPC cultures might indicate that once cells are committed to OL fate, they do not upregulate Olig2 in response to Shh. Similar *in vitro* results were reported for mouse embryonic stem cells. Treatment with Shh induced Olig2 expression in progenitor cells until oligodendrogenesis starts, but Shh was not required for the differentiation into later stages of OL lineage (Du et al., 2006). Similarly, in the spinal cord Shh also promotes proliferation of selected CNS precursors and blocked their differentiation (Rowitch et al., 1999).

SUBCELLULAR LOCALIZATION AND FUNCTION OF Olig2 IN HUMAN DEVELOPING OLs

We observed that Olig2 expression was generally nuclear in various cell types, consistent with its role as a transcription factor. However, with the progression of OLs development, Olig2 can also be observed in the soma of the cells. In late OPCs, labeled with O4 antibody, Olig2 is observed in the increasingly ramified cell processes in contrast to a restricted nuclear localization in early OPCs (**Figure 6A**). This finding supports our previous results on cellular localization of Olig2 in the human developing brain (Jakovcevski and Zecevic, 2005; Jakovcevski et al., 2009). The control of subcellular localization of transcription factors is considered to represent a regulatory mechanism of many signaling pathways (Ziegler and Ghosh, 2005). Thus, different subcellular localization of Olig2 at different OL maturation stages might be due to a different requirement of this transcription factor during OLs development. Recent reports on conditional knock out animals demonstrate that deletion of Olig2 in OLs progenitors (CNP$^+$cells) dramatically reduces differentiation into OLs, whereas Olig2 ablation in mature OLs (PLP$^+$) enhances

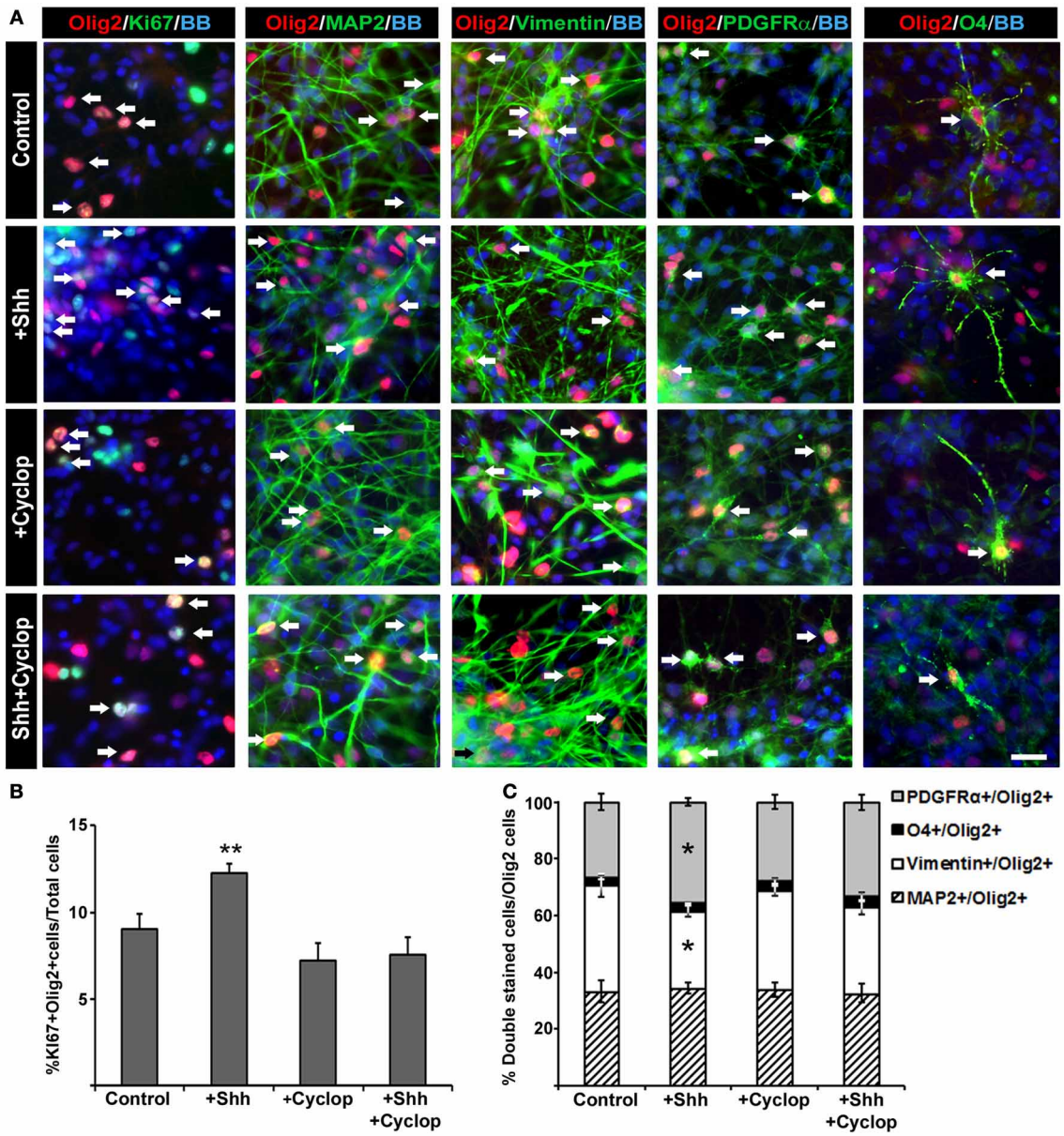

FIGURE 4 | The effect of Shh signaling on proliferation and differentiation of Olig2$^+$ cells *in vitro*. **(A)** In mixed cell cultures at midgestation, Olig2$^+$ cells (red) were co-labeled with Ki67 (cell proliferation marker), MAP2 (neuronal marker), vimentin (RGC marker), PDGFRα(early OPC marker) and O4 (late OPC marker) in green. Nuclear staining with bisbenzimide (BB) in blue. Scale bar: 20 μm. **(B)** Proliferation of Olig2$^+$ cells, estimated by double-labeling with Ki67, increased after Shh treatment, but did not change after either cyclopamine or combined cyclopamine and Shh treatments. **(C)** The composition of Olig2$^+$ cell population in 17 gw cortical mixed cell cultures changed with manipulation of Shh signaling. Graph shows the percentage of Olig2$^+$ neurons (Olig2$^+$/MAP2$^+$), RGCs (Olig2$^+$/Vimentin$^+$) or early (Olig2$^+$/PDGFRα$^+$) and late (Olig2$^+$/O4$^+$) OPCs out of the total number of Olig2$^+$ cells; note the selective increase of Olig2/PDGFRα$^+$ cells and the proportional decrease of Olig2/vimentin$^+$ cells after Shh treatment. Data are presented as mean ± s.e.m. $^*p < 0.05$, $^{**}p < 0.01$ vs. control.

maturation and increases myelination (Mei et al., 2013). This key role of Olig2 in OL specification and maturation might clarify why the transplantation of Olig2-overexpressing neural stem cells into demyelinating lesions significantly enhances the generation of OL but has only modest effects on remyelination (Copray et al., 2006; Maire et al., 2009; Kim et al., 2011; Hu et al., 2012).

CONCLUDING REMARKS

Numerous developmental studies tried to clarify regulatory mechanisms of Shh and Olig2 on oligodendrogenesis in animal models, but similar studies have not been done in developing human brain. We demonstrated that Shh promotes an expansion of human cortical progenitors, similar to its effect in rodents. Shh also influences progenitor fate by enlarging the pool of

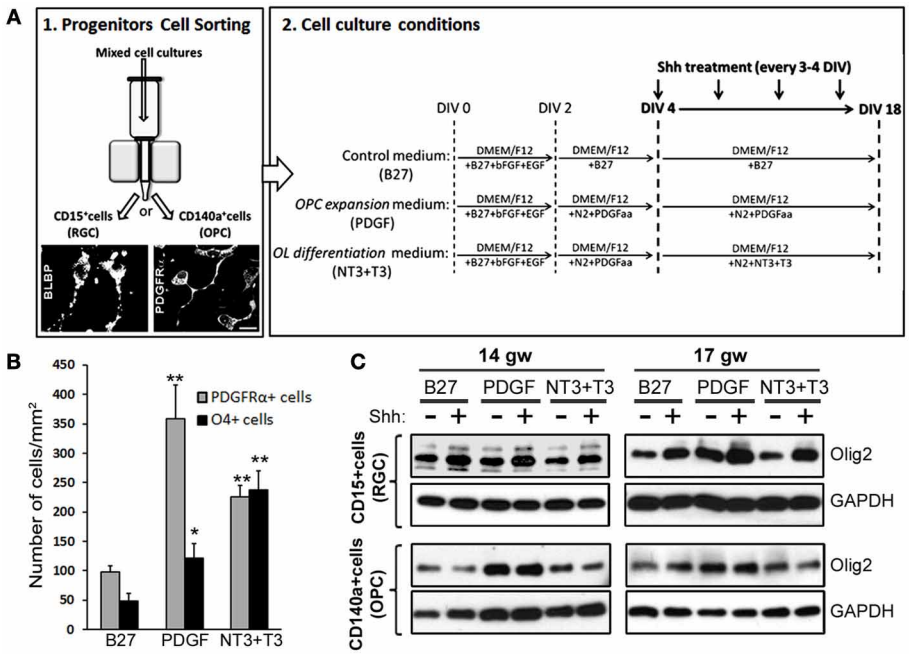

FIGURE 5 | The effect of Shh on selected cortical progenitors obtained from 14 and 17 gw cerebral cortex. (A) Scheme of the protocol for the immunosorting of human RGCs ($CD15^+$) and OPCs ($CD140a^+$); Representative image of $BLBP^+$ RGCs and $PDGFR\alpha^+$ early OPCs, 24 h after immunosorting. Sorted cells were cultured in three different media: Control medium (B27), PDGF and NT3+T3 media, and treated with Shh from 4-18 DIV. **(B)** Number of $PDGFR\alpha^+$ cells and $O4^+$ cells per surface area in 17 gw RGCs cultures with control (B27), OPC expansion (PDGF) and OLs differentiation (NT3+T3) medium. **(C)** Protein levels of Olig2 measured by Western blot in RGCs and OPCs cultures with and without Shh treatment in the three different media. The increase levels of Olig2 protein was only observed in RGC cultures, being specially high in OPC expansion medium, and not in OPC cultures; GAPDH-loading control. Data are presented as mean ± s.e.m. $^*p < 0.05$, $^{**}p < 0.01$ vs. control.

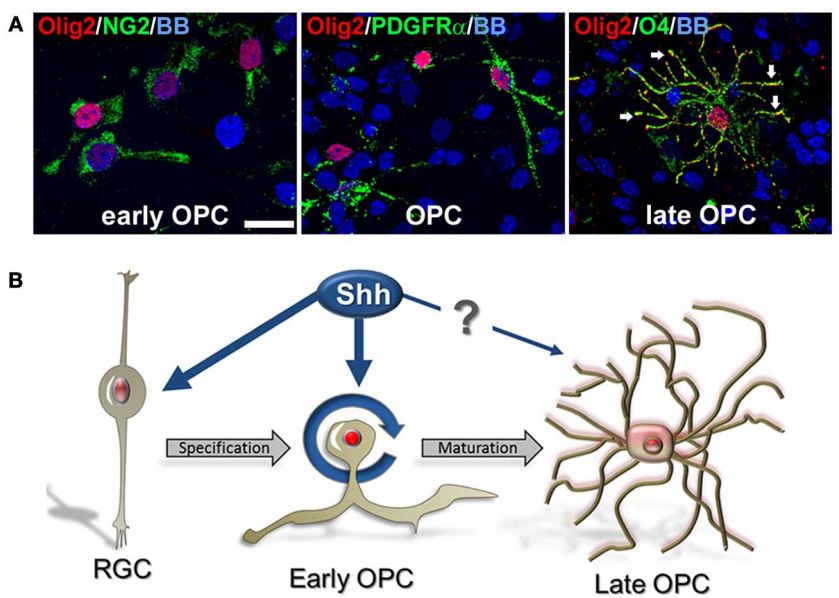

FIGURE 6 | Cellular distribution of Olig2 in different cell types. (A) Nuclear expression in early OPCs $NG2^+$ (1 DIV) and $PDGFR\alpha^+$ (14 DIV) cells and cytosolic expression in $O4^+$ late OPCs/immature OLs (14 DIV) (arrows). Nuclear staining with bisbenzimide (BB) in blue. Scale bar: 20 μm. **(B)** Schematic presentation of the possible effect of Shh on Olig2 expression (red) along the OLs lineage in human fetal cortical cultures. Shh might actively participate in proliferation and specification of early OPCs from RGCs in the cerebral cortex. However, the effect of Shh and Olig2 in the differentiation of early OPCs to mature OLs might not be required.

Olig2$^+$/PDGFRα$^+$ early OPCs. Based on our results we propose that Shh might actively participate in proliferation and specification of early cortical OPCs from RGCs, but its role in the transition from early OPCs to mature OLs is still unclear (**Figure 6B**). Better understanding of Shh and Olig2 function in human OL development is a critical point in developing new therapeutic targets for demyelinating diseases.

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NMDA modulates oligodendrocyte differentiation of subventricular zone cells through PKC activation

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Multipotent cells from the juvenile subventricular zone (SVZ) possess the ability to differentiate into new neural cells. Depending on local signals, SVZ can generate new neurons, astrocytes, or oligodendrocytes. We previously demonstrated that activation of NMDA receptors in SVZ progenitors increases the rate of oligodendrocyte differentiation. Here we investigated the mechanisms involved in NMDA receptor-dependent differentiation. Using functional studies performed with the reporter gene luciferase we found that activation of NMDA receptor stimulates PKC. In turn, stimulation of PKC precedes the activation of NADPH oxidase (NOX) as demonstrated by translocation of the p67phox subunit to the cellular membrane. We propose that NOX2 is involved in the transduction of the signal from NMDA receptors through PKC activation as the inhibitor gp91 reduced their pro-differentiation effect. In addition, our data and that from other groups suggest that signaling through the NMDA receptor/PKC/NOX2 cascade generates ROS that activate the PI3/mTOR pathway and finally leads to the generation of new oligodendrocytes.

Keywords: NMDA, PKC, NADPH oxidase, reactive oxygen species, multipotent cell differentiation

INTRODUCTION

Extracellular glutamate is one of the most important neurotransmitters and neuromodulators of the CNS. It participates in different functions such as synaptic plasticity, LTP and learning, glia-neuron communication, or transduction of sensory input at the periphery and nociceptive pathway (for a review, see Fellin and Carmignoto, 2004; Carozzi et al., 2008; Niswender and Conn, 2010). In addition, at high concentrations glutamate can contribute to excitotoxicity and apoptotic cell death. Glutamate exerts its function through two different subfamily of receptors, metabotropic and ionotropic, which are expressed in neurons, astrocytes, and oligodendrocytes as well (Matute et al., 2006; Matute, 2011a,b), but less is known about its role in modulating adult neurogenesis and gliogenesis.

Extracellular glutamate activates various ionotropic receptors. Depending on their molecular structure and pharmacology, they can be divided in AMPA (GluA1–GluA4), kainate (GluK1–GluK5), NMDA (GluN1, GluN2A–GluN2D, GluN3A-B) receptor subtypes. The receptors are heteromers and form ion channels, with fast response times, and generate an overall increase in Ca^{++} along with influx of Na^{+}. All functional NMDA receptors have the GluN1 subunit associated with other subunits. Moreover, NMDA receptor activation requires the co-activation of glycine (Kleckner and Dingledine, 1988), and the receptors bearing GluN3 subunits bear more affinity for this amino acid (Yao and Mayer, 2006).

Excessive stimulation of ionotropic glutamate receptors can trigger excitotoxic cell death (Matute, 2011b). For this reason the pharmacology of ionotropic glutamate receptors is important to develop new drugs for the treatment of neurological disorders such as brain ischemia, epilepsy, and more recently demyelinating diseases (Kim et al., 2011).

Depending on the stage of maturation, oligodendrocyte precursor cells (OPCs) and mature oligodendrocytes possess various combinations of glutamate receptor subtypes. Metabotropic receptors are highly expressed in OPCs but less in mature oligodendrocytes. This also would explain the higher sensitivity of OPCs to hypoxia with respect to mature oligodendrocytes (Deng et al., 2004). All mGluRs are expressed in OPCs but only receptors in group I are able to protect OPCs from excitotoxic death, by preventing ROS generation (Deng et al., 2004; Luyt et al., 2006). In turn, activation of mGluR can counteract the effects of NMDA receptors in oligodendrocyte differentiation (Cavaliere et al., 2012).

The exact role of NMDA receptors during oligodendrocyte maturation is unclear (Matute et al., 2006; Cavaliere et al., 2012). There is evidence suggesting that NMDA receptor signaling in oligodendrocyte progenitors is not required for oligodendrogenesis and myelination (De Biase et al., 2011). Thus, genetic deletion of structural GluN1 from OPCs does not result in changes in proliferation, differentiation, or myelination. More recently, we observed that strong activation of NMDA receptors in oligodendrocytes precursor cells derived from subventricular zone (SVZ) multipotent cells increases their differentiation and myelination rate *in vitro*. This data was confirmed using NMDA receptor antagonists or specific knockdown of GluN1 by RNA interference in OPCs which prevented differentiation induced by NMDA (Li et al., 2013).

Here we studied the intracellular pathway primed by NMDA receptor activation leading to oligodendrocyte differentiation in

a culture of rat SVZ neurospheres. We observed that activation of NMDA receptors stimulates differentiation via PKC/NADPH oxidase (NOX)-dependent ROS generation.

MATERIALS AND METHODS

NEUROSPHERE CULTURE

Cultures were prepared from 4 to 7-day-old Sprague-Dawley rat pups. The SVZ was isolated and minced with a McIllwain tissue chopper. SVZ tissue from two to three brains was digested for 10 min at 37°C in 5 ml of trypsin/EDTA (Sigma, Madrid, Spain). Digestion was stopped by adding an equal volume of trypsin inhibitor and 0.01% DNAse I (both from Sigma, Madrid, Spain) for 5 min at room temperature. The cell suspension was centrifuged for 10 min at 600 × *g* and the pellet mechanically dissociated 25 times in NeuroCult medium (Stem Cell Inc., Grenoble, France) using a glass Pasteur pipette and 20 times using 1 ml pipette tips. The cells that remained in suspension were decanted and the single cell suspension counted using the Neubauer method. Cells were seeded in proliferation medium (NeuroCult with 10% neural stem cell factors from Stem Cell Inc., 2 mM glutamine, penicillin/streptomycin mix, 20 ng/ml EGF (Promega, Madrid, Spain), 10 ng/ml bFGF (Promega), 10 ng/ml PEDF (Millipore, Madrid, Spain) at a density of 10^4 cells/cm^2 and cultivated in suspension for 7 days at 37°C, 5% CO2. EGF, bFGF, and PEDF were added fresh every 2–3 days.

OLIGODENDROCYTE DIFFERENTIATION

After 7 DIV (days *in vitro*), cells were aggregated as neurospheres. The neurospheres were maintained for 3 days in oligodendrocyte differentiation medium (ODM) composed of DMEM with 4.5 mg/ml glucose and sodium pyruvate (Gibco, Barcelona, Spain), SATO (100 μg/ml BSA, 100 μg/ml transferrin, 16 μg/ml putrescine, 40 ng/ml thyroxine, 30 ng/ml tri-iodothryronine, 60 ng/ml progesterone, 40 ng/ml selenium, all of from Sigma), 6.3 mg/ml *N*-acetyl-cysteine (Sigma), 0.5 mg/ml insulin (Sigma), 1 μg/ml CNTF (Peprotech, London, UK), and 10 μg/ml NT3 (Peprotech). This step was considered to be the pre-commitment stage before oligodendrocyte differentiation. After 3 DIV, floating

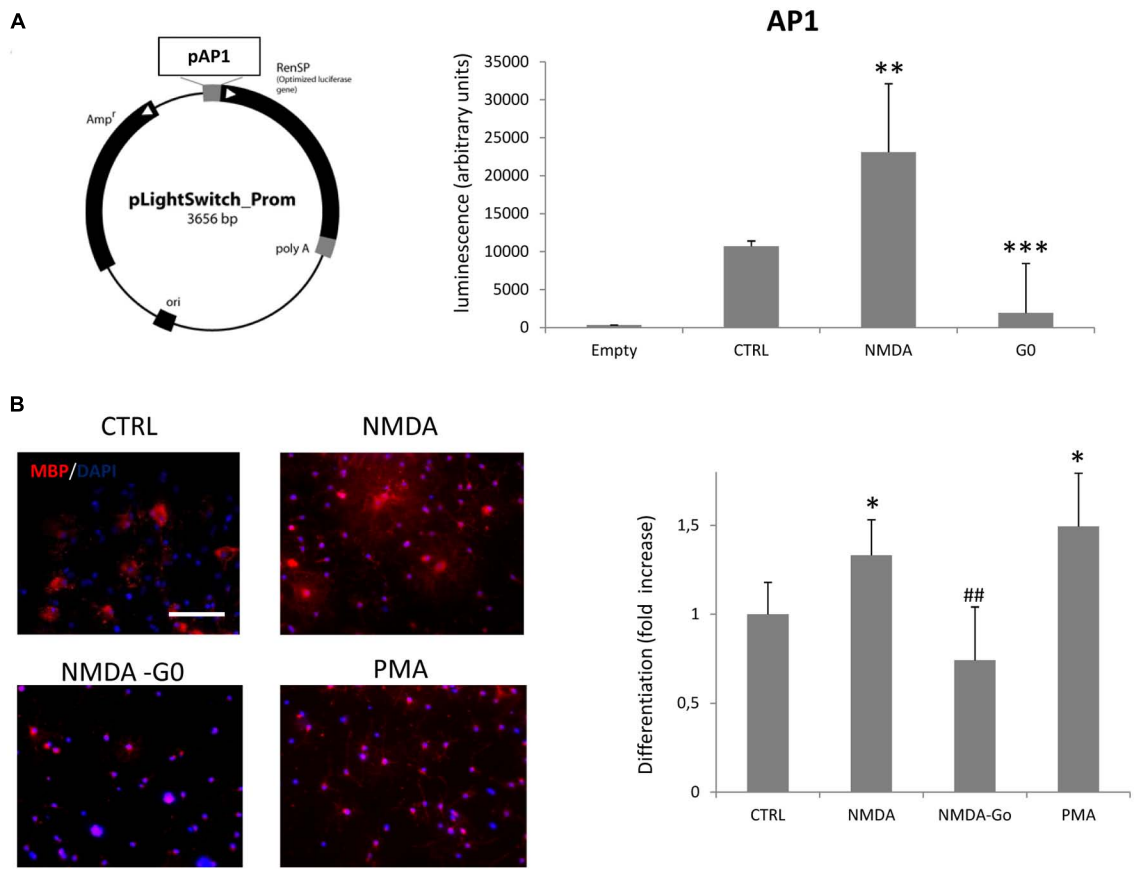

FIGURE 1 | Activation of NMDA receptors in neurospheres induces PKC activation and oligodendrocyte differentiation.(A) Pre-differentiated neurospheres were dissociated and transfected with 3 μg of pAP1-LightSwitch. Cells were differentiated for 12 h in the presence of 100 μM NMDA or 100 nM G0 6893. Empty vector, without AP1 promoter, was transfected as a negative control. **(B)** Neurospheres were differentiated to oligodendrocyte for 3–5 days in the presence of 100 μM NMDA, 100 nM G0 6983, or 10 nM PMA. Cells were fixed, immunostained with anti-MBP (red) and counterstained with DAPI (blue; left panel). Differentiation was evaluated as the ratio between MBP positive cells vs. total cells counterstained with DAPI and expressed as a fold increase respect to the control (bar graph). Counts represent means ± SEM ($n = 4$ independent experiments, five fields in each). ***$p < 0.001$, **$p < 0.01$ and *$p < 0.005$ vs. CTRL, ##$p < 0.01$ vs. 100 μM NMDA. Scale bar = 100 μm.

neurospheres were allowed to attach to cover slips previously treated with poly-ornithine in ODM and differentiated for 1–10 DIV in the presence of different compounds (G0 6983 from Tocris, Madrid, Spain; gp91 from Anaspec, Liege, Belgium). Differentiation was evaluated by immunofluorescence as a ratio of myelin basic protein (MBP, from R&D System, Madrid, Spain; used at 1:1000) positive cells determined by immunofluorescence to total nuclei determined by staining with DAPI. All experiments with NMDA were performed in the presence of 100 μM glycine.

ANIMAL CARE

All experiments were approved by the local Animal Care Committee of the University of Basque Country (Spain) Animal Ethics committee, as relevant, following European Communities Council Directive of 22 September 2010 (2010/63/EU). Every possible effort was made to minimize animal suffering and the number of animals used.

IMMUNOCYTOCHEMISTRY

Cell cultures on cover slips were fixed in 4% paraformaldehyde and permeabilized with 0.05% Triton and 5% normal goat serum in phosphate-buffered saline (PBS). Cells were incubated with MBP primary antibody at 1:1000 dilution for 2 h at room temperature and then washed three times with 0.05% Triton in PBS. All secondary antibodies at 1:200 were added and incubated for 1 h in the dark at room temperature (Molecular Probes, Barcelona, Spain). After three washes with 0.05% Triton in PBS, cells cultures were stained for 1 min at room temperature with DAPI and further washed with PBS. Finally, cover slips were mounted with Glycergel (Dako, Barcelona, Spain) and examined by fluorescence using the Apotome system (Zeiss, Goettingen, Germany).

mRNA EXTRACTION AND QUANTITATIVE RT-PCR

Total RNA from neurospheres (approximately 5000 neurospheres) after pre-differentiation was extracted with a commercial kit (Life technologies-Ambion, Madrid, Spain). The quality of the total RNA was determined by agarose gel electrophoresis and 1 μg was reverse transcribed at 60°C for 60 min using Superscript SSIII (Invitrogen, Madrid, Spain). A 2 μl aliquot of each mRNA were used for real-time quantitative PCR. Primers specific for rat NOX1, NOX2, and NOX3 were designed having the following sequence; NOX1fwd: TAC GAA GTG GCT GTA CTG GTT G, NOX1rev: CTC CCA AAG GAG GTT TTC TGT; NOX2fwd: GGT TCC AGT GCG TGT TGC T, NOX2rev: TCT TAT GGA AAG TAA GGT TCC TGT CC; NOX4fwd: GGA AGT CCA TTT GAG GAG TCA T, NOX4rev: TGG ATG TTC ACA AAG TCA GGT C. GAPDH and Hprt1 primers were used for normalization and the standard curves were determined using PrimerExpress software (Applied Biosystems, Madrid, Spain). Real-time quantitative PCR reactions were performed for 40 cycles at 60°C in a BioRad CFX96 amplification system (BioRad, Madrid, Spain).

LUMINESCENT ASSAY FOR PKC ACTIVITY

Pre-differentiated neurospheres were disgregated with Accutase (Sigma) and transfected by electroporation (Amaxa-Lonza, Madrid, Spain) with 3 μg of the plasmid pAP1-LightSwitch (SwitchGear genomics, Menlo Park, CA, USA). Cells were lysed at different times in the presence of the substrate for luciferase Luminescence produced by the luciferase reaction (luciferin + ATP + luciferase ⟶ AMP+light) was quantified with a luminometer (Sinergy HT by Biotek, Potton, Bedfordshire, UK)

STATISTICAL ANALYSIS

Data are presented as means ± SEM. Statistical analysis was carried out with the Student t test and, in all instances, at least a value of $p < 0.05$ was considered significant.

RESULTS

Our previous results demonstrated that overstimulation of NMDA receptors of SVZ multipotent cells induced an increase of oligodendrocyte differentiation through NOX-dependent generation of ROS (Cavaliere et al., 2012). Here we hypothesize that NOX activation is induced by PKC activation. After proliferation and the pre-differentiation protocol (see Materials and Methods) we transfected pre-differentiated neurospheres with a plasmid (pLightSwitch) carrying the reporter gene luciferase under the control of the PKC-activated promoter AP1. In cells transfected with pLightSwitch, if PKC becomes activated, expression from the AP1 promoter increases which results in increased luciferase activity. PKC activity can therefore be registered by monitoring luminescence intensity after the reaction with the substrate, luciferin. On monitoring luciferase activity over a time course of 12, 24, and 72 h after differentiation, we detected a maximal PKC activity at 12 h post transfection (data not shown). At this time point the treatment of neurospheres with 100 μM NMDA during differentiation increased the basal level of PKC activity by 2.15-fold (**Figure 1A**), while NMDA treatment in the presence of the PKC inhibitor G0 6983 almost completely inhibited its activity. To confirm the involvement of PKC in NMDA mediated oligodendrocyte differentiation we counted the number of MBP$^+$

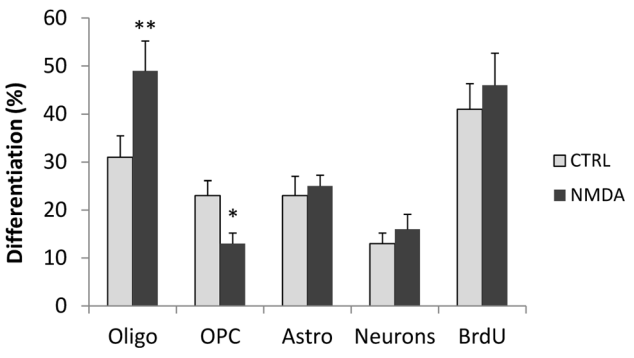

FIGURE 2 | Neurosphere cultures were differentiated for 3 days in the absence (CTRL) or presence of 100 μM NMDA. After 2 days, proliferating cells were primed with 10 μM BrdU for 24 h, fixed and stained with markers to label mature oligodendrocyte (O4), OPC (PDGFR/O4), astrocytes (GFAP$^+$/BrdU$^-$), neurons (βIII tubulin), and total proliferating cells (BrdU). Results are expressed as a percentage of stained cells vs. total cells, counterstained with DAPI. Counts represent means ± SEM ($n = 3$ independent experiments, five fields in each). $^{**}p < 0.01$, $^{*}p < 0.05$ vs. CTRL.

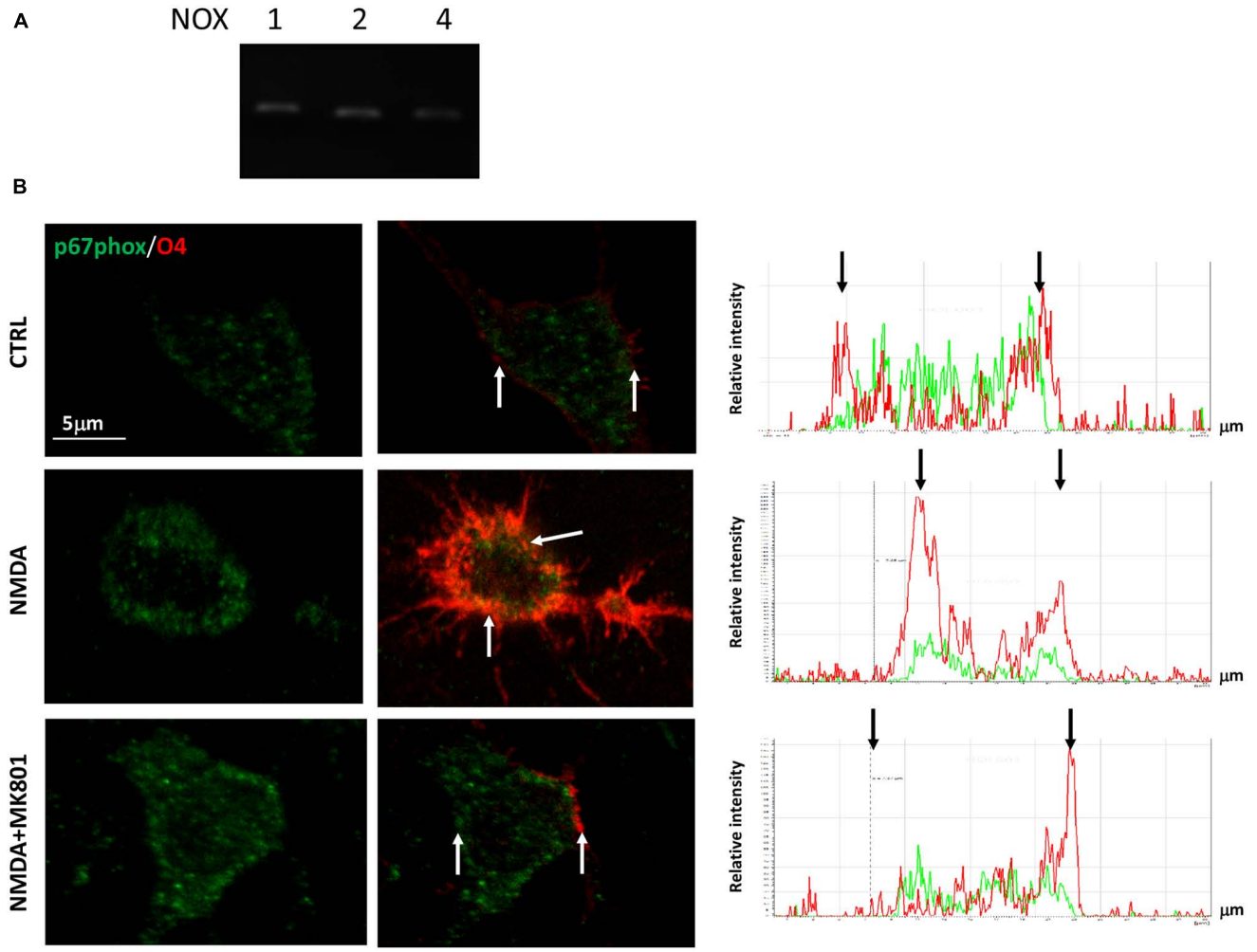

FIGURE 3 | (A) cDNA from pre-differentiated neurospheres was analyzed by quantitative RT-PCR with primers for NOX1–2 and 4. Quantification was calculated as $\Delta\Delta Dc_t$ relative values. Bar graphs represent the mean of four different experiments. **(B)** Neurospheres were differentiated to oligodendrocyte for three days in the presence of 100 μM NMDA and 50 μM MK801. Cells were fixed, immunostained with O4 (red) and p67phox (green). Bar = 5 μm. Analysis of colocalization shown on the right graphs was performed by the "Linescan" application of the LAS-AF software (Leica). Red and green peaks, belonging to O4 and p67 signal respectively, colocalized only in the NMDA treated cell, whereas in CTRL and NMDA+MK801 the p67 green signal is more dispersed along the cell body (black bar). White arrows point at cellular membrane sites corresponding to black arrows on the Linescan.

cells vs. the total cells counterstained with DAPI in the presence of NMDA alone or in conjunction with G0 6983. As previously observed (Cavaliere et al., 2012), NMDA stimulation increased the differentiation rate by 30%, and this effect was blocked by the PKC inhibitor G0 6983. As a positive control of PKC-dependent differentiation we used the PKC activator phorbol 12-myristate 13-acetate (PMA), which increased the basal differentiation by nearly 50% (**Figure 1B**).

In addition, we evaluated the effect of NMDA stimulation on the differentiation of neurons and astrocytes, as well as on the proportion of OPCs that did not differentiate into mature oligodendrocytes. Cell cultures were stained after 3 days of differentiation with antibodies to PDGF receptor (PDGFR), to label only OPCs and with O4 that label both OPCs and mature oligodendrocytes. Mature oligodendrocytes were only positive for O4 whereas OPCs were positive for both markers. Treatment of cells with NMDA during differentiation induced an increase in the number of differentiated oligodendrocyte, but a significative reduction on the OPCs number (**Figure 2**), demonstrating the effect of NMDA on differentiation from immature to mature oligodendrocyte. To evaluate astrocyte differentiation we labeled the proliferating cells with 10 μM BrdU and stained the cultures with anti-GFAP and anti-BrdU antibodies. GFAP is expressed in both proliferating SVZ progenitor cells and in non-proliferating mature astrocytes.To quantify mature astrocytes we counted only $GFAP^+/BrdU^-$ cells. No significant differences were found between control and NMDA treated cells. Likewise, no differences were found on the number of differentiated neurons as assessed with βIII tubulin staining, or in the total number of proliferating cells (BrdU+ cells; **Figure 2**).

In a previous study we demonstrated that NMDA-dependent oligodendrocyte differentiation occurred through the activation of NOX and the subsequent generation of intracellular ROS, which acted as a second messenger (Cavaliere et al., 2012). To establish the link between NMDA receptors, PKC and NOX activation, we first examined the expression profiles of the transcripts that encode the various NOXs expressed in the CNS (NOX1–2, 4) by quantitative RT-PCR. We found that all the NOXs examined are expressed in SVZ multipotent cells (**Figure 3A**). Furthermore, we observed that stimulation with NMDA (100 μM) during differentiation induced the traslocation of NOX subunit p67phox into the oligodendrocyte plasma membrane (Bedard and Krause, 2007), as demonstrated by confocal double immunofluorescence with O4 (**Figure 3B**). The use of the specific NMDA antagonist MK801 modulated the translocation, demonstrating the specificity of the NMDA effect. To get some insight as to which of the NOXs is involved in the modulation of oligodendrocyte differentiation we measured the differentiation rate after treatment of cells with NOX inhibitors. Gp91, which specifically inhibits NOX2, reverted the effect of NMDA during differentiation by 70% suggesting a pivotal role for NOX2 in oligodendrocyte differentiation (**Figure 4A**). The other general inhibitor, DPI, was toxic to the cells (data not shown). Finally, to further assess the involvement of the NMDA-PKC-NOX signaling cascade in modulating differentiation, we measured ROS generation during differentiation after stimulation with NMDA. We observed that activation of NMDA receptor induced a significant increase of ROS by 30% that is inhibited when the signaling in the NMDA-PKC-NOX pathway is blocked by the PKC inhibitor G06893 (**Figure 4B**).

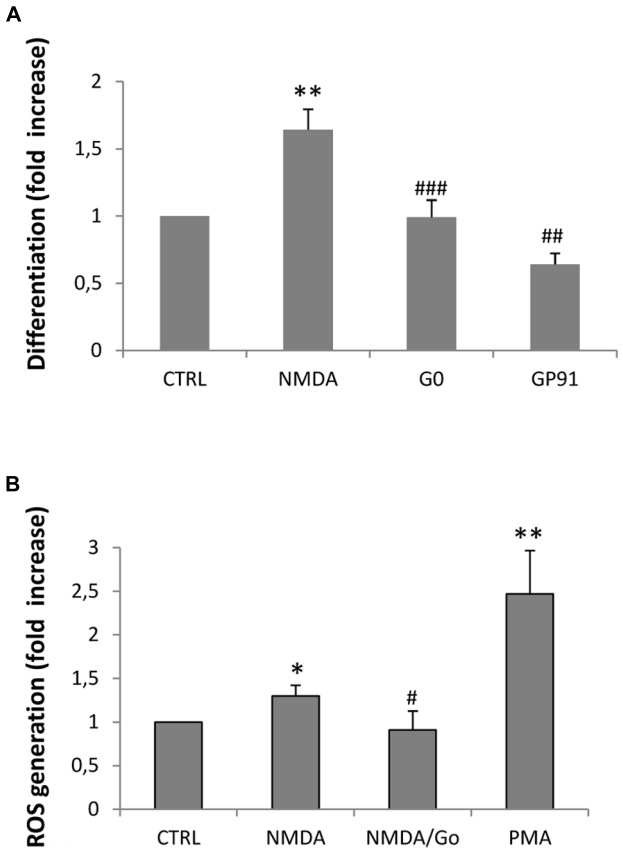

FIGURE 4 | (A) Neurospheres were differentiated to oligodendrocyte for 3–5 days in the presence of 100 μM NMDA, 100 nM G0 6983, or 1mM gp91. Cells were fixed, immunostained with anti-MBP and counterstained with DAPI. Differentiation was calculated as a ratio between MBP positive cells vs. total cells counterstained with DAPI and expressed as a fold increase respect to the control. Counts represent means ± SEM ($n = 4$ independent experiments, five fields in each). $^{**}p < 0.01$ vs. CTRL, $^{###}p < 0.001$ and $^{##}p < 0.01$ vs. 1 μM NMDA **(B)** ROS generation was calculated by luminescence after 3 days of differentiation in the presence of 100 μM NMDA and the PKC inhibitor G0 6983. The PKC activator PMA was used as a control for PKC-dependent ROS generation. Counts represent means ± SEM ($n = 4$ independent experiments, five fields in each). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. CTRL, $^{\#}p < 0.05$ vs. 1 μM NMDA.

DISCUSSION

In this study we provide evidence supporting the idea that NMDA stimulates oligodendrocyte differentiation via activation of PKC (see also **Figure 5**). Oligodendrocyte differentiation from SVZ multipotent stem cells is modulated by fine tuned regulation of NMDA receptor subtypes, especially by the expression of NR3 subunit (Cavaliere et al., 2012). SVZ multipotent cells express high levels of NR3 with high NR3/NR1 ratio during proliferation but after stimulating oligodendrocyte differentiation, the same ratio reverts with higher NR1 expression respect to NR3 (Cavaliere et al., 2012). This stoichiometry reveals a dominant negative role of the NR3 subunit which has a low affinity for glutamate but higher affinity for glycine (Yao and Mayer, 2006) resulting in a lack of NMDA receptor function during proliferation. In SVZ oligodendrocytes differentiate from type C cells (transit amplifying cells). Depending on the culture condition or environmental constraints, type C cells can typically differentiate into neuroblasts, or alternatively into OPCs. In turn, we found that NMDA stimulates the final differentiation from OPCs into mature oligodendrocytes. The effect of NMDA during oligodendrocyte differentiation looks cell specific since no significant differences were observed in neuronal and astrocytic differentiation.

As expected, overactivation of NMDA receptors can lead to activation of PKC. When transfected with the luciferase reporter gene, under the control of the AP1 promoter, differentiating oligodendrocytes showed increased luciferase expression when treated with NMDA (**Figure 1A**). These data were corroborated also by a negative modulation of the PKC inhibitor G0 6983 on oligodendrocyte differentiation (**Figure 1B**). Extensive stimulation of NMDA receptors induced the generation of ROS via NOX stimulation through the activation of PKC, which ultimately resulted in hippocampal neuronal death (Brennan et al., 2009). Similarly, during oligodendrocyte differentiation, PKC activation by overstimulation of NMDA receptors induces NOX activation. SVZ multipotent cells express similar levels of mRNA encoding NOX1, NOX2, and NOX4 (**Figure 2A**) and selective inhibition of NOX activity by apocyanin and gp91, the specific inhibitor of NOX2 in the presence of NMDA (but not by Dpi; Cavaliere et al., 2012 and **Figure 3B**) suggested that only NOX2 can be involved in oligodendrocyte differentiation. Unlike hippocampal neurons, the generation of NOX-dependent ROS

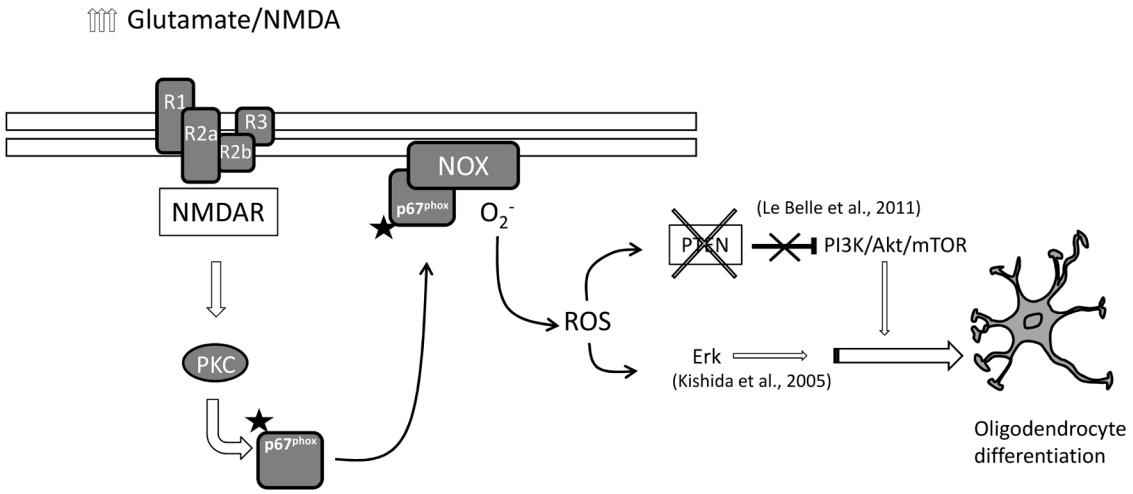

FIGURE 5 | Schematic representation of the hypothetical NMDA receptor-induced oligodendrocyte differentiation. High levels of extracellular glutamate trigger NMDA receptor responses which activate PKC/NOX/p67 signaling. Activated NOX generates intracellular ROS that in parallel can set off PI3K/mTOR and/or ERK pathways to induce oligodendrocyte differentiation.

did not induce oligodendrocyte death but acted as a second messenger to stimulate progenitor differentiation as observed by others (Le Belle et al., 2011; Li et al., 2013). Furthermore treatment with NMDA during oligodendrocyte differentiation generates an increase in ROS production in parallel with differentiation, which is modulated by apocynin and gp91 (Cavaliere et al., 2012 and **Figure 3**) and the specific NMDA antagonist MK801.

CONCLUSION

Results obtained from our group and others provide evidence for a mechanism by which mild excitotoxic insults promote oligodendrocyte differentiation derived from SVZ multipotent cells. These insults elevate the levels of ROS naturally generated in multipotent and pluripotent cells which act as a second messenger that induces oligodendrocyte differentiation through a proposed dual pathway (detailed in **Figure 4**). In one case, tumor suppressor protein PTEN is inactivated (Coant et al., 2010; Le Belle et al., 2011) and thus releases the blockade of the PI3/Akt/mTOR pathway (Li et al., 2013). In parallel, higher ROS levels can directly activate ERK pathway and stimulate differentiation as previously described (Kishida et al., 2005). In addition, a third mechanism has been recently suggested by which NMDA receptor activation regulates OPC migration and further differentiation by coupling to and activating the Tiam1/Rac1 pathway which is in turn is activated by PKC (Xiao et al., 2013).

