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Reduced EGFR signaling in adult progenitors of the subventricular zone attenuates oligodendrogenesis after demyelination

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Abstract

Neural progenitor cells expressing the NG2 proteoglycan are found in different regions of the adult mammalian brain, where they display distinct morphologies and proliferative rates. In the developing postnatal and adult mouse, NG2⁺ cells represent a major cell population of the subventricular zone (SVZ). NG2⁺ cells divide in the anterior and lateral region of the SVZ, and are stimulated to proliferate and migrate out of the SVZ by focal demyelination of the corpus callosum (CC). Many NG2⁺ cells are labeled by GFP-retrovirus injection into the adult SVZ, demonstrating that NG2⁺ cells actively proliferate under physiological conditions and after demyelination. In the *wa2* mouse, which is characterized by reduced EGFR signaling, NG2⁺ cell proliferation, under normal physiological conditions and after focal demyelination, is significantly attenuated. This results in reduced SVZ-to-lesion migration of NG2⁺ cells and oligodendrogenesis in the lesion. Expression of VEGF and EGFR ligands, such as HB-EGF and TGF- α , is upregulated in the SVZ after focal demyelination of the CC. EGF-induced oligodendrogenesis and myelin protein expression in cultured wild-type SVZ cells were significantly attenuated in *wa2* SVZ cells. Our results demonstrate that the NG2⁺ cell response in the SVZ and their subsequent differentiation in CC after focal demyelination are dependent upon EGFR signaling.

Keywords

NG2 progenitors; cell proliferation; cell migration; myelin; growth factors

INTRODUCTION

Axonal myelination is a complex process that occurs in the postnatal brain and requires an intricate series of tightly regulated cellular and molecular events, including oligodendrocyte progenitor cell (OPC) specification, proliferation, migration and differentiation (Baumann and Pham-Dinh, 2001; Levine et al., 2001; Nadarajah et al., 2001). These processes characterize specific stages of the oligodendrocyte lineage, and result in the transition of a proliferative and migratory OPC to a non-migratory, postmitotic, myelinating oligodendrocyte (McMorris and McKinnon, 1996; Chandross et al., 1999; Nadarajah et al., 2001). Defining the molecular mechanisms that control each aspect of the myelination sequence is not only important from a developmental perspective, but also for our understanding of several brain disorders or types of injury that indirectly or directly involve oligodendrocytes and myelin (Dubois-Dalcq et al., 2005; Keirstead, 2005; Nait-Oumesmar et al 2007).

The adult brain contains OPCs in the subventricular zone and in white matter regions (Levison and Goldman, 1997; Gensert and Goldman, 1997; Aguirre et al 2004, 2007; Menn et al., 2006). The current efforts in designing cell repair strategies that primarily target oligodendrocytes will unavoidably involve either targeting these endogenous adult OPCs, or progenitors isolated from the immature brain. Therefore, a crucial issue that relates to oligodendrocyte and myelin repair is to what extent regenerative events that occur in oligodendrocytes of the adult brain might recapitulate developmental processes.

Several cellular factors, including platelet-derived-growth factor (PDGF), fibroblast growth factor 2 (FGF2; Baron et al. 2000; Simpson and Armstrong, 1999; Murtie et al, 2005; Vana et al., 2007) and insulin-like growth factor 1 (IGF1; Leininger and Feldman, 2005; Zeger et al., 2007), play fundamental roles in oligodendrogenesis and myelination. We have recently utilized a CNP-hEGFR mouse, in which the human EGFR is overexpressed in neural progenitors that express the CNP gene (Ling et al., 2005; Aguirre et al., 2005; Aguirre et al., 2007). We showed that OPCs that express the membrane proteoglycan NG2 also display EGFR signaling (Aguirre et al., 2007). In the CNP-hEGFR mouse, we demonstrated that enhanced EGFR signaling promotes developmental myelination, as well as oligodendrogenesis and remyelination after focal demyelination of the corpus callosum (CC) (Aguirre et al., 2007).