In summary, our results highlight a novel signaling pathway directed by glutamate that drives oligodendrocyte development from SVZ, and that can favor remyelination in demyelinating diseases.

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AUTHOR CONTRIBUTIONS

Fabio Cavaliere, performed, conceived and designed the experiments; Monica Benito-Muñoz, performed the experiments; Mitradas Panicker, designed the experiments; Carlos Matute, designed the experiments.

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The role of EGFR and ErbB family related proteins in the oligodendrocyte specification in germinal niches of the adult mammalian brain

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In the adult brain, multipotent progenitor cells have been identified in three areas: the ventricular-subventricular zone (VZ-SVZ), adjacent to the striatal wall of the lateral ventricles, the subgranular zone (SGZ), located at the dentate gyrus of the hippocampus and the subcallosal zone (SCZ), located between the corpus callosum and the CA1 and CA2 regions of the hippocampus. The neural progenitor cells of these regions express the epidermal growth factor receptor (EGFR, ErbB-1 or HER1). EGF, the most important ligand for the EGFR, is a potent mitogenic agent that stimulates proliferation, survival, migration and differentiation into the oligodendrocyte lineage. Other ErbB receptors also activate several intracellular pathways for oligodendrocyte specification, migration and survival. However, the specific downstream pathways related to oligodendrogenesis and the hierarchic interaction among intracellular signaling cascades is not well-known. We summarize the current data regarding the role of EGFR and ErbB family signaling on neural stem cells and the downstream cascades involved in oligodendrogenesis in the neurogenic niches of the adult brain. Understanding the mechanisms that regulate proliferation, differentiation, migration of oligodendrocytes and myelination is of critical importance for the field of neurobiology and constitutes a crucial step in the design of stem-cell-based therapies for demyelinating diseases.

Keywords: neural stem cell, oligodendrocyte, epidermal growth factor, platelet-derived growth factor, myelin, NG2 glia

INTRODUCTION

Neurogenesis, the production of new neurons, occurs throughout the life of adult mammals. This finding ended the dogma that prevailed since the times of Santiago Ramón y Cajal (at the beginning of the 20th century). He declared that neurons cannot be generated in adult brain and that astrocytes only provided support for brain parenchyma and neurons; therefore, the first discoveries of neuronal mitosis in adult brain were not well-accepted by the scientific community. To date, adult neurogenesis has been fully demonstrated in the adult mammalian brain (Doetsch et al., 1999b; Gritti et al., 1999; Sanai et al., 2004; Kriegstein and Alvarez-Buylla, 2009) and some evidence has been observed in the human brain (Eriksson et al., 1998; Sanai et al., 2004; Quinones-Hinojosa et al., 2006).

Adult neurogenesis has been extensively studied in two proliferative niches of the adult brain: the ventricular-subventricular zone (VZ-SVZ), adjacent to the striatal wall of the lateral ventricles (Kriegstein and Alvarez-Buylla, 2009) and the subgranular zone (SGZ), located at the dentate gyrus of the hippocampus (Seri et al., 2004). Recently, a third proliferative region referred to as the subcallosal zone (SCZ) is located between the corpus callosum and dorsal hippocampus. In these three regions, germinal cells have been isolated which produce multi-potential cellular conglomerates (neurospheres) *in vitro*. When seeded on adherent substrates, these multipotent neurospheres can generate neurons and glial cells (Seri et al., 2006; Laskowski et al., 2007). Yet, only gliogenesis has been found in the SCZ *in vivo*. Remarkably, the primary neural progenitor of these three regions has astrocytic characteristics, such as: clear cytoplasm, gap junctions, condensed chromatin and multiple cytoplasmic ramifications. In addition, these multipotent cells express molecular markers related to the astrocytic lineage as: glial fibrillary acidic protein (GFAP), nestin, vimentin, astrocytic glutamate transporter (GLAST) or calcium binding protein S-100β. Further, these cells express receptors with intra-cellular domains of tyrosine kinase receptors, such as: platelet-derived growth factor alpha (PDGFRα), fibroblast growth factor (FGFR) and epidermal growth factor (EGFR) (Garcia-Verdugo et al., 1998; Doetsch et al., 2002; Jackson et al., 2006; Seth and Koul, 2008; Kriegstein and Alvarez-Buylla, 2009).

The EGFR and its main ligand, EGF, are some of the most important mitogens that also have significant effects on the survival, migration and differentiation rate of embryonic (Aguirre et al., 2007; Chong et al., 2008; Hu et al., 2010; Sinor-Anderson and Lillien, 2011) and adult neural precursors (Gonzalez-Perez and Alvarez-Buylla, 2011). Recent studies in the adult brain indicate that EGFR stimulates the proliferation of neural precursor cells and induces their differentiation toward oligodendrocyte lineages (Gonzalez-Perez et al., 2009; Aguirre et al., 2010; Gonzalez-Perez and Quinones-Hinojosa, 2010). However, the precise mechanisms by which the EGFR and its ligands can

induce such cellular differentiation are not entirely understood. The EGFR effects on differentiation seem to be driven by the heteromerization between the EGFR with other receptors (ErbB family receptor) and the subsequent signaling pathways activated by dimerization of them (Clark et al., 2012). This review summarizes the current information regarding the role of the ErbB signaling and the downstream signaling pathways involved in the oligodendrocyte specification in astrocytic neural stem cells. Additionally, we discuss neural disorders where the EGFR signaling in adult neural stem cells and oligodendrocyte precursors has been related to the pathophysiology of disease initiation and/or progression. Knowing the mechanisms that regulate proliferation, differentiation, migration and cell incorporation into the neural circuitry of stem-cell-derived oligodendrocyte progenitors is a crucial step in the design of therapies against demyelinating diseases.

NEUROGENIC REGIONS IN THE ADULT BRAIN

The largest neurogenic regions in the adult brain of mammals are the VZ-SVZ and the SGZ. A third region (SCZ) seems to retain predominantly glyogenic neural progenitors. In these three regions, there are neural stem cells with multi-potential and self-regenerative characteristics *in vitro*; however, the *in vivo* properties of these progenitors are quite different. The VZ-SVZ generates diverse interneurons, that incorporate into the olfactory bulb (Doetsch et al., 1999a), and oligodendrocytes that migrate to adjacent white matter (Menn et al., 2006). The SGZ only generates neurons that incorporate in the granular cells layer of the dentate gyrus (Seri et al., 2001; Abrous et al., 2005), while the SCZ appears to generate only oligodendrocyte precursors that migrate and settle in the corpus callosum (Seri et al., 2006).

VENTRICULAR-SUBVENTRICULAR ZONE (VZ-SVZ)

The adult VZ-SVZ is adjacent to the lateral walls of the lateral ventricles (**Figure 1**). This region has a complex cytoarchitecture delimitated by a layer of ependymal cells (E1- and E2-type cells) revisiting the ventricular cavity of the brain parenchyma (Mirzadeh et al., 2008). Adjacent to this layer of ependymal cells there are the type-B cells, which can be categorized in two subtypes: B1 cells and B2 cells. The B1 subtype are the *bona fide* neural stem cells (Doetsch et al., 1999b), while the B2 astrocytes appears to be supporting cells and constitute a limit between the neurogenic niche and the brain parenchyma (Mirzadeh et al., 2008). Type-B2 cells also form a net of glial tubes whereby the neuroblasts migrate toward the olfactory bulb (Lois and Alvarez-Buylla, 1994; Mirzadeh et al., 2008). Type-B1 cells have a cellular cycle of 17 h, with a 4.5-h length of S phase (Ponti et al., 2013). B1 cells also possess morphologic and ultrastructural characteristics of astrocytes (Doetsch et al., 1999b; Gritti et al., 1999) and express markers for several growth factors like PDGFRa, EGFR and FGFR-1 and -2 (Jackson et al., 2006; Frinchi et al., 2008; Danilov et al., 2009), as well as the class-IV intermediate filaments: vimentin, nestin and GFAP (Bonfanti and Peretto, 2007; Danilov et al., 2009).

The B1 cells give rise to transit-amplifying progenitor cells, also known as type-C cells (**Figure 1**). The type-C cells have a length of cell cycle between 18 and 25 h with a long S phase (12–17 h) and multiple cellular divisions (3 or 4 divisions) (Doetsch et al., 1999b; Gritti et al., 1999; Ponti et al., 2013). C cells express high levels of EGFR (Doetsch et al., 2002) and can be identified by the presence of the proneural gene Ascl1 (Ponti et al., 2013) and the transcription factor Dlx-2 (Danilov et al., 2009; Kriegstein and Alvarez-Buylla, 2009). Type C cells originate, in turn, type-A cells (migratory neuroblasts) that express the microtubular proteins doublecortin (DCX) and β-III tubulin (Tuj1), as well as the polysialylated neural cell adhesion molecule (PSA-NCAM) (Abrous et al., 2005; Bonfanti and Peretto, 2007). Type-A cells have a length of cell cycle of 18 h, with an S phase of 9 h (Ponti et al., 2013). These cells migrate tangentially through the rostral migratory stream (RMS) and reach the olfactory bulb (OB), where they disperse from the main cell stream, migrate radially and incorporate into the granular and periglomerular layers of the OB (Doetsch and Alvarez-Buylla, 1996; Alvarez-Buylla and Garcia-Verdugo, 2002; Hagg, 2005). Most of the SVZ-derived neuroblasts differentiate into GABAergic interneurons (Lois and Alvarez-Buylla, 1993, 1994), and a small percentage differentiate into dopaminergic (Basak and Taylor, 2009) or glutamatergic juxtaglomerular neurons that express the Neurog2, Trb1 and Trb2 transcription factors, as well as the vGluT1 and 2 glutamate-transporting proteins (Brill et al., 2009). The synaptic development and pruning of the VZ-SVZ-derived neurons are regulated by sensory activity (Saghatelyan et al., 2005) and experience-dependent plasticity long after maturation and integration (Livneh and Mizrahi, 2012). These newly generated neurons seem to function as neural mediators between sensory coding and brain plasticity (Adam and Mizrahi, 2010).

THE SUBGRANULAR ZONE (SGZ)

The SGZ is a neurogenic niche located in the dentate gyrus within the hippocampus (**Figure 2**) (Abrous et al., 2005). In the SGZ, there are two subtypes of astrocytes: the radial astrocytes and the horizontal astrocytes. The radial astrocytes, also known as type-1 cells, are the primary neuronal progenitors (Seri et al., 2001, 2004). Radial astrocytes express molecular markers as: GFAP, nestin, vimentin, S100β, musashi, 3-PGDH enzyme, and the transcription factor Sox-2 (Seri et al., 2001, 2004; Komitova and Eriksson, 2004; Balu and Lucki, 2009). SGZ astrocytes also express EGFR receptors, although in lower proportion as compared to the VZ-SVZ astrocytes (Jin et al., 2002). Radial astrocytes give rise to type-2 cells that are also referred as to type D cells (Seri et al., 2004). By their ultrastructural morphology, the type D cells can be subcategorized in type D1, type D2, and type D3, which express markers of immature neurons such as PSA-NCAM, DCX and Tuj1 (Seri et al., 2004; Lledo et al., 2006; Gonzalez-Perez et al., 2012). Type-D1 cells are small and ovoid. These cells give rise to D2 cell sub-type that possesses a short and thick process. According to the orientation of their cell processes, type D2 progenitors can be classified in three sub-types: D2v (vertically oriented), D2h (horizontally oriented) and D2i (pointing to the hilus) (**Figure 2**) (Seri et al., 2004). Type D3 cells have the morphology of immature granular neurons (Seri et al., 2004). Finally, the differentiation of type-D3 cells generates granular neurons (type-G cells) that can

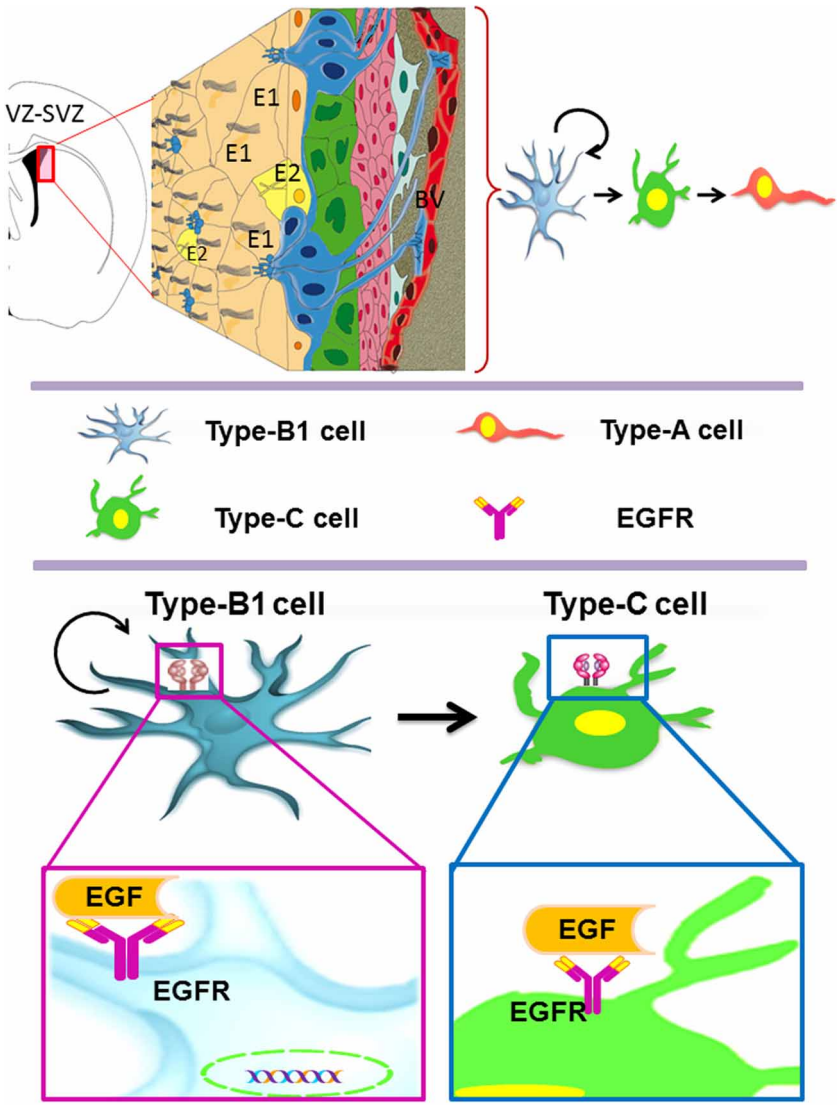


FIGURE 1 | The adult ventricular-subventricular zone (VZ-SVZ). 3-D reconstruction of this niche of neural stem cells located within the lateral wall of the lateral ventricles. Multiciliated ependymal cells, also called E2 cells, form pinwheel-like structures (in peach color) around the apical processes of type B1 cells (in blue). Biciliated ependymal cells as referred to E1 cells (in yellow). Type-C cells (in green) and type-A cells (in red). Type-B1 progenitors are neural stem cells that generate secondary progenitors (type-C cells), which in turn give rise to migrating neuroblast (type-A cells). Additionally, type-B1 cells generate oligodendrocyte progenitors *in vivo*. Both type-B and type-C progenitors express the EGFR. Note that type-B neural stem cells are in close contact with the cerebrospinal fluid and the adjacent blood vessels (BV).

be identified by the expression of NeuN, calretinin, and calbindin (Ming and Song, 2005). Throughout this differentiation process, the new neurons of the SGZ migrate small distances (Hagg, 2005) and project their axons to CA3 neurons and synapse axonal projections from entorhinal cortex (Abrous et al., 2005; Balu and Lucki, 2009). Current evidence suggests that the hippocampal neurogenesis plays an important role in the spatial memory acquisition-retention process (Barnea and Nottebohm, 1996; Gould et al., 1999; Feng et al., 2001; Shors et al., 2001) and may regulate emotional processes such as stress, social behavior and depression (Warner-Schmidt and Duman, 2006; Gheusi et al., 2009).

SUBCALLOSAL ZONE (SCZ)

The SCZ is the most recently described germinal region and there is scarce information about it. Interestingly, its cellular composition is similar to the adult VZ-SVZ (**Figure 3**). During the embryonic development, the SCZ is formed by the collapsing of the ventricular zone walls. Nevertheless, in the adult brain, the SCZ is no longer associated with the ventricular system (Seri et al., 2006). The SCZ is comprised by several cavities filled with cerebrospinal fluid that are located between the corpus callosum and the hippocampus. The cytoarchitecture of the SCZ includes a layer of ependymal cells (type-E cells) that line each cavity. Type-E cells are frequently surrounded by myelinated and unmyelinated

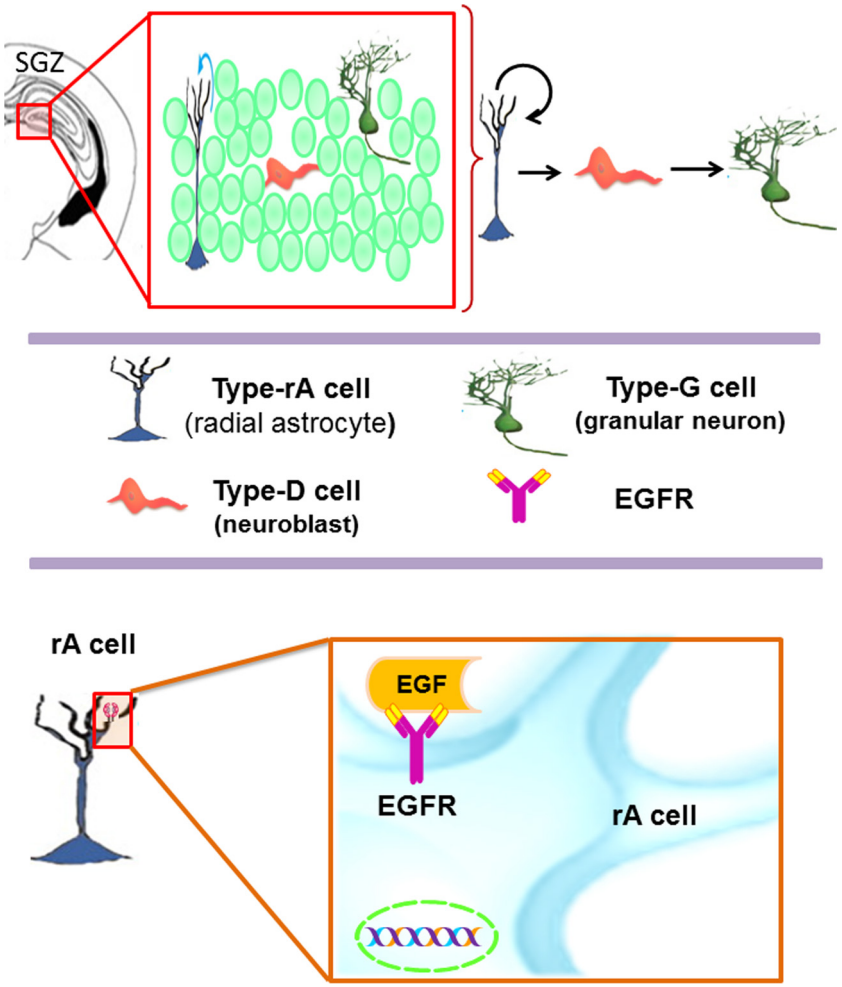


FIGURE 2 | The subgranular zone (SGZ) in the dentate gyrus of the adult hippocampus. Type-B1 cells (in blue) also known as type-1 cells or type-rA cells (radial astrocytes) are the neuronal progenitor cells in this region. Type-rA cells divide and produce type-D cells as referred to type-2 cells. Hippocampal neuroblasts migrate locally and incorporate into the granular layer where they differentiate in mature granular neurons (type-G cells). Type-B1 cells express EGFR and behave as putative neural stem cells *in vitro*.

axons. Near the type-E cells, there is a subpopulation of astrocytes (type-B cells) and a small population of type-C cells. In this region, migratory cells (type-A cells) that express PSA-NCAM have been shown to differentiate into oligodendrocytes in the adjacent corpus callosum (Seri et al., 2006). Interestingly, the SCZ type-B cells originate multi-potential neurospheres when exposed to EGF or bFGF *in vitro* (Seri et al., 2006). A recent study indicates that the SCZ can generate neurons, but they cannot reach the mature stage (Kim et al., 2011). However, in mutant mice lacking Bax expression, the immature neurons derived from the SCZ are able to reach mature stages (Kim et al., 2011, 2012). In summary, the EGFR expression has been described in type B and type C cells of the VZ-SVZ and in the radial astrocytes of the SGZ. Although the SCZ cells can respond to the presence of EGF *in vitro* (Seri et al., 2006), the specific cell lineage that expresses EGFR is not well defined. This ability to respond to EGF indicates that the EGFR constitutes an important signaling pathway for the adult neural stem cells found in the VZ-SVZ, the SGZ and the SCZ.

RECEPTOR OF THE EPIDERMAL GROWTH FACTOR (EGFR)

The EGFR, also known as erbB1 or HER-1, is a glycoprotein with a molecular weight of 170 kd that belongs to the related proteins family of c-erbB. There are three other members of this family: erbB2/HER2, erbB3/HER3 and erbB4/HER4 (**Table 1**). The EGFR can generate homodimerization or heterodimerization with all ErbB family members (Yarden and Sliwkowski, 2001). After homo or heterodimerization, the tyrosine kinase intracellular domains of ErbB monomers self-phosphorylate and recruit several proteins that, in turn, activate downstream signaling pathways with various cell functions (Schneider et al., 2008). Thus, the EGFR regulates proliferation, migration, tissue invasiveness, apoptosis inhibition, cell differentiation, circadian rhythm, puberty initiation and development of cognitive functions (Herbst and Bunn, 2003; Liu and Neufeld, 2004; Brandes et al., 2008).

The EGFR is synthetized as a precursor molecule that is integrated by a 1210-residue polypeptide. After cleavage in the

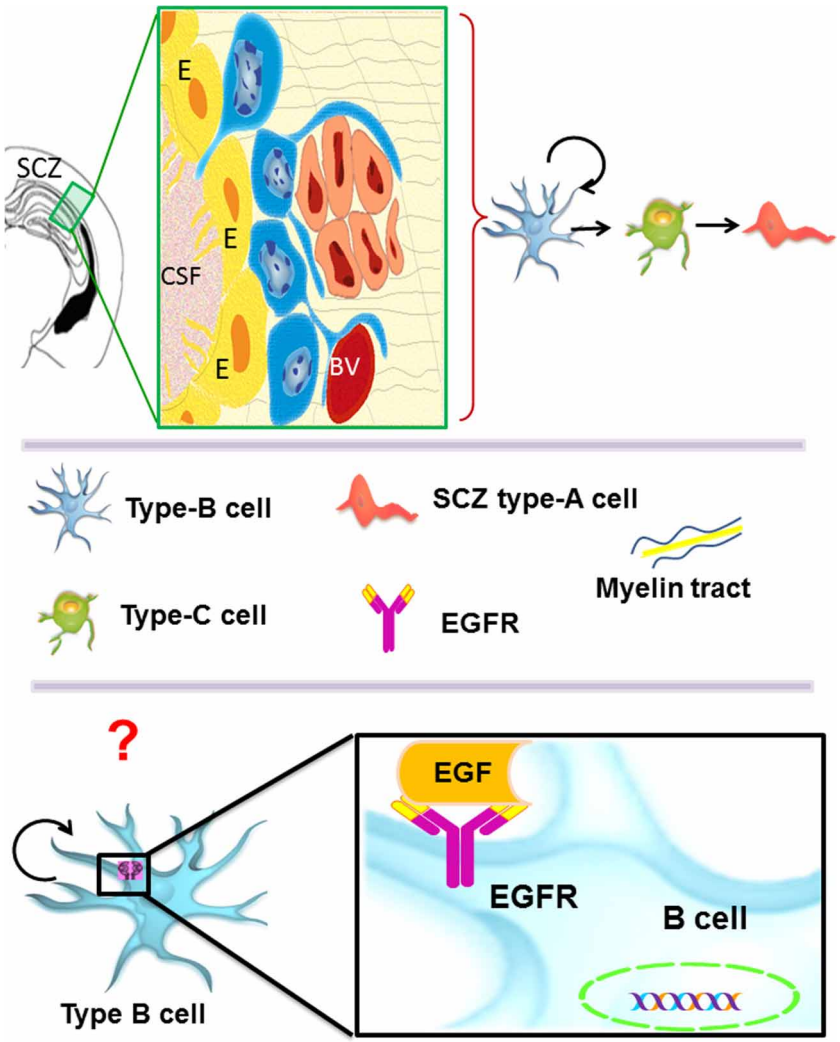


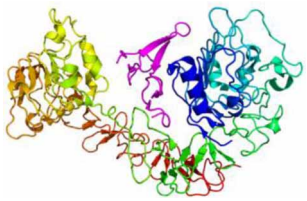
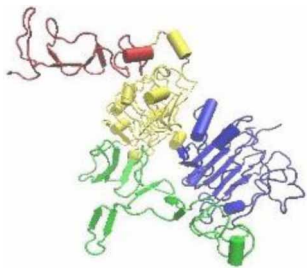
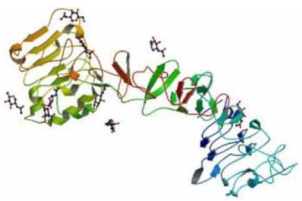
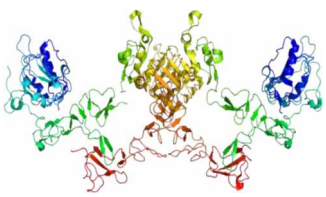
FIGURE 3 | The subcallosal zone (SCZ) is located between the hippocampus and the corpus callosum. The SCZ is a caudal extension of the VZ-SVZ that is no longer associated to the ventricular system. Type-B cells (in blue) generate type-C cells that, in turn, give rise to oligodendrocyte precursors (also called SCZ type-A cells) that migrate into the neighboring corpus callosum. Type-B and type-C cells isolated from the SCZ and cultured as neurospheres behave as neural stem cells *in vitro*. However, the cell type that expresses *in vivo* the ErbB family receptors is unknown.

N-terminal domain, a final fragment of 1186-amino acid protein is anchored on the cellular membrane surface (Jorissen et al., 2003). EGFR has a small transmembrane hydrophobic region and a tyrosine kinase intracellular domain (Yarden and Sliwkowski, 2001; Herbst and Bunn, 2003; Scaltriti and Baselga, 2006). The molecular structure of the EGFR is also characterized for four domains abundant in cysteine-rich (CR1 and CR2) and leucine-rich residues (L1 and L2 domains), which are the ligand binding regions (Jorissen et al., 2003; Flynn et al., 2009). EGFR ligands are the epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), β-cellulin, transforming growth factor alpha (TGF-α), amphiregulin (AREG), epiregulin (EREG), epigen (EPGN) and neuroregulin (NRG). All these ligands can activate the EGFR by autocrine or paracrine signaling (Schneider et al., 2008). The most important ligand of the EGFR is the EGF, a 5.5 kD peptide discovered in the 50s by Stanley Cohen when he was trying to purify the nerve growth factor (NGF) (Raivich and Kreutzberg, 1994; Nathoo et al., 2004). The EGF binds to high and low affinity sites in EGFR-expressing cells. Truncation of the CR1 domain, a loop that mediates dimerization, abolishes the high-affinity binding cell population (Jorissen et al., 2003). Yet, the precise mechanism that regulates these affinities is still unknown.

SIGNALING CASCADES OF THE ErbB RECEPTOR FAMILY

The strength and intensity of ErbB signaling depends principally on two biological mechanisms: (1) the homodimerization or heterodimerization among ErbB family receptors, and (2) the subsequent activation of downstream proteins (Jorissen et al., 2003; Clark et al., 2012). The ErbB receptor family may signal through PI3K/Akt (phosphoinositide 3-kinase/ v-akt murine thymoma), MAPK (the mitogen-activated protein kinases), STAT, PLC (phospholipase C) and many other pathways (**Figure 4**),

Table 1 | ErbB family members.

ErbB family member	Molecular weight	Description	Biological assembly
ErbB1, HER1 or EGFR	170 kDa	EGFR is the main member of the ERBB receptor tyrosine kinase family. It promotes protein tyrosine kinase activity due to an induced dimerization of the receptor, an essential part of the signal transduction pathway.	
ErbB2, HER2 or herstatin;	185 KDa	ErbB2 is the only ErbB family member that does not bind a known ligand. It works as a signal transducer following ligand dependent recruitment into heterodimer rs with ErbB1, ErbB3, or ErbB4	
ERbB3 or HER3	140 kDa	ErbB3 consists of four domains with structural homology to domains found in the type I insulin-like growth factor receptor. ErbB3 has a heregulin (HRG) or neuregulin binding domain but lacks intrinsic protein tyrosine kinase activity. Therefore, it can bind to the ligand but not convey the signal into the cell through protein phosphorylation. However, ErbB3 forms heterodimers with other EGF receptor family members which do have kinase activity	
ErbB4, HER4 or V-Erb-A Erythroblastic Leukemia Viral Oncogene Homolog 4	200 kDa	ERBB4 is a unique member of the ERBB family that can undergo regulated intramembrane proteolysis. ERBB4 is a single-pass type-1 transmembrane protein with multiple furin-like cysteine rich domains, a TK domain, a PI3K binding site and a PDZ domain binding motif. This receptor is activated by neuregulins-2 and -3, heparin-binding EGF-like growth factor and betacellulin	

Molecular subdomains are represented by colors: L1 (blue), L2 (yellow), CR1 (green), and CR2 (red).

thereby, regulating cell proliferation, apoptosis, differentiation or migration (Nathoo et al., 2004; Quesnelle et al., 2007). Following activation of EGFR, the SH2 domain of Grb2 can bind to the EGFR either directly (via Y1068 and Y1086) or indirectly via tyrosine phosphorylated Shc (Sasaoka et al., 1994). The association of Shc to EGFR through its PTB domain recruits Grb2 and constitutes one of the main steps in EGF-dependent induction of the Ras/MAPK pathway (Gong and Zhao, 2003). Heterodimerization between different ErbB family members generates a number of cellular effects (**Figure 5**). For instance, heterodimerization between ErbB1 and ErbB2 triggers mitosis (Yarden, 2001) and an undifferentiated cell stage (Ghashghaei et al., 2007). Heterodimerization between EGFR/ErbB4 is involved in dopamine neurons development (Iwakura et al., 2005), whereas ErbB2/ErbB3 is implicated in oligodendrocyte differentiation (Makinodan et al., 2012) by activation of PI3K/AKT pathway (Clark et al., 2012). Interestingly, ErbB2/ErbB3 heterodimerization can also promote glioma development (Clark et al., 2012). ErbB2/ErbB4 promotes cell differentiation in the embryonic hippocampus (Gerecke et al., 2004). ErbB1/ErbB4 activation mediated by betacellulin expands neural stem cells and neuroblasts in the postnatal brain (Gomez-Gaviro et al., 2012). In summary, the ErbB receptor family is involved in a myriad of biological effects, one of them is the generation and differentiation of oligodendrocytes from neural stem cells.

OLIGODENDROCYTE SPECIFICATION MEDIATED BY ErbB FAMILY IN THE EMBRYONIC BRAIN

In embryonic neural precursors, the EGFR is mainly associated with initiation of asymmetric divisions (Sun et al., 2005). The ErbB family regulates the maturation process of oligodendrocytes and myelin production during neural development (Aguirre et al., 2007). In rodents, gliogenesis occurs in the second week of postnatal development (Ivkovic et al., 2008), which is associated with the EGFR expression (Aguirre et al., 2007). ErbB1 and ErbB2 mRNAs are expressed in S100β+ and Olig2+ glial precursors, while ErbB3 mRNA expression coincides with the expression of 2′,3′-cyclic-nucleotide 3′-fosfodiesterase (a marker associated with mature oligodendrocytes) (Abe et al., 2009). Furthermore, ErbB2 appears to participate in the oligodendrocyte

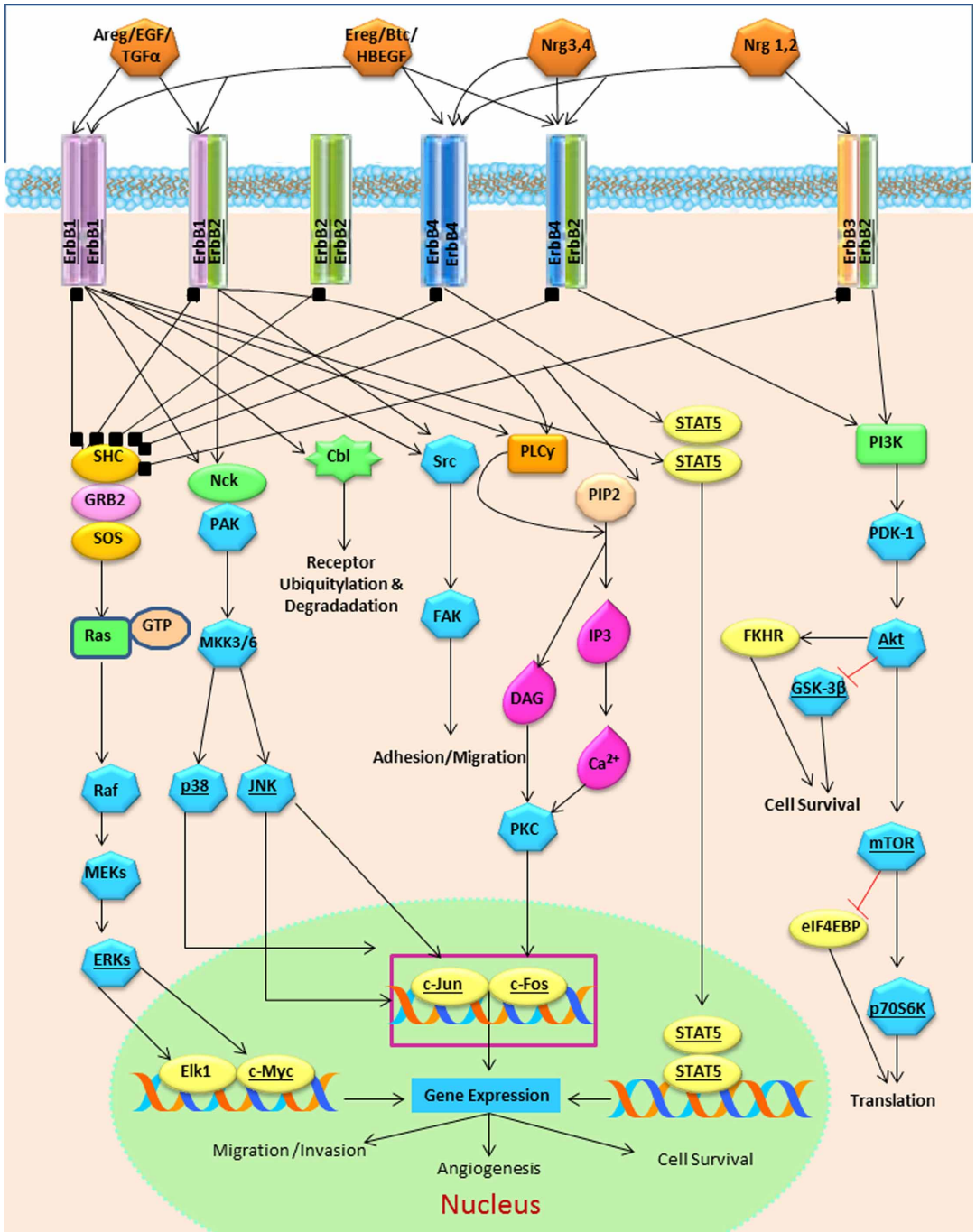


FIGURE 4 | The ErbB family receptors and their main cell signaling pathways: the Ras/MAPK, the PI3K/AKT and the PLCγ pathways.

differentiation process, while the expression of ErbB3 and ErbB4 is necessary for maturation of embryonic oligodendrocyte precursors (Sussman et al., 2005). Thus, a possible interaction between ErbB1 and ErbB3 signaling pathways may be involved in oligodendroglial specification.

AKT signaling is involved in myelination during early embryonic stages (Flores et al., 2008). SHP2 protein is involved in the AKT activation promoted by EGFR in oligodendrocyte precursors (Liu et al., 2011). Another targeting protein for the AKT signaling is Gab1; when Gab1 levels decrease in precursor cells of the spinal cord, the Olig-2 transcription factor expression decreases and the Pax7+ expression is inhibited (Hayakawa-Yano et al., 2007). This suggests that ErbB1 drives the Olig-2 expression via Gab1/AKT.

Neuroregulins (NRGs) are efficient ligands for EGFR, ErbB3, and ErBb4 proteins (Talmage, 2008; Clark et al., 2012). NRGs support the survival of oligodendrocytes and aged oligodendrocyte

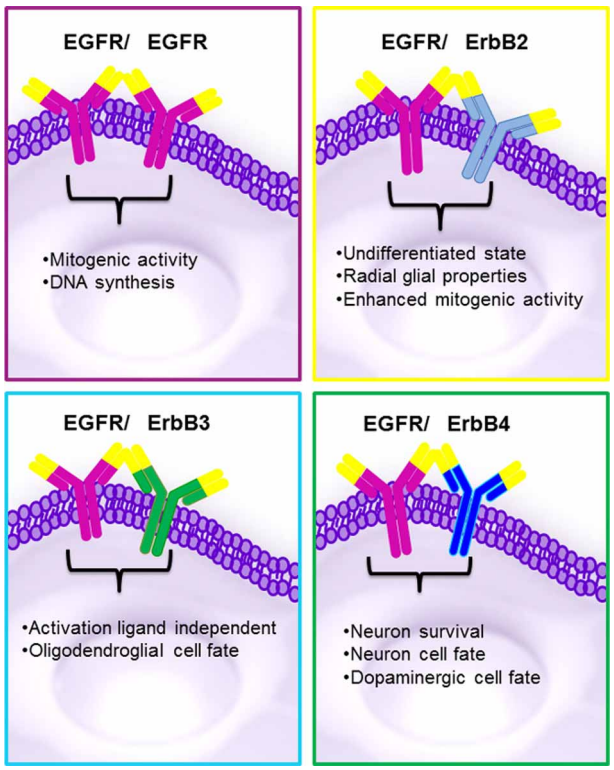


FIGURE 5 | Biological effects reported in ErbB family proteins. The homo or heterodimerization of the ErbB proteins may generate similar effects. Homodimerization between EGFR/EGFR can originates proliferation MAPK activation and the heterodimeriizations of EGFR/ErbB2 undifferentiated state and enhanced mitogenic activity; EGFR/ErbB3 oligodendroglial cell fate via AKT and EGFR/ErbB4 neuronal and survival cell fate.

precursor cells (Fernandez et al., 2000). NRGs induce axon-associated survival in developing oligodendrocytes through the PI3-kinase/Akt pathway (Flores et al., 2000). Inhibition of the signaling mediated by NRG/ErbB4, an important regulator of oligodendrocyte development, induces changes in the morphology, number and role of the oligodendrocytes *in vivo* (Roy et al., 2007). ErbB family members also promote myelination in the peripheral nervous system by increasing the expression of myelin protein zero (P0 or MPZ) in Schwann cells (Chen et al., 2006). Neuroregulin 1 (NRG-1) induces heterodimerization between ErbB2 and ErbB3 receptors and promotes myelination in the peripheral nervous system (Newbern and Birchmeier, 2010). Taken together this indicates that NRGs play an important role in oligodendrogenesis and myelination during CNS and PNS development.

ErbB EFFECTS IN THE OLIGODENDROCYTE SPECIFICATION IN ADULT NEURAL STEM CELLS

Increasing evidence indicates that EGFR ligands determine the cell fate of adult neural stem cell of the VZ-SVZ (Gonzalez-Perez and Alvarez-Buylla, 2011). At the postnatal day 3, EGFR overexpression in the VZ-SVZ generates intense hyperplasia. These cells express oligodendrocyte-type lineage markers characterized by the expression of NG2, Olig2, and PDGFRα (Ivkovic et al., 2008). In the adult VZ-SVZ, intracerebral administration of EGF induces strong cell proliferation and migration and drives differentiation into oligodendrocyte lineage (Gonzalez-Perez et al., 2009). In cell culture, VZ-SVZ astrocytes (type B cells) strongly proliferate and generate a number of O4+PDGFRα+ oligodendrocyte precursors after EGF exposure (Gonzalez-Perez and Quinones-Hinojosa, 2010). The astrocytic cells respond to the EGFR stimulation through several ligands and MAPK signaling pathway (Tournier et al., 1994). Interestingly, after three weeks in cell culture astrocytes do not respond to EGF stimulation, but when pre-treated with IL-6, astrocytes actively respond to EGF effects (Levison et al., 2000; Gonzalez-Perez et al., 2012). These findings indicate a synergy between IL-6 and EGF. A similar effect is also observed in spinal cord progenitor cells, where IL-6 produces a trans-activation of EGFR (Kang and Kang, 2008). IL-6 activates Jak/STAT signaling pathway (Scaltriti and Baselga, 2006) and stimulates oligodendroglial differentiation via the Jak/STAT pathway (Islam et al., 2009). STAT-3 is essential for the synergism between the chondroitin sulfate proteoglycan (CSPG) and EGF. In cell culture, CSPG and EGF activate PI3K and STAT-3 pathways, as well as promote the formation of neurospheres, cell survival and the phosphorylation of EGFR (Tham et al., 2010). The PI3K/AKT pathway is mainly activated when EGFR dimerizes with the ErbB3 monomer (Scaltriti and Baselga, 2006). EGFR-dependent STAT activation may be mediated by Src without JAK. Src is involved in the activation of PI3K/AKT via a p85 subunit binding (Jorissen et al., 2003) (**Figure 4**). Taken together, this suggests that Jak/STAT is important for cell-cycle progression and oligodendrogenesis.

ADPbetaS and UTP nucleotides activate EGFR and induce a rapid calcium influx in SVZ progenitor cells (Grimm et al., 2009). An increase in intracellular calcium levels promotes oligodendrocyte differentiation (Boscia et al., 2012; Paez et al., 2012). Oligodendrocyte differentiation and myelination is promoted by the NMDAR stimulation and by subsequent influx of calcium (Cavaliere et al., 2012). NRG/ErbB induces oligodendroglial differentiation (Roy et al., 2007) probably mediated by NMDAR activation (Brinkmann et al., 2008). The interaction between the ErbB and NMDAR during oligodendrocyte differentiation is mediated by intracellular levels of calcium (Brinkmann et al., 2008; Cavaliere et al., 2012). This indicates that the synergism between EGF and calcium ion channels controls oligodendrocyte specification.

Polidendrocytes, also known synantocytes or NG2 glia, are characterized by the expression of the NG2 chondroitin sulfate proteoglycan (Nishiyama, 2001). Many of the NG2-expressing cells are located in the corpus callosum and express the EGFR. In wild-type mice, re-myelination is mediated by NG2+Olig2+Mash1+ precursor cells (Aguirre et al., 2007). In contrast, mutant mice that constitutively express the human EGFR (hEGFR) in NG2 cells show an increase in the expression of Nkx2.2, Sox-9, and Sox-10 in a demyelinated area. Thus, an increase in the activity of EGFR drives oligodendrogenesis at the expense of astrogliogenesis (Aguirre et al., 2007). Consequently, the transcription factors Nkx2.2, Sox-9, and Sox-10 may regulate the oligodendrogenesis effects of EGFR in multipotent cells.

Sox-2 is a transcription factor that has homology with the SRY protein and determines the sex of individuals (Hu et al., 2010). Sox-2 is associated with oligodendrocyte-cell-fate commitment in the SVZ and SGZ precursor cells that express EGFR (Komitova and Eriksson, 2004; Baer et al., 2007; Balu and Lucki, 2009). Interestingly, changes in EGFR gene expression and EGFR protein occur at the time of puberty in the brain (Ma et al., 1994) and sex-dependent differences have been observed in myelination process (Kipp et al., 2012). Nevertheless, the biological relationship between ErbB family receptors and sexual hormones in oligodendrocyte specification remains to be elucidated.

Olig1 and Olig2 transcription factors participate in oligodendrogenesis in embryonic spinal cord and ventral telencephalon (Jakovcevski and Zecevic, 2005a,b). In the adult brain, a small population of type-B cells in the SVZ expresses Olig2 that may generate a discrete subpopulation of Olig2-expressing type-C cells. These Olig2+Dlx2+ type-C cells may be the oligodendroglial precursors of adult VZ-SVZ (Menn et al., 2006; Gonzalez-Perez and Quinones-Hinojosa, 2010). Interaction between Dlx2 and EGFR regulates proliferation and neurogenesis of type C cells in the VZ-SVZ (Suh et al., 2009).

MAPK pathway promotes neuronal differentiation and survival through phosphorylated CREB (CREB-p) that, in turn, induces Pax6 expression (Levison et al., 2000; Gampe et al., 2011; Herold et al., 2011; Yoo et al., 2012). Pax-6 reduces the EGFR expression in neural stem cells (Jia et al., 2011) and represses the expression of Olig-2 cells and glial lineage (Jang and Goldman, 2011). Thus, Pax-6 induces neuronal differentiation in embryonic and adult neural precursor cells (Kohwi et al., 2005; Osumi et al., 2008) and determines the neuronal cell fate while decreasing the expression of NG2 (Klempin et al., 2011). Hence, interaction between Pax-6 and Olig-2 seems to regulate the final phenotype of precursor cells by regulating EGFR activity. This intricate relationship among transcription factors and downstream signaling pathways activated by ErbB proteins regulates the oligodendrogenesis process. Therefore, molecular disruptions in any of these biological mechanisms may considerably alter the myelination process. ErbB-driven olidodendrogenesis has been implicated in psychiatric disorders and tumorigenesis.

ErbB RECEPTORS IN PSYCHIATRIC DISORDERS

Disruption in oligodendrogenesis and myelin formation has been associated with the pathophysiology of schizophrenia. Animals deprived of ErbB signaling show psychiatric-like behaviors (Roy et al., 2007). The brain of schizophrenic patients shows a significant alteration in myelination and oligodendrocyte generation (Chang et al., 2007), which is associated with a decrease in EGF levels (Futamura et al., 2002; Iwakura and Nawa, 2013). Mature oligodendrocytes express the dopamine receptors (D2 and D3) that are involved in myelin-sheath formation and oligodendrocyte turnover. A deficiency in expression of D2 and D3 receptors reduces the number of myelinating oligodendrocytes (Lee and Fields, 2009). In contrast, dopamine exposure promotes EGFR trans-activation, which increases the proliferation of neural precursor cells in the adult brain (Winner et al., 2009; O'keeffe and Barker, 2011). Therefore, ErbB receptor family is an important regulator of dopamine-induced oligodendrogenesis, which may explain some of the myelination disturbances observed in schizophrenia.

In major depression disease, a low density and reduced expression of oligodendrocyte-specific gene transcripts have been found in postmortem human brains (Edgar and Sibille, 2012). Patients with major depression often present with decreased levels of EGF (Tian et al., 2012). Oligodendrocyte precursor cells co-express serotonin receptors and EGF receptors (Schaumburg et al., 2008), while EGF has been shown to transcriptionally regulate transporters of serotonin 1 and 2 via ErbB1 (Gill et al., 2011) Taken together, this evidence suggests that ErbB receptors may be involved in the pathophysiology of mood and psychiatric disorders by controlling the oligodendrocyte cell population and serotonin brain levels.

EGFR ROLE IN HYPERPLASIA AND TUMORIGENESIS

In the adult brain, long-lasting EGFR stimulation induces polyp-like growths (Kuhn and Miller, 1996), leads to diffuse white-matter hyperplasia (Ivkovic et al., 2008) and promotes a strong invasive pattern of VZ-SVZ precursors (Gonzalez-Perez et al., 2009; Gonzalez-Perez and Quinones-Hinojosa, 2010). Remarkably, in all these experiments, the EGF-transformed cells appear to be related to oligodendroglia cell lineage, such as: NG2, Olig-2, O4, and PDGFRα. In brain tumors, there is evidence suggesting that EGFR signaling plays a role in tumor initiation and progression. Approximately 50% of high-grade astrocytomas show EGFR amplification that leads to glioblastoma malignization (Maher et al., 2001; Wechsler-Reya and Scott, 2001). Low-grade and high-grade oligodendroglial tumors also show an increased expression of EGFR mRNA and protein, which in the vast majority of cases is not produced by gene amplification (Reifenberger et al., 1996). Furthermore, EGFR overexpression is associated with mutations in the intracellular and extracellular domains, alterations in signaling cascade regulation and cross-signaling with other tyrosine kinase receptors (Schneider et al., 2008). This indicates that the EGFR may be activated by ligand-dependent and ligand-independent mechanisms (Scaltriti and Baselga, 2006). The amplification and overexpression of EGFR is found in approximately 50% of glioblastomas and promotes invasion, proliferation and apoptosis arrest (Clark et al., 2012). These effects are produced by AKT and ERK 1/2 (Clark et al., 2012). In approximately 14% of EGFR-expressing glioblastomas there is a mutation in the EGFRvIII gene, which is characterized by a lack of an extracellular domain and constitutive phosphorylation (Brandes et al., 2008). The EGFR contributes to the tumorigenic cellular diversity of glioblastoma by participating in the expansion of diverse cell types that initiate tumors (Mazzoleni et al., 2010). Whether *stem-cell-like* glioblastoma cells are initially exposed to EGF and bFGF, the EGFR-like activity continues despite the absence of a ligand. This suggests that other erbB family members, i.e., erbB2 and erBb3, may also be involved in glioblastoma cell expansion (Clark et al., 2012). Interestingly, EGFR overstimulation by itself is not enough to drive brain tumor formation. This indicates the genetic background plays an important role in tumorigenesis and the EGFR may only induce certain aspects of a malignant phenotype (e.g., proliferation and tissue invasion).

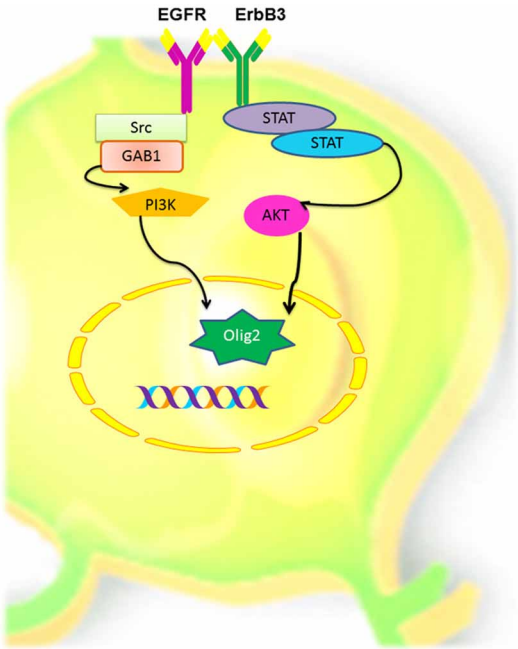


FIGURE 6 | Hypothetical model of oligodendrocyte cell signaling, via EGFR and ErbB3 in adult neural stem and progenitors cells. Homodimerization between these two ErbB members could activate the PI3K or the STAT pathways that in turn can activate AKT and induces the expression of Olig-2, which in turn may determine oligodendroglial lineage.

CONCLUSION

EGFR has different biological effects that can be attributed to: (1) the type of ErbB ligand (Jin et al., 2002; Cooper and Isacson, 2004; Gonzalez-Perez et al., 2009; Gonzalez-Perez and Quinones-Hinojosa, 2010; Gomez-Gaviro et al., 2012); (2) the homo or heterodimerization ErbB proteins (Iwakura and Nawa, 2013) and (3) the downstream signaling pathway that every ligand/dimmer may stimulate. ErbB proteins are important mediators of quiescence, proliferation, differentiation, phenotype specification, survival and migration of neural stem cells. In a number of independent reports, ErbB1 and Erbb3 are frequently involved in oligodendrocyte specification. Therefore, we hypothesize that the interaction between these receptors is one of the main promoters in the oligodendrocyte lineage (**Figure 6**). Yet, the role of the ErbB family members in adult neural stem cells is not completely understood. Elucidating the precise role of ErbB family members in oligodendrogenesis is a crucial step for designing stem-cell-based therapies for demyelinating diseases and other neurological disorders.

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Modulation of subventricular zone oligodendrogenesis: a role for hemopressin?

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Neural stem cells (NSCs) from the subventricular zone (SVZ) have been indicated as a source of new oligodendrocytes to use in regenerative medicine for myelin pathologies. Indeed, NSCs are multipotent cells that can self-renew and differentiate into all neural cell types of the central nervous system. In normal conditions, SVZ cells are poorly oligodendrogenic, nevertheless their oligodendrogenic potential is boosted following demyelination. Importantly, progressive restriction into the oligodendrocyte fate is specified by extrinsic and intrinsic factors, endocannabinoids being one of these factors. Although a role for endocannabinoids in oligodendrogenesis has already been foreseen, selective agonists and antagonists of cannabinoids receptors produce severe adverse side effects. Herein, we show that hemopressin (Hp), a modulator of CB1 receptors, increased oligodendroglial differentiation in SVZ neural stem/progenitor cell cultures derived from neonatal mice. The original results presented in this work suggest that Hp and derivates may be of potential interest for the development of future strategies to treat demyelinating diseases.

Keywords: oligodendrogenesis, subventricular zone, hemopressin, endocannabinoids, myelin pathologies

OLIGODENDROGENESIS

Oligodendrocytes are the myelin-forming cells of the central nervous system (CNS) of vertebrates. These glial cells possess cholesterol-rich membranes that compactly enwrap around neuronal axons building the so-called myelin sheath. This multilamellar spiral structure provides an electrical insulation and clustered distribution of ion channels in nodes and paranodes, which allows a fast and saltatory propagation of action potentials along the axon, an essential requirement for normal brain function. In addition, the myelin sheath sustains and protects the axons (reviewed in Nave and Trapp, 2008) and oligodendrocytes seem to play a role in signaling crosstalk with neurons (Bergles et al., 2010).

Oligodendrogenesis actively takes place during CNS development and following demyelinating insults, but also more silently throughout adulthood. Oligodendrocytes differentiate from oligodendrocyte precursor cells (OPCs), characterized by the presence of the neuron-glial antigen 2 (NG2) proteoglycan and therefore often called NG2 cells (Nishiyama et al., 2009). Forebrain OPCs are produced in three sequential waves during development, from different regions of the ventricular zone (VZ): at early embryonic stage (E12.5), from progenitors in the medial ganglionic eminence (MGE) and the anterior entopeduncular area (AEP); at E15.5, from progenitors in the lateral and/or caudal ganglionic eminence (LGE/CGE); and at birth (P0) from progenitors within the postnatal cortex (Kessaris et al., 2006). The highly proliferative progenitors migrate from these birth areas and colonize the developing gray and white matter. Herein, most of them exit the cell cycle and mature into myelinating oligodendrocytes. Some, however, remain as slowly dividing OPCs, widespread throughout the brain parenchyma, and constitute a stable population that comprises 3–8% of the total number of cells in the adult brain (Polito and Reynolds, 2005). Genetic fate mapping analysis

has demonstrated that the progeny of these OPCs is restricted to the oligodendrocyte lineage, including new myelinating oligodendrocytes and further NG2 cells (Dimou et al., 2008; Kang et al., 2010; Clarke et al., 2012). In addition, the subventricular zone (SVZ) bordering the lateral ventricles, constitutes a source of few oligodendrocyte progenitors that migrate and populate the corpus callosum, striatum and fimbria-fornix in the adult mouse brain (Menn et al., 2006; Ortega et al., 2013).

Oligodendrogenesis in the adult brain is stimulated by myelin pathologies. Evidence indicates that most of the remyelinating oligodendrocytes are derived from the widespread parenchymal OPCs. Upon a demyelinating injury, these cells are induced to proliferate and migrate to the demyelinated area, mature and reinvest the denuded axons, forming new myelin sheaths (reviewed in Franklin and ffrench-Constant, 2008). In addition, precursors in the rodent SVZ niche become activated and contribute to remyelination of nearby callosal axonal tracts (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002; Menn et al., 2006). Noteworthy, an increased activation of the SVZ niche was detected in *post-mortem* samples from multiple sclerosis (MS) patients suggesting that a similar response may occur in the human brain (Nait-Oumesmar et al., 2007).

Although robust remyelination upon acute MS lesions results in functional recovery, as disease progresses, this spontaneous reparative process starts to fail leading to axonal degeneration and neurological deficits. In many lesions, remyelination remains restricted to the edge of the lesion (reviewed in Kotter et al., 2011). In fact, remyelination in humans varies from patient to patient or even from lesion to lesion in the same patient. MS lesions are generally categorized according to the stage of the demyelinating activity and the presence of immune cells (Frohman et al., 2006); active demyelinating lesions are characterized by the presence of myelin-laden macrophages in the lesion or at the edge; smoldering lesions contain rare myelin-phagocytosing macrophages at the rim of the lesion whereas chronic inactive lesions display no evidence for ongoing demyelination although single T cells may be present in perivascular regions (Frohman et al., 2006). Recently, several efforts have been done to understand the causes for remyelination failure. It has been postulated that in human active lesions (where remyelination is possible), the density of fibroblast growth factor-2 (FGF-2) expressing macrophages and FGF receptor 1 (FGFR1)-OPCs increase, whereas the extracellular matrix (ECM) associated glycoprotein anosmin-1 is absent. In contrast, in chronic-lesions (where remyelination is mainly restricted to the periplaque), FGF-2 expression is limited to the macrophages/microglia in the periplaque, and expression of anosmin-1 is widespread throughout the core of the lesion and may be preventing remyelination (Clemente et al., 2011). Multiple causes seem to contribute to the decline in remyelination, including the failure of OPCs to differentiate and to loop around the nude neuronal axons (Franklin et al., 2002). Regenerative approaches for demyelinating diseases have been based on the stimulation of the endogenous self-repair mechanisms, or on the transplantation of myelinogenic cells, as for instance derived from neural stem cells (NSCs; Grade et al., 2013).

NSCs from the SVZ can be highly expanded in culture and give rise to neurons, astrocytes and oligodendrocytes. Importantly, SVZ-derived oligodendrocytes undergo all the oligodendrocyte cell lineage stages from the early bipolar OPC to the mature myelinating oligodendrocyte, recapitulating the developmental process (Levison and Goldman, 1993; Menn et al., 2006). Furthermore, these cells are able to remyelinate vulnerable axons when transplanted in animal models of demyelinating injuries (Keirstead et al., 1999; Smith and Blakemore, 2000; Akiyama et al., 2001; Pluchino et al., 2003; Cayre et al., 2006). However, under normal conditions, oligodendrocytes represent a small minority of the SVZ progeny both *in vitro* and *in vivo* (Menn et al., 2006). Excitingly, the last decade has been considerably rich in developing tools to guide or force cells to acquire a desired fate. Indeed, pharmacological or genetic tools may be used to direct NSCs to the oligodendrocyte cell lineage, thus increasing the proportion of oligodendrocytes in the expanded cultures, prior to transplantation. On the other hand, the weak endogenous remyelination at chronic stages of the disease may be assisted by fostering oligodendrocyte differentiation since this constitutes a limiting step for effective remyelination. Thus, much interest has been gathered on finding molecular cues for oligodendrocyte lineage specification and development.

MODULATION OF OLIGODENDROGENESIS

Oligodendrogenesis is regulated by intrinsic and environmental factors. A refined knowledge of these factors is necessary in order to be able to manipulate oligodendrogenesis for brain repair purposes in demyelinating diseases.

Importantly, the generation of induced OPCs (iOPCs) by direct lineage conversion was recently demonstrated. In fact mouse and rat fibroblasts can be reprogrammed by forced expression of oligodendrogenic transcription factors into iOPCs with morphologies and gene expression signatures resembling primary OPCs (Najm et al., 2013; Yang et al., 2013).

At the levels of gene expression, it has been shown that oligodendrogenesis is modulated by the histone deacetylase sirtuin 1 (SIRT1). Histone acetylation is mainly associated with active gene transcription. SIRT1 is a member of the sirtuin family and is expressed in adult mice SVZ and dentate gyrus (DG) in Sox2+ stem cells, proliferating cells and oligodendrocytes but not in mature astrocytes. Loss of SIRT1 deacetylase activity by conditional ablation of SIRT1 in nestin+ stem cells results in increased number of oligodendrocyte transcription factor 2 (Olig2)+ OPCs generated in the SVZ and migrating toward the septum and the striatum. In fact SIRT1 limits the expression of platelet-derived growth factor receptor alpha (PDGFRα) in nestin+ stem cells, involved in proliferation and differentiation of OPCs. Using chromatin immunoprecipitation, the authors showed that loss of SIRT1 increases histone 3 lysine 9 (H3K9) acetylation in the PDGFRα promoter, therefore allowing its transcription (Rafalski et al., 2013).

Expression of specific transcription factors is required to commit cells to the oligodendrocyte lineage. As an example, the basic-helix-loop-helix transcription factors Olig2 and Mash1 are key players in determining OPC specification. During postnatal oligodendrogenesis, OPCs double-labeled for Mash1 and Olig2, are found in the SVZ and corpus callosum. As maturation proceeds, OPCs express PDGFRα and down-regulate Mash1. Ablation

of Mash1 during perinatal oligodendrogenesis in mice decreases the number of mature oligodendrocytes, in the dorsal telencephalon and corpus callosum. In fact, Mash1 regulates the division of PDGFRα+ OPCs inducing the asymmetric division of OPCs into one OPC and one oligodendrocyte. In the absence of Mash1, OPCs divide symmetrically into 2 OPCs decreasing the generation of future myelinating oligodendrocytes (Nakatani et al., 2013). During the perinatal period, conditional forced expression of Olig2 in nestin+ cells of the SVZ increases the number of OPCs in the SVZ and of mature and myelinating oligodendrocytes in the corpus callosum (Maire et al., 2010). These data demonstrate that Olig2 expression is crucial for the specification of SVZ nestin+ stem cells and OPCs differentiation. Also, a recent study highlights the importance of Musashi1 function in oligodendrogenesis. In fact, Musashi1 mRNA binding protein is expressed in the SVZ stem cells and is required for the maintenance of the stem cell fate notably by inhibiting the translation of the Notch inhibitor Numb. Moreover, retroviral-mediated transduction of Musashi1 in lumbar dorsal funiculus of mouse pups decreases the proportion of mature oligodendrocytes (Dobson et al., 2008).

Many soluble factors have been shown to direct oligodendrogenesis from OPCs and NSCs. For instance, the intracerebroventricular infusion of epidermal growth factor (EGF) increases OPCs production in the SVZ, which migrate and differentiate into myelinating oligodendrocytes in the corpus callosum, striatum and fimbria-fornix (reviewed in Gonzalez-Perez and Alvarez-Buylla, 2011). The pigment epithelium-derived factor (PEDF) is another pro-oligodendrogenic factor. PEDF is secreted by endothelial cells and ependymal cells in the SVZ and acts on stem cells to promote self-renewal (Ramirez-Castillejo et al., 2006). Intracerebral injection of PEDF increases the number of OPCs in the SVZ without affecting proliferation, but rather suggesting a role for PEDF in promoting oligodendroglial specification. In fact, PEDF infusion induces the survival and maturation of local OPCs in the corpus callosum into myelinating oligodendrocytes. *In vitro*, PEDF increases the commitment of glial fibrillary acidic protein (GFAP)+ stem cells toward the oligodendrocyte lineage by inducing the expression of transcription factors related to oligodendrogenesis such as Sox10, Olig1 and Olig2 (Sohn et al., 2012). Similarly, FGF-2 signaling is crucial during the onset of perinatal oligodendrogenesis in the SVZ. Intraventricular injection of FGF-2 increases proliferation and OPCs production in the SVZ (Azim et al., 2012). Surprisingly conditional inactivation of FGFR-2 signaling in oligodendrocytes does not affect normal brain development in mice (Kaga et al., 2006). Nevertheless, FGFR1 and FGFR2 signaling play a role in the specification of OPCs from the embryonic ventral forebrain and regulates myelin sheath (Furusho et al., 2011, 2012). Also, it has been shown that infusion of PDGF-AA into the ventricle induces the hyper-proliferation of NSCs, the so-called type B cells, expanding and producing hyperplasias of Olig2+ OPCs. Type B cells, but not actively proliferative progenitors (type C cells), express the receptor PDGFRα and therefore are the cells that originate these glioma-like structures (Jackson et al., 2006). Intracerebroventricular injection of the morphogen sonic hedgehog in adult mice brain increases cell proliferation in the SVZ, septum, striatum and cerebral cortex, and maturation of oligodendrocytes in the corpus callosum and cortex (Loulier et al., 2006). The neuroprotective factor thymosin β4 (Tβ4) present in neurons and microglia induces differentiation of SVZ into myelinating oligodendrocytes expressing myelin basic protein (MBP) and 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase). Tβ4 inhibits both c-Jun expression and phosphorylation in SVZ cultures and therefore promotes oligodendrogenesis as phosphorylated c-Jun binds to myelin genes promoters such as MBP and CNPase to inhibit their expression (Santra et al., 2012). The thyroid hormone T3 is also a potent inducer of oligodendrocytic differentiation from SVZ cells (Whittemore et al., 1999; Grade et al., 2010).

The ECM also regulates oligodendrogenesis. During the perinatal wave of oligodendrogenesis, OPCs localize close to laminin-rich basal lamina of vessels in the SVZ. Genetic ablation of laminin alpha 2 subunit increases cell death of OPCs in the SVZ and in the corpus callosum during perinatal oligodendrogenesis. Later, by 3 weeks of age, an accumulation of OPCs but fewer mature oligodendrocytes is found and the myelin sheath is thinner. Therefore laminin is necessary for OPCs survival and maturation of OPCs into myelinating oligodendrocytes (Relucio et al., 2012). In fact, oligodendrocytes express a limited repertoire of integrins including α6β1, αvβ1, αvβ3, and αvβ5 that are the receptors for ECM laminin (α6β1), fibronectin and vitronectin. Sequential expression of these integrins regulates the timing of proliferation, migration and differentiation during oligodendrogenesis. Oligodendrocyte O-2A precursor cells are proliferative and migratory bipolar oligodendrocytes expressing α6β1, αvβ1, and αvβ3 integrins. Upon specific blocking of αv by Arg–Gly–Asp (RGD) peptides and anti-β1 antibodies, migration is blocked. During differentiation, oligodendrocyte precursors lose their ability to migrate which is correlated to a decrease of αvβ1 integrin expression (Milner and ffrench-Constant, 1994; Milner et al., 1996). At this step, αvβ3 integrin is transiently expressed and stimulates proliferation before being down-regulated. Indeed, enhanced proliferation is induced in CG-4 rat oligodendrocyte cell line engineered to express αvβ3 integrin and plated on vitronectin (and not on poly-D-lysine; Blaschuk et al., 2000). Then cells exit cell cycle and αvβ5 integrin is up-regulated for terminal differentiation and maturation (Milner and ffrench-Constant, 1994). In OPCs, integrins regulate biological outcomes of PDGF sensing. In fact, association of PDGFRα to αvβ3 integrin activates the Src kinase Lyn and triggers proliferation on fibronectin support. However, upon contact with axonal laminin to α6β1, the Src kinase Fyn is activated and potentiates prosurvival capacities of PDGFRα (Colognato et al., 2004). Moreover, α6β1 plays a crucial role in oligodendrocyte myelination. Numbers of wraps of myelin and amount of myelin protein MBP at axoglial contacts are dependent of the axon diameter. It has been shown that laminin substrate induces oligodendrocytes differentiation with increase in processes complexity and membrane expression of MBP as compared to oligodendrocytes plated on poly-D-lysine (Laursen et al., 2011). This effect is due to translation of MBP mRNA located in the myelinating nodes. The 3′UTR region of the MBP mRNA contains elements that repress its translation notably by binding the mRNA binding protein heterogeneous nuclear ribonucleoprotein-K (hnRNP-K). In the presence of active α6β1 integrin (through contact with

axonal laminin), this inhibition is abolished. By immunoprecipitation, it was shown that hnRNP-K interacts with α6β1 integrin in mature oligodendrocytes cultured on poly-D-lysine and this interaction is decreased on laminin substrate. Laursen et al. (2011) propose that upon activation of integrins, phosphorylation of hnRNP-K induces the release of MBP mRNA for translation and myelination.

MODULATION OF OLIGODENDROGENESIS BY ENDOCANNABINOIDS

Cannabinoids act on at least two types of receptors, type 1 and type 2 cannabinoid receptors (CB1R and CB2R), which are predominantly distributed in the CNS and immune system, respectively (Mackie, 2008). Although low levels of CB2R have also been detected in the CNS (Onaivi et al., 2006). In the brain, CBR are targeted by endocannabinoids such as anandamide (AEA) and 2-arachidonylglycerol (2-AG), which are molecules produced from membrane lipid precursors in response to cell activation, and therefore a reaction tightly controlled by neuronal activity (Galve-Roperh et al., 2008). Once generated, endocannabinoids act retrogradely mainly through presynaptic CB1R, blunting membrane depolarization and inhibiting neurotransmitter release (Galve-Roperh et al., 2008).

Interestingly, it was found that CB1R and CB2R are expressed *in vivo* in the SVZ and DG and in nestin+ cells from neural progenitor cultures (Jin et al., 2004; Aguado et al., 2005; Arevalo-Martin et al., 2007; Molina-Holgado et al., 2007). In fact, it was shown that CB1R and CB2R activation promotes NSC proliferation both in the hippocampus and SVZ (Jin et al., 2004; Aguado et al., 2005; Palazuelos et al., 2006; Molina-Holgado et al., 2007; Goncalves et al., 2008; Xapelli et al., 2013). Moreover, endocannabinoids have emerged as a potential target to modulate oligodendrogenesis, by modulating CB1R and CB2R. In fact, Arevalo-Martin et al. (2007) have found that postnatal treatment with a CB1R agonist increased the number of Olig2+ cells in the rat SVZ. Furthermore, recent studies suggest that endocannabinoids play a role in oligodendrocytic differentiation (Gomez et al., 2010; Solbrig et al., 2010; Gomez et al., 2011) and might modulate several neurodegenerative diseases (reviewed in Velayudhan et al., 2013). Very recently it was shown that CB1R activation promoted differentiation of OPCs and improved remyelination after stroke-induced demyelination (Sun et al., 2013). Despite the role of endocannabinoid receptors in several pathologies, selective agonists and antagonists produce severe adverse side effects. Indeed, activation of CB1 and/or CB2 receptor leads to increased psychoactivity, cardiovascular problems, obesity, diabetes, and inflammation (reviewed in Pacher and Kunos, 2013).

Hence it is of great importance to find alternative new drugs able to modulate cannabinoid receptors. Recent findings suggest that hemopressin (Hp), a nine residue-long peptide derived from the hemoglobin (Hg), modulates the cannabinoid receptors activity (Heimann et al., 2007; Gomes et al., 2010; Bomar and Galande, 2013). In fact, it was shown that Hp acts as a CB1R inverse agonist *in vivo* and inhibits appetite and induces antinociception (Heimann et al., 2007; Dodd et al., 2010) and importantly it was suggested that Hp exerts its effects by an alternative mode of action which might avoid adverse side effects (Dodd et al., 2013). Therefore, we investigated the role of Hp in SVZ oligodendrogenesis and found that this peptide promotes oligodendrocytic differentiation and maturation.

MATERIAL, METHODS AND RESULTS

All experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (86/609/EEC; 2010/63/EU).

We have used SVZ neurospheres prepared from early postnatal (P1-3) C57BL/6 mice in serum-free medium (SFM) supplemented with 10 ng/ml EGF and 5 ng/ml FGF-2 (both from Invitrogen, Carlsbad, CA, USA), as described previously (Xapelli et al., 2013). SVZ neurospheres were seeded for 48 h onto glass coverslips coated with 0.1 mg/ml poly-D-lysine in SFM medium devoid of growth factors. To evaluate cell death/proliferation or oligodendrocytic differentiation, the neurospheres were allowed to develop for further 2, 4, and 7 days at 37°C in the absence or presence of Hp (100 nM or 1 μM; Proteimax, São Paulo, SP, Brazil; **Figures 1A** and **2A**).

First we investigated whether Hp modified cell apoptosis, which was evaluated by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. SVZ plated neurospheres treated with Hp (100 nM or 1 μM) for 48 h (**Figure 1A**) were fixed in 4% paraformaldehyde (PFA) and reacted with the terminal deoxynucleotidyl transferase according to manufacturer's instructions (Roche, Basel, Switzerland). Apoptotic nuclei were labeled with fluorescein followed by nuclei counterstaining with Hoechst 33342 (all from Invitrogen). Finally, the preparations were mounted using Dakocytomation fluorescent medium (Dakocytomation, Carpinteria, CA, USA). We observed no significant differences in the percentage of TUNEL+ nuclei between control and Hp-treated cultures, indicating that Hp was not toxic to the cells (Control: 11.38 ± 1.15%, Hp 100 nM: 10.26 ± 1.70%, Hp 1 μM: 13.25 ± 1.56%; $n = 3$; **Figures 1B,C**).

To investigate the effect of Hp on cell proliferation, SVZ cells were exposed to 10 μM 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich), for the last 4 h of each Hp (100 nM or 1 μM) treatment (48 h). Then, SVZ cells were fixed in PFA and BrdU was immunolabeled with the anti-BrdU mouse monoclonal antibody (Clone MoBU-1) Alexa Fluor 594 (Invitrogen) as described previously (Xapelli et al., 2013; **Figure 1A**). We have found that Hp did not induce proliferation since the percentage of BrdU+ cells in Hp treated cultures were similar to control conditions (Control: 4.38 ± 0.21%, Hp 100 nM: 4.01 ± 0.81%, Hp 1 μM: 4.70 ± 0.53%; $n = 2$; **Figures 1D,E**).

Hp effect on oligodendroglial differentiation was investigated by incubating SVZ neurospheres for 2, 4, or 7 days with Hp (1 μM; **Figure 2A**) and western blotting analysis of oligodendrocyte markers at different stages of differentiation was performed: Olig2 (present during all oligodendrocyte development), PDGFRα (marker of OPC or pre-oligodendrocyte), Galactosylceramidase (GalC, pre-myelinating oligodendrocyte) and proteolipid protein (PLP, myelinating oligodendrocyte). Moreover, an immunocytochemistry for PDGFRα, GalC and PLP was performed following 2, 4, and 7 days of Hp treatment, respectively with the primary antibodies listed in **Table 1** and using a protocol described previously (Xapelli et al., 2013).

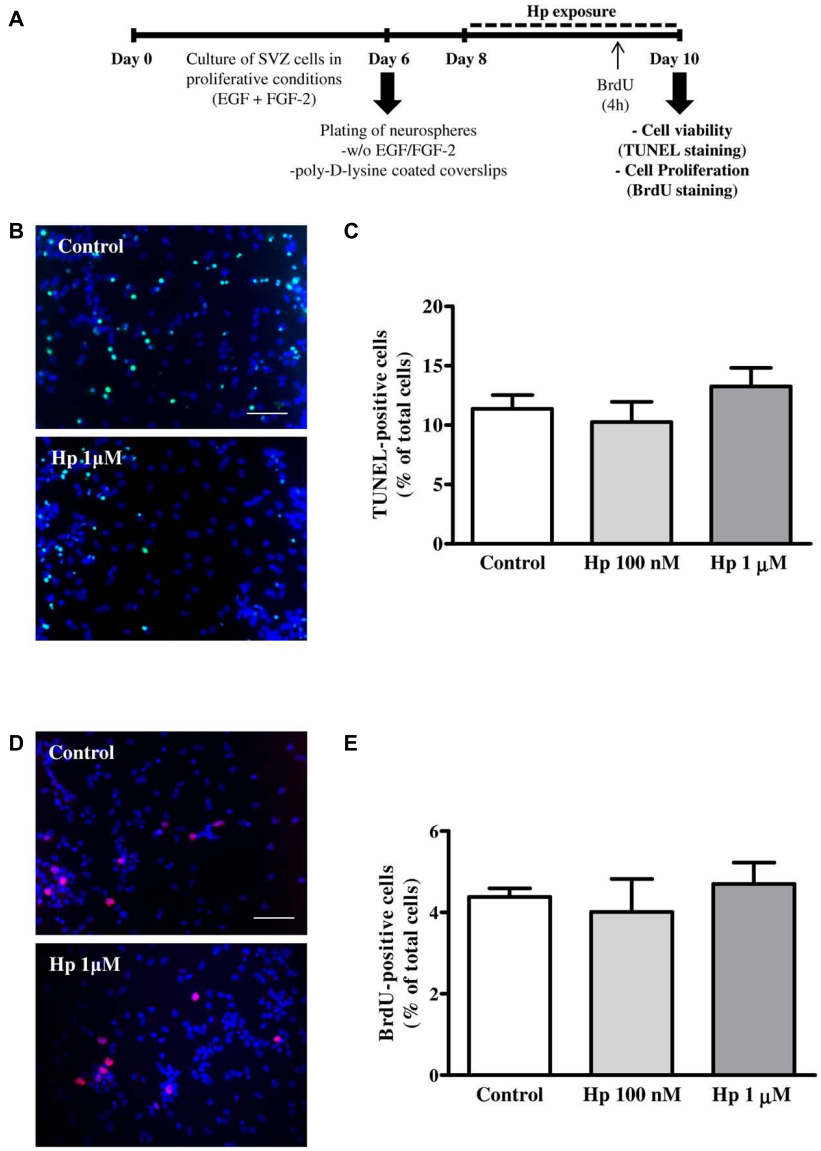

FIGURE 1 | Hp did not induce cell death nor cell proliferation. **(A)** Experimental protocol. **(B)** Representative fluorescence digital images of SVZ cell cultures treated for 48 h in the absence (control) or presence of Hp (1 μM) and stained using the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) method to reveal apoptotic nuclei (in green) and Hoechst 33342 (blue nuclei). **(C)** Bar graph depicts the percentages of TUNEL-stained nuclei. **(D)** Representative fluorescence digital images of SVZ cell cultures in the absence (control) or presence of Hp (1 μM) and immunolabeled for BrdU (5-bromo-2′-deoxyuridine, red nuclei) and Hoechst 33342 (blue nuclei). **(E)** Bar graph depicts the percentages of BrdU-immunostained nuclei. Data are expressed as a mean ± SEM of 2–3 independent experiments. Scale bar: 50 μm.

We have observed that although most of protein levels did not change when comparing with the respective control at the different time-points (**Figure 2B**), the morphology was very different in the cultures that were treated with Hp 1 μM (**Figure 2E**). In fact, PDGFRα and GalC immunoreactivity was increased following Hp treatment for 2 and 4 days respectively (**Figure 2E**). Interestingly, after 7 days of Hp incubation PLP-protein levels increased (to 165.3 ± 47.77% when compared with 100% Control 7 days, $n = 4$, $P < 0.05$; **Figure 2B**). Moreover, we observed that oligodendrocyte maturation was promoted upon Hp treatment for 7 days since the number of PLP+ cells increased when compared with control cultures (Control: 100 ± 0.00%, Hp 1 μM: 314.40 ± 76.06%; $n = 4$, $P < 0.05$; **Figures 2C–E**).

Functional oligodendroglial differentiation was evaluated by single cell calcium imaging (SCCI) to analyze the intracellular variations of calcium-free levels ($[Ca^{2+}]_i$) in single cells following stimulation with 50 mM KCl, 100 μM histamine (Sigma-Aldrich) and 0.1 U/ml thrombin (Sigma-Aldrich) as previously described (Grade et al., 2010). Briefly, SVZ cultures were loaded with Fura-2/AM, then were continuously perfused with Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES, pH 7.4) and

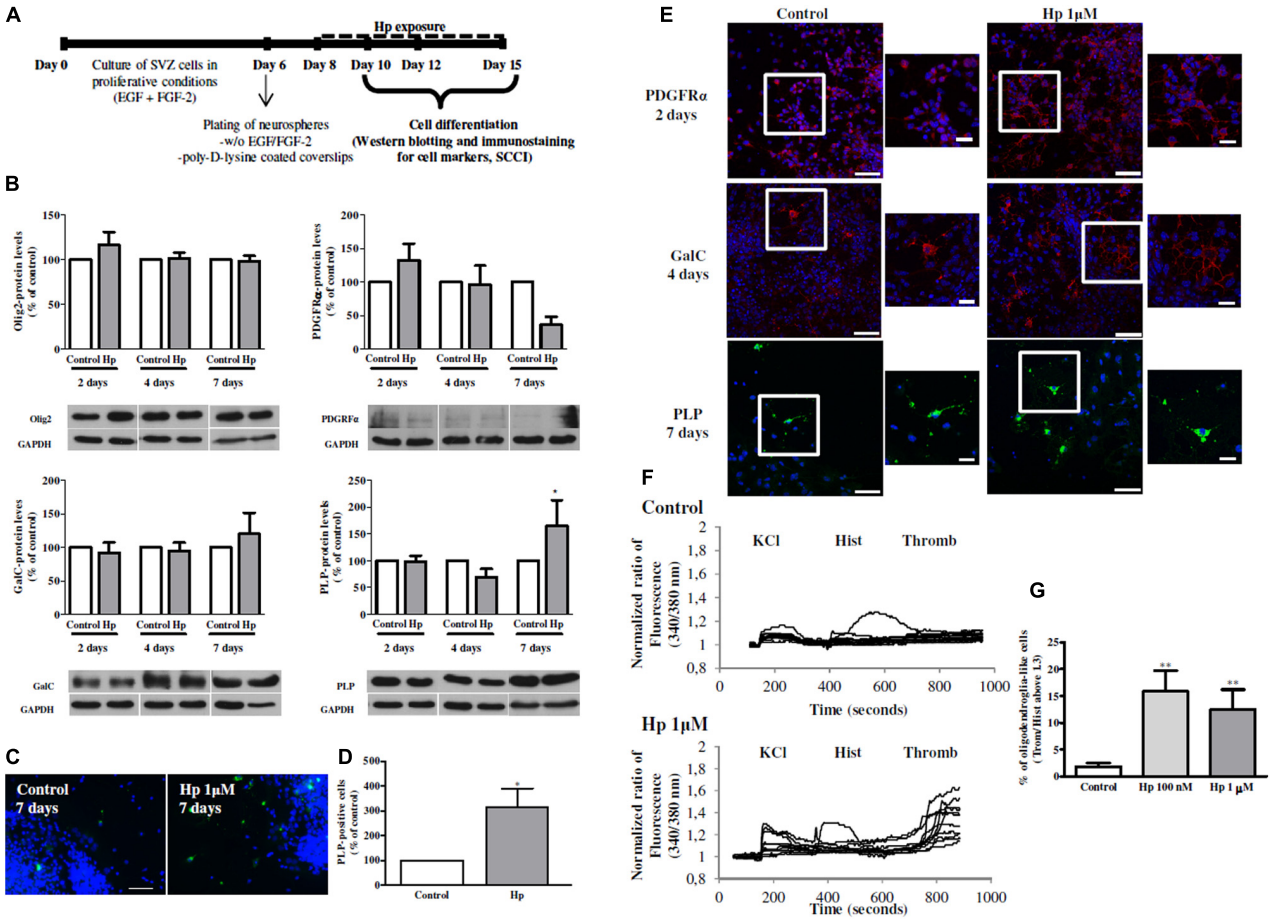

FIGURE 2 | Hp induced oligodendroglial differentiation in SVZ cultures. **(A)** Experimental protocol. **(B)** Bar graphs of oligodendrocyte transcription factor 2 (Olig2), Platelet-derived growth factor receptor alpha (PDGFRα), Galactosylceramidase (GalC) and Proteolipid Protein (PLP) protein levels of control and Hp 1 μM treated cultures for 2, 4, and 7 days. Data are expressed as mean ± SEM of 4–7 independent experiments. $*P < 0.05$; using Bonferroni's multiple comparison test for comparison with the respective control culture. **(C)** Representative fluorescence digital images of PLP+ oligodendrocytes (in green) and Hoechst 33342 staining (blue nuclei) in SVZ cultures. Scale bar: 50 μm. **(D)** Bar graph depicts the number of PLP+ cells, expressed as the total number of cells counted per culture, in control and Hp (1 μM) treated cultures for 7 days. Data are expressed as mean ± SEM of 4 independent experiments. $*P < 0.05$ using using unpaired student's *t*-test for comparison with control culture. **(E)** Representative confocal digital images of PDGFRα, GalC and PLP in control cultures and in cultures treated with Hp 1 μM for 2, 4, and 7 days of differentiation, respectively. PDGFRα (in red); GalC (in red), PLP (in green), and Hoechst 33342 (used to visualize cell nuclei, in blue). Scale bars = 50 μm. Squares are plans of higher magnifications. Scale bars = 20 μm. **(F)**: Representative single cell calcium imaging (SCCI) profiles of response of 8–10 cells in a control culture (no drug exposure) and, in a culture treated with Hp (1 μM) for 7 days. **(G)** Bar graph depicts the percentages of oligodendroglial-like responding cells in SVZ control cultures and in cultures exposed to Hp (100 nm and 1 μM). Data are expressed as mean ± SEM of 4–7 independent experiments. $**P < 0.01$; using Dunnett's multiple comparison test for comparison with SVZ control cultures.

stimulated with KCl, histamine and thrombin. $[Ca^{2+}]_i$ variations were evaluated by quantifying the ratio of the fluorescence emitted at 510 nm following excitation at 340 and 380 nm. KCl, histamine, and thrombin peaks given by the normalized ratios of fluorescence at 340/380 nm, were used to calculate the ratios of the responses to thrombin/histamine, as it was previously shown that ratios above 1.3 are consistent with oligodendroglial differentiation in SVZ cultures (Grade et al., 2010). Indeed it was shown that cells that increased their calcium levels specifically following thrombin stimulation are immunoreactive for O4 and PLP; and oligodendrocytes progenitors expressing the proteoglycan NG2 are responsive to thrombin (lesser than mature oligodendrocytes) but also to histamine consistent with a transition state between immature precursor and mature oligodendrocytes (Grade et al., 2010). In fact, Hp treatment increased the percentage of oligodendrocyte-like cells in SVZ cell cultures (Control: 1.83 ± 0.73%, Hp 100 nM: 15.84 ± 3.85%, Hp 1 μM:

Table 1 | Primary antibodies used for immunocytochemistry.

Antigen	Company	Host	Dilution
PDGFRα	BD Biosciences, San Diego, CA, USA	Rat	1:500
GalC	Millipore	Mouse	1:500
PLP	AbD Serotec, Kidlington, UK	Mouse	1:1000

12.44 ± 3.76%; $n = 4$, $P < 0.01$; **Figures 2F,G**). Moreover, using single cell imaging, we quantified the number of cells displaying a KCl/Histamine ratio of response inferior to 0.8 (consistent with mature neurons), and a ratio between 0.9 and 1 (consistent with mature GFAP+ astrocytes; Agasse et al., 2008). We found that Hp at 1 μM for 7 days increase neuronal differentiation (Control: 11.65 ± 1.58%; Hp 100 nM: 22.68 ± 7.99%; Hp 1 μM: 22.52 ± 5.68%; $n = 6$, $P < 0.05$), without altering the number of GFAP-positive astrocytes (Control: 40.01 ± 3.39%; Hp 100 nM: 34.40 ± 4.64 %; Hp 1 μM: 33.73 ± 3.32; $n = 6$; data not shown).

FUTURE DIRECTIONS

Due to the adverse side effects of synthetic cannabinoid agonists and antagonists (reviewed in Pacher and Kunos, 2013) it is very important to find other selective modulators that maintain therapeutic properties without producing side effects. Therefore, Hp, as a peptide modulator of the cannabinoid receptor, may be a potential therapeutic agent. In fact, it was shown that Hp inhibits appetite and induces antinociception without causing motor abnormalities or sedative effect (Heimann et al., 2007; Dodd et al., 2010, 2013).

Here we have observed that Hp treatment did not affect cell death and proliferation, but promoted oligodendrocytic differentiation and maturation of SVZ stem/progenitor cells. In light of these evidences, Hp and its derivates might be useful in future strategies to treat or prevent demyelinating disorders. Therefore, following steps urge at studying the effect of Hp in animal models of demyelinating diseases, from oligodendrocytic differentiation *in vivo* to amelioration of the disease symptoms.

AUTHOR CONTRIBUTIONS

Sara Xapelli: Conception and design; Administrative support; Provision of study material; Collection and/or assembly of data; Data analysis and interpretation; Manuscript writing; Final approval of manuscript. Fabienne Agasse: Conception and design; Financial support; Collection and/or assembly of data; Data analysis and interpretation, Manuscript writing; Final approval of manuscript. Sofia Grade: Manuscript writing. Liliana Bernardino: Financial support; Administrative support; Manuscript writing. Filipa F. Ribeiro and Clarissa S. Schitine: Provision of study material; Collection and/or assembly of data. Ricardo A. De Melo Reis: Conception and design; Provision of study material; Collection and/or assembly of data. Andrea S. Heimann and Emer S. Ferro: Conception and design; critical reading of manuscript. Ana M. Sebastião: Financial support; Final approval of manuscript. João O. Malva: Conception and design; Financial support; Data analysis and interpretation; Critical reading of manuscript; Final approval of manuscript.

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Enhancement of ventricular-subventricular zone-derived neurogenesis and oligodendrogenesis by erythropoietin and its derivatives

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In the postnatal mammalian brain, stem cells in the ventricular-subventricular zone (V-SVZ) continuously generate neuronal and glial cells throughout life. Genetic labeling of cells of specific lineages have demonstrated that the V-SVZ is an important source of the neuroblasts and/or oligodendrocyte progenitor cells (OPCs) that migrate toward injured brain areas in response to several types of insult, including ischemia and demyelinating diseases. However, this spontaneous regeneration is insufficient for complete structural and functional restoration of the injured brain, so interventions to enhance these processes are sought for clinical applications. Erythropoietin (EPO), a clinically applied erythropoietic factor, is reported to have cytoprotective effects in various kinds of insult in the central nervous system. Moreover, recent studies suggest that EPO promotes the V-SVZ-derived neurogenesis and oligodendrogenesis. EPO increases the proliferation of progenitors in the V-SVZ and/or the migration and differentiation of their progenies in and around injured areas, depending on the dosage, timing, and duration of treatment, as well as the type of animal model used. On the other hand, EPO has undesirable side effects, including thrombotic complications. We recently demonstrated that a 2-week treatment with the EPO derivative asialo-EPO promotes the differentiation of V-SVZ-derived OPCs into myelin-forming mature oligodendrocytes in the injured white matter of neonatal mice without causing erythropoiesis. Here we present an overview of the multifaceted effects of EPO and its derivatives in the V-SVZ and discuss the possible applications of these molecules in regenerative medicine.