In the present study we further analyzed oligodendrogenesis and remyelination in a mouse strain in which EGFR signaling is impaired. In the wa2 mutant mouse strain, EGFR is hypoactive in all cells, due to a mutation in the tyrosine kinase domain of the mouse EGFR (Luetteke et al., 1994). This results in a significant attenuation of ligand-dependent EGFR autophosphorylation and substrate phosphorylation (Luetteke et al., 1994). We wanted to determine whether demyelination-induced migration of activated neural progenitors from the SVZ into the CC lesion is impaired in the wa2 mouse, i.e. whether remyelination is attenuated, at least in part, because of defective progenitor migration from the SVZ. We also wanted to analyze in greater detail the role of different EGFR ligands that might modulate oligodendrogenesis after CC lesion, and whether enhanced EGFR signaling promotes neural progenitor response to these ligands. Therefore, we screened postnatal SVZ tissue for EGFR ligand expression after focal demyelination of the mouse CC, and tested the role of these ligands in oligodendrogenesis in vitro.

OBJECTIVE

We hypothesize that EGFR signaling plays a significant role in adult NG2⁺ cell progenitor proliferation, migration and differentiation to mature, myelinating oligodendrocytes. To test this hypothesis, we first analyzed NG2⁺ cell activation in the SVZ of the wa2 mouse - in which EGFR signaling is reduced - after focal demyelination of the CC. Secondly, we investigated regulation of EGFR ligand expression in the SVZ after focal CC demyelination, and their role in oligodendrogenesis in both WT and wa2 mouse neural progenitor cells.

METHODS

Transgenic and mutant mice

The generation and characterization of the CNP-EGFP mouse has been previously described (Yuan et al., 2002). Details on the generation and characterization of the CNP-hEGFR transgenic mice have been previously reported (Ling et al., 2005). Genotyping of these transgenic mice was performed by PCR. Transgenic mice were backcrossed >4 generations onto C57BL/6. In two lines of CNP-hEGFR mice, including the line used here, no obvious changes in brain or CC size were detected in the adult brain. Robust hEGFR expression was detected in total brain and spinal cord lysates from adult brain by using monoclonal anti-human EGFR antibody to probe Western blots after immunoprecipitation with a polyclonal anti-EGFR

antibody. Consistent with the idea that the CNP promoter drives expression in OLs, hEGFR expression was detected in OL lineage cells of the white matter and cerebral cortex in P8-P60 CNP-hEGFR mice, and in NG2⁺ progenitor cells of the SVZ. The wa2 EGFR-mutant mouse (waved-2 mutation; *Egfr^{wa2}*) was obtained from Jackson Labs (Bar Harbor, ME). The spontaneous waved-2 point mutation is a T-to-G conversion that results in glycine replacing valine at residue 743 in the amino terminal portion of the tyrosine kinase domain of mouse EGFR, thus rendering it hypoactive (Luetteke et al., 1994). All animal procedures were performed according to the Institutional Animal Care and Use Committee of Children's National Medical Center and the National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

Antibodies and immunohistochemistry

Freshly cut, floating tissue sections (50µm) P60 mice were prepared as previously described (Aguirre et al., 2004). Primary antibody dilutions were: 1:500 for anti-NG2 antibody (Chemicon, Temecula, CA), anti-MBP (Sternberger Monoclonals Incorporated, Lutherville, MD), anti-CNP (Sternberger Monoclonals Incorporated), anti-S100β (DAKO, Denmark; rabbit anti-human clone A5110); 1:10,000 for anti-GFAP (Sigma). Nuclear staining was performed using TO-PRO (Molecular Probes, Eugene, OR).