Keywords: ventricular-subventricular zone, neurogenesis, oligodendrogenesis, erythropoietin, regeneration, differentiation, neural stem cells

PRODUCTION OF NEURONAL AND OLIGODENDROCYTE PROGENITORS IN THE V-SVZ

NEW NEURON PRODUCTION IN THE V-SVZ

Neural stem cells (NSCs) in the ventricular-subventricular zone (V-SVZ), located at the lateral walls of the lateral ventricles, have been investigated as an endogenous cell source for neurons (Belvindrah et al., 2009; Kriegstein and Alvarez-Buylla, 2009; Ihrie and Alvarez-Buylla, 2011; Ming and Song, 2011) and oligodendrocytes (Nait-Oumesmar et al., 2008; Gonzalez-Perez and Alvarez-Buylla, 2011) in the postnatal brain. While maintaining themselves by self-renewing cell division, the NSCs produce actively proliferating intermediate progenitors called transit-amplifying cells, which generate immature new neurons, called neuroblasts. The neuroblasts born in the V-SVZ are characterized by their prominent migration capacity. They have a bipolar shape with leading and trailing processes, and in the rostral migratory stream, they migrate in chain-like aggregates for a long distance to the olfactory bulb, where they differentiate into interneurons to be integrated into the olfactory circuitry (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Petreanu and Alvarez-Buylla, 2002; Carleton et al., 2003).

These neurogenic cells are tightly associated with the vasculature. NSCs extend their process with an endfoot that makes contact with blood vessels (Mirzadeh et al., 2008; Tavazoie et al., 2008), the transit-amplifying progenitors reside very close to vessels (Shen et al., 2008; Tavazoie et al., 2008; Kokovay et al., 2010), and the neuroblasts frequently migrate along vessels (Snapyan et al., 2009; Whitman et al., 2009). Therefore, although its precise function remains unclear, the vasculature is thought to contribute to the neurogenic function of the progenitor cells and the efficient migration of neuroblasts (Ihrie and Alvarez-Buylla, 2011).

OLIGODENDROCYTE PRODUCTION IN THE V-SVZ

Oligodendrocytes form the myelin sheath, which wraps around axons to facilitate the rapid, saltatory conduction of electrical impulses along them. In early postnatal stages, V-SVZ is an important source of forebrain oligodendrocyte progenitor cells (OPCs) (Levison and Goldman, 1993; Luskin, 1993; Ivanova et al., 2003; Suzuki and Goldman, 2003). These OPCs differentiate into mature oligodendrocytes after they migrate to and colonize the parenchyma. However, a portion of the OPCs remain as progenitors, which are called NG2 glia due to their expression of the

OPC marker NG2 proteoglycan, and become a major provider of oligodendrocytes in later postnatal stages and throughout adulthood. (Stallcup and Beasley, 1987; Gensert and Goldman, 1997; Nishiyama et al., 2002; Greenwood and Butt, 2003; Fancy et al., 2004).

Recent studies revealed that the adult V-SVZ continuously produces OPCs, via a distinct group of transit-amplifying progenitors that express the oligodendrocyte lineage markers Olig2 and NG2 (Levison and Goldman, 1993; Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002; Hack et al., 2005; Menn et al., 2006). Although the adult V-SVZ-derived OPCs are a minor population among the entire OPC pool in the brain, they have a distinctive capacity to migrate actively over long distances into the corpus callosum, striatum, and fimbria fornix, where they differentiate into mature, myelin-forming oligodendrocytes (Nait-Oumesmar et al., 1999; Menn et al., 2006; Aguirre et al., 2007). Like the migrating neuroblasts, the migrating OPCs have a bipolar morphology with leading and trailing processes, however, they tend to migrate individually rather than by forming chain-like aggregates (Menn et al., 2006).

NEUROGENESIS AND OLIGODENDROGENESIS UNDER PATHOLOGICAL CONDITIONS

Cell proliferation in the V-SVZ is up-regulated in response to various pathological conditions that cause neuronal loss, such as ischemic stroke and neurodegenerative diseases. The neuroblasts generated in the V-SVZ migrate toward the injured area and differentiate into functional mature neurons (Yamashita et al., 2006; Kojima et al., 2010; Yoshikawa et al., 2010).

Myelin degeneration blocks conduction, whether it occurs in the context of oligodendrocyte-specific disorders, such as multiple sclerosis, or as a result of non-specific insults, including severe ischemia. The blocked conduction induces a variety of neurological impairments. After demyelination, new oligodendrocytes generated from the parenchymal OPCs contribute to remyelination (Reynolds et al., 2002; Aguirre et al., 2007; Fancy et al., 2009, 2011; Patel et al., 2010; Azim and Butt, 2011; Huang et al., 2011; Mi et al., 2011). In addition, in the brains of multiple sclerosis patients and of rodent demyelination models, new OPC production in the V-SVZ is significantly increased, and these OPCs also contribute to remyelination (Nait-Oumesmar et al., 1999, 2007). Furthermore, V-SVZ-derived migrating progenitors committed to the neuronal lineage can apparently change their fate to differentiate into oligodendrocytes in demyelinated areas (Jablonska et al., 2010).

The insult-induced spontaneous regeneration of neurons and oligodendrocytes is insufficient for full structural and functional restoration of the injured brain. Therefore, interventions to enhance these processes are being sought for future clinical applications. Various interventions that increase new neurons/oligodendrocytes have already been shown to improve neurological function (Leker et al., 2009; Lindvall and Kokaia, 2010; Nakaguchi et al., 2011; Christie and Turnley, 2012). Erythropoietin (EPO), a clinically used erythropoietic factor, is one of the promising candidate drugs that promote V-SVZ-derived neurogenesis and oligodendrogenesis.

ENDOGENOUS EPO ACTIVITY IN THE CENTRAL NERVOUS SYSTEM (CNS)

EPO AND ITS RECEPTOR

EPO is a single polypeptide glycoprotein hormone that is mainly produced in and secreted from fetal hepatocytes and interstitial fibroblasts in the adult kidney, to increase the number of circulating erythrocytes (Marti, 2004). It consists of 166 amino acids folded into 4 α-helices, and includes three *N*-glycosylation sites that each accommodate up to four sialic acid residues (Jacobs et al., 1985; Lin et al., 1985; Lai et al., 1986). The sialylation contributes to EPO's stability in the circulation (Fukuda et al., 1989).

EPO production dramatically increases in response to low partial oxygen pressure, mediated by the activation of hypoxia-inducible factor (Franke et al., 2013). In addition to hypoxia, metabolic stress, or proinflammatory cytokines can induce EPO production (Maiese et al., 2012). Upon binding to the homodimeric EPO receptor (EPOR) on erythroid progenitors in bone marrow (Marti, 2004), EPO induces JAK2 phosphorylation and the activation of downstream signaling pathways, including STAT5, PI3K/Akt, and MAPK, which enhance the proliferation, differentiation, and survival of the progenitors, thereby increasing the number of circulating erythrocytes (Quelle et al., 1996; Zhao et al., 2006). Cloned in 1985 (Jacobs et al., 1985; Lin et al., 1985), recombinant human EPO (rhEPO) has been used as a treatment for anemia (mainly caused by chronic renal failure) for more than 20 years.

EPO AND EPOR IN THE CNS

After EPO's hematopoietic functions were reported, subsequent studies have indicated that EPO is produced in other adult organs in addition to the kidney, such as the liver, spleen, and CNS, in response to hypoxia (Marti, 2004; Chateauvieux et al., 2011; Lombardero et al., 2011). The expression of EPO and EPOR in the brain is reported in rodents, monkeys, and humans during development and in adulthood (Marti et al., 1996; Juul et al., 1998; Knabe et al., 2004), and especially in the periventricular germinal zone of the fetal brain (Liu et al., 1997; Juul et al., 1999; Tsai et al., 2006). Although their expression decreases dramatically during the course of development, EPO and EPOR continue to be expressed in the adult V-SVZ (Liu et al., 1997).

Astrocytes were the first cells to be identified as EPO producers in the brain (Masuda et al., 1994; Marti et al., 1996). Later, neurons in several regions were also found to produce EPO (Bernaudin et al., 1999, 2000; Siren et al., 2001). Although much less EPO is produced in the brain than in the kidney (Tan et al., 1992), the locally produced EPO might play an important role in the brain, because EPO in the periphery cannot efficiently cross the blood-brain barrier (BBB) via lipid-mediated transport under normal conditions, due to its large molecular size.

Intriguingly, compared to the EPO in the circulation, brain-derived EPO has less sialylation, and consequently exhibits a smaller molecular weight and shorter plasma half-life, but it has a higher affinity for EPOR (Masuda et al., 1994). EPO production in the brain is induced by hypoxia, although the time course is quite different from that in the periphery. During hypoxia

exposure, the brain EPO mRNA level rises rapidly, and this increased level is sustained for more than 24 h, whereas the circulating EPO protein and EPO mRNA in the kidney quickly decline to basal levels even under conditions of continuous hypoxia (Chikuma et al., 2000). Taken together, these findings indicate that EPO in the brain has a distinct bioactivity and regulatory system from that in the circulation.

EPOR is also expressed in various cell types in the brain, including neurons, astrocytes, OPCs, microglia, and endothelial cells (Brines et al., 2000; Nagai et al., 2001; Sugawa et al., 2002; Marti, 2004). EPOR's expression is induced by hypoxia (Chin et al., 2000; Yu et al., 2002), proinflammatory cytokines (Nagai et al., 2001), and EPO (Chin et al., 2000), and its distribution corresponds to that of EPO, suggesting that brain EPO works in a paracrine/autocrine manner in response to hypoxia. In neuronal cells, in addition to the STAT5, PI3K/Akt, and MAPK pathways, NF-κB is involved in EPO-EPOR signaling (Digicaylioglu and Lipton, 2001; Yu et al., 2002).

FUNCTION OF ENDOGENOUS EPO SIGNALING IN THE BRAIN

While a lack of EPO-EPOR signaling causes embryonic lethality with severe anemia (Wu et al., 1995; Lin et al., 1996), the brain-specific knockout of EPOR from the late embryonic phase (using EPOR-floxed mice crossed with human GFAP-Cre mice) and selective EPOR knock-in in the hematopoietic tissues of EPOR-null mice cause only a small defect in brain development (Suzuki et al., 2002; Tsai et al., 2006; Chen et al., 2007), suggesting that EPO signaling is not deeply associated with brain development. However, interestingly, EPOR is expressed in nestin-expressing NSCs in the ganglionic eminence, including in the area that develops into the postnatal V-SVZ, and hypoxia-induced EPO expression in NSCs *in vitro* promotes neuronal differentiation (Shingo et al., 2001). EPOR is also expressed in EGFR-expressing neuronal progenitors (mostly transit-amplifying progenitors) in the adult V-SVZ. In an ischemic stroke model, brain-specific EPOR knockdown did not affect the infarct volume, but it suppressed reactive cell proliferation in the V-SVZ and the migration of neuroblasts to the injury site (Tsai et al., 2006). These findings indicated that endogenous EPO-EPOR signaling in the brain is involved in controlling neurogenesis under physiological and pathological conditions.

CNS PROTECTION BY EPO TREATMENT

PROTECTIVE EFFECTS OF EPO ON NEURONS AND OLIGODENDROCYTES

A number of studies have revealed beneficial effects of EPO administration in various animal models for CNS diseases (Ghezzi and Brines, 2004; Maiese et al., 2004; van der Kooij et al., 2008) and in patients with ischemic stroke, schizophrenia, and multiple sclerosis (Siren et al., 2009). Recombinant human EPO (rhEPO) treatment directly protects neurons from hypoxia, excitotoxins, and metabolic stresses *in vitro* through multiple pathways, such as by blocking calcium influx-induced glutamate release, enhancing anti-apoptotic and anti-oxidant protein production, and suppressing pro-apoptotic protein and free radial production (Ghezzi and Brines, 2004; Marti, 2004; van der Kooij et al., 2008; Maiese et al., 2012). The peripheral administration or intraventricular infusion of rhEPO protects neurons in a number of experimental disease models, including those for ischemic stroke, traumatic injury, neurodegenerative diseases, seizure, and schizophrenia, by modifying the immune reaction and degree of inflammation, protecting the BBB, promoting angiogenesis to restore the oxygen supply, and suppressing brain atrophy and secondary gliosis, in addition to EPO's direct neuroprotective activities (Ghezzi and Brines, 2004; Maiese et al., 2004; Noguchi et al., 2007; van der Kooij et al., 2008; Chateauvieux et al., 2011).

rhEPO treatment also protects oligodendrocytes and OPCs and prevents demyelination in animal models of multiple sclerosis, spinal cord injury, and stroke (Zhang et al., 2005; Savino et al., 2006; Vitellaro-Zuccarello et al., 2007; Mizuno et al., 2008). While several studies support the finding that EPOR is expressed on OPCs (Nagai et al., 2001; Sugawa et al., 2002; Kato et al., 2011), whether or not it is expressed on mature oligodendrocytes is still controversial. It is possible that EPO stimulates other EPOR-expressing cell type(s), thereby indirectly promoting the survival of oligodendrocytes.

With regard to how peripherally administered EPO reaches the brain, both receptor-mediated active transport and extracellular pathways are used to pass EPO through the BBB (Brines et al., 2000; Banks et al., 2004; Ehrenreich et al., 2004; Juul et al., 2004; Xenocostas et al., 2005). After a single high-dose intravenous injection, EPO is detectable in the brain within hours, reaching a peak concentration at 3–4 h in the brain or cerebrospinal fluid in humans and other animals (Banks et al., 2004; Ehrenreich et al., 2004; Juul et al., 2004; Xenocostas et al., 2005). Although the influx of EPO is restricted in the healthy brain, the permeability of the BBB is significantly increased by brain insults such as ischemic stroke (Yang and Rosenberg, 2011), which can increase the ability of peripherally administered EPO to cross into the brain.

RECEPTORS FOR EPO IN CYTOPROTECTION

In addition to the homodimeric EPOR, EPO binds to a heterodimeric receptor consisting of the classical EPOR and the beta common receptor (βcR), a subunit also known as CD131 and shared by several cytokine receptors, including those for interleukin (IL)-3, IL-5, and granulocyte-macrophage colony stimulating factor (Hanazono et al., 1995; Jubinsky et al., 1997). While the hematopoietic activity of EPO depends on the homodimeric EPOR, other effects appear to be mediated by the heterodimeric receptor, which has a lower affinity for EPO. For example, EPO does not exert its protective effect on spinal cord injury in mice lacking βcR (Brines et al., 2004). Moreover, EPO derivatives that do not bind the homodimeric EPOR can still protect tissue and improve neurological function similarly to EPO in stroke, spinal cord injury, and demyelination models (Brines et al., 2004; Leist et al., 2004; Savino et al., 2006; King et al., 2007; Villa et al., 2007; Wang et al., 2007b), indicating that the tissue-protection function of EPO might be mediated by the βcR-containing heterodimeric receptor.

On the other hand, the expression level of βcR in the brain is relatively quite low, and its localization does not correspond to the expression of EPO/EPOR or to the cell types protected by EPO treatment in culture or in a pilocarpine-induced epilepsy model (Nadam et al., 2007; Um et al., 2007; Sanchez et al., 2009). EPO inhibits apoptosis in neuron-like cell lines in which the βcR

expression is undetectable, and the interaction between EPO and homodimeric EPOR appears to be essential for this effect (Um et al., 2007). These data suggest that EPO needs to interact with classical homodimeric EPOR to exert its neuroprotective effect. However, the functional difference between the homodimeric and heterodimeric EPOR in the V-SVZ has not been demonstrated.

DERIVATIVES OF EPO USED FOR CYTOPROTECTIVE TREATMENT

Many derivatives of EPO have a cytoprotective effect similar to that of EPO itself (Jerndal et al., 2010). Daberpoetin-alpha, an EPO derivative with 3 times the circulation half-life of endogenous EPO, due to having more sialic acid moieties, is not only beneficial for treating anemia, but also has a neuroprotective activity similar to that of EPO in stroke, intracerebral hemorrhage, and acute ethanol intoxication models (Seymen et al., 2013; Belayev et al., 2005; Grasso et al., 2009). On the other hand, the hematopoietic activity of EPO and its derivatives sometimes causes polycythemia and thrombosis, making them inappropriate for clinical use to treat CNS disorders. Therefore, the effect of derivatives with reduced or no hematopoietic activity has been investigated in several disease models (Leist et al., 2004; Villa et al., 2007).

Asialo erythropoietin (AEPO), which is generated by the total enzymatic desialylation of rhEPO, binds to the homodimeric EPOR with a similar affinity as EPO, but is rapidly cleared from the circulation by hepatic cells, due to the lack of sialic acid at the terminals of its oligosaccharides (plasma half life, EPO: 5.6 h, AEPO: 1.4 min by intravenous administration) (Fukuda et al., 1989; Imai et al., 1990). Notably, *in vitro* experiments revealed that, while the induction of hematopoiesis requires EPO stimulation of long duration, only 5 min of EPO exposure is sufficient for neuroprotection (Morishita et al., 1997). We and others further revealed that repeated AEPO administration biweekly for more than 1 month in adult mice (Erbayraktar et al., 2003) or once a day for 2 consecutive weeks in neonatal mice (Kako et al., 2012) did not enhance erythropoiesis. Nevertheless, AEPO protects neurons (Erbayraktar et al., 2003; Wang et al., 2004b; Grasso et al., 2006) and oligodendrocytes (Savino et al., 2006) in stroke, spinal cord injury, sciatic nerve injury, and multiple sclerosis models. Since AEPO is a natural physiological metabolite of EPO, it appears to be a promising and safe drug for clinical applications.

Carbamylated erythropoietin (CEPO) is a derivative produced by chemically replacing all the lysine residues in EPO with homocitruline, a process called carbamylation. CEPO has a similar plasma half-life as EPO. Although CEPO does not bind to homodimeric EPOR, which is responsible for EPO-induced hematopoiesis (Leist et al., 2004), CEPO treatment protects neurons and oligodendrocytes from apoptosis, and it protects tissue and suppresses inflammation in brain and spinal cord injury models and cultures, as effectively as EPO (Brines et al., 2004; Leist et al., 2004; Montero et al., 2007; Villa et al., 2007; Wang et al., 2007b; Liu et al., 2011; Xiong et al., 2011). CEPO can bind to the EPOR and βcR heterodimeric receptor, and this binding was reported to mediate CEPO's cytoprotective effect in spinal cord injury (Brines et al., 2004). However, because CEPO is not a natural metabolite, careful investigation is needed to evaluate its safety for clinical applications.

PROMOTION OF NEUROGENESIS/OLIGODENDROGENESIS IN THE V-SVZ BY EPO TREATMENT

While most previous studies suggest that EPO and its derivatives should be administered before or immediately after injury to elicit their tissue-protective effects in various animal models (van der Kooij et al., 2008), delaying administration for 24 h or even for several days after the insult can enhance neurogenesis and/or oligodendrogenesis, and lead to improved neurological symptoms weeks to months after the insult (Wang et al., 2004a; Iwai et al., 2010; Zhang et al., 2010; Kako et al., 2012) (**Table 1**). Although the functional aspects of the new neurons/oligodendrocytes are still unclear, infusion of an anti-mitotic agent, Ara-C, which inhibits neurogenesis, effectively abolished the functional recovery after EPO treatment in a traumatic injury model (Zhang et al., 2012), suggesting that the EPO-induced functional improvement depends, at least in part, on the production of new neurons/oligodendrocytes after the injury.

PROMOTION OF NEUROGENESIS BY EPO TREATMENT

rhEPO administration promotes neurogenesis under both physiological and pathological conditions. In the intact brain, rhEPO infusion for 6 days into the lateral ventricle increases the number of neuronal progenitor cells, with a concomitant decrease in the number of NSCs, suggesting that EPO enhances neuronal differentiation (Shingo et al., 2001). Furthermore, in stroke and neonatal hypoxia/ischemia models, peripherally administered rhEPO increases the numbers of both NSCs and neuronal progenitors by promoting their proliferation and neuronal differentiation, and it enhances the migration of neuroblasts to the injury site (Wang et al., 2004a; Iwai et al., 2007). The intraventricular infusion of rhEPO in 6-OH-DOPA-injected Parkinson's disease model animals also enhances neuroblast production in the V-SVZ and increases the number of neuroblasts within the striatum (Kadota et al., 2009).

With regard to how EPO affects neurogenesis, *in vitro* studies suggest that it has direct effects on NSCs. For example, in NSCs dissociated from the adult V-SVZ, EPO treatment increases Akt activity, which promotes proliferation, differentiation, and neurite outgrowth; neurogenin-1 is involved in this pathway (Wang et al., 2006b). In a similar culture system, CEPO activates sonic hedgehog to induce the expression of Mash1, a bHLH transcription factor that increases neurogenesis (Wang et al., 2007a). In embryonic NSCs, EPO enhances the nuclear translocation of NF-κB to promote neuronal differentiation and induces Mash1 expression (Shingo et al., 2001).

EPO can also enhance neurogenesis through the protection and remodeling of the cerebral vasculature. As previously mentioned, the vasculature is involved in maintaining the function of NSCs and transit-amplifying neuronal progenitors in the V-SVZ. In addition, after ischemia, endothelial cells secrete attractive molecules such as SDF-1 and angiopoietin-1, which guide neuroblasts expressing receptors for these molecules toward the injury (Imitola et al., 2004; Ohab et al., 2006; Robin et al., 2006). Blood vessels also appear to act as a scaffold for the migrating neuroblasts in the striatum after stroke (Yamashita et al., 2006; Kojima et al., 2010) and for V-SVZ-derived progenitors that give rise to oligodendrocytes in the demyelinated corpus callosum

Table 1 | EPO's effects on V-SVZ neurogenesis/oligodendrogenesis.

Model	Age	EPO treatment	Effects on neurogenesis, oligodendrogenesis, and angiogenesis	References
Rat, stroke	Adult	rhEPO, i.p., 1-8 dpi	V-SVZ: increase in NSCs and neuroblasts St, Cx: increase in neuroblasts and blood vessels	Wang et al., 2004a, 2006a
Rat, stroke	Adult	rhEPO, i.p., 0-4 dpi	V-SVZ: increase in cell proliferation and OPCs Cx: increase in angiogenesis	Kim and Jung, 2010
Rat, stroke	Adult	rhEPO, i.p., 1-8 dpi	(Lentivirus injection into V-SVZ 3 d before injury) V-SVZ: increase in OPCs and OPC proliferation St: increase in virus-labeled OPCs	Zhang et al., 2010
Mouse, stroke	Adult	rhEPO, with hydrogel, epicortical, 4 or 11 dpi	V-SVZ: increase in neuroblasts St, Cx: increase in neuroblasts	Wang et al., 2012
Rat, stroke	Neonate (P 10)	rhEPO, i.p. 0 dpi	St: increase in mature new neurons	Gonzalez et al., 2007
Rat, stroke	Neonate (P 7)	rhEPO, i.p. 0, 1, 7 dpi	(Lentivirus injection into LV at P1) St, Cx: increase in virus-labeled neuroblasts and OPCs, decrease in virus-labeled astrocytes	Gonzalez et al., 2013
Rat, H/I	Neonate (P 7)	rhEPO, i.p. 0, 2, 4, 6 dpi	V-SVZ: increase in proliferating cells and neuroblasts St, Cx: increase in neuroblasts, mature new neurons, and blood vessels	Iwai et al., 2007
Rat, H/I	Neonate (P 7)	rhEPO, i.p. 2, 4, 6, 9, 13 dpi	St, Cx: increase in neuroblasts St, Cx, CC: increase in OPCs and oligodendrocytes Cx, CC: increase in BrdU-labeled oligodendrocytes	Iwai et al., 2010
Mouse, H/I	Neonate (P 9)	rhEPO, i.p. 0, 1, 2 dpi	V-SVZ: increase in proliferating cells (only in females)	Fan et al., 2011
Mouse, H/I	Neonate (P 5)	AEPO, i.p.,5-19 dpi	(Retrovirus injection into V-SVZ at P5) CC: promotion of oligodendrocyte differentiation	Kako et al., 2012
Rat, 6-OHDA	Adult	rhEPO, i.c.v. for 7 d	V-SVZ: increase in neuroblast St: increase in neuroblasts	Kadota et al., 2009
Mouse, intact	Adult	rhEPO, i.c.v., for 6 d	V-SVZ: decrease in NSCs, increase in NPCs and neuroblasts	Shingo et al., 2001
Mouse, intact	Adult	EPOR-CKO	V-SVZ: decrease in cell proliferation	Tsai et al., 2006
Mouse, stroke	Adult	EPOR-CKO	V-SVZ: lack of stroke-induced increase in cell proliferation	Tsai et al., 2006
Mouse, intact	Adult	Conditionally rescued *EPOR*$^{-/-}$	V-SVZ: decrease in cell-proliferation	Chen et al., 2007

Previous studies that report the effects of EPO treatment and EPO signaling modification on V-SVZ-associated neurogenesis and oligodendrogenesis. i.c.v., intracerebroventricular; i.p., intraperitoneal; dpi, days post injury; St, striatum; Cx, cortex; P, postnatal day; H/I, hypoxia/ischemia; CKO, conditional knockout.

(Cayre et al., 2013). EPO treatment suppresses the apoptosis of vascular endothelial cells in which EPOR expression is strongly induced by ischemia (Bernaudin et al., 1999; Chong et al., 2002), and promotes angiogenesis to restore blood flow in the ischemic area (Li et al., 2007). This angiogenesis is reported to be mediated by the enhanced endothelial expression of VEGF, a critical growth factor for generating and remodeling the vasculature (Wang et al., 2004a). EPO also induces matrix metalloproteinase (MMP)-2 and MMP-9 in endothelial cells, via activation of the Akt and ERK1/2 pathway, to promote neuroblast migration (Wang et al., 2006a). Interestingly, EPO-treated neurospheres derived from the adult V-SVZ promote the capillary-like tube formation of cultured

endothelial cells by secreting VEGF (Wang et al., 2008). Therefore, EPO's efficient enhancement of neurogenesis and oligodendrogenesis appears to be tightly associated with vascular function.

PROMOTION OF OLIGODENDROGENESIS BY EPO TREATMENT

In addition to preventing myelin degeneration (Zhang et al., 2005; Savino et al., 2006; Vitellaro-Zuccarello et al., 2007; Liu et al., 2011), several studies have reported that delaying EPO treatment by 1–7 days after the insult, which doesn't protect the tissue from injury, efficiently enhances the production of new oligodendrocytes (Iwai et al., 2010; Zhang et al., 2010; Kako et al., 2012) and promotes white matter reorganization (Li et al., 2009) leading to improved neurological function. While the underlying mechanism remains unknown, these observations suggest that EPO treatment promotes remyelination to alleviate neurological dysfunction.

Since oligodendrogenesis takes place not only in localized areas, but also widely throughout the brain even in adulthood, especially after demyelination, a region-specific cellular labeling method is needed to determine the effect of EPO treatment on the behavior of V-SVZ-derived OPCs/oligodendrocytes. For this purpose, viral vectors were stereotaxically injected into the rodent V-SVZ (Zhang et al., 2010; Kako et al., 2012). The lentivirus vector is efficiently integrated into the genome of both proliferating and non-proliferating cells, permanently labeling them and their progenies. Using this system, a 7-day treatment with EPO starting 24 h after ischemic stroke was shown to increase the OPCs in the V-SVZ, and to promote the recruitment to the injury site of lentivirus-labeled cells that express a mature oligodendrocyte marker (Zhang et al., 2010). In addition, in a neonatal stroke model, EPO treatment was shown to promote the proliferation of lentivirus-labeled NSCs, which preferentially produced neurons and oligodendrocytes rather than astrocytes in the injured striatum (Gonzalez et al., 2013).

To investigate the specific effect of EPO treatment on V-SVZ-derived OPCs, we combined a retrovirus injection into the V-SVZ (which labels proliferating cells and their progenies) with a lineage-specific fate-mapping method for OPCs, using tamoxifen-induced recombination, in a neonatal ischemic injury model (Kako et al., 2012). Even though V-SVZ-derived OPCs showed extensive migration in the corpus callosum toward the injured white matter, only about 30% of them underwent maturation in the subsequent 19 days. The maturation ratio was significantly lower than that in the intact brain, in which more than 60% of OPCs differentiated into mature oligodendrocytes over the same time period. Interestingly, this maturation defect in the injured area was relatively mild, when we considered the entire OPC population, in which parenchymal OPCs predominate and the percentage of OPCs derived from the V-SVZ is small. These data suggest that V-SVZ-derived OPCs/oligodendrocytes are more susceptible to an inflammatory environment than are parenchymal OPCs. Delayed AEPO treatment given for a duration of 2 weeks, but not for 3 days, could almost completely prevent this maturation defect and neurological impairment. Taken together, these findings indicate that EPO treatment enhances OPC production in the V-SVZ and promotes the migration and differentiation of V-SVZ-derived OPCs to supply new oligodendrocytes in the injured brain.

In summary, many studies support the idea that EPO enhances neurogenesis and/or oligodendrogenesis at multiple steps after brain insults (**Figure 1**). However, in most of these studies, EPO was administered using the same protocols as those of the previous studies that demonstrated EPO-induced neuroprotection; thus, the optimal dosage, timing, and duration of EPO treatment for promoting regeneration have not been carefully investigated. Considering that regeneration is a continuous process occurring over months to years, which is much longer than the process of cell death, which mostly takes place during the acute phase after insult, the optimal EPO treatment schedule for efficient regeneration should be different from that used for cytoprotection.

PERSPECTIVES

EPO and its derivatives appear to be promising drugs for promoting neuron and oligodendrocyte regeneration by V-SVZ-derived progenitors in various CNS disorders, as discussed above. However, fundamental questions remain to be addressed. For example, it is not known whether the V-SVZ-derived oligodendrocytes induced by the EPO treatment are functionally similar to those derived from parenchymal OPCs. In addition, the effect of EPO on neurogenesis and oligodendrogenesis in the primate V-SVZ remains to be studied. Furthermore,

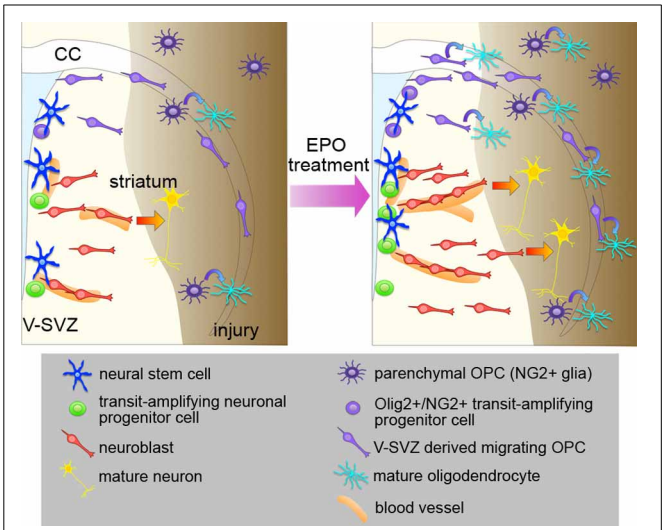

FIGURE 1 | Effects of EPO treatment on the V-SVZ after brain injury. Schematic representation of the effects of EPO treatment on neurogenesis, oligodendrogenesis, and angiogenesis in the brain after injury. EPO treatment enhances the proliferation of NSCs and neuronal progenitors to increase the number of neuroblasts after injury, and promotes recruitment of the neuroblasts to the injured area, where they differentiate into mature neurons. EPO treatment increases the production of migrating OPCs from the Olig2+/NG2+ transit-amplifying progenitors in the V-SVZ, and promotes the differentiation of V-SVZ-derived OPCs into oligodendrocytes in the injured area. The proliferation and differentiation of the parenchymal OPCs are also enhanced by EPO. EPO also enhances angiogenesis, which might be involved in the EPO-induced neurogenesis and oligodendrogenesis after injury.

the underlying mechanisms of EPO's effects are still largely unknown, especially those responsible for EPO's effect on V-SVZ oligodendrogenesis.

Notably, systemic rhEPO treatment has several potential risks, including not only polycythemia and thrombosis, due to its hematopoietic activity, but also hypertension due to increased vascular smooth muscle contraction (Vaziri et al., 1995; Miyashita et al., 2004) and the promotion of malignant tumor growth by enhanced tumor cell survival and angiogenesis (Yasuda et al., 2003). Therefore, further studies to determine the mechanisms of each effect of EPO and its derivatives should help guide the development of appropriate treatments that specifically promote neurogenesis and oligodendrogenesis without causing adverse events.

AUTHOR CONTRIBUTIONS

Naoko Kaneko: conception and design, collection and assembly of data, and manuscript writing, Eisuke Kako: collection and assembly of data, Kazunobu Sawamoto: conception and design, manuscript writing, and financial support

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Strategies for repair of white matter: influence of osmolarity and microglia on proliferation and apoptosis of oligodendrocyte precursor cells in different basal culture media

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The aim of the present study has been to obtain high yields of oligodendrocyte precursor cells (OPCs) in culture. This is a first step in facilitation of myelin repair. We show that, in addition to factors, known to promote proliferation, such as basic fibroblast growth factor (FGF-2) and platelet derived growth factor (PDGF) the choice of the basal medium exerts a significant influence on the yield of OPCs in cultures from newborn rats. During a culture period of up to 9 days we observed larger numbers of surviving cells in Dulbecco's Modified Eagle Medium (DMEM), and Roswell Park Memorial Institute Medium (RPMI) compared with Neurobasal Medium (NB). A larger number of A2B5-positive OPCs was found after 6 days in RPMI based media compared with NB. The percentage of bromodeoxyuridine (BrdU)-positive cells was largest in cultures maintained in DMEM and RPMI. The percentage of caspase-3 positive cells was largest in NB, suggesting that this medium inhibits OPC proliferation and favors apoptosis. A difference between NB and DMEM as well as RPMI is the reduced Na^+-content. The addition of equiosmolar supplements of mannitol or NaCl to NB medium rescued the BrdU-incorporation rate. This suggested that the osmolarity influences the proliferation of OPCs. Plating density as well as residual microglia influence OPC survival, BrdU incorporation, and caspase-3 expression. We found, that high density cultures secrete factors that inhibit BrdU incorporation whereas the presence of additional microglia induces an increase in caspase-3 positive cells, indicative of enhanced apoptosis. An enhanced number of microglia could thus also explain the stronger inhibition of OPC differentiation observed in high density cultures in response to treatment with the cytokines TNF-α and IFN-γ. We conclude that a maximal yield of OPCs is obtained in a medium of an osmolarity higher than 280 mOsm plated at a relatively low density in the presence of as little microglia as technically achievable.

Keywords: oligodendrocyte progenitors, proliferation, osmolarity, microglia, culture medium, cytokines

INTRODUCTION

Cell cultures are used extensively to produce cell populations for tissue repair, and also for the investigation of the properties and interactions of populations of specific cells. Earlier cultures needed growth factors contained in fetal calf serum to allow cells to survive, but later serum free media were developed for cell growth under defined conditions. A first serum-replacing medium supplement contained five principal factors (insulin, transferrin, progesterone, selenium, and putrescine at optimized concentrations, termed N2). It was developed by Bottenstein and Sato for culturing B104 neuroblastoma cells (Bottenstein and Sato, 1979). Romijn et al. developed a more elaborate medium containing additional ingredients, including hormones and essential fatty acids (Romijn et al., 1984) which was then further refined to a medium called B18 by Brewer and Cotman to sustain the survival of neurons (Brewer and Cotman, 1989). Later further changes in the supplement were made (B27) and a new basal medium, providing the necessary electrolytes, amino acids, and vitamins (termed Neurobasal -NB) was developed to optimize maintenance of hippocampal neurons at a low background of astrocytes (Brewer et al., 1993). This medium has been widely used for culturing various cell types, as evident from more than 90 citations per year in the last decade. Problems with the quality of the commercially available supplements have recently led to the development of a slightly modified formulation, which contains 21 ingredients (NS21) (Chen et al., 2008).

Apart from culturing neurons the B27/NB medium can also be used to culture oligodendrocytes. For example, it has been used to investigate effects of inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) on oligodendrocyte precursor cell (OPC) survival, differentiation, and ion channel expression in culture (Feldhaus et al., 2004a; Mann et al., 2008). A methodological study comparing the viability of OPC cultures in B27/NB medium and N1/DMEM medium provided evidence, that the B27/NB medium was significantly more effective in maintaining viable cells and in supporting

oligodendrocyte proliferation than the combination N1/DMEM (Yang et al., 2005). The composition of the basal medium as well as the different supplements could have led to the higher success rate of the B27/NB combination. To study the influence of the basal culture medium on oligodendrocyte lineage cells we here systematically investigated the yield of surviving cells, their bromodeoxyuridine (BrdU) incorporation as measure for cell proliferation as well as caspase-3 immunofluorescence as a measure for the percentage of preapoptotic cells after culturing cells under three conditions differing only in the choice of the basal medium, [Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), and Neurobasal Medium (NB)].

Furthermore, cultures obtained by differential adhesion contain microglia (Hewett et al., 1999). Microglia may secrete factors that are deleterious or supportive for the cultured oligodendrocytes. In order to investigate the influence of the remaining microglia we further investigated the impact on OPC proliferation and survival of adding surplus microglia to the culture.

This surplus of microglial cells and factors secreted from neighboring OPCs may also impact the responses of cultured OPCs to pharmacological treatments. This is demonstrated in a series of experiments investigating the response of OPC cultures of various densities to a standardized treatment with the cytokines TNF-α and IFN-γ.

METHODS

PREPARATION OF MIXED GLIAL CULTURES

The protocol for the preparation of glial cultures followed the general procedures described by McCarthy and de Vellis (1980) and Armstrong (1998) with some modifications. Postnatal 0–3 day-old Wistar Hannover rat pups were decapitated and the whole brain rostral of the cerebellum was removed and placed in a phosphate buffered saline (PBS)containing 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. After removal of inner and outer meninges the brains were passed successively through nylon meshes with pore sizes of 125 and 36 μm to remove neurons from the cell suspension. The dissociated cells were centrifuged [10 min, 900 rpm at room temperature (RT)] and the cell pellet resuspended in 5 ml glial mixed medium (GMM) composed of DMEM:Ham's F12 (1:1) supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin [all PAA, Germany)]. The obtained suspension was transferred into an uncoated T-75 flask, so that the cells of 1.5 brains were plated per flask. Ten milliliter of GMM were added, cultures were then allowed to grow at 37°C and 5% CO_2 in a Haereus B5060 incubator (Hanau, Germany). Three to four days after plating the supernatant was centrifuged (5 min, 900 rpm), resuspended in fresh GMM and cells plated back onto the cell layer. Thereafter, the medium was replaced every 3–4 days without recycling cells from the supernatant.

ISOLATION OF OLIGODENDROCYTE PROGENITOR CELLS

Cells were allowed to proliferate up to day 10–12 after preparation until the culture was composed of a confluent astrocyte layer adherent to the bottom of the flask with microglial cells and oligodendrocytes growing on top. The first step to obtain purified oligodendrocyte precursors was to remove the microglia by shaking the flasks (180 rpm) for 3 h on an orbital shaker ES-W (Kühner AG, Birsfelden, Switzerland). The supernatant, containing mostly microglia, was rejected, flasks were washed once with PBS, then fresh GMM was added and the flasks were shaken for another 18 h at 180 rpm to detach the oligodendrocytes from the astrocyte layer. The supernatant was centrifuged (5 min, 1700 rpm) and the cells contained in the pellet preplated in GMM (1 ml per flask) in non-coated petri dishes for 45 min at 37°C and 5% CO_2 to remove remaining astrocytes from the suspension. The supernatant, containing mostly oligodendrocyte progenitors, was then centrifuged (5 min, 1000 rpm), the pellet resuspended in 1 ml GMM and cells counted in a Neubauer chamber. Cells were adjusted thereafter to densities of 5000, 20,000, and 80,000 cells per coverslip or Petri dish in GMM. Coverslips and Petri dishes were precoated with poly-L-lysine (5 μg/ml) to enhance attachment of the cells to the coverslips/Petri dishes. In the Petri dishes cells were first plated in sterile glass rings with the diameter of the coverslips (12 mm). After at least 1 h of adhering to the poly-L-lysine coated surfaces (at 37°C, 5% CO_2) media were replaced by specialized media promoting proliferation.

Proliferation medium (PM) contained: Neurobasal (NB) (Invitrogen, Carlsbad, USA) medium, Roswell Park Memorial Institute (RPMI) (PAA, Cölbe, Germany), or Dulbecco's Modified Eagle's Medium (DMEM) (PAA, Cölbe, Germany) supplemented with 1xB27 (Sigma, Steinheim, Germany) without antioxidants, 100 U/ml penicillin (PAA), 100 μg/ml streptomycin (PAA, Cölbe, Germany), 10 ng/ml platelet derived growth factor (rHuPDGF-AA, Biomol, Hamburg, Germany, product no 50363), that inhibits differentiation and drives proliferation of O-2A glial progenitor cells into mature oligodendrocytes (Noble et al., 1988; Raff et al., 1988; Pringle et al., 1989) and10 ng/ml basic fibroblast growth factor (FGF-2, rHuFGF-basic, Biomol, Hamburg, Germany, product no 50361) that induces proliferation of OPCs (Bogler et al., 1990; McKinnon et al., 1991; Grinspan et al., 1993), see Bansal et al. (2002) for review. FGF-2 supplementations have been shown to saturate proliferation of O2-A precursor cells at 5–10 ng/ml (Besnard et al., 1989). For the present experiments we thus used the same concentrations of 10 ng/ml of PDFG and FGF-2 as employed by Back et al. (1998). Since in the experiments described here we attempted to study effects of basal media we did not study in more detail whether the yield of cells could be further optimized by varying the concentration of these growth factors or by adding further potential proliferation promoting factors, such as hepatocyte growth factor (HGF) (Yan and Rivkees, 2002), heregulin (HRG) (Canoll et al., 1996), insulin like growth factor-1 (IGF-1), (Zeger et al., 2007), or sonic hedgehog (Gao, 2006). In some experiments the osmolarity of the NB-medium (205 mOsm, as measured with a Knauer Type-ML osmometer) was increased to the osmolarity of the DMEM-medium (305 mOsm) by increasing the osmolarity of the NB medium by 100 mOsm by the addition of 52.5 mM NaCl or 100 mM mannitol.

After 3 days in PM cell culture media were exchanged to differentiation promoting media (DM). The media consisted of NB, RPMI, or DMEM supplemented with 100 U/ml penicillin (PAA),

100 μg/ml streptomycin (PAA, Cölbe, Germany), 45 nM triiodo-L-thyronine (T3) (Barres et al., 1994), 5 μM forskolin (Back et al., 1998), 10 ng/ml ciliary neurotrophic factor (CNTF) (Lopes-Cardozo et al., 1989), and 1xB27 without antioxidants (Sigma, Steinheim, Germany) per 50 ml.

Cytokine-treatment was performed 24 h after seeding for 48 h in PM/RPMI with recombinant rat IFN-γ (20 ng/ml) and TNF-α (10 ng/ml, both PeproTech, Hamburg, Germany). This cytokine mixture was chosen because in a preceding investigation on purified oligodendrocyte progenitors we had observed in accordance with Andrews et al. (1998), Melcangi et al. (2000), and Buntinx et al. (2004), that a combination TNF-α and IFN-γ is more potent in inducing apoptosis than either factor in separation.

ISOLATION OF MICROGLIA

Microglia were isolated from the mixed glia culture in the first shaking step during OPC isolation (see above). The supernatant containing the microglia was centrifuged (5 min, 1000 rpm at RT). The pellet was resuspended in 5 ml fresh GMM. Cells were plated in a T12 flask. Immediately before co-cultivation, the flasks were shaken for 5–10 min (180 rpm) to suspend the microglia.

CO-CULTIVATION OF MICROGLIA AND OPCs

The number of microglia in cultures with a seeding density of 80,000 cells was determined from an OX-42 staining. The evaluated density (2700 cells) of microglia was added to OPC-cultures with seeding densities of 5000 cells per coverslip/Petri dish. The glia cells were then co-cultivated for 3 days in PM. Control cultures were cultivated for 3 days in PM without additional microglia.

CULTIVATION OF OPCs WITH CONDITIONED MEDIUM

Supernatants from 3 day old cultures with a seeding density of 80,000 cells per cover slip/petri dish were collected. Then fresh OPC-cultures seeded with a density of 5000 cells per coverslip/Petri dish were cultured for 3 days in either control PM or in the conditioned medium.

IMMUNOCYTOCHEMISTRY

Oligodendrocyte precursor cells (OPCs) were labeled with antibodies against A2B5 (Schnitzer and Schachner, 1982; Raff et al., 1983; Levi et al., 1987) and oligodendrocytes starting myelin production (OLs) were visualized by antibodies against myelin oligodendrocyte specific protein (MOSP). By immunoprecipitation anti MOSP antibodies have been shown to specifically bind to a 48 kDa membrane surface protein with a pI of 6.7 that is highly conserved in rodents, cats, monkeys, and humans (Dyer et al., 1991). Molecular masses of other myelin proteins such as myelin/oligodendrocyte glycoprotein (MOG) amount to 26–28 kDa, myelin basic protein (MBP) from 14 to 21.5 kDa, 2′,3′- cyclic nucleotide 3′- phosphodiesterase (CNPase) to 45 kDa, oligodendrocyte specific protein (OSP/claudin-11) to 22 kDa, and myelin associated glycoprotein (MAG) to 69 kDa (www.uniprot.org). MOSP expression correlates with increases in microtubular structures in oligodendrocytes and its initial expression occurs at the stage in development when oligodendrocyte processes have formed but membrane sheets have not yet been elaborated. This occurs shortly after galactocerebroside and sulfatide expression and 3 days before the expression of MOG (Mu and Dyer, 1994). In contrast to MOSP, OSP/claudin-11 expression, that is controversially discussed as a target for autoantibodies in multiple sclerosis (Aslam et al., 2010) occurs from the early progenitor stage and continues in mature oligodendrocytes (Bronstein et al., 2000). MOSP mediates signals that appear to increase the thickness and numbers of microtubular structures within oligodendrocyte membrane sheets and the preincubation of oligodendrocyte cultures with anti-MOSP antibodies leads to its redistribution from a uniform surface staining to lacy networks overlying microtubular structures that have CNPase colocalized along them. In contrast, incubation of cultures with antibodies against MOG induces an accumulation of MOG over internal domains of MBP, indicating that these sets of proteins play a distinct role in the cytoskeletal organization of oligodendrocytes (Dyer and Matthieu, 1994). A similar co-localization of MOSP and CNPase has later also been observed in the adult rhesus monkey brain where it was used as a marker for ongoing myelin formation (Sloane et al., 2003). Since MOSP belongs to the class of proteins involved in structuring the cytoskeleton of myelin (Dyer and Matthieu, 1994), it has later been used as marker for myelin producing oligodendrocytes (Gomes et al., 2003; Hoenicka et al., 2010; Iseki et al., 2011). We chose to use MOSP as marker to count the percentage of oligodendrocytes starting to produce myelin, since we had previously shown, that in cultures grown using the same protocol on the basis of NB-medium, as used here, MOSP stained cells with elaborate membrane processes appeared in parallel with an increase in MBP, MAG and CNPase [Figures 2B,C in Feldhaus et al. (2004a)] and since it showed a particularly clear specific staining of the membrane surfaces.

Mouse anti-A2B5 antibodies and anti-MOSP antibodies were obtained from Millipore [anti A2B5, clone A2B5-105 (MAB312), anti-oligodendrocytes, clone CE-1 (MAB328)], Alexa Fluor 488 labeled IgM and IgG anti-mouse antibodies and Alexa Fluor 594-labeled IgG anti-rabbit secondary antibodies were from Invitrogen, Carlsbad, CA. Primary and secondary antibodies were diluted in PBS to a concentration of 1:250 for first antibodies and 1:500–1:1000 for second antibodies. At the conclusion of each experiment the medium was removed, cells were then saturated with PBS with 3% goat serum for 30 min at RT. Afterwards cells were washed once with PBS, followed by the incubation with the first antibody for 1 h at RT. In the experiments shown in **Figure 2**, this was done in Krebs-Ringer (Krebs-Ringer-HEPES consisting of 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM $NaHCO_3$, 16 mM HEPES), whereas later A2B5 stainings were performed in PBS. To remove the excess of first antibody cells were washed once with PBS. After labeling the extracellular epitopes cells were then fixed with 4% paraformaldehyde (PFA) for 20 min. at RT. After washing cells twice with PBS, cells were incubated with secondary antibody for 1 h at RT during shaking. For counting progenitor cells one has to bear in mind that all antibodies staining progenitor cells, i.e., NG2, PDGFRα, and A2B5 do not specifically bind to bipolar progenitors but far beyond that point up to cells expressing various processes. Therefore progenitor cells referred to in the experiments investigating cytokine-effects shown in **Figure 9** were

identified morphologically. In preliminary experiments phase dark cells of bipolar shape bearing up to four unbranched processes had been identified to be 100% A2B5-positive. Using this definition the progenitor cell population counted in **Figure 9** comprised a subpopulation of 70% of the A2B5-positive cells.

To determine the percentage of proliferating cells in culture, BrdU incorporation stainings were performed. For the BrdU staining cells were first incubated with BrdU (20 μM) for 20 h. After fixing the cells for 20 min with 4% PFA, they were washed with PBS. Cells were incubated with 37°C HCl (1M) for 1 h to uncover the DNA. Then cells were washed once with PBS. Afterwards cells were washed for 10 min with 100 mM borate buffer (15 mM sodium borate in distilled water, pH 8.3) to neutralize the remaining acid. Then the cells were incubated with a block buffer consisting of PBS-T (PBS with 0.1% Triton X-100) and 5% goat serum, followed by an overnight incubation with the primary antibody (*rat* anti-BrdU, Accurate chemicals OBT0030G) at 7°C used 1:750 in PBS. Thereafter cells were washed twice with PBS. The cells were then incubated with the secondary antibody (Alexa Fluor® 488 donkey anti-rat IgG, Invitrogen), that was used 1:750 in PBS. The duration of the incubation was 1.5 h at RT under shaking. Cells were then washed with PBS.

To determine the percentage of preapoptotic and apoptotic cells, caspase-3 and cleaved-caspase-3 stainings were performed. For the caspase-3 and the cleaved-caspase-3 staining cells were first fixed for 20 min with 4% PFA at RT, then submerged with hot citrate buffer (10 mM, pH 6 at 95°C) and incubated for 25 min at RT, followed by 3 washing steps, each 5 min, with PBS-T (PBS with 0.01% Triton X-100). Then the cells were incubated for 1 h at RT in a block buffer, consisting of PBS-T (PBS with 0.01% Triton-X) and 5% goat serum. Next cells were incubated with the first antibody (rabbit anti-Caspase-3, Cell Signaling, 9662) for the caspase 3 staining and with rabbit anti cleaved-caspase-3 antibody (New England Biolabs, 9661) overnight at 7°C. The antibodies were diluted 1:200 in block buffer. Cells were then washed three times with PBS, followed by the incubation with the second antibody [AlexaFluor® 594 goat anti-rabbit IgG, Invitrogen (A11012) diluted 1:500 in PBS] for 1 h at RT under shaking. Afterwards the cells were washed with PBS.

To identify DNA fragmentation in oligodendrocytes (*in vitro*) a TUNEL detection Kit (Roche 11684795910) was used. Cells, which had been fixed in 4% PFA for 20 min at RT were washed in PBS and incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium-citrate) for 2 min on ice. After washing three times in PBS, the TUNEL mixture was prepared: The labeling solution (Fluorescein labeled nucleotides) was diluted in a ratio 1:4 and the enzyme solution (terminal desoxynucleotidyl transferase) in a ratio 1:50 in dilution buffer supplied by the Kit (11966006001). Each coverslip was incubated for 1 h at 37°C with 50 μl of this TUNEL mixture. Thereafter cells were washed with PBS and double stained with 4′,6 Diamidin-2-Phenylindol (DAPI 100 ng/ml 5 min at RT).

To calculate the amount of microglia in OPC cultures, OX-42 staining was performed. Cells were first fixed for 20 min with 4% PFA at RT, then incubated with block buffer, consisting of PBS-T (PBS with 0.1% Triton X-100) and 3% goat serum. Then cells were incubated with the primary antibody [mouse anti-rat CD11b IgG (anti-OX-42), Millipore, CBL1512Z], diluted 1:200 in PBS for 1 h at RT. Afterwards cells were washed with PBS, following the incubation with the secondary antibody (AlexaFluor® 488 goat anti-mouse IgG, Invitrogen, A11001), that was used 1:1000 in PBS. The incubation ensued 1 h at RT.

Finally nuclei of all cells were visualized by staining for 20 min at RT with DAPI (Sigma, Steinheim, Germany, diluted 1:2000 in earlier experiments) or Hoechst-33258 dye (10 ng/ml in PBS, Sigma-Aldrich, in later experiments). Fluorescence microphotographs were taken using a 20x objective on an Olympus (IX 51) microscope equipped with analysis[B] software for earlier experiments and cellSens software for later experiments (Olympus) and a ColorView 12 camera. The higher light sensitivity of the more recently used microscope system could potentially account for overall larger numbers of BrdU positive and caspase-3 positive cells found in more recently performed experiments compared with earlier experiments. Results from an earlier experiment are shown for example in **Figure 3**. However, apart from overall larger percentages of stained cells counted more recently, all BrdU stainings qualitatively led to the same result, that cells cultured in NB show significantly lower BrdU incorporation rates. In contrast, changes in percentages of caspase-3 immunopositive cells got only significant in the more recent experiments, showing more than 5% positively stained cells.

For cell counts 8–10 frames of randomly chosen, non-overlapping fields were photographed. Cell numbers were determined by counting Hoechst-33258 or DAPI stained nuclei in each frame. Numbers of cells on a coverslip or in a Petri dish were then calculated by determining the average cell number per frame and multiplied by the conversion factor between the area of the frame and the area of the coverslip. The percentage of BrdU and caspase positive cells was determined by divison of the number of the stained cells through the total number of the nuclei from morphologically identified oligodendrocyte lineage cells. Statistical analysis was performed using One-Way ANOVA with Tukey *post-hoc* test or paired Student's *t*-test if only two columns were compared. Results are given as mean ± standard error. Results shown in **Figures 1**, **5**, **6** as significant using ANOVA showed even stronger significances when the single columns under treatment conditions were compared with controls using Student's *t*-test.

RESULTS

INFLUENCE OF BASAL MEDIA ON PROLIFERATION AND DIFFERENTIATION OF PURIFIED OPCs

The first goal of this study was to investigate the impact of basal culture media on the number of OPCs surviving in culture, leaving the other supplements (B27 without antioxidants plus PDGF and FGF-2 in PM and forskolin, T3 and CNTF in differentiation medium) unchanged. Following an initial increase in cell number in NB-based PM the number of cells in the culture remained almost constant during the following 6 days in differentiation medium (**Figure 1A**). In contrast, cells grown for d1–d3 in RPMI-based PM and further 6 days in RPMI-based differentiation medium showed a continuous increase in cell number that had more than doubled after 9 days in culture. Every day *in vitro* from d1 to d9 the cell number was higher in RPMI based medium than in NB based medium, the difference reaching significant values from d3 on ($^*p < 0.005$). In many studies DMEM is used as

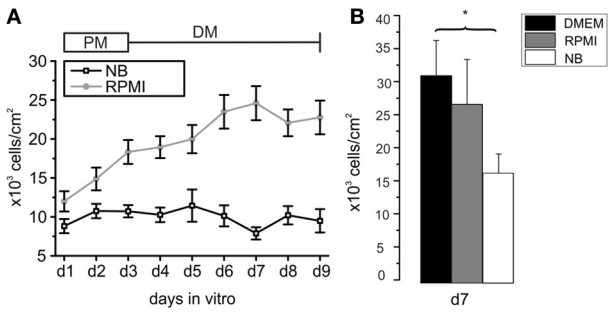

FIGURE 1 | Development of cell densities with time in culture in different basal media. (A) Oligodendrocyte precursor cell cultures incubated from d1 to d3 in proliferation medium (PM) and for an additional 6 days in differentiation medium (DM) based on NB (black line) or RPMI medium (gray line) after purification. Each data point obtained by counting DAPI-stained nuclei from at least 8 photographed frames per preparation. Note the higher cell number from d1 to d9 [d3 to d9 ($*p < 0.05$)] in RPMI based medium compared with NB based medium (13 preparations in RPMI and 7 preparations in NB investigated). **(B)** Comparison of number of cells/cm^2 surviving after 7 days in serum free media differing exclusively in the choice of the basal medium. Note that the highest rate of surviving cells was found in DMEM. ($*p < 0.05$, error bars indicate ± s.e.m.).

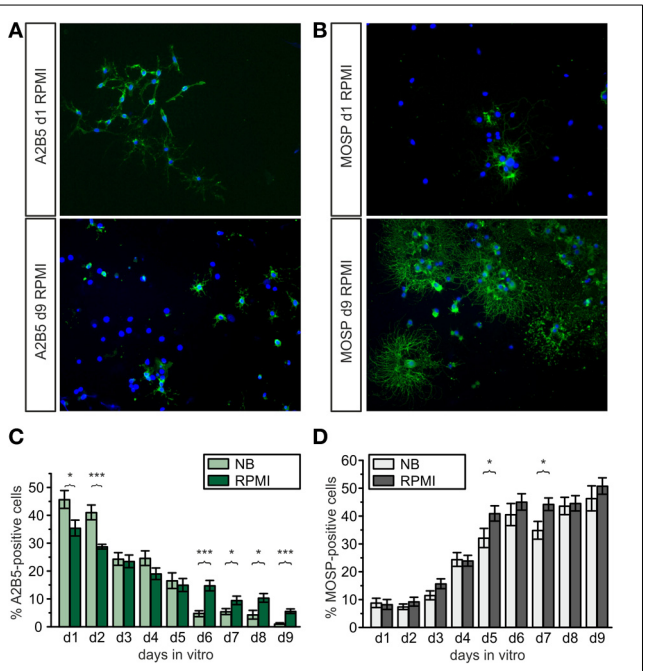

FIGURE 2 | Development of A2B5-positive progenitors and MOSP-positive oligodendrocytes in proliferation and differentiation media of different composition. (A,B): Examples for A2B5- and MOSP-immunopositive cells cultured in RPMI vs. NB and stained at d1 and d9 after isolation. **(A)** Immunostained pictures of A2B5-positive cells, showing approximately 35% A2B5-positive cells at d1 (top) and 5% at d9 (bottom). **(B)** Immunostained pictures (right) of approximately 5% MOSP-positive cells at d1 (top) and almost 50% at d9 (bottom). Nuclei counterstained with DAPI. **(C)** Direct comparison of percentage of A2B5-positive cells cultured in Neurobasal and RPMI based media, **(D)** direct comparison of MOSP-positive cells cultured in either Neurobasal or RPMI based media ($*p < 0.05$, $***p < 0.005$; error bars indicate ± s.e.m., paired Student's *t*-test between pairs of bars).

basal medium for oligodendrocyte cultures. We performed additional experiments in which we compared the outcome of sister cultures maintained for 3 days in PM and further 4 days in differentiation medium based on NB, RPMI as well as DMEM. The highest yield of cells was found in cultures maintained in DMEM, as shown in **Figure 1B**.

To obtain further information how different basal culture media may influence the maturational stage of the oligodendrocyte lineage cells cultures on cover slips were stained with A2B5- and MOSP-antibodies (**Figure 2**) after growth in NB and RPMI based media. Earlier investigations have already shown, that the percentage of A2B5 positive OPCs progressively decreases while the number of MOSP-positive cells increases in parallel with increased levels of the myelin markers CNPase, myelin associated glycoprotein (MAG) and myelin basic protein (MBP) within 6 days in NB based differentiation medium [see Figure 2 in Feldhaus et al. (2004a)]. In the present experiments in both media the total number as well as the percentage of A2B5-positive cells decreased with progression of maturation of the OPCs (in NB from 46 to 1%, in RPMI from 35 to 6%, **Figure 2C**) and the total number as well as percentage of MOSP-positive cells increased (in NB from 9 to 46%, in RPMI from 8 to 51%) from d1 to d9 (see **Figure 2D**). Although in both media about 50% of the cells had differentiated to MOSP-positive cells after 6 days in differentiation medium, some differences emerged: Cells incubated in NB medium showed significantly higher percentages of A2B5-positive progenitor cells at d1 and d2. However, from d6 to d9 cells grown in RPMI-based medium displayed a significantly higher percentage of progenitor cells (**Figure 2C**). This observation is consistent with the finding shown in **Figure 1**, indicating that the total number of cells cultured in RPMI increased continuously. Hence this basal medium sustains a higher background of proliferating A2B5-positive progenitor cells.

EFFECT OF BASAL CULTURE MEDIA ON PROLIFERATION AND APOPTOSIS

A larger number of surviving cells and a larger number of remaining A2B5 positive cells in RPMI compared with NB could have resulted either from a decreased proliferation or an increased apoptosis in the later medium. In order to investigate whether one or both possibilities apply we tested, whether the percentage of OPCs showing BrdU incorporation as an indicator of cell proliferation differs after culturing the cells for 3 days in either of the three basal media. As shown in **Figure 3** at all time points investigated OPCs cultured in DMEM or RPMI showed significantly higher percentages of cells that had incorporated BrdU than cells cultured in NB medium, suggesting that the NB medium inhibits OPC proliferation. A further investigation of the percentage of caspase-3 positive, proapoptotic cells as well as cleaved caspase-3 positive cells, that indicate a further advanced step in the process of apoptosis, yielded a higher percentage of labeled cells after 3 days in NB medium in comparison with the other media (**Figure 4**). This suggests that in NB medium fewer cells proliferate and more cells undergo apoptosis than in the other two media.

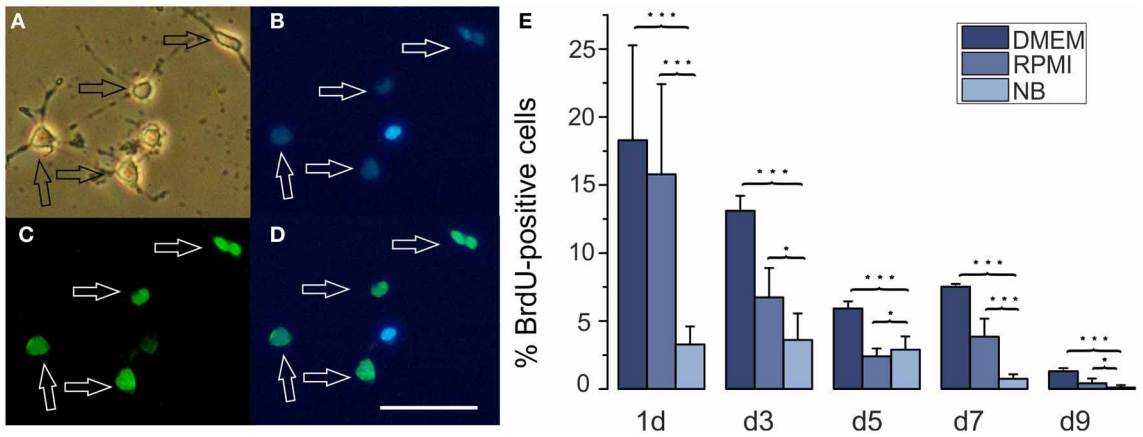

FIGURE 3 | Effects of different basal culture media on BrdU incorporation. (A–D) Examples of a BrdU stained culture **(A)** phase contrast picture, **(B)** Hoechst 33258 staining of the same image, **(C)** BrdU stain, **(D)** merged pictures **(B)** and **(C)**, calibration bar represents 50 μm. Cells had been cultured in DMEM for 3 days. **(E)** Percentage of BrdU positive cells in cultures maintained for the days indicated below the columns in NB, DMEM as well as RPMI based media (*$p < 0.05$, ***$p < 0.005$; error bars indicate ± s.e.m.).

EFFECTS OF OSMOLARITY ON OPC PROLIFERATION AND APOPTOSIS

A prominent difference between RPMI, DMEM, and NB is the lower osmolarity of the NB medium. The commercial NB medium used contained only 205 mOsm, compared with 280 mOsm of RPMI and 305 mOsm of DMEM. To test whether the lower osmolarity of NB might have been the cause for the lower proliferation rate and the higher rate of apoptotic cells we increased the osmolarity of the culture medium by increasing the NaCl concentration by 52.5 mM. As shown in **Figure 5** this procedure resulted in an increase in the percentage of BrdU incorporating cells (from 36 ± 2% in NB to 43 ± 4% in NB supplemented by NaCl) as well as in a decrease in the percentage of caspase-3 positive cells [from 20 ± 1% in NB to 12 ± 2% in NB supplemented with NaCl ($p < 0.05$), $n = 4$ preparations investigated]. The increase in the proliferation rate of the OPCs could potentially be explained by a larger amino acid uptake through Na^+-coupled transporters in an extracellular solution containing a larger concentration of Na-ions and thus a larger driving force for Na^+ influx into the cells. To test, whether this explanation is feasible we increased the extracellular osmolarity of the NB medium by adding 100 mM mannitol. This procedure led to an even larger increase in the percentage of cells showing BrdU incorporation (to 51 ± 7% in NB supplemented by mannitol, $p < 0.005$) as well as to a slightly larger decrease in the percentage of caspase-3 positive cells to 10 ± 3% in NB supplemented with mannitol, $p < 0.005$, $n = 4$ preparations investigated (**Figure 5**). Hence we conclude, that the lower osmolarity of the NB medium inhibits proliferation and favors apoptosis of OPCs.

INFLUENCE OF CELL DENSITY ON DIFFERENTIATION OF OLIGODENDROCYTES

To determine optimal cell seeding densities for maximal proliferation rates *in vitro* we investigated potential effects of the cell density on the further development of OPCs. Cells were seeded at four densities (2500, 5000, 7500, and 10000 cells/cm^2). Then OPCs were incubated either for 2 days in PM on the basis of RPMI to examine the percentage of A2B5-positive OPCs in dependence of cell density or for 6 days in RPMI based differentiation medium to investigate potential cell density effects on the percentage of MOSP-positive differentiated oligodendrocytes. With increasing cell density the percentage of A2B5-positive cells decreased (see **Figure 6Aa**). After 2 days in PM 40 ± 3% of the cells in the culture were A2B5-positive in the lowest density culture examined. The percentage of A2B5-positive cells decreased with increasing cell density showing the lowest percentage of 29 ± 2% (40 ± 3% to 29 ± 2%, $p < 0.005$) at a cell density of 10,000 cells/cm^2. This indicates that in lower density cell cultures either more cells proliferate or fewer cells differentiate or undergo apoptosis. In case of a significant increase in proliferation the ratio of seeded to surviving cells should be larger in the lower density cultures. Indeed, the ratio of the number of cells counted after 2 days to the number of cells seeded was 6% higher, amounting 33% in low density cultures (2500 cells/cm^2) compared with 27% in high density cultures. The percentage of A2B5-positive cells was even 12% higher in the low density cultures, suggesting that the higher cell density indeed induces differentiation of OPCs. To further investigate whether the higher cell density exerts a differentiating effect, OPCs were seeded at densities ranging from 2500 to 10,000 cells/cm^2 and directly cultured in differentiation medium for 6 days. At d6 in differentiation medium the percentage of MOSP-positive cells varied from 26 ± 2% (2500 cell/cm^2) to 38 ± 1% (10000 cells/cm^2) ($p < 0.005$). As illustrated in **Figure 6Ab** our results indicate that an increasing cell density promotes maturation at the expense of the proliferating A2B5-positive phenotype.

Since the cell density could influence OPC proliferation and/or apoptosis we performed an additional series of experiments in DMEM based PM (see **Figure 6A**) at a later stage of the experiments to further substantiate the initial findings of the influence of cell density in RPMI based medium, also expanding the range of cell densities studied to higher plating densities (**Figure 6B**). Immunostaining for A2B5 confirmed, that in DMEM based medium an increase in plating density leads to a decreased percentage of A2B5 positive progenitor cells as well (**Figure 6Ba**).

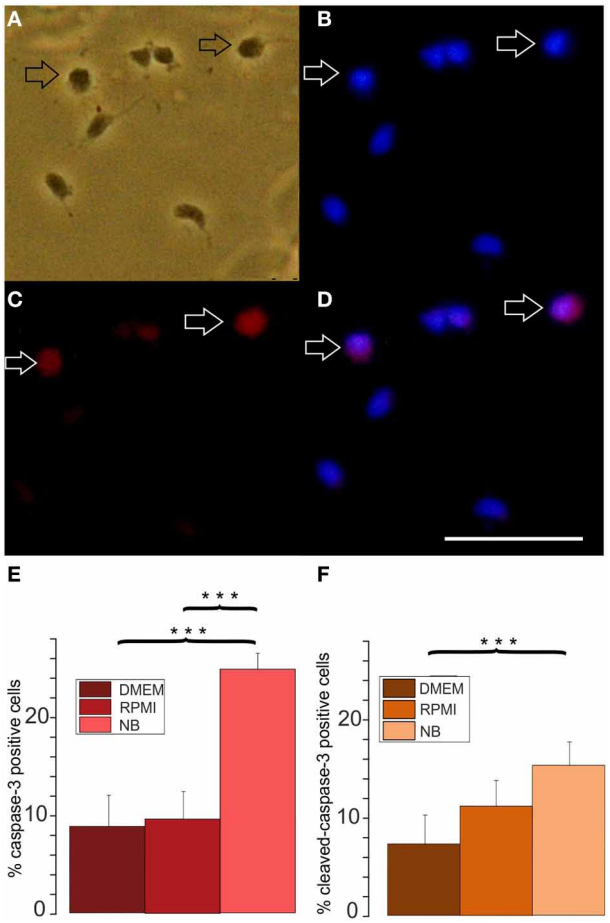

FIGURE 4 | Effects of different basal culture media on caspase-3 immunopositivity. (A–D) Examples of a caspase-3 stained culture: **(A)** phase contrast picture, **(B)** Hoechst 33258 staining of the same image, **(C)** caspase-3 immunofluorescence, **(D)** merged pictures **(B)** and **(C)**, calibration bar indicates 50 μm. Cells stained after culture in proliferation medium based on DMEM for 3 days **(E)** percentage of caspase-3 positive cells and **(F)** cleaved caspase-3 positive cells after 3 days of culture in NB, DMEM as well as RPMI based media, (8 coverslips from 5 preparations evaluated, $^{***}p < 0.005$; error bars indicate ± s.e.m.).

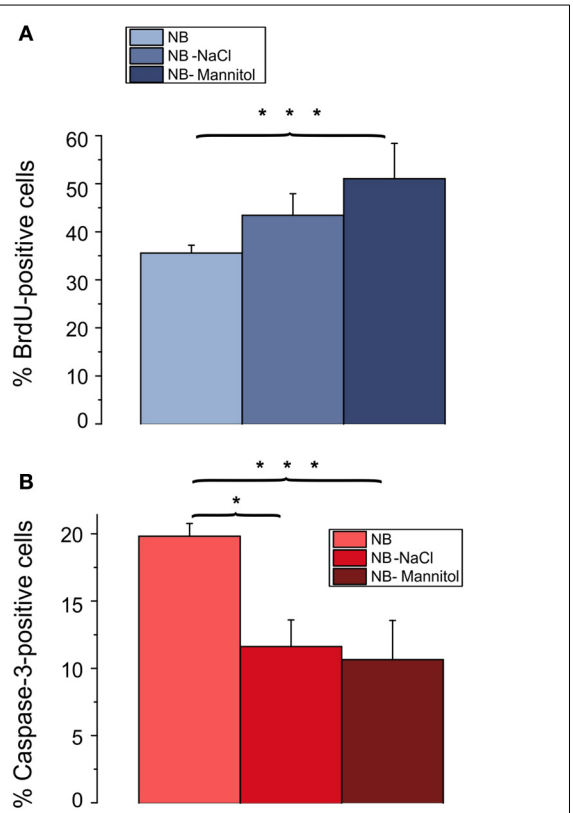

FIGURE 5 | Effects of osmolarity on percentage of BrdU and caspase-3 positive cells. Oligodendrocytes maintained for 3 days in NB based culture that had been supplemented with 52.5 mM NaCl or 100 mM mannitol to increase the osmolarity of NB to the osmolarity of DMEM. Note, that a change in osmolarity by either mannitol or NaCl results in an increase in proliferating, BrdU positive cells **(A)** and a decrease in caspase-3 positive, proapoptotic cells **(B)** (4 coverslips from 4 preparations counted, $^{*}p < 0.05$, $^{***}p < 0.005$; error bars indicate ± s.e.m., One-Way ANOVA followed by Tukey *post-hoc* Test).

The increased plating density led to a parallel significant decrease in the percentage of BrdU positive proliferating cells (**Figure 6Bb**) indicating that the enhanced cell density inhibits proliferation. Furthermore, a significant increase in the percentage of caspase-3 positive proapoptotic cells was observed, indicating that an increase in plating cell density also leads to an enhanced apoptosis rate in the cultures.

EFFECTS OF CONDITIONED MEDIA ON PERCENTAGE OF A2B5 POSITIVE, PROLIFERATING, AND PROAPOPTOTIC CELLS

To investigate, whether soluble cell secreted factors might be the cause for the increased differentiation as well as increased apoptosis of the OPCs in higher density cultures, OPCs were first seeded at a high density of 80,000 cells/cm^2 in DMEM based medium. After 3 days in culture the medium was removed and added to low density cultures containing 5000 cells/cm^2 on cover slips or seeded into the center of Petri dishes. Counts of cells immunopositive for BrdU (**Figures 7Aa,Ba**), A2B5 (**Figures 7Ab,Bb**), and caspase-3 (**Figures 7Ac,Bc**) performed after 3 days in PM showed, that the conditioned medium selectively inhibited the proliferation of the OPCs. Although the percentage of A2B5 positive cells was not significantly affected, the conditioned medium exerted a differentiating effect, as evident by a morphologically more mature appearance of the A2B5 positive cells (**Figure 7C**). The factors contained in the conditioned medium however, did not change the percentage of caspase-3 positive preapoptotic cells. No obvious differences were observed for OPCs seeded on cover slips or in Petri dishes, suggesting that the higher dilution of the secreted factors in the larger fluid volumes of the Petri dishes did not influence the results.

EFFECTS OF SURPLUS MICROGLIA ON PERCENTAGE OF A2B5 POSITIVE, PROLIFERATING, AND PROAPOPTOTIC CELLS

The preceding results suggest that secreted factors from neighboring OPCs inhibit proliferation and induce differentiation. However, the impact of the high plating density on caspase-3

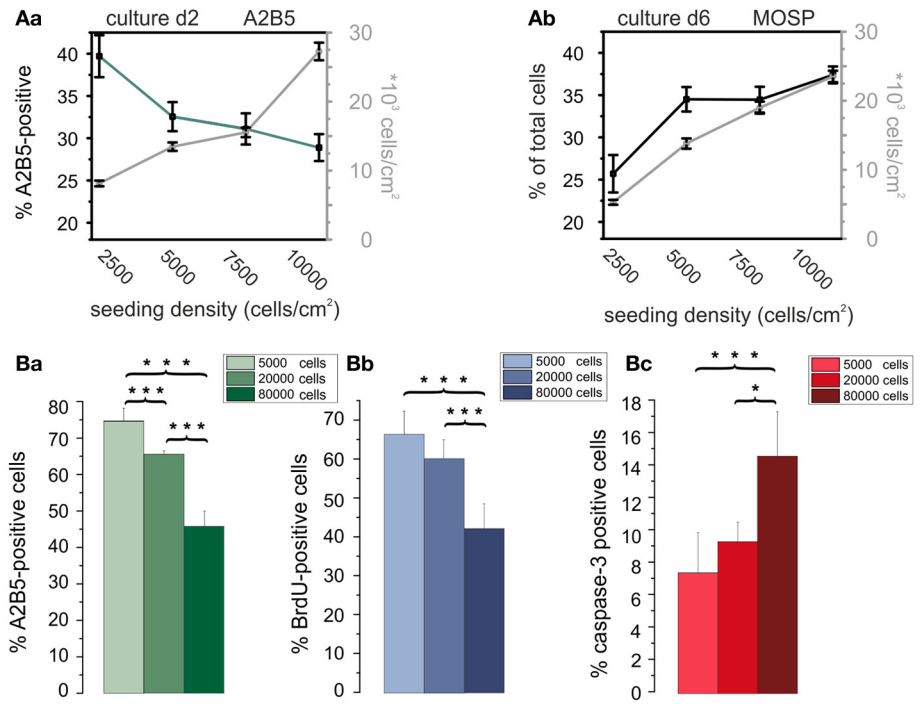

FIGURE 6 | Influence of cell density on differentiation, proliferation, and apoptosis of OPCs. (Aa) Cell density (data connected by gray lines) and percentage of A2B5-positive cells (data connected by green line) vs. plating density at day 2 in culture. Note, that the percentage of immature OPCs negatively correlates with cell density. **(Ab)** Total cell density (gray line) and percentage of MOSP-positive cells (black line) vs. cell density after 6 days in differentiation medium. Note the larger percentage of differentiated cells in higher plating density. Data shown in **(A)** obtained from RPMI based cultures. Data shown in every bar collected from $n = 100$ frames from 5 preparations. Data for A2B5 as well as MOSP staining between first and last plating density significantly different with $p < 0.005$. **(Ba)** Percentage of A2B5 positive OPCs from a second series of experiments, confirming a higher yield of immature cells in cultures with a larger plating density. **(Bb)** Percentage of BrdU positive cells in dependence of plating density. Note that a higher plating density decreases proliferation rate. **(Bc)** Influence of plating density on percentage of caspase-3 positve cells. Note, that the larger plating density increases the percentage of proapoptotic cells. Experiments shown in **(B)** performed after 3 days of culture in DMEM based medium [2 coverslips counted from 4 preparations ($*p < 0.05$, $***p < 0.005$; error bars indicate ± s.e.m.)].

positive cells could not be explained by these experiments. Since a larger plating density also leads to more microglia in the respective cultures we performed an additional series of experiments in which additional microglia were added to low density OPC cultures. For these experiments the number of microglia per coverslip, respectively Petri dish, was determined from OX-42 stained cultures. Cultures seeded at a density of 80,000 cells/coverslip contained a low percentage of microglia (3.6 ± 0.2%, $n =$ 4 preparations), which amounted to 2700 cells per coverslip. Cultures seeded at a low density of 5000 cells/coverslip were co-cultivated with an additional number of 2700 microglia, amounting to nearly a microglia to OPC ratio of 1:2. As shown in **Figure 8** the presence of additional microglia significantly inhibited BrdU incorporation, albeit to a smaller extent than the conditioned medium. In contrast to the conditioned medium, the extra dosage of microglia significantly increased the percentage of caspase-3 positive proapoptotic OPCs. This finding was confirmed with a TUNEL assay on coverslip cultures, showing a similar percentage of apoptotic cells. These results show that the microglia present in the higher density cultures could explain the higher rates of proapoptotic cells observed in high density cultures.

EFFECTS OF CELL DENSITY ON CYTOKINE-EFFECTS ON THE POPULATIONS OF A2B5- AND MOSP-POSITIVE CELLS

Cultures offer the advantage that cell types can be purified and this makes it possible to study drug actions on cell populations of varying composition, and to work out interactions between different cell types in a way that is not possible *in vivo*.

We have previously described an inhibition of OPC survival and differentiation after 2 days of treatment of proliferating OPCs in culture with the cytokines TNF-α and IFN-γ (Feldhaus et al., 2004a; Mann et al., 2008). Since the results shown above indicated that the percentage of A2B5 positive cells in the OPC culture depends to some extent on cell density and basal medium we here repeated our previous treatment protocol of OPCs using different plating densities in RPMI based proliferation and differentiation medium. Cells were maintained for 3 days in PM, the last two of which included cytokine-treatment, followed by a differentiation phase of further 6 days in cytokine-free differentiation medium. As shown in **Figure 9** the cell density was larger in cultures with a high seeding density. However, as already observed in **Figure 6A**, the increase in surviving cells was smaller than the increase in plating density. Here an increase in seeding density by a factor of 17 only increased the yield of cells by a factor of 4. Furthermore,

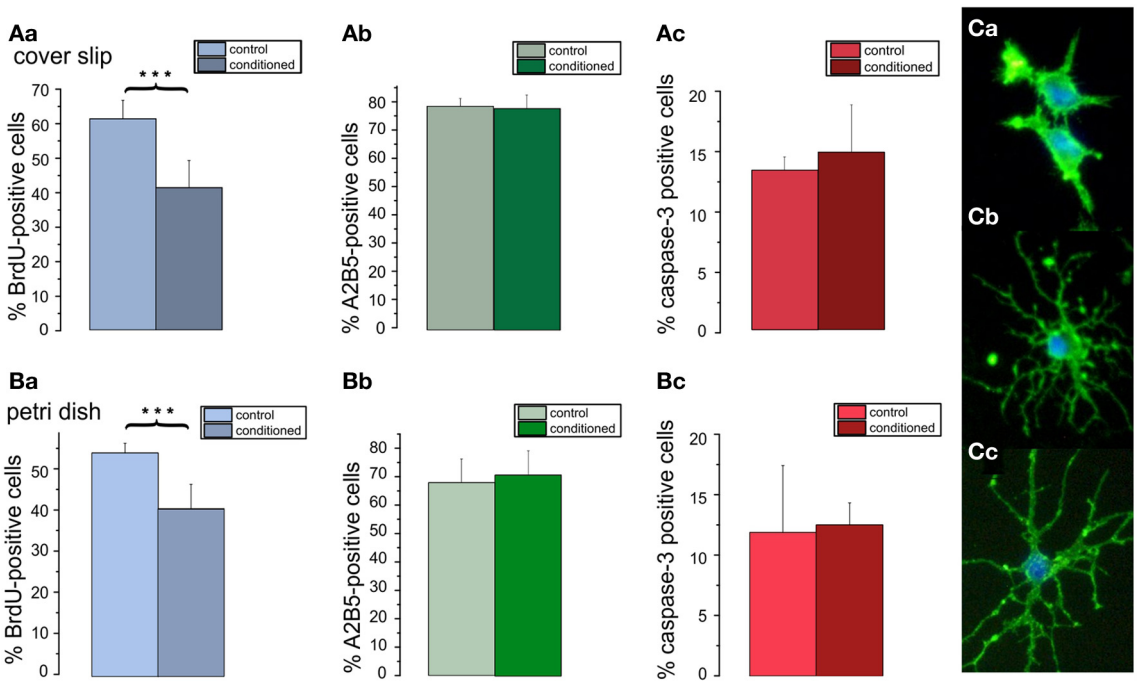

FIGURE 7 | Effects of conditioned media on percentage of A2B5 positive cells, proliferation, and apoptosis in low density cultures. Cells plated with a seeding density of 5000 cells/coverslip cultured in DMEM based proliferation medium for 3 days (control) or in parallel with the supernatant of high density cultures (80000 cells/coverslip) (conditioned). Bar graphs shown in **(A)** represent results of immunostainings for **(Aa)** BrdU, **(Ab)** A2B5 **(Ac)** caspase-3 performed on of 5 coverslips from 3 preparations. Bar graphs shown in **(B)** represent results from analogous stainings performed in Petri dishes, both after 3 days in proliferation media. Note, that the conditioned medium decreases the proliferation rate of the cells (***$p < 0.005$; error bars indicate ± s.e.m.). **(C)** A5B5 immunostainings of OPC from control cultures show a less mature morphology than those from conditioned cultures **(Cb,Cc)**.

in accordance with our observations shown in **Figure 6Aa**, the percentage of progenitor cells declined with increasing number of cells seeded from 13.5% at the lowest cell density to less than 0.7% at the highest seeding density. In further agreement with the results shown in **Figure 6Ab** the percentage of MOSP-positive cells increased in the higher density cultures.

In further agreement with previous results (Feldhaus et al., 2004a) a cytokine-treatment of the proliferating cells for 48 h induced a significant decrease in the number of the surviving cells. At all densities investigated, the percentage of the MOSP-positive cells was significantly lower and the percentage of progenitor cells was significantly higher after 6 days in differentiation medium following cytokine-treatment. The cytokine-effect on cell survival as well as block of differentiation was largest in the highest density culture. However, in the lowest density culture the cytokine-treatment had no effect on cell number, but still significantly increased the percentage of progenitor cells ($p < 0.005$) and decreased the percentage of MOSP-positive cells ($p < 0.05$) (**Figure 9B**). The stronger effect of the cytokine treatment in the high density cultures could be explained by the small percentage of about 5% microglia in the culture which reach higher absolute numbers in the high density cultures. Since the cytokine treatment could have additionally activated microglia to secrete further cytokines, the effect of the cytokine treatment might have been amplified by the residual microglia in the high density cultures.

DISCUSSION

DIFFERENCES IN BASAL MEDIA

The main novel result of the present investigation is that the Neurobasal based culture medium strongly reduces the proliferation of OPCs and enhances apoptosis. After a preculture period in PM and an additional incubation in NB-based differentiation medium the number of A2B5-positive cells was reduced to less than 3%. In contrast, when the cultures were incubated with identical serum free medium supplements on the basis of RPMI, the number of cells in the culture continued to increase during the first week leading to a much higher yield of surviving and of differentiated MOSP positive cells. The higher yield of cells in the cultures could be explained by the continued maintenance of a proliferating progenitor cell pool as evidenced by the higher rate of BrdU positive cells in cultures maintained in DMEM or RPMI in addition to a decrease in apoptotic cells as evidenced from the decreased percentage of caspase-3 positive cells in the latter media. Hence, if a culture with a minimal percentage of A2B5-positive cells after differentiation is required, NB medium is the basal medium of choice for studying differentiated oligodendrocytes. If a larger absolute number of surviving cells is required, basal media such as RPMI or DMEM are recommended.

Since NB differs from RPMI as well as DMEM by a strong difference in osmolarity we investigated whether adding NaCl or mannitol to the culture medium could influence BrdU

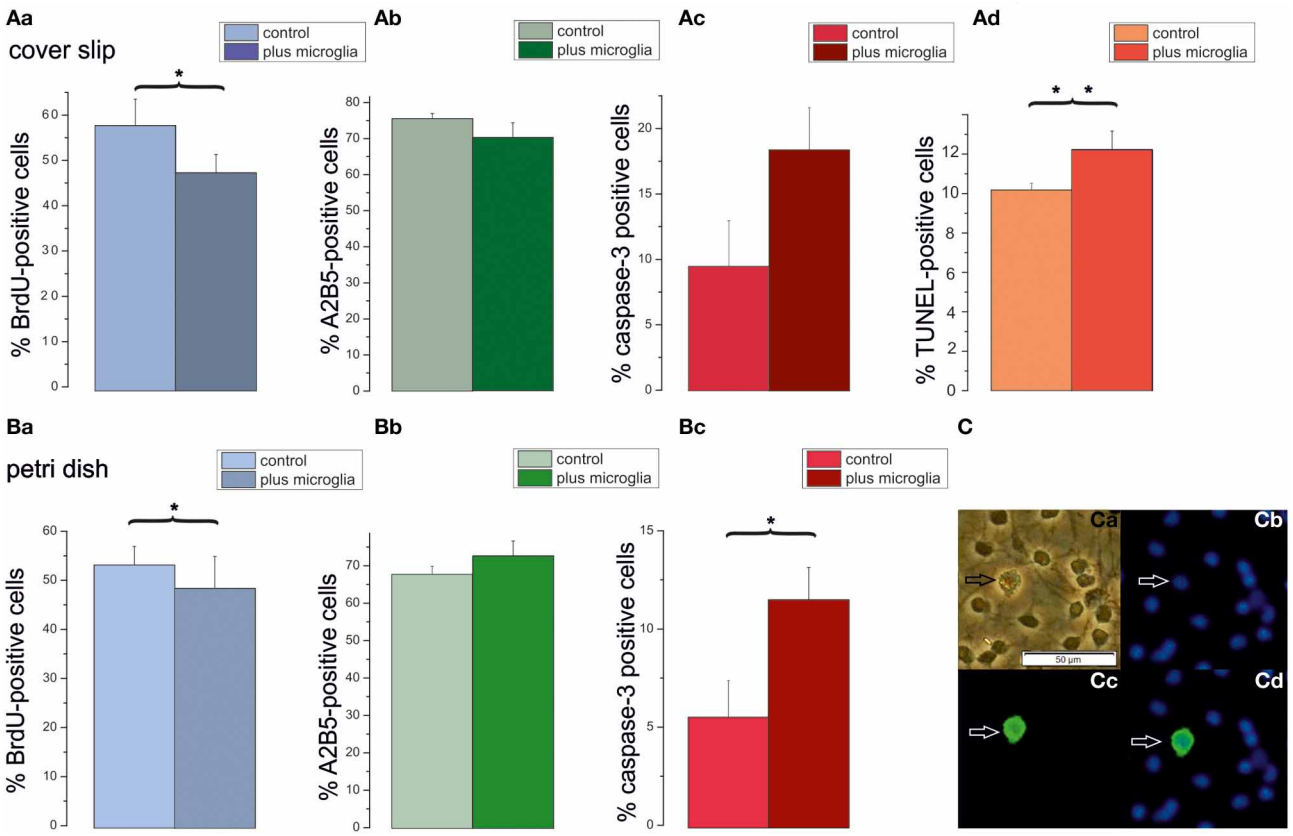

FIGURE 8 | Impact of additional microglia on OPCs cultured on cover slips or petri dishes. Cells plated with a seeding density of 5000 cells/coverslip were cultured in DMEM based proliferation medium for 3 days (control) or co-cultivated with an added density of microglia (plus microglia), as counted in high density cultures (80,000 cells/coverslip) in proliferation medium. Bar graphs **(Aa–Ad)** display percentages of OPCs cultured on coverslips. **(Aa)** BrdU-positve cells. **(Ab)** A2B5-positive cells. **(Ac)**: Caspase-3-positive cells, **(Ad)** TUNEL positive cells, 6 cover slips from 3 preparations investigated. Bar graphs in **(Ba–Bc)** display percentages of OPCs cultured on Petri dishes. **(Ba)**: BrdU-positive cells. **(Bb)** A2B5-positive cells. **(Bc)** Caspase-3-positive cells; 6 cover slips from 3 preparations investigated. Error bars indicate ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$; ANOVA followed by Tukey *post-hoc* Test. **(C)** Examples of OX-42 stained microglia after 3 days in DMEM based proliferation medium, seeding density 80,000 cells/coverslip **(Ca)** phase contrast picture, **(Cb)** Hoechst 33258 staining of the same image, **(Cc)** OX-42 immunofluorescence, **(Cd)** merged pictures, calibration bar indicates 50 µm.

incorporation as well as Caspase-3 immunopositivity. In a first series of experiments we found that the addition of NaCl strongly increased the percentage of BrdU positive cells to near the values found in DMEM (data not shown). In a second series of experiments we studied, whether this effect was due to the reduced Na^{+} concentration in the NB medium or induced by changes in osmolarity. Our results showed that the BrdU incorporation rate showed an even stronger increase and the percentage of caspase3-positive cells was even lower if mannitol was added to the culture medium, suggesting that an increase in osmotic pressure increases OPC proliferation. Although the inventors of the Neurobasal medium noticed a reduction of gliosis in the culture they did not investigate in more detail the effect of reducing the osmotic pressure on the proliferation of oligodendrocytes (Brewer and Cotman, 1989; Brewer et al., 1993). Although to our knowledge this effect has not been noticed before on oligodendrocyte lineage cells, increases in cell volume have been observed in proliferating fibroblasts, mesangial cells, lymphocytes, HL-60 cells, GAP A3 hybridoma cells, smooth muscle cells, and HeLa cells (cited in Lang et al., 1998). However, in most of these cell types, cell swelling has been associated with proliferation whereas cell shrinkage rather correlated with apoptosis (Lang et al., 1998). This is somewhat in contrast to the present findings, since the hypoosmolar NB medium should at first induce a cell swelling. Potentially, our results could be explained by an overcompensation of the transient short term osmotic effects during a culture period of several days. Alternatively, in hypoosmolar solutions, cyclic volume changes might be reduced, resulting in an inhibition of ion fluxes required for cell proliferation, such as volume activated anion channels (see e.g., He et al., 2012).