Lysolecithin-induced focal demyelination

CNP-hEGFR, wa2, and WT mice (2–3 months old) were deeply anaesthetized with ketamine/xylazine cocktail (10 mg/g) and positioned in a stereotaxic frame (Stoelting, USA). A needle was attached to a 5µl Hamilton syringe and mounted on a stereotaxic micromanipulator. Focal demyelination was induced by stereotaxic injection of 2 µl of a solution of 1% lysolecithin (LPC; Calbiochem) in 0.9% NaCl. The demyelinating agent was injected unilaterally into the CC using stereotaxic coordinates of 2.5 mm anterior to the bregma, 1 mm lateral and 2.5 mm deep from the skull surface. The needle was kept in place for 5 min to reduce reflux along the needle track. Controls were injected with 2 µl of saline. The day of LPC injection was designated day 0 (0dpl). Mice were processed for histology at different time points (2, 5, 10dpl) after LPC injection. The LPC demyelination lesion boundaries were defined based on MBP expression in all mouse strains analyzed (Aguirre et al., 2007). Immunostaining with anti-S100b, anti-MBP, and anti-CNP was used to demonstrate loss of OLs and demyelination at different times after LPC injection. Repopulation of the demyelinated lesion area by endogenous neural progenitors was analyzed by immunostaining progenitors with the following OPC and OL cell lineage markers: anti-NG2, anti-S100b, anti-CNP, and anti-CC1. Anti-GFAP was also used to identify astrocytes. To determine cell density, antibody-positive cells were quantified in the lesion area using unbiased stereological morphometric analysis, as described by Aguirre and Gallo (2004). The cell density (cells/mm³) was estimated as described above. Percentages of cells from each cell population were also determined at different times after lesion. The areas of lesion and of remyelination were examined in at least 4–5 microscopic fields from the entire lesion area in each of 5–6 sections/brain. Data were obtained from at least three brains for each time point, and results were compared between CNP-hEGFR, wa2, and WT mice.

Semiquantitative RT-PCR analysis

In order to determine TGFα, HB-EGF, VEGF, PDGFA, PDGFB and IGF1 expression levels within the SVZ after demyelinating lesion, stereotaxic injections of LPC in the CC of the C57BL/6 mice were performed as described above. Saline and tripan blue (Sigma) were injected ipsilaterally as controls. Two, five and 10 days after injection, SVZ tissue was microdissected from 300µm-thick coronal sections. Corresponding saline-injected was used as control and SVZ was dissected as LPC injected mice. RNA was isolated from SVZ tissue

using Trizol (Invitrogen). RNA (1µg) from each sample was reverse-transcribed using the SuperScript™ First-Strand cDNA Synthesis kit (Invitrogen). The mouse gene-specific primers used were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). TGFα, HB-EGF, VEGF, PDGFA, PDGFB and IGF1 mRNA levels were expressed as arbitrary units after normalization with actin using control levels as reference. RT-PCR primer sequences were as follows: TGFα: sense 5'-CGCTGGGTATCCTGTTAG-3', antisense 5'-ATGGCTTGCTTCTTCTGG-3', VEGF: sense 5'-CTGCTCTCTTGGGTCCACTGG-3' antisense 5'-CACCGGGTTGGGTTGTCACAT-3', IGF1: sense 5'-CATCTCTCTCGCATCTCTT-3' antisense 5'-TTGAGAGGCGCACAGTACATC-3', PDGF-A: sense 5'-TCAAGGTGGCCAAAGTGGAG-3' antisense 5'-CTCGTGACAAGGAAGCT-3', PDGF-B: sense 5'-ATCGCCGAGTGCAAGACGCG-3' antisense 5'-AAGCACCATTGGCCGTCCGA-3', and Actin: sense 5'-CGTGGGCCGCCCTAG GCACCA 3', antisense 5'-AACATGCAGCCTTCTGTTCTGC-3'. Genes were amplified by denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 35 cycles. PCR products were resolved by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Retrovirus stock and injection

Dividing cells in the SVZ were labeled using a 5'LTR-driven bicistronic GFP retrovirus by direct injection 48hr prior to LPC-induced demyelination (Aguirre et al., 2007). The virus plasmid, pNIT, contains a cDNA fragment of enhanced green fluorescent protein (EGFP) downstream of the tetracycline operon enhancer-promoter. Retrovirus production and titer determination were performed as previously described (Kakita and Goldman, 1999). P60-90 wa2, CNP-hEGFR and C57BL/6 (WT) mice were injected with the GFP-retrovirus stock (2µl; titer of $1-2 \times 10^6$ cfu/ml, as assayed with NIH3T3 cells). Injections were performed stereotactically at the following coordinates (anteroposterior relative to bregma, mediolateral, and dorso-ventral from surface of the brain) for SVZ (0.5, 1.5, 3.0mm). Brains were processed for histology at 2, 5, and 12 days after LPC injection, and sections were immunostained for CC1, CNP, and S100β for oligodendrocytes, GFAP for astrocytes, and NG2 for neural progenitors.

Dissociation and cell culture of SVZ progenitor cells

SVZ tissue was microdissected from 300µm-thick coronal sections of P15 mouse brains. The SVZ was dissected out with fine forceps to avoid tissue contamination from surrounding areas. Cells were dissociated and seeded (20,000 cells/well) on coated coverslips in 24-well plates (BDFalcon, Franklin Lakes, NJ). Cells were cultured for 4 days in vitro (DIV) in stem cell medium (DMEM/F12, Invitrogen®) with daily addition of either T3 (10ng/ml), PDGF, EGF or TGFalpha (20ng/ml, Upstate, Charlottesville, VA). Four days later plating, coverslips were processed for immunocytochemistry with the following primary antibodies: anti-NG2, anti-CNP and anti-GFAP (American Type Culture Collection, Manassas, VA).

Microscopy and cell counting

An Olympus BX60 fluorescence inverted microscope was used to visualize immunofluorescence in cultured cells. Images were acquired using a 40x objective. For analysis in tissue sections, a Bio-Rad MRC 1024 confocal laser-scanning microscope (Hercules, CA) equipped with a krypton-argon laser was used for image localization of FITC (488nm laser line excitation; 522/35 emission filter), Texas Red (568nm excitation; 605/32 emission filter), and of Cy5 (647 excitation; 680/32 emission filter). Optical sections ($Z=0.5\mu\text{m}$) of confocal epifluorescence images were sequentially acquired using a 40x (NA=1.35), 60x (NA=1.40), or 100x oil objective (NA=1.35) with Bio-Rad LaserSharp v3.2 software. ImageJ software was used to merge images, which were processed in Photoshop 7.0

with minimal manipulation of contrast. For in situ cell counting, cells were analyzed in z-series confocal scanning images [20 μ m thickness; step size=0.5 μ m between successive images of the same field (228 μ m²)]. An average of 15–20 sections was counted for the SVZ and CC to obtain an estimate of the total number of cells. Percentages of cells expressing different antigens were determined by scoring the number of cells double- or triple-labeled with the marker in question. Cell counting data in tissue sections are expressed as mean $\pm$ s.e.m. For cell counting of cultured cells, means were obtained from three separate sets of cultures. In each culture, at least 10 separate microscopic fields (248 μ m² field) were analyzed. Statistical analysis was performed using unpaired t-tests.

RESULTS

Retroviral infection of NG2-expressing progenitor cells in the adult SVZ after focal demyelination demonstrates proliferation and migration after focal demyelination

NG2-expressing progenitors are found in different regions of the developing postnatal and adult brain (Aguirre et al., 2004; Aguirre and Gallo, 2004; Chittajallu et al., 2004). In particular, NG2⁺ cells that display distinct morphologies and proliferation rates are found in the SVZ, corpus callosum (CC) and cerebral cortex (Figure 1a1–a4) (see also Aguirre et al., 2004; Chittajallu et al., 2004). In the lateral and anterior SVZ, NG2⁺ cells display a migratory morphology, characterized by one leading process (Figure 1a1 and a2), whereas in CC they are mostly multipolar, with processes oriented along axons (Aguirre et al., 2007; Chittajallu et al., 2005). Finally, in cerebral cortex, NG2⁺ progenitors have larger cell bodies and are multipolar, with considerable process arborizations (Chittajallu et al., 2004).

We have previously demonstrated that NG2⁺ progenitors proliferate in the SVZ throughout postnatal development into adulthood (Aguirre et al., 2004; Aguirre et al., 2005; Aguirre et al., 2007; Jablonska et al., 2007). Lysolecithin (LPC)-induced focal demyelination of the CC induced activation of SVZ cells, promoting both proliferation and migration of NG2⁺ progenitors into the CC (Aguirre et al., 2007). In the adult SVZ of the WT (data not shown) and of the CNP-hEGFR mouse (Figure 1b1–b4), injection of the pNIT GFP-tagged retrovirus results in a significant number of infected NG2⁺ cells that are also GFP⁺ at 2 days after LPC injection (2 days post lesion; 2dpl). At 2dpl, no GFP⁺ cells can be detected in the CC of either the WT or the CNP-hEGFR mouse (Aguirre et al., 2007), indicating that labeled progenitors were recruited from the SVZ, rather than from a local pool. Consistent with this notion, at 5 dpl, GFP⁺NG2⁺ cells can be detected in the CC of the CNP-hEGFR mouse (Figure 1a5 and 1c1–c4), indicating direct migration of these progenitors from the SVZ into the white matter.