FURTHER FACTORS INFLUENCING SURVIVAL AND PROLIFERATION OF OPCs

Our results, showing that OPCs proliferate better in DMEM compared with NB, are in contrast to the observations of Yang et al. who showed that B27/NB medium was significantly more effective in maintaining viable cells and in supporting oligodendrocyte proliferation than the combination N1/DMEM (Yang et al.,

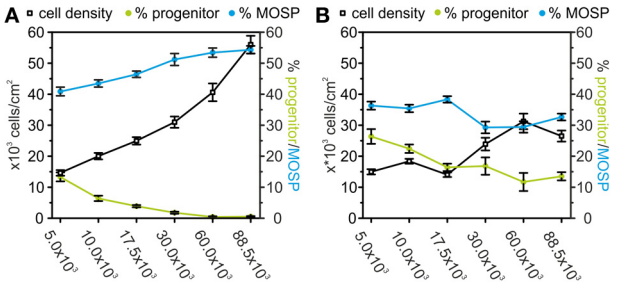

FIGURE 9 | Effects of TNF-α and IFN-γ treatment on A2B5-positive progenitors and MOSP-positive oligodendrocytes after 9 days in serum-free culture. (A) Dependence of the number of surviving cells (counted from DAPI-positive nuclei—black), progenitor cells (green), and MOSP-positive oligodendrocytes (blue) after 3 days of incubation in proliferation medium and 6 days in differentiation medium based on RPMI. Note the decrease in the percentage of bipolar progenitor cells and increase of MOSP-positive cells with increased cell density at time of seeding. **(B)** Dependence of the number of surviving cells (counted from DAPI-positive nuclei—black), bipolar progenitor cells (green), and MOSP-positive oligodendrocytes (blue) under identical conditions as used for data shown in **(A)** with the exception of adding 10 ng/ml TNF-α and 20 ng/ml IFN-γ from d2–d3. Note the increase in the percentage of progenitor cells and the decrease in the percentage of MOSP-positive cells at all cell densities studied. Error bars indicate ± s.e.m.

2005). Since our present results provide strong evidence that the lower osmolarity of NB inhibits proliferation, we suggest, that the additional ingredients in B27 compared with N1 obviously overcome the inhibitory effect of NB, in sum resulting in the enhanced proliferation observed by Yang et al., 2005.

In addition to the factors contained in B27 (Brewer et al., 1993) or NS21 (Chen et al., 2008), further factors have been shown to promote OPC proliferation, such as the supplement of PDGF (Noble et al., 1988; Raff et al., 1988; Pringle et al., 1989; Barres et al., 1992) as well as FGF-2 (Bogler et al., 1990; McKinnon et al., 1991; Grinspan et al., 1993) used here to induce OPC proliferation. These factors not only play a role in OPC proliferation *in vitro* but have been shown to be involved in OPC proliferation *in vivo* as well. The current concept is that FGF functions downstream of sonic hedgehog (Shh) which induces ventrally-derived OPCs (Nery et al., 2001) and acts predominantly via the FGF receptors FGFR1 und FGFR2 to supply OPCs *in vivo* (Furusho et al., 2011). Most prominently, FGFR1 and FGFR2 seem to be required *in vivo* to assemble the normal number of myelin wraps around axons in the course of maturation (Furusho et al., 2012) using the ERK1/2- MAPK downstream signaling cascade (Ishii et al., 2012). Activation of ERK1/2 is, however, not only responsible for extensive myelination but also essential in the earlier step of OPC generation (Ishii et al., 2013). In accordance with the prominent role of FGF-2 *in vivo* is the finding of enhanced FGF-2 levels in multiple sclerosis lesions, which could originate from activated astrocytes and microglia and help to repopulate the tissue with OPCs (Clemente et al., 2011).

Besides adding PDGF and FGF-2 to the PM alternative methods have been described to further stimulate OPC proliferation in culture. Hence it has been shown that conditioned medium from B104 neuroblastoma cells stimulates OPC proliferation by secretion of a PDGF AA and AB dimer independent factor, which could be especially useful for expanding adult brain derived OPCs (Hunter and Bottenstein, 1991). Conditioned medium from B104 neuroblastoma cells was also successfully used to induce OPC proliferation on astrocyte feeder layers and increase the yield of isolated OPCs (Niu et al., 2012) as well as to induce OPCs from embryonic mouse neurosphere cultures (Chen et al., 2007). However, other authors came to the conclusion, that the combination of FGF-2 and PDGF is more efficient than the combination of B104 conditioned medium with FGF-2 to expand OPCs from E16 rat spinal cord (Fu et al., 2007). Further antioxidants could exert protective effects on cultured OPCs (Cammer, 2002) and additional regulatory factors, which are released *in vivo* for e.g., from axons (Barres and Raff, 1999) continue to be identified (Sher et al., 2008). Such potential factors include hepatocyte growth factor (HGF) (Yan and Rivkees, 2002), which potentiates the proliferation enhancing effect of heregulin (HRG), a glial growth factor (Canoll et al., 1996). Further factors, such as brain derived neurotrophic factor (BDNF) (Vondran et al., 2010), neurotrophin-3 (NT-3) (Kahn et al., 1999) as well as insulin-like growth factor type 1 (IGF-1) (Zeger et al., 2007) have been identified as factors promoting proliferation of oligodendrocytes. Furthermore, it would be useful to test, whether the addition of Shh which increases OPC proliferation (Merchán et al., 2007; Wang et al., 2008) could additionally increase the yield of OPCs in culture. In order to further optimize culture media yielding maximal numbers of OPCs in addition to PDGF and FGF-2 further factors will have to be added to the PM.

EFFECTS OF CELL DENSITY ON MATURATION OF OLIGODENDROCYTES

Additional factors secreted paracrinely from OPCs could also explain our second observation, that the density of the cells at the time of seeding had a clear influence on proliferation and differentiation in culture. Thus, the percentage of A2B5-positive cells decreased already under conditions favoring proliferation, and the percentage of differentiated MOSP-positive cells increased after incubation in differentiation promoting solution with increasing density of oligodendroglial cells. The influence of the conditioned medium on BrdU incorporation showed that OPCs secrete factors that inhibit proliferation and promote differentiation. Furthermore cell death in very low density cultures suggests that the cells require additional paracrinely secreted survival signals not present in sufficient concentration in our serum replacement formulation. These findings are in agreement with results by Levi and Agresti (1991) who found that O-2A progenitors cultured at high densities largely differentiated into mature oligodendrocytes. Furthermore they suggested that O-2A progenitors secrete high molecular weight, non-mitogenic factors capable of inducing a rapid differentiation (Levi et al., 1991). Such autocrinely or paracrinely released factors, influencing survival and differentiation of OPCs could e.g., include endocannabinoids (Molina-Holgado et al., 2002), as well as TGF-β which could promote differentiation (McKinnon et al., 1993).

EFFECTS OF MICROGLIA ON OPCs

We observed that the addition of microglia to the cultures resulted in a weak inhibition of BrdU incorporation and a strong increase in the percentage of caspase-3 expressing preapoptotic and TUNEL-stained apoptotic cells. This is surprising, since

microglia secreted factors should have also been present in the conditioned medium, which only influenced proliferation and not apoptosis. This discrepancy could be explained by the fact, that OPCs could secrete TGF-β (McKinnon et al., 1993), which in turn has been shown to inactivate microglial cells (Spittau et al., 2013). Our results confirm previous observations that activated microglia may act deleterious to OPCs (Miller et al., 2007; Pang et al., 2010). Likewise, Taylor et al. (2010) observed an inhibition of proliferation by microglia and identified interleukin-6 and tumor necrosis factors (TNFs) as potential factors involved. Selmaj et al. (1991) additionally identified TNF-α and IFN-γ as factors released from microglia and astrocytes and (Agresti et al., 1996) showed that these factors inhibit proliferation of OPCs.

CYTOKINE-EFFECTS AT DIFFERENT CELL DENSITIES

A major disease effecting OPCs in neurodevelopment is periventricular leukomalacia (PVL), which can lead to spastic palsy and mental retardation (Dammann and Leviton, 1997; Benarroch, 2009). PVL can be caused by infection and inflammation or by hypoxic-ischemic episodes (Berger and Garnier, 1999; Du Plessis and Volpe, 2002; Ellison et al., 2005) and is therefore considered to be of multifactorial origin. Increased numbers of reactive INF-γ positive astrocytes (Folkerth et al., 2004) as well as increased levels of TNF-α, have been found in autopsies of children with PVL (Kadhim et al., 2001), suggesting that these cytokines could contribute to the generation of this disease. Studies performed on cultured purified oligodendrocyte progenitor cells provided evidence that the exposure of OPCs to INF-γ and TNF-α in fact induces cell death and additionally prevents the morphological differentiation of oligodendrocyte progenitors as well as the expression of myelin-specific proteins (Agresti et al., 1996; Andrews et al., 1998; Cammer and Zhang, 1999; Feldhaus et al., 2004a; Chew et al., 2005) confirming the concept that these factors could possibly be involved in the generation of PVL. In addition, further cytokines, like IL-1β, which are released as well as act on oligodendrocytes (Blasi et al., 1999; Vela et al., 2002) have also been shown to cause PVL like symptoms and axonal damage after systemic administration (Favrais et al., 2011).

Previous evidence indicates that the treatment with INF-γ and TNF-α not only impairs survival but leads to a larger population of undifferentiated cells than observed in control cultures after one week of differentiation (Cammer and Zhang, 1999; Feldhaus et al., 2004a). Cytokine-treated cultured oligodendrocytes express less myelin proteins at the mRNA (Feldhaus et al., 2004b) as well as the protein level and maintain their immature complement of voltage-gated ion channels (Mann et al., 2008). Furthermore after OPC transplantation into lipopolysaccharide-lesioned rat brain tissue the transplanted OLs show a reduced differentiation (Webber et al., 2009), which could be explained by cytokine actions, as observed in culture.

On might argue, that the impaired differentiation observed in cell culture experiments stems from the dilution of the differentiation promoting factors secreted at high cell densities (see **Figures 6**, **7**), which would increase with an increasing cytokine induced cell death in the culture. The impairment of differentiation observed after cytokine treatment would then be an indirect effect caused secondarily to the reduction in cell density. To learn more about potential cell density effects on the cytokine actions we thus performed a 48 h treatment with 10 ng/ml TNF-α and 20 ng/ml IFN-γ in cultures seeded at different cell densities (**Figure 9**). Our results show, that even at the lowest plating density, at which no cytokine effects on the number of surviving cells was observed, the cytokine treatment increased the percentage of immature cells and decreased the percentage of differentiated cells. This suggests, that combined treatment with INF-γ and TNF-α directly inhibits differentiation of OPCs.

Most interestingly, the increase in the number of bipolar progenitor cells and the reduction of the percentage of surviving cells as well as of MOSP-positive differentiated cells in response to the cytokine treatment was more pronounced in high density cultures than in low density cultures. In accordance with the results of (Hewett et al., 1999; Pang et al., 2010) this effect could be explained by the larger absolute number of microglia cells in the high density cultures, which could be stimulated by the cytokines to release further deleterious cytokines thus amplifying the cytokine effects on OPC survival and differentiation.

CONCLUSIONS

In order to work out concepts for myelin repair strategies to obtain high yields of cultured OPCs are desirable. These cells can then be used to perform pharmacological as well as biochemical investigations to investigate the properties of purified OPCs in isolation. Furthermore, purified OPC cultures could potentially be useful for transplantation experiments, as already performed by Bambakidis and Miller (2004) and Webber et al. (2009). Further differentiation of transplanted OPCs *in vivo* might, however, be compromised by signals from the native environment, preventing the further differentiation into myelinating oligodendrocytes (Lü et al., 2010; Sypecka and Sarnowska, 2013). It will have to be worked out in future, whether the addition of further factors (see e.g., Bambakidis and Miller, 2004) could potentially help to restore functional myelination.

To obtain satisfactory numbers of OPCs *in vitro* adequate culture conditions have to be worked out. The first step would be to start with a high number of purified OPCs. To obtain purer cultures immunopanning (Barres et al., 1992), generation from neurospheres (Itoh, 2002; Pedraza et al., 2008), which can also be used to obtain OPCs from mice (Chen et al., 2007) and Magnetic Activated Cell Sorting (MACS) (Dincman et al., 2012) methods have been developed which, however reduce the yield of cells. An interesting alternative, leading to much higher numbers of OPCs to start with and causing lower costs is a recently developed expansion of OPC growth on an astrocyte feeder layer with neuroblastoma cell conditioned media (Niu et al., 2012).

In addition to expanding cells with proliferation promoting factors such as PDFG and FGF-2 which were used in the present experiments, further factors, such as HGF (Yan and Rivkees, 2002), HRG (Canoll et al., 1996) or IGF-1 (Zeger et al., 2007) or Shh (Gao, 2006; Merchán et al., 2007) wait to be tested in order to clarify whether a different combination of growth factors could further increase the yield of OPCs. Here, we worked out, that, additionally, the basal culture medium has a significant influence on proliferation and apoptosis of OPCs. Particularly we

observed, that a decrease in the osmolarity of the medium reduces proliferation and increases apoptosis of OPCs. If a culture of differentiated oligodendrocytes with a low number of remaining OPCs is desired, the low osmolarity Neurobasal medium would be the medium of choice. If a high yield of OPCs is required a medium with higher osmolarity, favoring proliferation, such as DMEM or RPMI is recommended.

Furthermore, the experiments shown here confirm that oligodendrocyte lineage cells secrete differentiation promoting factors. If a high proliferation rate is desired cells should be maintained at cell densities of about 5000 cells/cm^2.

Since cells secrete paracrinely acting factors some pharmacological effects observed may to some extent depend on the cell density in the cultures. This was demonstrated here by a series of experiments showing that cytokines exert stronger effects on survival and differentiation of oligodendrocyte lineage cells in higher than in lower density cultures. This can be explained by the activation of a small percentage of 3–5% residual microglial cells, amplifying the cytokine effects by releasing additional cytokines upon activation.

Taken together, the highest yield of oligodendrocytes is obtained in media of high osmolarity, seeded at low density in the presence of a low concentration of microglia. Since microglia have been shown to be not only deleterious but also secrete survival promoting factors (Miller et al., 2007), it has to be worked out, whether it would be beneficial to inactivate, but not totally remove remaining microglia, for instance by supplementing TGF-β (Spittau et al., 2013).

AUTHOR CONTRIBUTIONS

Karolina Kleinsimlinghaus, Romy Marx, Meray Serdar, Ivo Bendix, and Irmgard D. Dietzel conceived and designed the experiments, which were carried out by Karolina Kleinsimlinghaus, Romy Marx, and Meray Serdar assisted by the undergraduate students listed in the Acknowledgement. Karolina Kleinsimlinghaus, Romy Marx, and Irmgard D. Dietzel predominantly wrote the manuscript. All authors have read the manuscript before submission.

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The generation of oligodendroglial cells is preserved in the rostral migratory stream during aging

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The subventricular zone (SVZ) is the largest source of newly generated cells in the adult mammalian brain. SVZ-derived neuroblasts migrate via the rostral migratory stream (RMS) to the olfactory bulb (OB), where they differentiate into mature neurons. Additionally, a small proportion of SVZ-derived cells contribute to the generation of myelinating oligodendrocytes. The production of new cells in the SVZ decreases during aging, affecting the incorporation of new neurons into the OB. However, the age-related changes that occur across the RMS are not fully understood. In this study we evaluate how aging affects the cellular organization of migrating neuroblast chains, the proliferation, and the fate of the newly generated cells in the SVZ-OB system. By using electron microscopy and immunostaining, we found that the RMS path becomes discontinuous and its cytoarchitecture is disorganized in aged mice (24-month-old mice). Subsequently, OB neurogenesis was impaired in the aged brain while the production of oligodendrocytes was not compromised. These findings provide new insight into oligodendrocyte preservation throughout life. Further exploration of this matter could help the development of new strategies to prevent neurological disorders associated with senescence.

Keywords: neuroblast migration, subventricular zone, rostral migratory stream, olfactory bulb, neurogenesis, oligodendrogenesis, aging

INTRODUCTION

Neurogenesis persists in the subventricular zone (SVZ) of the lateral ventricles throughout life. SVZ astrocytes continuously proliferate and give rise to intermediate progenitor cells, which then differentiate into neuroblasts forming large chains ensheathed by gliotubes of astrocytes (Doetsch et al., 1997; Ponti et al., 2013). These migratory structures emerge from the SVZ and coalesce into the rostral migratory stream (RMS) that ends in the olfactory bulb (OB) (Lois et al., 1996; Peretto et al., 1997; Alvarez-Buylla and Garcia-Verdugo, 2002). Thus, neuroblasts migrate tangentially from the SVZ and incorporate into the OB. Neuroblasts in the RMS retain their ability to proliferate (Smith and Luskin, 1998; Poon et al., 2010), but once they reach the OB, neuroblasts begin radial migration and mature into interneurons that integrate in preexisting functional circuits (Lois and Alvarez-Buylla, 1994; Luskin et al., 1997; Carleton et al., 2003; Imayoshi et al., 2008; Kelsch et al., 2010; Lazarini and Lledo, 2011). Although most SVZ precursor cells generate neuroblasts to support OB neurogenesis, a small subpopulation of precursors gives rise to cells in the oligodendroglial lineage, which are able to migrate toward the corpus callosum, striatum, or septum to differentiate into myelinating oligodendrocytes (Nait-Oumesmar et al., 1999; Menn et al., 2006; Gonzalez-Perez et al., 2009; Gonzalez-Perez and Alvarez-Buylla, 2011; Capilla-Gonzalez et al., in press).

During aging, the proliferative potential of the rodent SVZ decreases due to a partial depletion of neural precursor cells, with a subsequent disruption of OB neurogenesis (Maslov et al., 2004; Luo et al., 2006; Molofsky et al., 2006; Bouab et al., 2011; Conover and Shook, 2011; McGinn et al., 2012). Similarly, the human SVZ maintains its proliferative and neurogenic potential in adult life but it is drastically decreased when compared to fetal and pediatric stages (Sanai et al., 2004; Quinones-Hinojosa et al., 2006; Guerrero-Cazares et al., 2011; Sanai et al., 2011). However, the effects of aging on the genesis of new cells have mostly been studied at the SVZ level with little emphasis on the migratory pathway that the SVZ-derived cells follow to reach the OB (Bouab et al., 2011; Shook et al., 2012). Here, we investigate the age-related changes occurring across the RMS of mice. We demonstrate that this migratory pathway is deeply altered during aging and tends to disappear, resulting in a disruption of the OB neurogenesis. Interestingly, we found that the production of oligodendrocytes in the SVZ-OB system is not compromised by aging. Our study provides a better interpretation of the neurogenesis decline occurring in the aged brain, which could help to understand neurological disorders associated with senescence.

RESULTS

THE RMS CYTOARCHITECTURE IS DISRUPTED BY AGING

Consistent with previous studies (Luo et al., 2006; Bouab et al., 2011), the SVZ of mice showed a loss of cells during aging, particularly in the population of neuroblasts forming migratory chains (Figure S1). To evaluate if those effects from aging were

also present in the RMS, sagittal sections of the brain were first stained with DAPI nuclear dye. We observed that the population of cells that form the RMS connecting the SVZ with the OB was diminished in the aged brain (**Figures 1A,B** and Figure S2). The astrocytic and neuroblast populations within the RMS were examined using the glial fibrillary acidic protein (GFAP) and doublecortin (DCX) markers, respectively. The RMS of aged mice preserved GFAP+ cells that were stained more intensively than those from young mice (**Figures 1C,D**). In contrast, the expression of DCX was severely reduced during aging (**Figures 1E,F**). This data indicates that the aged brain maintains the astrocytes that constitute gliotubes in the young brain, but it does not preserve chains of migrating neuroblasts.

To investigate these findings further we used transmission electron microscopy. The analysis of RMS coronal sections revealed a notable loss of cells during aging, which resulted in a significant decrease of the area occupied by the RMS compared to young animals (Young 1217.7 μm^2 vs. Aged 218.7 μm^2, $p = 0.003$) (**Figures 2A–C**). Remaining cells in the aged RMS were found to form small groups of cells that appeared isolated. Unlike the young mice, occasional cells were found in the intrabulbar part of the anterior commissure of the aged brain, where axons are located (**Figure 2C**). At higher magnifications, we detected that the reduction in the area occupied by the RMS was primarily due to a loss of migrating neuroblasts (**Figures 2D–G**). We did not observe ultrastructural differences in the remaining neuroblasts of the aged RMS compared to those from young mice. However, we found abundant dense bodies in the cytosol of astrocytes and frequent microglial cells close to the RMS in the aged brain (Figure S3).

PROLIFERATIVE CELLS WITHIN THE RMS DECREASE IN THE AGED BRAIN

To study the proliferative capacity of remaining cells in the aged RMS, animals received a single dose of 5-bromo-2-deoxyuridine (BrdU) 2 h before sacrifice. We observed an 83% reduction in the number of BrdU+ cells per section in the RMS of aged mice (Young 23.6 ± 0.4 cells vs. Aged 4 ± 0.8 cells, $p < 0.001$) (**Figure 3A**). These proliferative cells were found in small groups of cells that were preserved in the aged RMS. Given that BrdU is only incorporated by cells in S-phase, we also used the proliferation marker Ki67 that is present during all active phases of the cell cycle (G1, S, G2, and mitosis). Consistently, we observed frequent Ki67+ cells in the young RMS, while they were occasional in aged mice ($n = 3$ in all groups), supporting the results from the BrdU assay (**Figures 3B,C**). To determine the identity of these proliferative cells, we performed double immunostaining against Ki67-GFAP or Ki67-DCX. In the aged RMS, some proliferative cells were found to express GFAP (**Figure 3B** and Figure S4), however, proliferating DCX+ cells were not detected (**Figure 3C**). In addition, to evaluate if proliferative cells were from the oligodendroglial lineage, we used

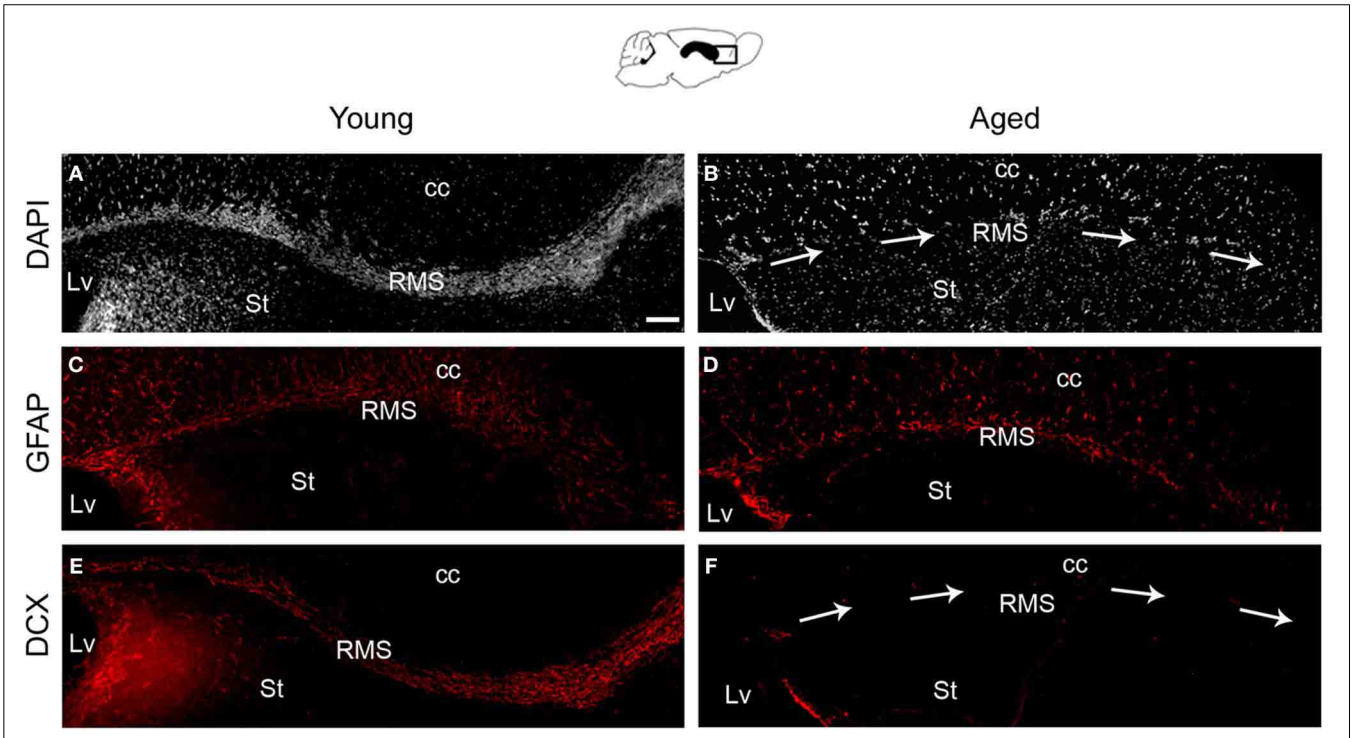

FIGURE 1 | The population of neuroblasts within the RMS is disrupted during aging. Sagittal sections of the RMS in young and aged mice. **(A)** Young RMS stained with DAPI. **(B)** Aged RMS stained with DAPI, showing a notable reduction of cells in the migratory pathway (arrows). **(C)** Young RMS immunolabeled with GFAP. **(D)** Aged RMS immunolabeled with GFAP did not reveal remarkable differences in the population of GFAP+ cells. **(E)** Young RMS immunolabeled with DCX. **(F)** Aged RMS (arrows) immunolabeled with DCX, showing a notable reduction of neuroblasts. cc, corpus callosum; St, striatum; Lv, lateral ventricle. Scale bar: 100 μm

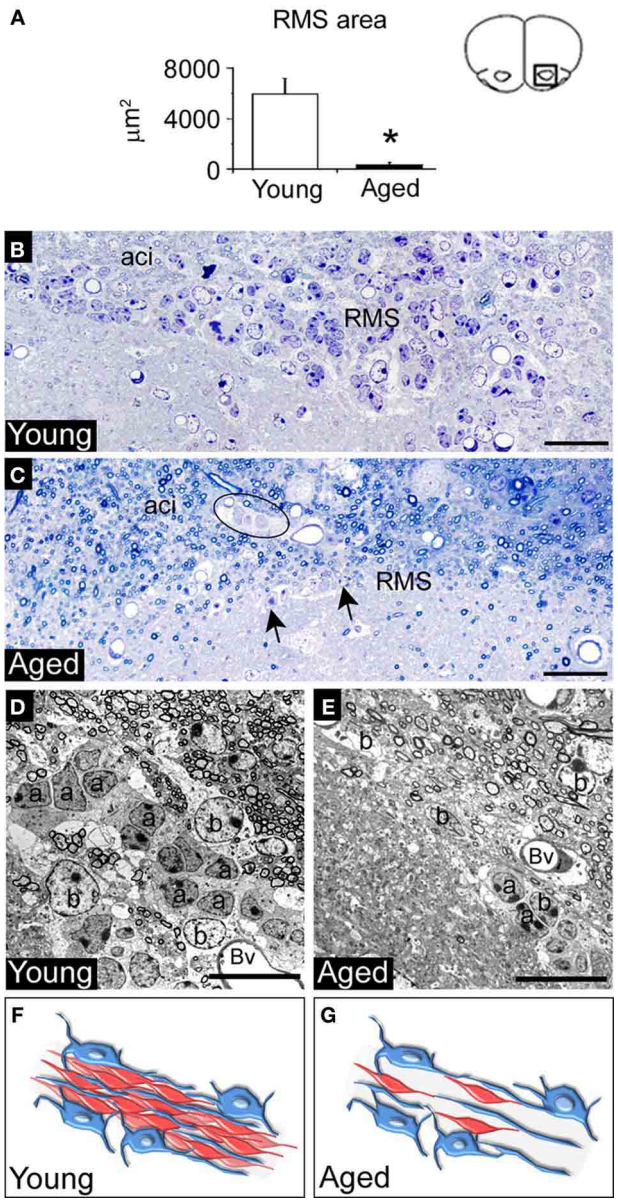

FIGURE 2 | Cytoarchitecture of aged RMS reveals a loss of migrating neuroblasts into the gliotubes. Analysis of the RMS by using light and electron microscopy. **(A)** Bar graph depicting a significant reduction of the area occupied by the RMS in aged mice. **(B)** Semithin section of the young RMS showed multiples chains of neuroblasts. **(C)** Semithin section of the aged RMS showed a notable loss of migratory chains, but dispersed cells remained (arrows). Note the presence of groups of neuroblasts and astrocytes within the intrabulbar part of the anterior commissure (delineated). **(D)** Electron microscopy image of a young RMS showing a detail of the neuroblasts chains surrounded by astrocytic gliotubes. **(E)** Electron microscopy image showing a detail of the aged RMS, where neuroblasts were severely reduced. **(F)** Schematic representation of the RMS cytoarchitecture in young mice. Chains of neuroblasts (red) migrating through gliotubes, which were formed by astrocytes (blue). **(G)** Schematic representation of the RMS cytoarchitecture in aged mice. Note the loss of neuroblast migrating through the gliotubes. a, neuroblast; aci, intrabulbar part of the anterior commissure; b, astrocyte; Bv, blood vessel. Scale bar: **B,C** = 20 µm, **D,E** = 10 µm. *$p < 0.01$.

the transcription factor Olig2. Surprisingly, we found that both groups of animals presented an equal number of BrdU/Olig2+ cells per section (Young 1.01 ± 0.5 cells vs. Aged 0.8 ± 0.2 cells, $p = 0.692$). Given that the overall number of BrdU+ cells declines over time, there was a resulting increase in the proportion of BrdU+ cells that expressed the Olig2 marker in the aged RMS (Young $3.5 \pm 1.9\%$ vs. Aged $16.5 \pm 4.7\%$, $p = 0.0117$) (**Figures 4A–D**). These findings suggest that remaining proliferative cells in the aged RMS could be supporting oligodendrogenesis.

NEWLY GENERATED CELLS IN THE AGED RMS BECOME OLIGODENDROCYTES

In order to evaluate the proliferative potential of the RMS cells in a longer period of time and to determine the fate of the newly generated cells by ultrastructural analysis, a group of mice was injected with tritiated thymidine (^{3}H-Thy, 1 dose/day) over a 10-day period and euthanized after 6 weeks (**Figure 5A**). The ^{3}H-Thy+ cells found in the aged RMS displayed irregular contours and light cytoplasm with few intermediate filaments. Their nuclei were fusiform and contained dense, peripherally-distributed chromatin. These ^{3}H-Thy+ cells had ultrastructural features bearing a resemblance to astrocytes and oligodendrocytes (**Figure 5B**). We also detected ^{3}H-Thy+ oligodendrocytes displaying a smooth contour, round nucleus, and dark cytoplasm that contained short cisternae of rough endoplasmic reticulum and an absence of intermediate filaments (**Figure 5C**). The lack of dense bodies in the cytoplasm of these cells discarded the possibility of them being microglia cells. Neuroblasts labeled with ^{3}H-Thy were not detected in any group, likely due to the fact that these cells had already migrated to the OB at the time of euthanasia. Together, these findings support the hypothesis that the incorporation of neuroblasts into the OB is impaired during aging, while oligodendrogenesis is preserved.

NEWLY GENERATED CELLS IN THE AGED OB SUPPORT THE OLIGODENDROGLIAL LINEAGE

To evaluate if neurogenesis in the OB was disrupted in the aged brain, animals were injected with 4 doses of BrdU, each dose injected with a 2 h interval, and euthanized after 30 days (**Figure 6A**). Quantitative analysis of OB coronal sections showed a significant decrease of BrdU+ cells in the aged group (Young 87.31 ± 7.84 cells vs. Aged 5.31 ± 0.33 cells, $p < 0.001$; **Figure 6B**). In accordance, a decrease in the neuroblast population was observed in the aged OB, which was more pronounced in the medial region (Figure S5). To verify if newly generated cells in the OB were differentiating into mature neurons, we performed a double immunostaining against BrdU and NeuN markers. The number of BrdU/NeuN+ cells per section was significantly reduced in aged mice (Young 75.6 ± 13.01 cells vs. Aged 3.5 ± 0.3 cells, $p = 0.01$), while the percentage of BrdU+ cells that expressed NeuN did not differ across the two experimental groups (Young $90.12 \pm 2.5\%$ vs. Aged $83.7 \pm 2.9\%$, $p = 0.228$). Next, the production of new cells from the oligodendroglial lineage was evaluated using the Olig2 marker. Contrarily to the decrease in neurogenesis, aged mice displayed an increase

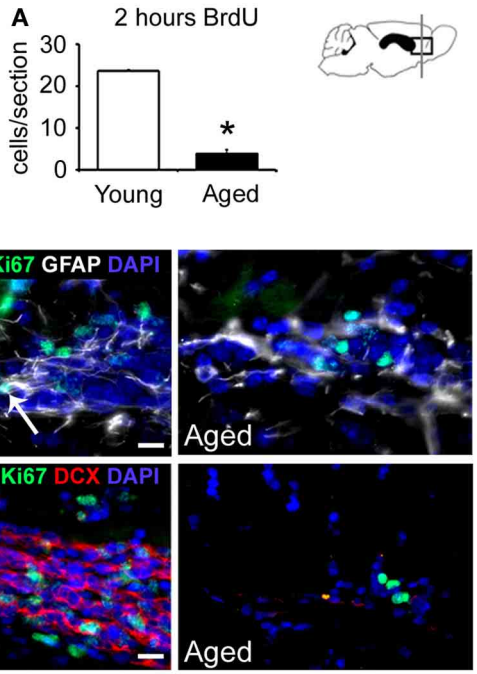

FIGURE 3 | Aging decreases the population of proliferating cells in the RMS. (A) Bar graph depicting the number of BrdU+ cells in coronal sections of the RMS, 2 h after BrdU administration. Note the significant decrease of proliferative cells in the aged RMS. **(B)** Immunoassay against Ki67 (green) and GFAP (white) in sagittal sections of the RMS. The number of Ki67+ was drastically reduced in aged mice, while GFAP+ cells were maintained during aging. Note the presence of double Ki67/GFAP+ cells (arrow) in the young RMS. **(C)** Immunoassay against Ki67 (green) and DCX (red) in sagittal sections of the RMS. The number of both Ki67+ and DCX+ cells was drastically reduced in aged mice. Scale bar: **B,C** = 10 μm. $^*p < 0.01$.

in the percentage of cells that co-expressed BrdU and Olig2 markers (Young $3.5 \pm 1.1\%$ vs. Aged $42.7 \pm 0.7\%$, $p < 0.001$), but did not show a difference in the number of BrdU/Olig2+ cells (Young 2.7 ± 0.6 cells vs. Aged 2.4 ± 0.2 cells, $p = 0.675$) (**Figures 6C–F**). The generation of new oligodendroglial cells was also examined in the corpus callosum, striatum, and SVZ of the aged mice, finding a consistent increase in the proportion of cells co-expressing BrdU and Olig2 in the SVZ (Figure S6). Interestingly, the aged OB showed occasional cells co-expressing BrdU, Olig2, and NeuN markers (**Figure 6G**). Despite the reduction in neurogenesis with aging, these results suggest that the production of oligodendrocytes is maintained across the aged SVZ-OB system.

In order to test the possibility that the new cells found in the OB were endogenously generated from local progenitors (Gritti et al., 2002), we examined the OB of animals that received a single dose of BrdU and were euthanized 2 h later. Immunostaining against BrdU revealed that proliferation was significantly decreased in the aged OB (Young 21.1 ± 4.6 cells vs. Aged 5.1 ± 0.4 cells, $p < 0.001$). This reduction of proliferative cells was more pronounced in the medial region of the OB, where migrating neuroblasts arrive. Interestingly, proliferative cells were found to express Olig2 in the young OB, but not in the aged OB (Figure S7). These results suggest that the new oligodendroglial cells within the aged OB were not originated from local progenitors. Nonetheless, more experiments would be required to conclusively study the origin of the new oligodendroglial cells.

DISCUSSION

During aging, the SVZ undergoes a severe decline in its proliferative capacity which subsequently affects neurogenesis in the OB (Maslov et al., 2004; Luo et al., 2006; Bouab et al., 2011; McGinn et al., 2012). However, the age-related changes induced in the migratory pathway that the newly generated cells follow had not been completely described. In this study we present important findings on the effects of aging across the SVZ-OB system. We demonstrated that proliferation declines at the RMS and OB levels during aging, similar to the SVZ. We observed a decrease in the number of neuroblasts migrating across the RMS and an altered cellular organization of this migratory pathway. Finally, while we confirmed an OB neurogenesis decline with aging, oligodendrogenesis across the RMS was surprisingly not compromised.

Newly generated cells in the SVZ migrate a long distance through the RMS before reaching the OB, where they differentiate into interneurons to replace old cells (Lois and Alvarez-Buylla, 1994). The SVZ maintains this neurogenic capacity throughout life, but the number of cells that ultimately integrate into the OB decrease over time (Bouab et al., 2011; Shook et al., 2012). Consistent with these findings, we described a remarkable depletion of the migratory chains of neuroblasts in the SVZ during senescence, which was mimicked in the RMS. While the gliotubes of astrocytic cells were maintained in the aged brain, the number of migrating neuroblasts was drastically reduced. Subsequently, we found a decrease of newly generated cells in the aged OB, in accordance with previous publications (Bouab et al., 2011; Shook et al., 2012). These effects on OB neurogenesis could be a direct consequence of altering the SVZ niche. Prior studies from our group support this hypothesis as we previously demonstrated that the disruption of the SVZ by exposure to N-ethyl-N-nitrosourea leads to an impairment in the incorporation of new cells into the OB (Capilla-Gonzalez et al., 2012). Similarly, the radiation impact on the SVZ neurogenic niche interrupted neuroblast migration to the OB (Lazarini et al., 2009; Achanta et al., 2012). Another explanation for OB neurogenesis decline is that aging could impair the migratory capacity of neuroblasts, however, a recent publication suggests that the capacity of neuroblasts to exit the SVZ and reach the OB is not affected by age (Mobley et al., 2013). Based on these reports, we consider that SVZ-derived cells are able to migrate in the aged brain, but the number of cells that finally incorporate into the OB is drastically reduced due to the age-related effects on the SVZ niche.

Contrary to the decline in neurogenesis during senescence, we demonstrated, for the first time, that the production of oligodendroglial cells is maintained in the aged SVZ-OB system. An increase in oligodendrocyte-generation has been observed in other regions of the central nervous system, such as the spinal cord and neocortex of rodents (Levison et al., 1999; Lasiene et al., 2009), or the fornix of monkeys (Peters et al., 2010). Concurrent with these reports, a more recent study indicate that

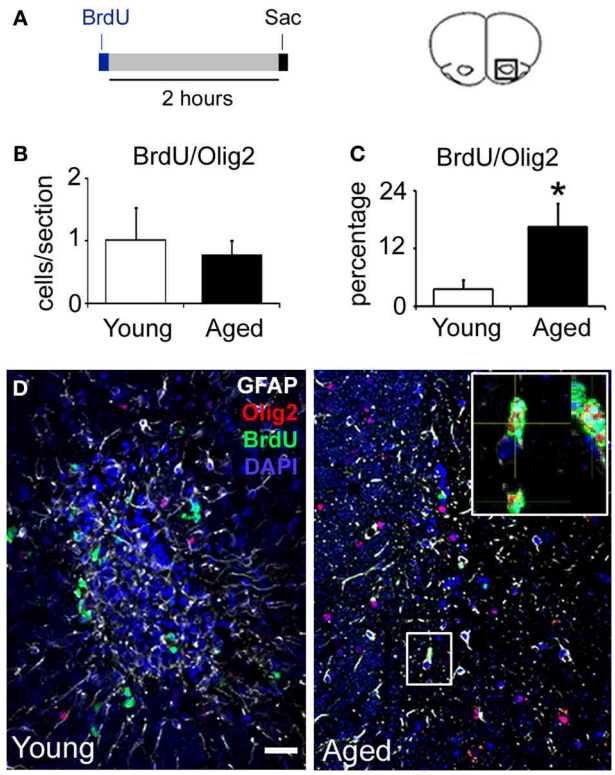

FIGURE 4 | A high percentage of the RMS proliferative cells pertain to the oligodendroglial lineage. (A) Animals received a single dose of BrdU and were euthanized 2 h after. **(B)** Bar graph depicting the number of BrdU/Olig2+ cells in the RMS. Note that there is not a significant difference between young and aged mice. **(C)** Bar graph depicting the percentage of BrdU/Olig2+ cells in the RMS. **(D)** Immunostaining against BrdU (green), Olig2 (red), and GFAP (white) in coronal sections of the RMS. Note the presence of cells co-expressing BrdU and Olig2 markers. Sac, sacrifice. Scale bar: **C** = 20 μm. $^*p < 0.05$.

NSCs, derived from young and middle-aged SVZ, present similar ability to produce oligodendrocytes *in vitro,* when they were differentiated in absence of exogenous growth factors (Bouab et al., 2011). In our study, we found that the capacity of the SVZ to produce Olig2+ cells *in vivo* is not diminished by aging. The maintained SVZ ability to produce Olig2+ cells in the aged brain could subsequently help to preserve the oligodendrogenesis in the RMS-OB. This would require that a group of SVZ-derived cells become oligodendrocytes instead of differentiating into neurons. This idea is supported by previous reports showing that DCX+ cells from the SVZ can be redirected from neuronal to glial fates following a demyelination process, and generate new oligodendrocytes in the corpus callosum (Jablonska et al., 2010). Another possible explanation for the maintenance of the oligodendrogenesis in the aged brain is that local stem cells within the OB are contributing to the production of oligodendroglial cells (Gritti et al., 2002; Rivers et al., 2008). In fact, stem cells isolated from the OB were found to produce more oligodendrocytes *in vitro* than those from the SVZ (Gritti et al., 2002). On the other hand, it could be possible that those local precursors within the OB may support neurogenesis to serve as a compensatory mechanism for the loss of newly arriving neuroblasts from the SVZ. Indeed, it has been described that oligodendrocyte progenitors in the OB are also able to produce new neurons in transgenic mice, after the blockage of the Platelet-Derived Growth Factor Receptor-alpha (Rivers et al., 2008). In accordance with this finding, we observed cells co-expressing BrdU, NeuN, and Olig2 markers in the aged OB. However, the reduced number of proliferative cells detected in the OB of aged mice conflicts with the possibility that most newly generated cells, either neurons or oligodendrocytes, were produced from local precursors instead of being SVZ-derived. These modifications in cell production may respond to age-related changes in cell signaling pathways. For instance, the balance between neurogenesis and gliogenesis in the SVZ niche is controlled by the BMP and noggin signaling (Lim et al., 2000; Mekki-Dauriac et al., 2002; Bilican et al., 2008). SVZ astrocytes express BMP inducing gliogenesis, while ependymal cells express noggin that promotes neurogenesis. Since the SVZ niche is disrupted during aging (Bouab et al., 2011; Shook et al., 2012), the BMP-noggin balance could be modified and contribute to the neurogenesis decline and favor oligodendrogenesis. Further studies are required to fully understand the mechanisms associated to the age-related changes described here.

Although the existence of the RMS in the adult human brain remains highly controversial (Sanai et al., 2004, 2007; Curtis et al., 2007; Kam et al., 2009; Wang et al., 2011), the presence of neural stem cells within the SVZ is widely accepted (Sanai et al., 2004; Quinones-Hinojosa et al., 2006). Nevertheless, the function of the newly generated cells in the human brain is currently unknown. A recent publication demonstrated that the postnatal production of new neurons in humans is minimal, while there is a continuous turnover of non-neuronal cells, which could contribute to the production of oligodendrocytes (Bergmann et al., 2012). Hence, the neurogenic system of aged mice becomes a better model for comparisons with adult human beings than the neurogenic system from young rodents. The maintained oligodendrogenesis provides new insight into the relevance of the oligodendrocytes throughout life, which could be a response to myelin damaged during aging (Nait-Oumesmar et al., 1999; Jablonska et al., 2010; Gonzalez-Perez and Alvarez-Buylla, 2011; Capilla-Gonzalez et al., in press). Moreover, oligodendrogenesis could be beneficial for the proper function of the remaining neurons in the aged brain. All of these possible functions could be crucial to the survival and maturation of newly generated oligodendrocytes.

In conclusion, we have demonstrated that the decrease of SVZ proliferation during aging is mimicked in the RMS-OB system. Neuroblast migratory chains are severely disrupted across the whole RMS of aged mice, consistent with OB neurogenesis impairment. Furthermore, oligodendrogenesis is maintained in the SVZ-OB system of the aged brain, but the origin of these new cells is still unknown. These findings provide new insight into the events occurring during aging on the genesis of new cells. Modifying the source and subsequent fate of these newly generated cells in the aged brain could serve as the foundation for new therapeutic strategies for brain disorders associated with senescence.

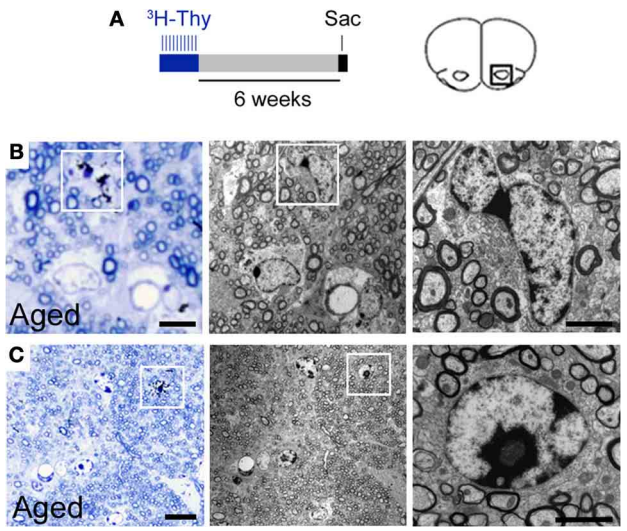

FIGURE 5 | Thymidine incorporation assay supports the generation of new oligodendrocytes in the aged RMS. (A) Animals received ten consecutive doses of 3H-Thy cells (dose/day) and were euthanized 6 weeks after. **(B)** Light and electron microscopy images showing a labeled cell (boxes) with irregular contours and light cytoplasm, with few intermediate filaments. The nucleus was fusiform and contained dense chromatin that was peripherally distributed. This cell contained features which bore a resemblance to astrocytes and oligodendrocytes. **(C)** Light and electron microscopy images showing a typical oligodendrocyte labeled with 3H-Thy (boxes). Sac, sacrifice. Scale bar: **B** = 5 μm, detail in **(B)** 2 μm, **C** = 10 μm, detail in **(C)** = 2 μm.