In WT mice, GFP⁺CC1⁺S100b⁺ oligodendrocytes were detected in CC at 10dpl (Figure 1d1–d4). In the CNP-hEGFR mouse, an increased number of pNIT-infected progenitors in the SVZ (Aguirre et al., 2007) resulted in a larger number of GFP⁺CC1⁺S100b⁺ oligodendrocytes generated in the CC (Figure 1e1–e4).

These results indicate that NG2⁺ cells are present in the adult SVZ as a proliferative and migratory cell population, and that they generate oligodendrocytes in CC in response to EGFR signaling under pathological conditions, i.e. after white matter demyelination.

Characterization of neural progenitors in the SVZ of the wa2 mouse

We have previously characterized the SVZ neural progenitor cell population that generates oligodendrocytes in CC after focal demyelination, and demonstrated that these cells express NG2, Olig2 and Mash1 (Aguirre et al., 2007). Therefore, we compared this neural progenitor population in wa2 and WT mice. Figure 2 shows that a significant reduction was observed in the total number of Olig2⁺, Mash1⁺, NG2⁺ cells in the wa2 SVZ (Figure 2a–e). This decrease

in cell number was accompanied to a reduction in total SVZ cell proliferation, as demonstrated by BrdU incorporation (Figure 2e). Moreover, the number of proliferating Olig2⁺Mash1⁺BrdU⁺ and NG2⁺Mash1⁺BrdU⁺ progenitor cells was also significantly reduced in the wa2 mouse SVZ, as compared to WT (Figure 2f). These results indicate that the oligodendrocyte progenitor pool is reduced in the adult wa2 SVZ.

Reduced EGFR signaling prevents SVZ-to-lesion migration of neural progenitors

In contrast to the results obtained with the CNP-hEGFR mouse, injection of the pNIT-GFP virus into the SVZ of the wa2 mouse resulted in the labeling of a more limited number of progenitor cells, as compared to WT and CNP-hEGFR mice (Figure 3a1–a3). After LPC-induced focal demyelination, a smaller number of GFP⁺ cells were found either in the SVZ-CC migratory pathway (Figure 3a1 and 3d1) or in the CC itself (Figure 3a1, c1 and 3d1). The ratios between number of cells found in CC vs. SVZ were estimated in both WT and wa2 mice, as an overall index of migration. This ratio was 4.0 and 0.6 in WT and wa2 mice, respectively (2 independent experiments), indicating decreased migration upon reduced EGFR signaling. In the wa2 mouse, reduced migration of the pNIT-infected progenitors resulted in the persistence of GFP⁺ cells in striatum at 10dpl (Figure 3a1, and 3b1–b4), and in a decrease in the number of differentiated GFP⁺CC1⁺S100b⁺ cells in the CC (Figure 3c1–c4 and 3d2).

These results demonstrate that reduced EGFR signaling significantly attenuates SVZ progenitor activation and delays their migration and differentiation in CC.

RT-PCR analysis of the SVZ demonstrates enhanced growth factor expression after focal demyelination

We have previously demonstrated that expression of EGFR ligands is upregulated in the lesion area after focal demyelination of the CC (Aguirre et al., 2007). In the present study, we wanted to determine whether the neural progenitor cell expansion observed in the SVZ after focal demyelination of the CC could be attributed, at least in part, to enhanced expression of growth factors in the SVZ. Therefore, we screened postnatal SVZ tissue to identify EGFR-related ligand expression after focal demyelination. SVZ tissue was collected from WT mice at 2, 5 and 10dpl and microarray gene profiling was performed to compare ipsilateral with contralateral SVZ at the same ages (Aguirre and Gallo, in preparation).

RT-PCR analysis confirmed an increase in a number of growth factors in ipsilateral vs. contralateral SVZ. These included HB-EGF, TGF- α , VEGF, PDGF A and B, and IGF-1 (Figure 4a and b). Interestingly, all these changes were detectable between 2 and 10 dpl (Figure 4a), i.e. within the time window of demyelination and remyelination of the lesion (Aguirre et al., 2007).

Altogether, these results demonstrate that EGFR ligands and other growth factors known to play a role in oligodendrocyte development are upregulated in the adult mouse SVZ after focal demyelination of the CC, pointing to a functional role for these factors in oligodendrocyte repair and regeneration.

Reduced EGFR signaling in SVZ neural progenitors decreases oligodendrogenesis induced by EGFR ligands

In order to examine the direct effects of those growth factors that were found to be upregulated in the SVZ after focal demyelination, we tested their role in oligodendrogenesis in vitro. We isolated SVZ cells from postnatal day 15 (P15) WT and wa2 mice, and cultured these cells for 4 days in the presence of T3 hormone, PDGF, EGF and TGF- α . Cells were isolated from P15 tissue, in order to favor oligodendrogenesis (Aguirre et al., 2007), and T3 hormone was

used as a positive control for a factor that strongly promotes oligodendrocyte maturation (Bass et al., 1998).

Figure 5 shows that CNP⁺, mature oligodendrocytes were observed at a higher percentage in WT cells cultured in the presence of EGF (Figure 5a3 and 5c) or TGF- α (data not shown) than in the presence of T3 or PDGF (Figure 5a1, a2 and 5c) ($p < 0.001$ EGF vs. PDGF or T3). Conversely, in SVZ cells obtained from the wa2 mouse, EGF and TGF- α did not significantly promote oligodendrogenesis (Figure 5b3 and 5c; data not shown), but PDGF appeared to be still effective (Figure 5b2 and 5c; compare wa2 PDGF vs. wa2 T3) ($p < 0.05$ PDGF vs. T3).

Western blot analysis of MBP expression in SVZ cells cultured under the same conditions confirmed the cellular results. In WT cells, EGF enhanced MBP expression to a larger extent than T3 or PDGF (Figure 5d), and reduction of EGFR signaling in wa2 cells significantly attenuated EGF-induced MBP expression (Figure 5d).

Altogether, these cellular and biochemical results consistently show that oligodendrogenesis in SVZ cells is regulated by EGFR signaling.

CONCLUSIONS

- Neural progenitors expressing the proteoglycan NG2 are present in the adult mouse SVZ and proliferate in this neurogenic region, as demonstrated by retroviral labeling.
- Adult NG2-expressing progenitors are induced to proliferate and migrate out of the SVZ after focal demyelination of the CC.
- Reduction of EGFR signaling in NG2-expressing progenitors decreases their proliferative and migratory response to demyelination.
- Focal demyelination of the CC induces an increase in growth factor expression in the adult SVZ.
- Reduction of EGFR signaling in NG2-expressing progenitors decreases oligodendrogenesis promoted by EGF in vitro.

DISCUSSION

Due to their large number and to their presence in neurogenic regions of the adult brain, NG2⁺ progenitors have become the subject of much interest, with regard to their cellular and developmental properties, and to their regenerative potential. NG2⁺ cells have been found both in the SVZ and hippocampus, and analysis of their developmental potential has demonstrated that they are not only a source of oligodendrocytes (OLs), but also have neurogenic properties (Aguirre et al., 2004; Dayer et al., 2005; Ohori et al., 2006; Rogelious et al., 2006; Tamura et al., 2007).

Several developmental studies reported the presence of NG2-expressing progenitors in the postnatal and adult SVZ (Levison et al., 1999; Aguirre et al., 2004; Loulier et al., 2006; Rogelious et al., 2006). Using lineage-tracing retroviral labeling in the SVZ of the postnatal rat, Levison et al. (1999) found that, at two days after infection, the SVZ contained a significant number of infected cells with small cell bodies with unipolar or bipolar processes. Using different cellular markers for glial and neuronal cell types, the authors demonstrated that a significant percentage of infected cells were actually NG2⁺, and that after several weeks these infected cells migrated out into white and gray matter regions of the postnatal brain (Levison et al., 1997). Studies from the same lab using an immunohistochemical approach (Levison et

al 1999; Kakita et al., 2003) demonstrated that approximately 2% of the total SVZ cell population was NG2⁺.