EXPERIMENTAL PROCEDURES

ANIMALS

The animals used were 2 and 24-month-old C57BL/6 male mice ($n_{young} = 20$, $n_{aged} = 22$) from National Institute on Aging (Baltimore, MD, USA) and Charles River Laboratory (Barcelona, Spain). Animals were housed under a 12-h light/dark cycle with food and water available *ad libitum*. All animal procedures were performed according to the European Communities Council (86/609/EEC) guidelines and approved by the Johns Hopkins Animal Care and Use Committee and followed standard animal care and use protocols.

ADMINISTRATION OF 5-BROMO-2-DEOXYURIDINE

5-bromo-2-deoxyuridine (BrdU, Sigma Aldrich, Dorset, UK) is an exogenous marker, which is incorporated into the newly synthesized DNA of replicating cells, during the S phase. To study the proliferation, animals received a single intraperitoneal injection of BrdU (50 mg/Kg b.wt.) and were euthanized 2 h after ($n_{young} = 4$, $n_{aged} = 6$). To assess cell migration, animals received 4 intraperitoneal injections of BrdU, separated by 2 h, and were euthanized 30 days later ($n_{young} = 3$, $n_{aged} = 3$).

TRITIATED THYMIDINE ADMINISTRATION

For ultrastructural identification of the proliferative cells and newly generated cells, animals received a single daily dose of 1.67 μl/g b.wt. of 1 mCi tritiated thymidine (^{3}H-Thy; specific activity 5 Ci/mmol; Amersham Biosciences, Uppsala, Sweden), for 10 consecutive days, and were euthanized 6 weeks after the last injection ($n_{young} = 4$, $n_{aged} = 4$).

BRAIN TISSUE FIXATION

Animals were anesthetized by an intraperitoneal injection of 2:1 ketamine/xylazine (5 μl/g of weight) and subjected to an intracardiac perfusion using a peristaltic pump. As fixative, we used 4% paraformaldehyde for immunohistochemistry, or 2% paraformaldehyde and 2.5% glutaraldehyde for electron microscopy. Prior brain dissection, heads were removed and post-fixed in the same fixative overnight.

IMMUNOHISTOCHEMISTRY

After post-fixation, brains were washed in 0.1 M PB and cut into serial 10 μm thick coronal ($n_{young} = 7$, $n_{aged} = 9$) or sagittal ($n_{young} = 3$, $n_{aged} = 3$) sections using a cryostat (Leica, CM 1900). One series (5–8 sections) from each animal was used in each immunostaining. Sections were incubated in blocking solution for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies (see Table S1). Then, sections were washed and incubated with the appropriate secondary antibodies conjugated with either biotin or fluorophores. After the secondary biotinylated antibody, sections were incubated with ABC Elite complex (Vector, Burlingame, CA, USA) and treated with diaminobenzidine (DAB, 0.05%; Sigma Aldrich). Measurement of BrdU incorporation during DNA synthesis was carried out in coronal sections by quantification of BrdU+ cells under an Eclipse E200 light microscope (Nikon, Tokyo, Japan), and were expressed as cells/section. Fluorescence samples were examined under an Olympus IX81 confocal microscope and imaged using the Olympus Fluoview software version 3.1 (Center Valley, PA, USA). Three-dimensional images were obtained using the Slidebook™ software (Intelligent Imaging Innovations, Denver, CO, USA). Quantification of BrdU double immunostaining with NeuN or Olig2 was expressed as double+ cells per section and as percentage (100 × BrdU-NeuN+ cells/total BrdU+ cells and 100 × BrdU-Olig2+ cells/total BrdU+ cells).

TRANSMISSION ELECTRON MICROSCOPY

Post-fixated brains were washed in 0.1 M phosphate buffer (PB; pH 7.4), cut into 200 μm sections with a VT 1000 M vibratome (Leica, Wetzlar, Germany) and treated with 2% osmium tetraoxide in 0.1 M PB for 2 h. Then sections were rinsed, dehydrated through increasing ethanol solutions and stained in 2% uranyl acetate at 70% ethanol. Following dehydration, slices were embedded in araldite (Durcupan, Fluka BioChemika, Ronkokoma, NY, USA). To study cell organization of the RMS, we cut serial 1.5 μm semithin sections with a diamond knife and stained them with 1% toluidine blue. To identify individual cell types, 60–70 nm ultrathin sections were cut with a diamond knife, stained with lead citrate, and examined under a Spirit transmission electron microscope (FEI Tecnai, Hillsboro, OR, USA). Changes in the area of the RMS were determined by measuring the area occupied by the RMS in semithin sections of 3 different levels per animal ($n_{young} = 6$, $n_{aged} = 6$), using light microscopy. The analysis was

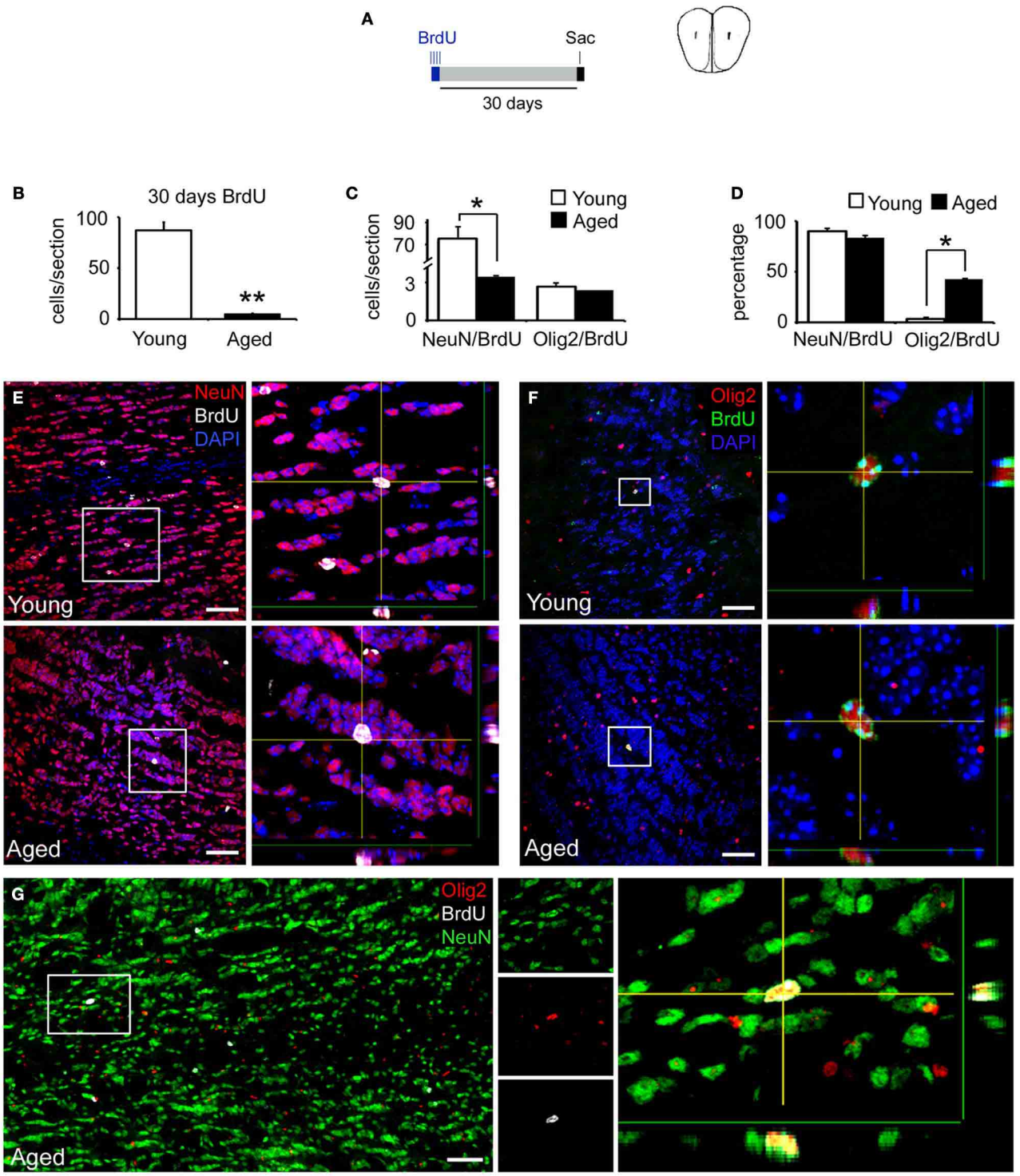

FIGURE 6 | Aging decreases neurogenesis, but not oligodendrogenesis in the OB. (A) The animals received 4 doses of BrdU, separated by 2 h, and were euthanized 30 days after. **(B)** Bar graph depicting a significant reduction of BrdU+ cells in the aged OB, 30 days after treatment. **(C)** Bar graph depicting the number of NeuN and Olig2+ cells co-expressing BrdU marker. Note the decrease of newly generated neurons in aged mice, while the number of new oligodendrocytes remained unchanged. **(D)** Bar graph depicting the percentage of NeuN and Olig2+ cells co-expressing BrdU marker. **(E)** Immunostaining against NeuN (red) and BrdU (white) markers representing bar graphs in **(B,C)**. **(F)** Immunostaining against Olig2 (red) and BrdU (green) markers representing bar graph in **(B,C)**. **(G)** Immunostaining against BrdU (white), Olig2 (red), and NeuN (green) showing BrdU/Olig2/NeuN+ cells in the OB of aged mice. Sac, sacrifice. Scale bar: 40 μm. $^{*}p < 0.05$, $^{**}p < 0.01$.

performed with Image Tool software (Evans Technology, Roswell, GA, USA).

TRITIATED THYMIDINE AUTORADIOGRAPHY

Brains treated with ^{3}H-Thy were processed for transmission electron microscopy as described above. Subsequently, semithin sections were dipped in LM-1 hypercoat emulsion (Amersham Biosciences), dried in the dark, and stored at 4°C for a month (Doetsch et al., 1997). Autoradiography was developed using standard methods and counterstained with 1% toluidine blue. Selected semithin sections, with a total of 40 labeled cells, were processed for ultrathin sections to be analyzed using electron microscopy.

STATISTICAL ANALYSIS

Data were expressed as mean ± SEM. After testing for normal distribution with Shapiro–Wilke Test, a Student's *t*-test was performed using SigmaPlot 11.0 software (Jandel Scientific, San Rafael, CA, USA). For samples that were not normally distributed, the non-parametric Mann Whitney *U*-Test was used. Differences were considered significant at a *p*-value lower than 0.05.

AUTHOR CONTRIBUTIONS

Vivian Capilla-Gonzalez, collection and assembly of data, concept and design, data analysis and interpretation, manuscript writing, final approval of manuscript; Arantxa Cebrian-Silla and Hugo Guerrero-Cazares, collection of data, data analysis and interpretation, final approval of manuscript; Jose M. Garcia-Verdugo and Alfredo Quiñones-Hinojosa, concept and design, data analysis and interpretation, final approval of manuscript, financial support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/CellularNeuroscience/10.3389/fncel.2013.00147/abstract

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Mechanisms of oligodendrocyte regeneration from ventricular-subventricular zone-derived progenitor cells in white matter diseases

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White matter dysfunction is an important part of many CNS disorders including multiple sclerosis (MS) and vascular dementia. Within injured areas, myelin loss and oligodendrocyte death may trigger endogenous attempts at regeneration. However, during disease progression, remyelination failure may eventually occur due to impaired survival/proliferation, migration/recruitment, and differentiation of oligodendrocyte precursor cells (OPCs). The ventricular-subventricular zone (V-SVZ) and the subgranular zone (SGZ) are the main sources of neural stem/progenitor cells (NSPCs), which can give rise to neurons as well as OPCs. Under normal conditions in the adult brain, the V-SVZ progenitors generate a large number of neurons with a small number of oligodendrocyte lineage cells. However, after demyelination, the fate of V-SVZ-derived progenitor cells shifts from neurons to OPCs, and these newly generated OPCs migrate to the demyelinating lesions to ease white matter damage. In this mini-review, we will summarize the recent studies on extrinsic (e.g., vasculature, extracellular matrix (ECM), cerebrospinal fluid (CSF)) and intrinsic (e.g., transcription factors, epigenetic modifiers) factors, which mediate oligodendrocyte generation from the V-SVZ progenitor cells. A deeper understanding of the mechanisms that regulate the fate of V-SVZ progenitor cells may lead to new therapeutic approaches for ameliorating white matter dysfunction and damage in CNS disorders.

Keywords: oligodendrogenesis, oligodendrocyte precursor cells, vascular dementia, multiple sclerosis, demyelination, subventricular zone, neural stem/progenitor cells

INTRODUCTION

During embryogenesis and development, germinal zones form stem cell niches, where multi-potential progenitor cells generate new neurons, astrocytes, and oligodendrocyte lineage cells. In the adult brain, the ventricular-subventricular zone (V-SVZ) of the lateral ventricle and the subgranular zone (SGZ) in the dentate gyrus of hippocampus retain neural stem/progenitor cells (NSPCs) to form the largest germinative areas for new neurons and glial cells (Gonzalez-Perez and Alvarez-Buylla, 2011; Ihrie and Alvarez-Buylla, 2011; Falcao et al., 2012). In addition, recent studies suggest that NSPCs also reside in the nonconventional zones outside of the V-SVZ and SGZ, such as the cerebral cortex (Nakagomi et al., 2009; Ohira et al., 2010), white matter (Nunes et al., 2003), and pia mater (Nakagomi et al., 2011, 2012). Among these germinative areas, the V-SVZ generates the most abundant number of stem cells in the adult brain that are capable of migrating to a long distance.

NSPCs play important roles in many CNS diseases as endogenous recovery mechanisms in injured brains areas (Ohab et al., 2006; Curtis et al., 2007, 2012; Nait-Oumesmar et al., 2007, 2008; Bedard et al., 2010; Lazarov and Marr, 2010; Ekonomou et al., 2011). Although NSPC responses are often thought to represent attempts to ameliorate neuronal loss in gray matter, emerging data now suggest that NSPCs may also be involved in endogenous recovery mechanisms in white matter. White matter dysfunction occurs in a wide spectrum of neurodegenerative conditions including multiple sclerosis (MS) and vascular dementia. Within damaged white matter areas, the fate of NSPCs may shift from neurons to oligodendrocyte lineage cells in order to compensate for oligodendrocyte death and myelin loss. The precise mechanisms underlying the fate determination are still mostly unknown. However, several factors have been proposed as key modulators in promoting the NSPC differentiation into oligodendrocyte lineage cells. In this mini-review, we will summarize extrinsic and intrinsic factors that regulate the fate and behavior of NSPCs in the V-SVZ under normal and diseased conditions.

OLIGODENDROCYTE GENERATION FROM NEURAL STEM/PROGENITOR CELLS (NSPCs)

Oligodendrocytes, one of the major glial cells in the CNS, produce a lipid-rich membrane called myelin. Each oligodendrocyte can enwrap up to 60 axonal segments, thereby enabling fast and salutatory nerve impulse conduction (Baumann and Pham-Dinh, 2001). During development, oligodendrocyte precursor

cells (OPCs) are first generated in the germinal zones where they will proliferate. They then migrate to both grey and white matter areas where most will differentiate into mature oligodendrocytes and form myelin sheaths. Although myelinated tracts are formed early in life, renewal of myelin/oligodendrocyte continues throughout adult life (Paus et al., 1999; Dimou et al., 2008; Young et al., 2013). In addition, myelin in the adult CNS maintain some plasticity in response to changes in neural activity (Scholz et al., 2009) and brain injury (Nait-Oumesmar et al., 2008).

Under normal conditions in the adult brain, most V-SVZ progenitor cells give rise to neuronal linage cells. They migrate along the rostral migratory stream (RMS) to the olfactory bulbs, where they terminate and differentiate into mature interneurons (Gonzalez-Perez and Alvarez-Buylla, 2011; Ihrie and Alvarez-Buylla, 2011; Falcao et al., 2012). Oligodendrocytes can also be generated from V-SVZ cells in the adult brain, and newly generated OPCs migrate towards the corpus callosum and the white matter tracts of striatum and fimbria fornix (Menn et al., 2006). However, the ratio of V-SVZ progenitor cells differentiating into oligodendrocyte linage cells decrease after early postnatal period (Gonzalez-Perez and Alvarez-Buylla, 2011). Interestingly, in the V-SVZ area, neuronal and oligodendroglial progenies constitute separate lineages under physiological conditions. Using continuous live imaging and single-cell tracking of NSPCs, Ortega et al. (2013) have demonstrated that a single NSPC and its offsprings in the subventricular zone (SVZ) cannot show both neuronal and oligodendroglial progenies (Ortega et al., 2013). Furthermore, the adult SVZ is highly regionalized. The neuronal progeny of distinct identity is generated at different areas along the dorsoventral and rostrocaudal axes (Hack et al., 2005; Merkle et al., 2007). In addition, clones fated to generate oligodendrocytes are prevalent in NSPCs isolated from dorsolateral SVZ. On the contrary, ventrolateral SVZ regions consist of both neuronal and astroglial progenies with few oligodendroglial progeny (Costa et al., 2011; Ortega et al., 2011, 2013).

V-SVZ progenitor cells in the adult brain show some lineage plasticity under pathological conditions. After CNS damage, a number of progenitors migrate out of the RMS to the injured site. The fate of these progenitor cells can be dynamically altered according to the disease type. The fate of V-SVZ progenitor cells can shift from NSPCs to OPCs after demyelination, and these newly generated OPCs proliferate and migrate to the lesion areas (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002; Jablonska et al., 2010; Gonzalez-Perez and Alvarez-Buylla, 2011). In a model of experimental autoimmune encephalomyelitis (EAE), enhanced proliferation and migration of SVZ NSPCs are observed, and these mobilized cells give rise to oligodendrocytes and astrocytes without neurons in the injured white matter (Picard-Riera et al., 2002). In addition, demyelination would change the fate of glutamic acid decarboxylase 65 (GAD65)/doublecortin (Dcx)-expressing NSPCs derived from the adult SVZ to generate oligodendrocytes, rather than neurons, in corpus callosum (Jablonska et al., 2010). This process may restore developmental myelination to some extent; NSPCs that generate oligodendrocytes migrate from SVZ to developing white matter, where they stop dividing to differentiate and myelinate axons (John et al., 2002; Jablonska et al., 2010).

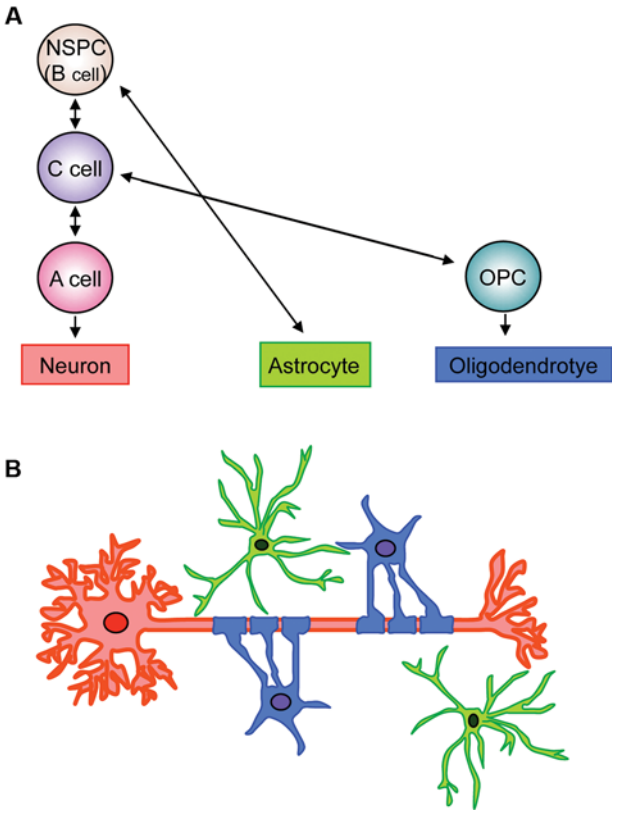

FIGURE 1 | Schematic of fate of neuronal, astroglial, oligodendrocytic lineage cells in V-SVZ. **(A)** Type B cells retain neuroepithelial trait and function as NSPCs in the V-SVZ. Type B cells slowly divide and give rise to rapidly dividing type C cells (IPCs), which generate neuroblasts (type A cells). The type B cells can also generate astrocytes and Olig2-expressing type C cells. The Olig2-expressing type C cells give rise to highly migratory OPCs, which differentiate into myelinating oligodendrocytes. **(B)** Neurons, astrocytes, and oligodendrocytes, derived from V-SVZ NSPCs (type B cells), interact with each other to maintain proper neural function. Red: neuron, green: astrocyte, blue: oligodendrocyte.

Past studies have extensively examined the process of NSPC differentiation into oligodendrocyte lineage cells in the V-SVZ area (**Figure 1**). The V-SVZ contains a subpopulation of cells with astroglial properties (type B cells) that retain neuroepithelial trait and function as NSPCs. Type B cells slowly divide and give rise to rapidly dividing intermediate progenitor cells (IPCs) or transient amplifying progenitors (type C cells), which divide further to generate neuroblasts (type A cells). Although at a lower population, type B cells can also generate Olig2-expressing type C cells that give rise to highly migratory OPCs. These OPCs leave the V-SVZ and migrate to the corpus callosum and the white matter tract in the striatum and fimbria fornix (Menn et al., 2006; Gonzalez-Perez and Alvarez-Buylla, 2011; Ihrie and Alvarez-Buylla, 2011; Falcao et al., 2012; Fuentealba et al., 2012).

NSPCs in the V-SVZ display diverse interactions with their neighboring environments (Falcao et al., 2012; Fuentealba et al., 2012; **Figure 2**). On one side of V-SVZ, type B cells are surrounded by multiciliated non-dividing ependymal cells, which form pinwheel-like structures on the ventricular surface. These

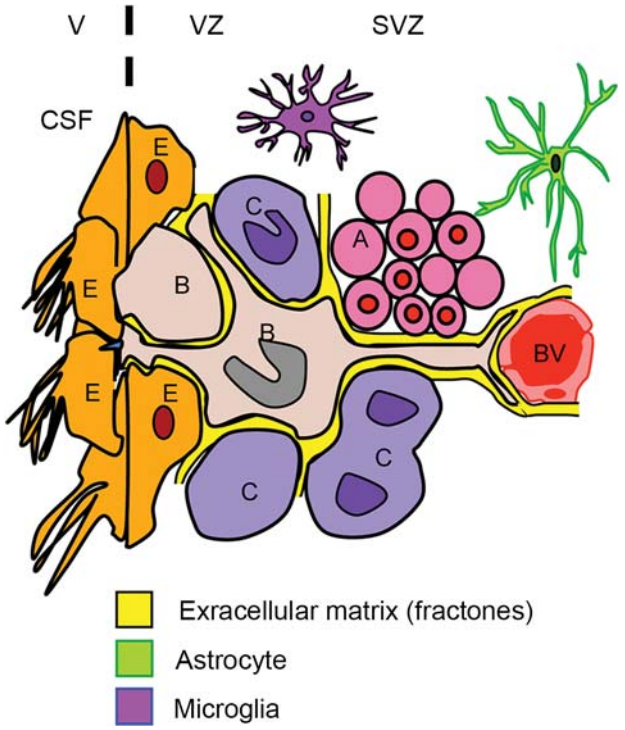

FIGURE 2 | Schematic of interplay for SVZ cells. The subventricular zone (SVZ) and ventricular zone (VZ) are lining the lateral ventricles (V) in the brain. Type B cells (B) contact the ventricle (V) containing CSF through specialized apical processes. The processes contain a single primary cilium, which is surrounded by a rosette of ependymal cells (E) with large apical surfaces forming pinwheel-like structures. On the other side, the type B cells have long basal processes with specialized endings that frequently contact BV. The type B cells also contact their progeny, i.e., type C cells (C) and the chains of migrating type A neuroblasts (A). The V-SVZ includes ECM (fractones) that contacts all the cell types including BV, microglia, and astrocytes in this region.

cells are in direct contact with the cerebrospinal fluid (CSF) by a short non-motile primary cilium that extends towards the ventricle. On the other side, type B cells interact with the extensive network of blood vessels (BV) with a long basal process. Type B cells also attach to type C cells and chains of young neurons (type A cells) by the extracellular matrix (ECM). Proliferating type C cells are closely located to their progenitors, and are also often in close proximity to BV (Shen et al., 2008). Type B cells interact with one another by gap and adherens junctions, the same as ependymal cells (Mirzadeh et al., 2008). Furthermore, the adult V-SVZ possesses a highly organized basement membrane, which is absent in other areas of the brain (see the Section Extracellular Matrix).

Overall, the V-SVZ is poised to receive informational inputs via cell-cell and cell-matrix contacts. The integration of these multifaceted external cues (e.g., extracellular signals from the vasculature, ECM, and the cerebrospinal fluid) to intrinsic factors leads to the determination of the fate and behavior of each cell lineage. In the next section, we will discuss the components that can potentially shift the fates and behaviors of NSPCs towards oligodendrocyte lineage cells.

EXTRINSIC FACTORS THAT PROMOTE OLIGODENDROCYTE GENERATION

VASCULATURE

The vasculature is an integral component of the V-SVZ stem cell niche that possesses specialized properties in regulating stem cell proliferation and regeneration (Shen et al., 2008; Tavazoie et al., 2008). Endothelial cells can secrete factors that contribute to stem cell self-renewal or proliferation. Co-culture of endothelial cells with NPSCs enhance the *in-vitro* neurosphere generation from embryonic progenitors (Shen et al., 2004). NSPCs were shown to have direct coupling with cerebral endothelial cells (Teng et al., 2008), and various kinds of perivascular regulators, including growth factors, purinergic signaling, nitric oxide signaling, and chemokines, contribute to cell genesis and fate determination in the V-SVZ (Goldman and Chen, 2011). Here, we will focus on specific vascular features of the V-SVZ.

Dividing progenitor cells (type B cells) and their transit-amplifying type C cells lie adjacent to the extensive planar vascular plexus in the V-SVZ. Approximately 47% of dividing type B cells and 46% of type C cells are found within five microns of the vasculature. During homeostasis and regeneration, type B cells and type C cells directly contact SVZ BV sites devoid of astrocyte end-feet and pericyte coverage (Shen et al., 2008; Tavazoie et al., 2008). Most dividing type B and type C cells are close to these sites, highlighting the importance of vasculature in supporting progenitor cell function. By contrast, most migrating neuroblasts are more distal to the vasculature (only 14% are within 5 μm) compared to type B and type C cells, even though BV run parallel to the aggregates of migrating neuroblasts chains in the dorsal aspect of the SVZ and in the RMS. However, it still remains to be understood whether neuronal differentiation occurs in response to leaving the vascular bed or whether cells leave the vasculature after they are differentiated (Tavazoie et al., 2008).

BV in the V-SVZ region also serve as a scaffold for long-distance migration of neuroblasts from V-SVZ to the olfactory bulb, potentially through the release of chemoattractant (e.g., BDNF, vascular endothelial growth factor (VEGF)) and chemorepulsive factors (e.g., semaphorins, ephrins) (Bovetti et al., 2007; Snapyan et al., 2009; Whitman et al., 2009; Kojima et al., 2010). Migrating neuroblasts are ensheathed by a layer of astrocyte processes and use each other as guides in the migration process toward the olfactory bulb. Similarly, in animal stroke models (Ohab et al., 2006) and human stroke patients (Jin et al., 2006), a long-distance migration of newly born immature neurons from SVZ to peri-infarct cortex is observed. Stromal derived factor-1/C-X-C chemokine receptor 4 (SDF-1/CXCR4) signaling assists BV- and astrocyte-associated migration of adult SVZ progenitors after cortical injury (Saha et al., 2013). Recent studies have identified that SDF-1/CXCR4-mediated signaling is a critical homing factor in the V-SVZ niche. CXCR4 is expressed by all progenitor cells in the SVZ. SDF1 is expressed in the SVZ BV, but the ependymal cells that line the lateral ventricles express higher level of SDF-1 to create a concentration gradient. SDF1 increases integrin α6 and epidermal growth factor receptor (EGFR) expression in activated type B and type C cells, enhancing their activated state and ability to bind laminin in the vascular niche (Kokovay et al., 2010). SDF1 also increases the motility of type A neuroblasts.

These type A cells express lower levels of integrin $\alpha 6$, which might promote evacuating from the vascular niche.

As noted, beside the role as a conduit for blood delivery for brain, cerebral BV (cerebral endothelial cells) support neighboring cells by secreting trophic factors. Recent studies confirmed that cerebral endothelial cells regulate the function of oligodendrocyte lineage cells. In co-culture system of endothelium with NSPCs, the chemokine C-C motif chemokine 2/monocyte chemotactic protein-1 (CCL2/MCP-1) mediates the interaction between endothelium and neural precursor cells to promote the differentiation of NSPCs into oligodendrocytes (Chintawar et al., 2009). Another study used the *in vitro* media-transfer system to show that conditioned medium from endothelial cells promotes the differentiation of NSPCs into oligodendrocyte lineage cells (Plane et al., 2010). In addition, cerebral endothelial cells and OPCs may provide an oligovascular niche to promote the proliferation and migration of OPCs (Arai and Lo, 2009a; Hayakawa et al., 2011, 2012). This endothelium-to-OPC supportive signaling would be attenuated by excessive oxidative stress (Arai and Lo, 2009b), supporting the idea that oligodendrocyte/myelin maintenance and renewal is disturbed under pathological conditions.

EXTRACELLULAR MATRIX (ECM)

The vascular and extravascular basal lamina (BL) are composed of the ECM proteins such as laminin, heparan sulfate proteoglycans (HSPG), and collagen-IV. The BL determine inductive microenvironments for adjacent stem cells by providing, storing, and compartmentalizing growth factors and cytokines. These factors can be concentrated in the extravascular BL and bind to cellular receptors present on the cells in direct contact with the BL (Roberts et al., 1988; Yayon et al., 1991). The extravascular BL are continuous with the surrounding local BV (i.e., vascular BL). However, the BL project into the V-SVZ independently from BV and terminate underneath the ependyma. Notably, compared with the cellular volume in the V-SVZ, the V-SVZ extravascular BL occupy a smaller volume, but are folded, branched, and fractionated to increase contact surface with the cellular environment. As these features are characteristics of a fractal structure, the V-SVZ extravascular BL was termed "fractone" (Mercier et al., 2002). Anatomically, the fractone is very efficient in contacting an enormous number of cells in the wall of the ventricle. Therefore, this structure may help V-SVZ progenitors receive blood/CSF-borne information from virtually all brain sites, circumventricular organs, and peripheral organs.

ECM proteins themselves are also active molecules for V-SVZ progenitor cells. Mice lacking laminin $\alpha 2$ subunit (LAMA2-/-) have fewer OPCs both in the dorsal SVZ and an adjacent developing white matter tract, coupled with high levels of OPC death (Relucio et al., 2012). Furthermore, defects in the spatial organization of IPCs in the perinatal V-SVZ niche lead to defective oligodendrocyte maturation and myelination (Relucio et al., 2012). These findings indicate that laminin promotes the survival of OPCs in the gliogenic niche, allowing the appropriate numbers of OPCs to populate their target white matter tracts. This survival-promoting effect is partly due to localizing or enhancing trophic factor signals.

Taken together, fractones, perivascular, and subpial BL constitute an ideal anatomic mechanism for exchanging growth factors and cytokines between extraparenchymal and NSPCs in the V-SVZ. This environment may also prevent extensive diffusion of these signaling molecules in the extracellular environment.

CEREBROSPINAL FLUID (CSF)

Type B cells in the V-SVZ extend an apical primary cilium toward the brain ventricular space. The space is filled with CSF and the composition of CSF can modulate the self-renewal, proliferation, and differentiation of V-SVZ progenitor cells (Falcao et al., 2012). CSF is secreted mainly from the choroid plexus (CP), located in the caudal regions of the lateral ventricle. The adult CP expresses and secretes numerous trophic factors and cytokines, which could influence the dynamics of V-SVZ progenitor cells (Falcao et al., 2012). For example, CP-secreted IL-1β binds to IL-1 receptors on the surface of type B cells to upregulate vascular cell adhesion molecule 1 (VCAM1) expression. This change promotes the adhesion of type B cells to the neural stem cell niche and the pinwheel architecture of ependymal cell rosettes via maintenance of redox homeostasis by NADPH oxidase 2 (NOX2) activation. In turn, inhibition of VCAM1 stimulates quiescent Type B cells to proliferate and advance through the cell lineage to type A neuroblasts that migrate to the olfactory bulb (Kokovay et al., 2012).

Factors in CSF or CP may also affect V-SVZ progenitor cells even under pathological conditions. In a Lysophosphatidylcholine (LPC)-induced demyelination model, intraventricular infusion of epidermal growth factor (EGF) dramatically promoted the proliferation and migration of SVZ NSPCs as well as their differentiation into oligodendrocytes (Gonzalez-Perez et al., 2009). Intraventricular infusion of the bone morphogenetic protein (BMP) inhibitor Noggin also increased the number of Olig2-positive oligodendrocytes after cuprizone-induced demyelination in mice (Cate et al., 2010). Additionally, CP is also a source of chemorepulsive factors, including Slits, Semaphorins, and ephrins, which can influence V-SVZ NSPCs migration. For instance, the infusion of the Ephrin-B2 ligand in the lateral ventricles disrupts the migratory chain of neuroblasts and increases the proliferation of type B cells. Another report has shown that the ciliary beating of ependymal cells in the wall of lateral ventricle generates CSF flow, which forms a concentration gradient of chemoattractants secreted by the CP. Such guidance molecules gradients may contribute to the directional migration of neuroblasts to the olfactory bulbs (Sawamoto et al., 2006).

INTRINSIC FACTORS THAT PROMOTE OLIGODENDROCYTE GENERATION

TRANSCRIPTIONAL FACTORS

A dynamic combination of transcription factors may modulate oligodendrocyte maturation (Nicolay et al., 2007). The different stages of oligodendrocyte development (specification, proliferation, differentiation, and myelination) are spatially and temporally regulated by various transcription factors under the control of multiple signaling pathways, such as Wnt (Fancy et al., 2009), sonic hedgehog (Shh; Lu et al., 2000), BMP (Samanta and Kessler, 2004; Jablonska et al., 2010), and Notch (Wang et al., 1998; Nicolay et al., 2007). In this section, we will overview key

transcription factors that regulate the function of oligodendrocyte lineage cells.

The basic helix-loop-helix (bHLH) transcription factors Olig1 and Olig2 have been extensively studied in oligodendrocyte development (Lu et al., 2002; Zhou and Anderson, 2002). An *in vivo* gain-of-function study has shown that the inducible overexpression of Olig2, but not Olig1, in SVZ progenitor cells increases the generation of OPCs. These newly generated OPCs migrate and differentiate into mature oligodendrocytes in the corpus callosum, cortex, and olfactory bulb. Subsequently, these cells lead to precocious myelination with an increase in the number of astrocytes in the corpus callosum at postnatal CNS myelination stage (Maire et al., 2010). Olig2-expressing cells may represent a transition state between type B and C cells. Olig2 over-expression directs SVZ progenitors towards oligodendrocyte and astrocyte fates, while it opposes the neurogenic role of Pax6 and represses neuronal lineages (Hack et al., 2005; Marshall et al., 2005). In addition, the interactions between Olig and Id proteins have been reported to mediate the inhibitory and promoting effects of BMP4 on oligodendrogenesis and astrogliogenesis, respectively (Samanta and Kessler, 2004).

Many other factors have also been reported to regulate oligodendrocyte specification and development (Nicolay et al., 2007). Firstly, an oligodendrocyte-specific zinc finger transcription repressor Zfp488, a downstream effector of Olig1, favors oligodendrocyte maturation in concert with Olig2 (Wang et al., 2006). Retrovirus-mediated Zfp488 overexpression in SVZ NSPCs can increase the number of oligodendrocytes in the corpus callosum and leads to functional recovery after cuprizone-induced demyelination in mice (Soundarapandian et al., 2011). Secondly, a proneural transcription factor Ascl1/Mash1 operates in genetic interaction with Olig2 during OPC specification in the embryonic telencehpalon and the loss of Ascl1 reduces embryonic oligodendogenesis (Parras et al., 2007; Sugimori et al., 2007). Recent conditional deletion and lineage tracing study has demonstrated that Ascl1 positively regulates OPC specification from SVZ progenitors. The study also shows that Ascl1 controls the proper differentiation into oligodendrocytes during postnatal myelination stage and remyelination after LPC-induced demyelination (Nakatani et al., 2013). In parallel with the above findings, postmortem examination of human periventricular MS lesions confirmed that Ascl1 expression is a hallmark of OPCs involved in myelin repair (Nakatani et al., 2013). In addition, Ascl1 induces Notch-mediated repression of the neurogenenic determinants Dlx1/2, which may promote oligodendrogenesis at the expense of an astrocytic fate (Nakatani et al., 2013). Thirdly, members of the SRY-box (Sox) transcription factors have also emerged as crucial regulators of oligodendrocyte behavior. Sox8, 9, and 10 induce early postnatal SVZ NSPCs toward the oligodendrocyte lineage fate (Pozniak et al., 2010), while Sox4, 5, and 6 have inhibitory roles in timing oligodendrocyte specification and terminal differentiation (Potzner et al., 2007). The gain-of-function approach has shown that Sox17 overexpression in oligodendrocyte lineage cells promotes postnatal oligodendogenesis and prevents cell loss after LPC-induced demyelination by increasing oligodendrocyte lineage cells (Ming et al., 2013). Another study has also demonstrated that the suppression of Wnt/β-catenin signaling by Sox17 enhances progenitor cell maturation (Chew et al., 2011). Additionally, nuclear factor 1A (NF1A) NF1A is expressed in OPCs, but not in mature oligodendrocytes during mouse embryonic development. Similarly, NF1A is observed in only OPCs in demyelinated white matter lesions of human neonatal hypoxic-ischemic encephalopathy (HIE) or adult MS. During development or neonatal/adult remyelination after injury, NF1A suppresses OPC differentiation via direct repression of myelin gene expression (Fancy et al., 2012). The role of NF1A on OPCs during remyelination is a recapitulation of development (Fancy et al., 2012). These findings may indicate that downregulation of NF1A stimulates OPC differentiation while deregulation of NF1A contributes to the suppression of remyelination in white matter disorders.

Taken together, various transcription factors combined with multiple other cofactors and signaling pathways lead to the determination of cell fate under developmental stage and during post-injury remyelination. In turn, factors that regulate oligodendrocyte lineage cells can exert the opposing effects for the fate of neuronal and astroglial lineage cells. It still remains largely unknown how niche-provided signals modulate transcription factor expression. However, the mechanisms and expression patterns of transcription factors during developmental myelination may have some similarities with those during remyelination after myelin loss or oligodendrocyte death.

EPIGENETIC MODULATORS

During development, the crosstalk between transcription factors and epigenetic modulators of gene expression is essential for the acquisition of specific cell fates (Hemberger et al., 2009). The epigenetic regulation also influences the multiple steps of oligodendrocyte generation (Liu and Casaccia, 2010); i.e., from embryonic stem cells to OPCs via multipotential neural precursors or even from OPCs to myelinating oligodendrocytes. The epigenetic modulators represent post-translational modifications of nucleosomal histones, changes in histone variants, chromatin remodeling enzymes, DNA methylation, and microRNAs (miRNAs). Among them, we will mainly focus on histone deacetylases (HDACs) and miRNAs in this section.

Persistent histone acetylation in OPCs alters their lineage choice decision by suppressing the acquisition of the oligodendroglial identity. The histone acetylation also favors a conformation of chromatin that is consistent with the establishment of a neuronal or astroglial phenotype (Liu et al., 2007). The oligodendrocyte identity of OPCs is dependent on HDAC enzymatic activity. When HDAC is high, the epigenetic memory of specified progenitors in oligodendrocyte is established by repressing neuronal and astroglial genes. By contrast, when HDAC activity is inhibited, the progenitors are unable to establish an oligodendrocyte-specific program of gene expression and as a response to neurogenic or astrogliogenic signals they are reprogrammed into a multipotential state (Liu et al., 2007). Hence, HDAC inhibition may erase the epigenetic memory of oligodendrocyte in the progenitor cells. In turn, the HDAC inhibition allows the cells to acquire a pattern of gene expression consistent with neuronal and astroglial lineage. In accordance with this phenomenon, global histone acetylation is detected in

precursor cells during the early stages of brain development, which are associated with neurogenesis and astrogliogenesis. In contrast, histone deacetylation prevails in OPCs during the later stages of postnatal development and coincides with the onset of myelination (Shen et al., 2005). Furthermore, a genetic ablation loss-of-function study has shown that HDAC1 and HDAC2 are required for oligodendrocyte differentiation (Ye et al., 2009). HDAC1/2 also controls the Wnt signaling pathway, which is known as an inhibitory signal for oligodendrocyte differentiation (Ye et al., 2009). Notably, some extrinsic factors can regulate oligodendrocyte differentiation, at least in part by modifying histone acetylation. For example, Shh induces histone deacetylation via HDACs to promote oligodendrocyte differentiation, while BMP4 blocks the deacetylation and inhibits oligodendrogenesis (Wu et al., 2012). Therefore, one of the major roles of HDACs may repress certain gene expressions that normally blocks OPC differentiation thus allowing NSPCs to mature into myelinating oligodendrocytes (Zuchero and Barres, 2013).

miRNAs are also important epigenetic regulators of various aspects of CNS development and homeostasis by responding to environmental cues and cellular states. miRNAs have an advantage over mRNAs as they are more stable (Jung et al., 2010). A number of miRNAs have been recently shown to play a critical role in oligodendrogenesis, i.e., cell proliferation, differentiation, and myelin formation (Barca-Mayo and Lu, 2012). For example, miR-219 and miR-338 are increased at the onset of oligodendrocyte myelination and play a positive role in the OPC differentiation to mature oligodendrocytes (Dugas et al., 2010; Zhao et al., 2010). miR-219 and miR-338 suppress the expressions of platelet-derived growth factor receptor α (PDGFRα), hairy and enhancer of split 5 (Hes5), and Sox6 that are known to inhibit OPC differentiation and to maintain OPC in the proliferative state (Dugas et al., 2010; Zhao et al., 2010). The two miRNAs also inhibit Zfp238, FoxJ3 and NeuroD1, which shift the fate of NSPCs from OPCs to neuron lineage (Dugas et al., 2010; Zhao et al., 2010). Thus, the interplay of transcription factors and epigenetic modifiers should be required for the precise regulation of the NSPC-to-oligodendrocyte transition.

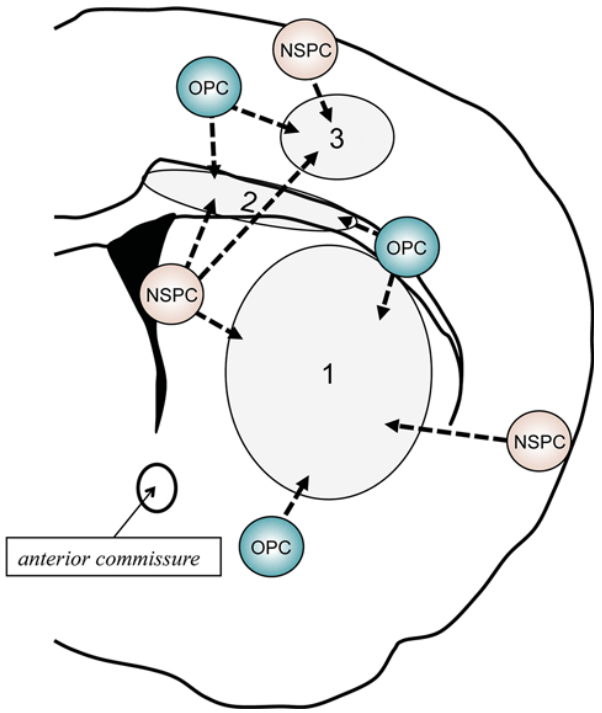

FIGURE 3 | Schematic of behavior of NSPCs and OPCs after demyelination. In response to myelin loss or oligodendrocyte death, both NSPCs and OPCs would attempt to repair the white matter damage by proliferating, migrating to the injured areas, and restoring myelinating oligodendrocytes. If the damaged area is restricted in the corpus callosum (2), V-SVZ-derived NSPCs would shift from neuronal lineage cells to oligodendroglial lineage cells. In addition, residing OPCs adjacent to the damaged area may also contribute to the repairing. Although the V-SVZ-derived NSPCs could travel to the cortex (1) or striatum (3), the recruitment of local OPCs/NSPCs outside of the V-SVZ (e.g., pia matter and/or cortical layer 1) to the lesion area would be more important when the demyelination occur in these areas.

OLIGODENDROGENESIS AFTER WHITE MATTER DAMAGE

Both NSPCs in V-SVZ and OPCs outside of V-SVZ exhibit endogenous repair attempts in response to demyelination (Menn et al., 2006; **Figure 3**). In this section, we will overview oligodendrocyte regeneration attempts in human and small animals, focusing on the pathophysiological conditions in MS and vascular dementia.

MULTIPLE SCLEROSIS (MS)

MS is characterized by inflammation, demyelination, and axonal damage in the CNS with different degrees of autoimmune involvement (Sospedra and Martin, 2005; Fugger et al., 2009). The typical disease course after the first attack consists of remissions and relapses with slow onset of disability (Fugger et al., 2009). In general, the extent of remyelination varies from patient to patient and from lesion to lesion. The remyelination attempt is mostly restricted to a thin rim around the lesion edge and decreases as the disease progresses. However, non-negligible remyelination may occur after white matter damage in MS patients. Post-mortem studies have revealed that the density of glial fibrillary acidic protein (GFAP)-positive astrocytes and early progenitors in the SVZ is increased in MS patients. In the subependymal region, these progenitors express early glial markers such as Sox9, Sox10, and Olig2. Similar progenitors prevail in periventricular lesions. The polysialylated neuronal cell adhesion molecule (PSA-NCAM) is a marker for developing and migrating neuronal progenitors in the immature vertebrate nervous system. The presence of PSA-NCAM-positive progenitors with a bipolar morphology in the lesion area may suggest their potential migration within or away from the SVZ to oligodendrocyte renewal/repairing (Nait-Oumesmar et al., 2007). As mentioned, remyelination attempts often fail in chronic MS patients. While the underlying mechanisms are still mostly unknown, the failure might correspond to reduced recruitment and/or disturbed maturation of OPCs (Nait-Oumesmar et al., 2008; Kotter et al., 2011). Recently, TAT-interacting protein 30 kDa (TIP30), a proapoptotic factor, was proposed as a new pathogenic factor in MS. In human chronic MS lesions, Notch1 is activated in OPCs and Contactin is abundantly expressed on demyelinated axons. This noncanonical pathway

(Notch1-F3/Contactin) is important for OPC differentiation and axon myelination. The pathologic upregulation of TIP-30 blocks the nuclear transport of Notch1 intracellular domain, thus leading to disruption of the noncanonical pathyway (Nakahara et al., 2009).

Analysis of lesion-induced oligodendrogenesis in experimental rodent models would be needed to design repair strategies in white matte-related diseases (Keough and Yong, 2013). There are several animal models of MS, including EAE model, targeted EAE (tEAE) model, and cuprizone model. In most animal models of MS, activation of the V-SVZ (i.e., increase in the number of SVZ-NSPCs) is confirmed, and NSPCs in the V-SVZ have been shown to migrate and undergo oligodendrogenesis in demyelinated lesions (Picard-Riera et al., 2002). In addition, remyelination failure in the MS models is not attributed to an absence or a reduction of OPCs in the lesion area. Rather, the failure is a result of the lack of positive signals for oligodendrocyte maturation/myelination or the overactivation of inhibitory signals from immune cells for the myelination program (Back et al., 2005; Sloane et al., 2010; Kotter et al., 2011). However, under some conditions, endogenous microglia and infiltrating macrophages would work for promoting the oligodendrocyte remodeling/repairing (Napoli and Neumann, 2010). The myelin debris is generated during demyelination and the containing proteins inhibit OPC differentiation, but microglia and macrophages try to remove the myelin debris. These immune cells also secrete soluble mediators, which attract the phagocytic and repair-promoting effectors. In addition, TNF-α dearth may lead to a significant delay in remyelination with a reduction of proliferation and maturation of OPCs in mouse MS models. Analysis with mice lacking TNF-R1 or TNF-R2 has demonstrated that TNF-α signaling through TNF-R2 promotes the accumulation of proliferating OPCs (Arnett et al., 2001). Furthermore, a transcriptomic analysis in a mouse cuprizone model of MS reveals that microglia can exhibit the phenotype of supporting remyelination. These microglia produce a rich repertoire of cytokines and chemokines to recruit endogenous OPCs to the lesion site for repairing the damaged myelin sheathes (Olah et al., 2012).

Small animal models are useful to examine the precise mechanisms of oligodendrogenesis after white matter injury. However, it should be noted that behavior of NSPCs (lineage commitment, migration, maturation/myelination) and glial activation are different among animal models. For example, compared to myelin oligodendrocyte glycoprotein (MOG)-induced EAE model in C57BL/6, a model using SJL mice shows persistent activation of microglia in the forebrain, which is similar to current observations in the cortex of MS patients (Kutzelnigg et al., 2005; Rasmussen et al., 2007). In this model, NSPCs proliferate and engage in repair during the acute phase of EAE, but this capacity is lost during the chronic phase of the disease. As the number of microglia is in an inverse relationship with the proliferative activity of SVZ cells in the SJL model, chronic microglial activation in the SVZ may have a tonic inhibitory role on NSPC proliferation (Rasmussen et al., 2011). Ultimately, preclinical studies are required to be conducted in multiple animal models of MS.

VASCULAR DEMENTIA

Although not typically thought of as a demyelinating disease, white matter injury comprise a critical part of vascular dementia pathophysiology. Vascular dementia accounts for 20% of the 25 million people with dementia worldwide. It is also increasingly recognized that Alzheimer's disease and vascular dementia may belong to a continuous spectrum of diseases based on vascular pathologies (Viswanathan et al., 2009). In addition, 25% of older stroke patients develop dementia within 3 months of a stroke (Censori et al., 1996), and there is a 10-fold increased risk of delayed dementia over the subsequent 5 years in stroke survivors compared to people of the same age (Kokmen et al., 1996). Postmortem human brain analyzes have demonstrated a significant increase of progenitor cells with nestin, PSA-NCAM, and Dcx expression in the SVZ and peri-infarct regions in vascular dementia patients (Ekonomou et al., 2011). Another postmortem human brain study has shown that in ischemic white matter lesions of vascular dementia patients, OPCs were increased, but oligodendocytes were decreased (Miyamoto et al., 2010). These studies may suggest that to some extent the endogenous regenerative attempts in oligodendrogenesis occur in the brain of vascular dementia patients. However in most cases, complete recovery of function cannot be achieved, probably due to various inhibitory factors related to chronic ischemic lesions. The detailed mechanisms that suppress oligodendrocyte regeneration in patients with vascular dementia still remain unclear. However, recent efforts using rat or mouse models of vascular dementia have proposed some promising cues in understanding the pathophysiology of vascular dementia.

Vascular dementia is characterized by cognitive impairment, cerebrovascular white matter changes, and cerebral hypoperfusion. In this regard, rat and mouse models of prolonged cerebral hypoperfusion have been developed and widely used (Farkas et al., 2007; Ihara and Tomimoto, 2011). The rat model is accompanied with cognitive impairment and cholinergic deficits (Wakita et al., 1994; Ni et al., 1995). These animals develop demyelination with axonal damage (Wakita et al., 2002), which appears similar to that found in human cerebrovascular white matter lesions. This model also shows an increase of OPCs in the demyelinating lesions (Miyamoto et al., 2010; Chida et al., 2011). The extent of demyelination is in inverse correlation with cognitive function (Chida et al., 2011), and therefore treatments that can enhance remyelination may ameliorate the cognitive dysfunction under prolonged cerebral hypoperfusion. A mouse model of prolonged cerebral hypoperfusion is achieved by narrowing the bilateral common carotid arteries (CCAs) with newly designed micro-coils (Shibata et al., 2004, 2007). This model demonstrates good reproducibility of the white matter changes seen in clinic, including blood-brain barrier disruption, glial activation, oxidative stress, and oligodendrocyte loss. In this mouse model, the cerebral white matter is selectively damaged, and the integrity of the gray matter (including hippocampal) remains intact at a month after the surgery if the bilateral CCAs are appropriately placed by 0.18 mm internal diameter micro-coils (Shibata et al., 2004, 2007). Recent study using this model has demonstrated that after induction of prolonged cerebral hypoperfusion, the numbers of newborn oligodendrocytes and their precursors are transiently

increased in 2-month old mice (Miyamoto et al., 2013a). On the contrary, these endogenous repairing attempts are significantly dampened in older mice (8-month old) partly due to defects in cyclic AMP response element-binding protein (CREB) signaling (Miyamoto et al., 2013a). In fact, activating CREB signaling by the treatment of phosphodiesterase (PDE)-III inhibitor cilostazol increased the oligodendrogenesis in the older mice (Miyamoto et al., 2013a). More recently, another study using the mouse model has shown that excessive oxidative stress under prolonged cerebral hypoperfusion may disrupt the differentiation from OPCs to oligodendrocytes (Miyamoto et al., 2013b). These findings may suggest that drugs that promote oligodendrocyte regeneration can be useful for vascular dementia patients. To note, effects of those drugs on white matter remodeling after injury should be carefully examined in preclinical studies before testing them in clinical trials. In addition, further studies are also warranted to elucidate the regulatory mechanisms in NSPCs-to-OPC transition under cerebral hypoperfusion conditions.

CONCLUSION

The V-SVZ region possesses the specialized microenvironments that enable NSPCs to have efficient and dynamic interactions with the V-SVZ components. The fate of NSPCs under physiological conditions is tightly regulated by the combined actions of intrinsic and extrinsic factors. After demyelination, the balance would be changed to promote the endogenous repairing attempts in oligodendrogenesis. On the other hand, as the disease conditions progress, the NSPC-OPC-oligodendrocyte transition becomes disrupted, mainly due to the decrease of pro-oligodendrogenesis signals and the increase of anti-oligodendrogenesis signals. A deeper understanding of the underlying mechanisms in oligodendrocyte generation from NSPCs may lead us to effective therapeutic approaches for white matter related diseases. But it should be noted that factors that regulate the fate of NSPCs sometimes exhibit opposing effects between neurogenesis and gliogenesis/oligodendrogenesis. Ultimately, we may need to consider the balance between neurogenesis and gliogenesis/oligodendrogenesis in pursuing therapeutic strategies for ameliorating white matter damage and dysfunction in CNS disease.

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Niche derived oligodendrocyte progenitors: a source of rejuvenation or complementation for local oligodendrogenesis?

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With the recent revelations that strikingly plastic cytogenic and migratory processes take place in the adult mammalian brain, it cannot anymore be considered as an organ static or utterly unequipped against injury. The Subependymal Zone (SEZ) or otherwise called with its embryonic equivalent as the subventricular zone, is one major cytogenic area located at the lateral wall of the lateral ventricles (Luskin, 1993) and is known to continuously contribute new cells to different brain areas both during homeostasis and following injury (Kazanis, 2009). A well-documented role of the SEZ in rodents is the constant supplementation of neural progenitors to the olfactory bulb (OB), through the rostral migratory stream (Lois and Alvarez-Buylla, 1994). Once in the OB, SEZ-derived neural progenitors differentiate into GABAergic (mainly) and glutamatergic neurons, depending on their exact anatomical origin in the niche (Brill et al., 2009). Cellular integration and survival depends on sensory olfactory inputs (i.e., exposure to new odors), as well as on the ability of these neural progenitors to form the appropriate synaptic connections with other cells (Mouret et al., 2008). This supply of new neurons to the OB is not only necessary for olfactory learning and memory but also for successful social and mating encounters in rodents (Oboti et al., 2011). Despite its initially identified neurogenic output, the SEZ is now known to also generate oligodendrocyte progenitor cells (OPCs) from Olig2-expressing transit-amplifying progenitors in the niche (TaPs/ also known as type C cells). During early post-natal stages stem and progenitor cells of the SEZ generate oligodendrocyte progenitors that migrate in a wide range of brain areas, including the corpus callosum, the cerebral cortex and the striatum, where they give rise to myelinating oligodendrocytes (Zerlin et al., 1995). After the SEZ assumes its mature structure (Alves et al., 2002) and switches to an adult phenotype (Jablonska et al., 2007), the adult niche-derived OPCs seem to migrate shorter distances, such as to the closely located corpus callosum (Cayre et al., 2006; Menn et al., 2006) (**Figure 1**), but in response to demyelination can migrate further afield such as to the striatum (Capilla-Gonzalez et al., 2013b). However, currently little is known about the characteristics of niche-derived adult OPCs and the functions they might serve.

In contrast to the less studied SEZ-derived adult OPCs, much more is known about adult parenchymal OPCs. During perinatal development OPCs are generated in different waves (Kessaris et al., 2006), firstly from sonic hedgehog (shh)-responsive ventral progenitors and at later stages from dorsal progenitors in a shh-independent manner (Fogarty et al., 2005). Subsequently, perinatal OPCs migrate away to populate the gray and white matter and to give rise to the myelinating cells of the central nervous system, the oligodendrocytes. Once early post-natal myelination program is concluded, some OPCs remain to give rise to the parenchymal adult OPCs which are equally distributed around the brain and account for a striking 5% of the total cell population with the majority of them (>80%) being mitotically active (Polito and Reynolds, 2005; Young et al., 2013). These parenchymal OPCs are capable of responding to demyelinating insults by quickly migrating to the injured site where they proliferate and give rise to new myelinating oligodendrocytes, a process called remyelination (Franklin and Ffrench-Constant, 2008). In light of the abundance of adult parenchymal OPCs and their capacity to successfully replace damaged oligodendrocytes following injury, the role of adult SEZ-derived OPCs in the brain remains a puzzle, especially because there is no evidence to support that adult niche and parenchymal OPCs behave in any significantly different way (Caillava et al., 2011).

One approach to address this puzzle is to look into the development of adult parenchymal OPCs. Experiments performed in the late 80's suggested that adult OPCs originate from a fraction of the late-embryonic and perinatal OPC population (Zerlin et al., 1995) that escapes terminal differentiation to oligodendrocytes (Wren et al., 1992). Over time, these perinatal OPCs switch to a significantly different adult-like behavior with an almost 4-fold slower cell cycle as compared to perinatal OPCs (Shi et al., 1998; Tang et al., 2000; Young et al., 2013). Moreover, adult and perinatal parenchymal OPCs respond differently to growth factors such as platelet derived growth factor (PDGF) and neuregulin *in vitro* (Shi et al., 1998); downregulate membrane ion channel expression (Clarke et al., 2012); and have different migratory and remyelinating properties (Windrem et al., 2004). With ageing, increasing numbers of adult parenchymal OPCs start expressing various markers of senescence (Kujuro et al., 2010) and their efficiency in regenerating damaged oligodendrocytes significantly deteriorates

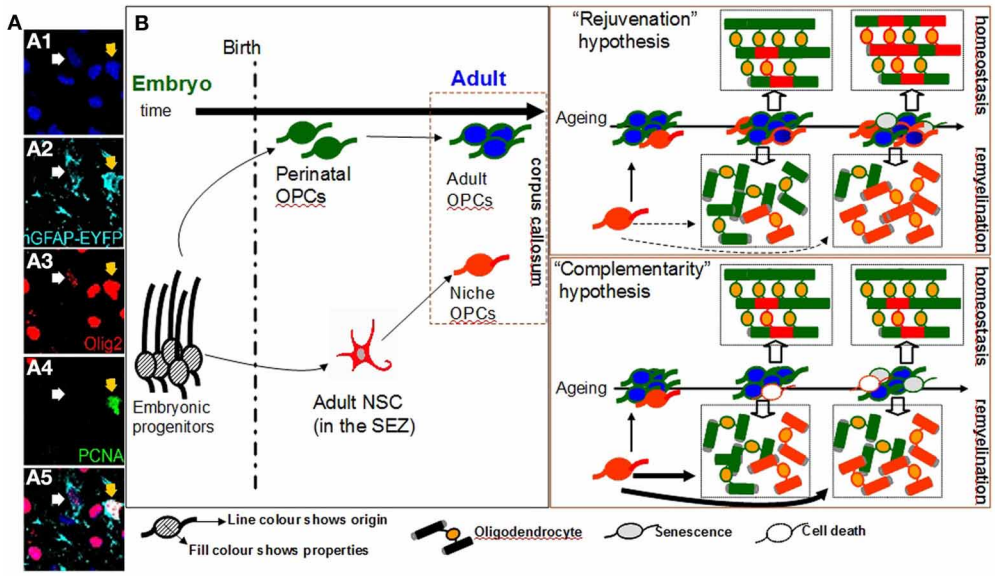

FIGURE 1 | Oligodendrogenesis from niche and parenchymal progenitors in the adult corpus callosum. (Panel **A**) Numerous cells of the oligodendroglial lineage (both OPCs and oligodendrocytes, here all identified by expression of Olig2, in red) populate the adult corpus callosum (cc). A fraction of these is generated within the adjacent SEZ cytogenic niche (here identified by the expression of EYFP, in cyan, with two examples shown with arrows). OPCs are a mitotically active cell population as indicated by the expression of the mitotic marker PCNA (in green; note that the yellow arrow shows a proliferating OPC of SEZ origin). (Panel **B**: Diagram) A schematic illustration of the possible role of niche-derived OPCs in oligodendrogenesis in the adult cc. One fraction of OPCs in the cc is derived from perinatal OPCs (cells of green outline) that have switched to an adult, self-renewing mode of division (cells of blue fill); whilst, another is derived from adult neural stem cells located in the SEZ (cells of red outline). According to the "rejuvenation hypothesis", niche OPCs migrate in the cc and gradually acquire properties similar to those of parenchymal adult OPCs (such as self-renewing potential). According to this scenario the main role of niche OPCs is to provide freshly-born self-renewing progenitors, thus partially compensating for the increase in the numbers of adult parenchymal OPCs that show signs of senescence over ageing (cells of gray fill). These niche OPCs contribute to the constant remodeling of myelin during homeostasis and to the generation of oligodendrocytes after demyelinating insults (such as during the course of diseases like multiple sclerosis). Within the ageing cc the contribution of the "younger" niche OPCs is increased (note the generation of higher numbers of red oligodendrocytes over time). According to the alternative "complementarity hypothesis", the two sources of OPCs co-exist, albeit operating independently and with niche OPCs generating oligodendrocytes directly in response to local demand but without exhibiting long-term self-renewing capacity. In this scenario the contribution of niche-driven oligodendrogenesis is overall lower than that expected based on the "rejuvenation hypothesis", especially regarding the homeostatic myelin remodeling. [Images in panel **A** show immunostained adult mouse brain cryosections. The double transgenic mice hGFAP-Cre^{ERT2} x Rosa26-EYFP were used. In these mice EYFP expression is induced in neural stem cells of the SEZ and is maintained in all of their progeny].

(Sim et al., 2002). This phenomenon has been linked both with the deterioration of health in patients suffering from chronic demyelinating diseases such as multiple sclerosis (Franklin and Ffrench-Constant, 2008), but also with cognitive decline in the elderly (Sullivan et al., 2010). Therefore, one possible scenario is that, the main function of SEZ-derived OPCs is to replenish the ageing pool of adult parenchymal OPCs, with freshly-born self-renewing progenitors from adult neural stem cells. This "rejuvenating hypothesis", according to which a functional convergence between adult niche and parenchymal OPCs exists, leads to a clear prediction, that the adult OPC pool in the supra-ventricular corpus callosum should gradually become dominated by self-renewing OPCs of SEZ origin. Recently, it was suggested that low-level oligodendrocyte turnover occurs under physiological conditions in the corpus callosum (a process named myelin remodeling)(Rivers et al., 2008; Clarke et al., 2012; Young et al., 2013); therefore, the numbers of oligodendrocytes generated by SEZ-derived progenitors in the homeostatic corpus callosum should also significantly increase over time. Notably, the contribution of SEZ-driven oligodendrogenesis should become more apparent in cases of extensive generation of oligodendrocytes, such as during remyelination (**Figure 1**).

An alternative scenario regarding the fate of SEZ-derived OPCs could be drawn by looking at the neurogenic output of the SEZ. Progenitors of neuronal commitment (neuroblasts) are generated within the niche by stationary adult neural stem cells and TaPs. Subsequently, they migrate in chains to the OB where they disperse radially to the different neuronal layers (Lois and Alvarez-Buylla, 1994; Brill et al., 2009), where a small fraction survives long-term by fully differentiating and integrating into existing networks, while the rest die. There is no evidence for long-term self-renewal potential of neuroblasts and the levels of SEZ-driven neurogenesis at the OB are controlled by the proliferative activity of stem cells into the distant niche, as well as by the rate of migration and differentiation/integration of the neuroblasts. Notably, cellular integration

and survival of SEZ-derived neuroblasts depends on sensory olfactory activity. Long-term exposure to odor enriched environments or to odor discrimination learning protocols, dramatically increases the survival of SEZ-derived neuroblasts in the murine OB (Mouret et al., 2008; Oboti et al., 2011). This activity-dependent neurogenesis is also prominent in the other major stem cell niche of the adult brain, the subgranular zone (SGZ) of the dentate gyrus (DG). New neurons (but no OPCs) are constantly born in the SGZ and subsequently migrate and populate the granule cell layer of the DG where they differentiate into glutamatergic granule cells. It has been documented that various environmental cues ranging from voluntary exercise to environmental enrichment and training on associative tasks, significantly increase adult DG neurogenesis (Ma et al., 2009). If this scenario also applies to SEZ-driven oligodendrogenesis, then niche-derived OPCs enter the corpus callosum and either differentiate to mature oligodendrocytes or die, depending on local demands. According to this "complementarity hypothesis", in which niche and parenchymal oligodendrogeneses co-exist but operate in an independent and supplemental way, the predictions would be i) a lack of SEZ-derived OPC accumulation within the pool of OPCs in the homeostatic adult corpus callosum (in contrast to the "rejuvenating hypothesis"), ii) a limited involvement of SEZ-generated oligodendrocytes to the suggested low-level homeostatic myelin remodeling (Clarke et al., 2012; Young et al., 2013), and iii) in cases of remyelination, a dramatically accelerated contribution of adult niche OPCs directly migrating by the niche (**Figure 1**). If this hypothesis is correct, then the overall oligodendrogenic output of the SEZ over time will be lower than that of the "rejuvenating hypothesis" due to the lack of contribution from corpus callosum self-renewing OPCs of niche origin. In support of this scenario in experimentally-induced corpus callosum demyelination, oligodendrogenesis is also driven by neuron-committed neuroblasts that are capable of escaping their route to the OB and of migrating toward the lesion where they switch cell fate and establish synaptic contacts with axons (Etxeberria et al., 2010) similarly to parenchymal OPCs during development (Káradóttir et al., 2005; Kukley et al., 2007; Ziskin et al., 2007). Subsequently they differentiate into myelinating oligodendrocytes with chordin and netrin1 having been identified as regulatory signals in this process (Jablonska et al., 2010; Cayre et al., 2013). This response-mediated oligodendrogenic plasticity of the SEZ has also been documented in experimental autoimmune encephalitis (an animal model of multiple sclerosis) with increased oligodendrogenesis from the niche in the expense of neurogenic fates (Tepavcevic et al., 2011). But if this is how SEZ-derived oligodendrogenesis occurs, does it actually present any advantages over the parenchymal oligodendrogensis?

As old age seems to be the major factor underlying the decreased efficiency of remyelination in the CNS (Sim et al., 2002; Hampton et al., 2012; Ruckh et al., 2012), the existence of an active neural stem cell population that can either constantly supply new-born OPCs (rejuvenating hypothesis), or can directly generate high numbers of oligodendrocytes upon demand (complementary hypothesis), provides a valuable explanation for SEZ-driven oligodendrogenic potential (see the ageing scenarios in **Figure 1**). Notably, neural stem cells located in the SEZ retain their differentiation potential during ageing (Ahlenius et al., 2009; Capilla-Gonzalez et al., 2013a in this volume of FCN) and increase their homeostatic mitotic activity to compensate for the gradual depletion of their population (Shook et al., 2012), properties that confer to the niche a level of resistance to ageing. Significant questions remain to be addressed concerning the exact role of SEZ-driven oligodendrogenesis under physiological and pathological conditions. It is certainly possible that the role of the SEZ falls into both the above-stated hypotheses with not only a role in rejuvenating the adult parenchymal pool of OPCs, but also constantly responding to various environmental cues by providing a cellular boost to the areas in need. There is a definite necessity for a better understanding of adult human oligodendrogenesis and neurogenesis and whether age-related brain pathology is strongly linked to defects in the neurogenic niches.

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Oligodendrogenesis after cerebral ischemia

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Neural stem cells in the subventricular zone (SVZ) of the lateral ventricle of adult rodent brain generate oligodendrocyte progenitor cells (OPCs) that disperse throughout the corpus callosum and striatum where some of OPCs differentiate into mature oligodendrocytes. Studies in animal models of stroke demonstrate that cerebral ischemia induces oligodendrogenesis during brain repair processes. This article will review evidence of stroke-induced proliferation and differentiation of OPCs that are either resident in white matter or are derived from SVZ neural progenitor cells and of therapies that amplify endogenous oligodendrogenesis in ischemic brain.

Keywords: cerebral ischemia, oligodendrocytes, oligodendrocyte progenitor cells, neural stem cells, microRNAs

INTRODUCTION

More than 80% of stroke is ischemic stroke triggered by blockage of blood flow within major cerebral arteries by clots, which leads to infarction in white and gray matter (Dewar et al., 2003; Cui et al., 2009; Karki et al., 2009). Studies from experimental stroke and patients with stroke show that adult brain has the capability to self-repair in response to stroke. However, the spontaneous brain repair process is constrained with limited improvement of neurological outcome (Benowitz and Carmichael, 2010). Thus, stroke remains the leading cause of adult disability around the world (Demaerschalk et al., 2010).

Neurogenesis, oligodendrogenesis, angiogenesis, and astrogliosis are major brain repair processes during stroke recovery (Zhang and Chopp, 2009). Cerebral ischemia induces neurogenesis and angiogenesis in the adult human and rodent brains, which have been studied in depth (Jin et al., 2001, 2006; Zhang et al., 2001; Arvidsson et al., 2002; Parent et al., 2002; Macas et al., 2006; Minger et al., 2007; Curtis et al., 2011). In contrast, stroke-induced oligodendrogenesis in ischemic brain has not been broadly studied (Dewar et al., 2003; Pham et al., 2012). Oligodendrocytes, myelin forming cells in the central nervous system (CNS), are vulnerable to cerebral ischemia (Pantoni et al., 1996; Dewar et al., 2003). Loss of oligodendrocytes and their myelin impairs axonal function (Franklin, 2002). New oligodendrocytes are required to form myelin sheaths for sprouting axons during brain repair processes after stroke because mature oligodendrocytes do not proliferate in the adult brain and injured oligodendrocytes no longer form new myelin sheets (Gensert and Goldman, 1997; Gregersen et al., 2001; Franklin, 2002; Menn et al., 2006; Franklin and Ffrench-Constant, 2008; McTigue and Tripathi, 2008). New oligodendrocytes are derived from non-myelinating oligodendrocyte progenitor cells (OPCs; Gensert and Goldman, 1997; Franklin, 2002). Using an inducible cre-lox fate mapping strategy in adult neural progenitor cells of transgenic mice, recent studies demonstrate that OPCs originating from neural progenitor cells in the subventricular zone (SVZ) of the lateral ventricle differentiate into myelin forming oligodendrocytes under physiological and ischemic conditions (Li et al., 2010; Zawadzka et al., 2010; Zhang et al., 2011, 2012; Rafalski et al., 2013). Preclinical studies show that enhancement of endogenous oligodendrogenesis in ischemic brain by cell-based and pharmacological therapies facilitates brain repair processes and reduces neurological deficits (Li et al., 2005; Zhang and Chopp, 2009; Morris et al., 2010; Zhang et al., 2010b, 2012). These findings have led to a hope for a neurorestorative treatment of stroke which aims to manipulate endogenous neurogenesis, angiogenesis and oligodendrogenesis and thereby to enhance brain repair. In this article, we will review proliferation and differentiation of OPCs in adult rodent brain after focal cerebral ischemia and therapies that amplify endogenous oligodendrogenesis in ischemic brain. Molecular mechanisms that mediate oligodendrogenesis after stroke will also be reviewed.

STROKE INDUCES OLIGODENDROGENESIS

Oligodendrocyte progenitor cells identified by the chondroitin sulfate proteoglycan NG2 and platelet-derived growth factor receptor α (PDGFRα) comprise 3–9% of the total cell number in the adult CNS and are the majority of proliferating cells (Dawson et al., 2003; McTigue and Tripathi, 2008). These OPCs continuously differentiate into mature myelinating oligodendrocytes throughout the gray and white matter of the adult brain (Gensert and Goldman, 1997; Franklin, 2002; McTigue and Tripathi, 2008; Fancy et al., 2011). OPCs are locally present in the corpus callosum, the striatum, and the cortex and are derived from neural progenitor cells in the SVZ (Nait-Oumesmar et al., 1999; Roy et al., 1999; Picard-Riera et al., 2002; Fancy et al., 2004; Menn et al., 2006). The SVZ contains glial fibrillary acidic protein (GFAP) expressing neural stem cells that generate intermediate progenitors (Alvarez-Buylla et al., 2008). Retroviral lineage tracking studies show that although the majority of the neural progenitor cells give rise to homogeneous neuronal progeny, some progenitor cells generate OPCs in the adult brain (Menn et al., 2006). OPCs

generated from the SVZ migrate to white matter tracts of corpus callosum, fimbria fornix and striatum (Menn et al., 2006). Studies of inducible Cre recombination transgenic mice confirm Ascl1- and nestin-expressing neural stem and progenitor cells in adult brain generate OPCs (Li et al., 2010; Zhang et al., 2011, 2012). Early retroviral lineage tracking studies show that the production of OPCs by adult SVZ neural stem cells is limited (Menn et al., 2006). However, using nestin CreER; mT/mG mice in which Cre recombination leads to deletion of a membrane-targeted tomato fluorescent protein and expression of a membrane-targeted green fluorescent protein in adult neural stem and progenitor cells, Rafaski et al. (2013) recently demonstrated that a substantial fraction of OPCs are generated from adult SVZ nestin-expressing neural stem and progenitor cells under physiological condition. *In vitro*, tracking the lineage progression of primary adult SVZ neural stem cells, Ortega et al. (2013) recently demonstrated that the adult neural stem cells generated oligodendrogliogenic clones which gave raise to NG2 positive OPCs by symmetric cell division or NG2 positive OPC and GFAP positive astroglial progeny by asymmetric cells division. However, oligodendrogliogenic clones do not generate OPC and neuronal progeny. These data suggest that adult neural progenitor cells contain oligodendrogliogenic and neuronal lineages and they are distinct and not shared (Ortega et al., 2013).

Stroke acutely induces mature oligodendrocyte damage, leading to loss of myelin, which is associated with loss of axons (Pantoni et al., 1996; Dewar et al., 2003). However, during stroke recovery there is a significant increase in generation of OPCs and some of them become mature myelinating oligodendrocytes in peri-infarct gray and white matter where sprouting axons are present (Gregersen et al., 2001; Zhang et al., 2011, 2012; Ueno et al., 2012). An increase in mature myelinating oligodendrocytes observed after stroke in the adult brain likely result from new oligodendrocytes differentiated from OPCs. Studies in adult transgenic mice that express a tamoxifen-inducible form of Cre recombinase under control of an Ascl1 or nestin promoter have shown that OPCs located in the gray and white matter and OPCs from the SVZ are involved in generation of new oligodendrocytes (Zhang et al., 2011, 2012). During the first 2 weeks after stroke, a robust increase in Ascl1-expressing OPCs was observed in ischemic boundary regions of the gray and white matter. However, 2 months after stroke, the Ascl1 or nestin lineage cells in per-infarct white matter exhibited myelin sheet morphology and expressed protein components of myelin, cyclic nucleotide 3'-phosphodiesterase (CNPase) and myelin basic protein (MBP; Zhang et al., 2011, 2012). In addition to resident OPCs in white matter, stroke recruits SVZ neural progenitor cell-generated OPCs by attracting them from the SVZ to the ischemic striatum and corpus callosum (Zhang et al., 2010b, 2011, 2012). It is uncertain whether SVZ neural progenitor cells share neuronal and oligodendrocyte lineages after experimental induction of demyelination, although under physiological conditions they do not (Ortega et al., 2013). Demyelination in the corpus callosum produced by lysolecithin induces SVZ generated doublecortin (DCX) lineage neuroblasts to differentiate into OPCs (Jablonska et al., 2010). However, DCX lineage OPCs were not detected in adult ischemic brain, although stroke greatly increases DCX lineage neuroblasts (Liu et al., 2009; Zhang et al., 2009). These data indicate that stroke induces oligodendrogenesis by recruiting resident OPCs in white and gray matter and OPCs generated by SVZ neural progenitor cells and that new oligodendrocytes generated after stroke become mature myelinating oligodendrocytes. SVZ generated OPCs are present in humans after demyelination (Nait-Oumesmar et al., 2007). Stromal-derived factor 1α (SDF-1α) and vascular endothelial growth factor (VEGF) secreted by activated cerebral endothelial cells in the ischemic boundary region are likely involved in OPC migration to peri-infarct gray and white matters (Zhang et al., 2000; Imitola et al., 2004; Ohab et al., 2006; Robin et al., 2006; Hayakawa et al., 2012; Bain et al., 2013; de Castro et al., 2013). Glutamatergic inputs from damaged axons in the corpus callosum may also trigger migration of OPCs from the SVZ to peri-infarct areas (Etxeberria et al., 2010).

In addition to serving as a source to generate myelination oligodendrocytes, OPCs act as a surveillance network to detect brain injury (Hughes et al., 2013). Molecules produced by reactive astrocytes and microglia after brain injury trigger OPC proliferation and regulate OPC differentiation (Moore et al., 2011; Miron et al., 2013). For example, insulin-like growth factor-1 (IGF-1) and bone morphogenic proteins (BMPs) secreted by astrocytes promotes and inhibit, respectively, the generation of myelinating oligodendrocytes (Moore et al., 2011). Transformation of pro-inflammatory (M1) microglia and macrophages to anti-inflammatory (M2) drives OPCs to differentiate into mature oligodendrocytes in a model of focal demyelination (Miron et al., 2013). However, the role of such cross-talk in mediating stroke-induced oligodendrogenesis has not been investigated.

THERAPIES ENHANCE ENDOGENOUS OLIGODENDROGENESIS IN THE ISCHEMIC BRAIN

Stroke increases OPC generation in the SVZ, yet endogenous oligodendrogenesis from SVZ neural stem and progenitor cells in response to stroke is limited. Emerging preclinical studies show that cell and pharmacological based therapies initiated at days after stroke enhance endogenous oligodendrogenesis and axonal outgrowth (Li et al., 2005; Zhang and Chopp, 2009; Morris et al., 2010; Zhang et al., 2010b, 2012). Erythropoietin (EPO) regulates neural stem and progenitor cell function through interaction with its receptor EPOR in the adult SVZ (Shingo et al., 2001; Tsai et al., 2006; Chen et al., 2007; Wang et al., 2007). Administration of recombinant human EPO (rhEPO) 24 h after stroke induced sustained OPC proliferation in the peri-infarct white matter and the SVZ (Zhang et al., 2010b). Moreover, rhEPO treatment substantially amplified myelinating oligodendrocytes and increased myelinated axons in peri-infarct white matter (Jiang et al., 2006; Zhang et al., 2010b). Thymosin β4 (Tβ4) is a G-actin binding protein (Goldstein et al., 2005). Administration of Tβ4 24 h after stroke robustly increased NG2 positive OPCs in the SVZ and mature myelinating oligodendrocytes and OPCs in the peri-infarct striatum and corpus callosum 2 months after stroke (Morris et al., 2010). Treatment of stroke with cerebrolysin, a mixture of neurotrophic peptides, amplified generation of OPCs in the SVZ and mature oligodendrocytes in white matter of the peri-infarct region (Zhang et al., 2010a, 2013). Furthermore, administration

of mesenchymal stromal cells (MSCs) even at 7 days after stroke substantially increased NG2 positive OPCs in the SVZ and MBP positive oligodendrocytes in the peri-infarct striatum and corpus callosum 4 months after stroke (Li et al., 2005). The effect of MSCs on enhancement of oligodendrogenesis appears specific because the treatment with MSCs significantly reduced GFAP positive astrocytes in peri-infarct regions (Li et al., 2005). These data indicate that cell and pharmacological based therapies amplify stroke-induced oligodendrogenesis. Aging reduces oligodendrocytes in rodent and human brains (Sim et al., 2002; Pelvig et al., 2008; Shen et al., 2008a,b). By tracking progeny of SVZ nestin lineage neural progenitor cells in ischemic brain of transgenic mice at age of 12 months, a study shows that stroke increased nestin lineage OPCs and oligodendrocytes and that administration of sildenafil, a potent phosphodiesterase type 5 (PDE5) inhibitor, further augmented nestin lineage OPCs and oligodendrocytes in peri-infarct corpus callosum and striatum (Zhang et al., 2012). These data suggest that even in aged animals, oligodendrogenic potential is present in SVZ neural progenitor cells in response to stroke and the treatment. More importantly, increases in new oligodendrocytes are closely associated with augmentation of sprouting axons in peri-infarct areas, which may contribute to functional recovery after stroke, although further studies are warranted to demonstrate newly generated oligodendrocytes myelinate axons in peri-infarct regions.

SIGNALING PATHWAYS MEDIATE OLIGODENDROGENESIS IN THE ISCHEMIC BRAIN

Sonic hedgehog (Shh) is a member of the family of the hedgehog proteins and binds to the transmembrane receptor protein, patched (ptc), which, in the absence of Shh, exerts an inhibitory effect on the seven transmembrane receptor smoothened (Smo; Ingham and McMahon, 2001; Gutierrez-Frias et al., 2004). In the canonical way, binding of Shh to ptc blocks the inhibitory effect of ptc on Smo. Once activated, Smo induces complex series of intracellular reactions that targets the Gli family of transcription factors (Ruiz i Altaba et al., 2002). In addition to neurogenesis, the Shh signaling pathway regulates oligodendrogenesis by inducing a basic helix-loop-helix transcription factor Olig2 that is required for specification of NG2 positive OPCs and mediates OPC differentiation (Arnett et al., 2004; Ligon et al., 2004, 2006; de Castro et al., 2013; Ferent et al., 2013). In the adult brain, Olig2-expressing neural progenitor cells in the SVZ give rise to OPCs that migrate to the white matter (Arnett et al., 2004; Ligon et al., 2006; Menn et al., 2006). Stroke upregulated Shh signal in SVZ neural progenitor cells and blockage of the Shh pathway with cyclopamine, a specific inhibitor of Smo, suppressed stroke-induced neural progenitor cell proliferation and attenuated EPO-increased neural progenitor cell proliferation (Wang et al., 2007; Liu et al., 2013a). Furthermore, administration of cyclopamine to ischemic animals abolished cerebrolysin-enhanced oligodendrogenesis (Zhang et al., 2013). Consistent with stroke, in a model of focal demyelination induced by lysolecithin in the corpus callosum of adult mice, the blocking of Shh signaling with its physiological antagonist, hedgehog interacting protein, led to a decrease of OPC proliferation and differentiation (Ferent et al., 2013). Together, these data suggest that the Shh pathway in SVZ neural progenitor cells plays an important role in mediating oligodendrogenesis in the ischemic brain.

Wnt signaling in adult neural progenitor cells regulates oligodendrogenesis (Rafalski et al., 2013). Overexpression of Wnt3 in SVZ neural progenitor cells results in a substantial and selective increase of PDGFRα positive OPCs in adult mouse brain (Rafalski et al., 2013). However, the canonic Wnt signaling pathway negatively regulates OPC differentiation (Fancy et al., 2011). The relevance of Wnt signaling to ischemia-induced oligodendrogenesis remains to be established.

Phosphatidylinositol 3-kinase (PI3K) and its downstream target, Akt, affect multiple cellular functions such as cell survival,proliferation, and differentiation (Vojtek et al., 2003). Mitogen-activated protein kinases (MAPKs) belong to families of Ser/Thr-specific kinases activated by extracellular stimuli through protein phosphorylation (Rubinfeld and Seger, 2005). Extracellular signal-regulated kinases (ERKs), ERK1 and ERK2, are MAPKs (Rubinfeld and Seger, 2005). The PI3K/Akt, p38MARK and ERK1/2 signals are involved in OPC differentiation (Chew et al., 2010; Fyffe-Maricich et al., 2011; Santra et al., 2012; Rafalski et al., 2013). Inhibition of either p38 MAPK signaling with the inhibitor SB202190 or PI3K with the inhibitor LY294002 significantly reduces adult neural progenitor cells generated OPCs (Santra et al., 2012; Rafalski et al., 2013). Inactivation of p38MAPK with the inhibitor SB202190 in adult neural progenitor cells activated ERK1/2 and abolished Tβ4-increased OPCs (Santra et al., 2012). Therefore, activation of PI3K/Akt and p38MAPK signals and cross-talk between p38MAPK and ERK signals in neural progenitor cells appear to be important in regulating generation of OPCs.

MICRORNAs MEDIATE PROCESSES OF OLIGODENDROGENESIS AFTER STROKE

MicroRNAs (miRNAs), small non-coding RNAs, regulate neural stem cell function and play a pivotal role in controlling processes of OPC generation and differentiation (He et al., 2012). Dicer is an endoribonuclease that cleaves double-stranded RNA and pre-miRNA into short double-stranded RNA (Krol et al., 2010). During development, disruption of miRNA biogenesis by conditional ablation of Dicer in nestin lineage neural progenitor cells results in neural progenitor cell death and abnormal neuronal OPC differentiation (Kawase-Koga et al., 2009). Conditional deletion of Dicer in Olig2 lineage cells led to impairment of OPC differentiation (Dugas et al., 2010; Nave, 2010; Zhao et al., 2010), whereas ablation of Dicer in proteolipid protein (PLP) lineage oligodendrocytes resulted in dysmyelination (Shin et al., 2009). These data suggest that in addition to neural progenitor cells, miRNAs are required for maintaining OPCs in the undifferentiated state and for preserving myelin in mature oligodendrocytes. Indeed, studies on miRNA profiles and functions show that OPCs and oligodendrocytes express distinct sets of miRNAs (Dugas et al., 2010; Nave, 2010; Zhao et al., 2010). For example, over expression of miR-219 and miR-338 in OPCs promoted oligodendrocyte differentiation by repressing targeting genes including PDGFRa, Sox6, Zfp238, FoxJ3, and Hes5 (Dugas et al., 2010; Zhao et al., 2010).

During development, the miR17-92 cluster, a cluster of seven miRNAs, regulates processes of proliferation and survival of

CNPase-expressing oligodendrocytes via upregulation of PTEN and thus inactivation of Akt (Budde et al., 2010). The adult SVZ neural progenitor cells expressed the miR17-92 cluster and stroke substantially upregulated this cluster expression (Liu et al., 2011, 2013a,b). Attenuation of members of the miR17-92 cluster, miR18a and miR19a, or elevation of this cluster in adult SVZ neural progenitor cells suppressed or enhanced neural progenitor cell proliferation, respectively, via alteration of PTEN protein levels (Liu et al., 2013a). Thus, the miR17-92 cluster may regulate processes of oligodendrogenesis in adult brain. The Shh signaling pathway functionally interacts with the miR-17-92 cluster in neural progenitor cells in mediating cell proliferation (Northcott et al., 2009; Uziel et al., 2009). *In vitro* and *in vivo* studies show that activation and suppression of the Shh signaling pathway down- and up- regulated, respectively, expression of the miR17-92 cluster in SVZ neural progenitor cells under non-ischemic and ischemic conditions (Liu et al., 2013a,b).

In addition, miR-9 and miR-200b are likely involved in stroke-induced oligodendrogenesis by targeting serum response factor (SRF; Buller et al., 2012). Stroke considerably downregulated miR-9 and miR-200b in white matter. Overexpression of miR-9 and miR-200 in OPCs suppressed SRF expression and inhibited OPC differentiation (Buller et al., 2012). Collectively, these findings demonstrate that miRNAs are involved in processing stroke-induced oligodendrogenesis.

HISTONE DEACETYLASES AND STROKE-INDUCED OLIGODENDROGENESIS

Classes I and II histone deacetylase (HDAC) activity is required for oligodendrocyte differentiation during brain development (Shen and Casaccia-Bonnefil, 2008; Shen et al., 2008a,b). Pharmacological inhibition of HDAC activity and conditional ablation of HDAC1 and HDAC2 in the oligodendrocyte lineage cells lead to reduction of OPCs and mature oligodendrocytes (Shen and Casaccia-Bonnefil, 2008; Shen et al., 2008a,b; Ye et al., 2009). There are few studies that have examined the role of classes I and II HDACs in mediating processes of oligodendrogenesis in ischemic brain. Stroke increased HDAC 1 and HDAC2 proteins in OPC nuclei and cytoplasmic HDAC4 proteins in OPCs, which was accompanied by reduction of the acetylation levels of histones H3 and H4 (Kassis et al., 2013). Interestingly, treatment of stroke with valproic acid, a pan HDAC inhibitor, considerably increased OPCs and new oligodendrocytes in the adult rat (Liu et al., 2012). These data suggest that HDACs are involved in stroke-induced oligodendrogenesis. The sirtuins, a family of NAD-dependent histone deacetylases, regulate crucial metabolic pathways and are linked to lifespan (Penner et al., 2010; Yu and Auwerx, 2010). Inactivation of SIRT1 in SVZ neural progenitor cells expanded OPCs, which was mediated by activation of Akt and p38 MAPK signaling (Rafalski et al., 2013). However, additional studies are needed to investigate the specific roles of individual HDACs and SIRT1 in proliferation and differentiation of OPCs during adult brain repair.

SUMMARY

Stroke induces oligodendrogenesis. OPCs resident in white matter and OPCs derived from neural progenitor cells contribute to generation of mature myelination oligodendrocytes that interact with axons and astrocytes during post stroke brain remodeling. Potential mechanisms underlying stroke-induced oligodendrogenesis are emerging. Recent studies show that in addition to facilitating salutatory conduction, myelination in adult brain contribute to maintaining axonal integrity, neural plasticity and circuitry function (Fields, 2008; Nave, 2010; Fancy et al., 2011; Zatorre et al., 2012; Young et al., 2013). It is essential for future studies to investigate mechanisms that temporally and spatially coordinate controlling oligodendrogenesis at multiple stages, and to study relevance of remyelination by oligodendrogenesis to neuronal circuitry, which will greatly enhance the development of new therapies for stroke and other demyelination diseases.

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