These earlier studies formed the basis of our developmental analysis of NG2⁺ progenitors in the postnatal and adult SVZ. Our studies demonstrate that, at any postnatal age, at least 50% of the NG2⁺ cells in the SVZ are proliferative and that Cdk2 is a major molecular regulator of their proliferation throughout postnatal development (Belachew et al., 2002; Jablonska et al., 2007). The results of our previous studies also defined the following cellular and molecular properties of NG2⁺ cells in the SVZ (Aguirre et al. 2004; Aguirre and Gallo, 2004, Chittajallu et al., 2004 and 2005; Jablonska et al., 2007): i) NG2⁺ cells are found scattered through the wall of the lateral ventricle, but they are more abundant in the anterior SVZ; ii) NG2⁺ cells in the SVZ constitute approximately 12% and 3% of the total cell population at P8 and P30-60, respectively; iii) NG2⁺ cells in the SVZ are highly proliferative, as demonstrated by the percentages of NG2⁺Ki67⁺ cells in the SVZ - 56 and 32% at P8 and P30, respectively; iv) NG2⁺ cells of the SVZ express cellular markers of neural progenitor cells and stem cells, including the Lewis X antigen (LeX) (Capela and Temple, 2003), and the transcription factors Mash1, Olig2, and Dlx (Doetsch et al., 2002; Parras et al., 2004; Marshall et al., 2005; Menn et al., 2006; Kohwi et al., 2007; Parras et al., 2007); and v) NG2⁺ cells of the SVZ display a type-C cell phenotype, including expression of the EGFR, PSA-NCAM, and nestin (Capela and Temple, 2002; Doetsch et al., 2002). Consistent with our findings, other reports have also described the presence of NG2⁺ cells in the SVZ, and demonstrated diverse cellular and molecular properties of these progenitors in this neurogenic region, including co-expression of DCX (Aguirre and Gallo, 2004; Tamura et al., 2007), responsiveness to sonic hedgehog (Loulie et al., 2006), and Islet-1-induced migration into striatum (Rogelius et al., 2006). Thus, it appears that properties of NG2⁺ cells in the postnatal and adult SVZ are readily amenable to genetic manipulations through the expression of exogenous genes.

In the present study we further confirm the presence of NG2⁺ cells as a proliferative cell population in the adult SVZ and demonstrate their migratory potential. We show that a significant percentage of retrovirally-labeled cells in the SVZ express NG2, consistent with a study by Men et al. (2006) in which adenovirally-infected NG2⁺ cells were found in the SVZ. In this same study, the authors also demonstrated that, in the SVZ, NG2⁺ cells are derived from type B stem cells, and that NG2⁺ progenitor cells migrate out to the CC, where they differentiate into myelinating oligodendrocytes (Menn et al., 2006). These findings are also in agreement with our developmental analysis of NG2⁺ cells in the adult SVZ, demonstrating that this progenitor cell population gives rise to myelinating oligodendrocytes in CC (Aguirre et al., 2007).

Based on the large and heterogeneous postnatal/adult SVZ progenitor cell population capable of giving rise to neurons and glia, recent studies have focused on the activation of neural progenitors in the SVZ after various types of brain injury. For example, it has been demonstrated that, after hypoxic/ischemic brain injury, significant SVZ progenitor cell activation occurs, including an increase in proliferation rate (Yang and Levison, 2007). Importantly, this proliferative effect was combined with a significant increase in neurogenesis in striatum (Yang and Levison, 2007).

Recent studies have also focused on the mobilization of SVZ neural progenitors into demyelinated lesions, particularly in CC. Using an EAE mouse model, Picard-Riera et al. (2002) demonstrated enhanced migration of SVZ progenitor cells into the lesioned CC, combined with an increase in newly generated oligodendrocytes and astrocytes (Picard-Riera et al., 2002). Importantly, a recent study from the same group also demonstrated an increased density of cells expressing PSA-NCAM and GFAP in the SVZ of human multiple sclerosis (MS) tissue. This finding is consistent with the idea that, as in mouse models of demyelination,

activation of SVZ progenitors also occurs in MS, and that this might cause an increase in cell migration from the SVZ into lesioned areas, where SVZ progenitors could significantly contribute to oligodendrogenesis (Nait-Oumesmar, et al., 2007).

Consistent with the studies described above, we have recently demonstrated that manipulation of a specific progenitor cell population in the SVZ via the CNP gene promoter accelerates remyelination and regeneration of the demyelinated CC (Aguirre et al., 2007). Using a mouse model of focal demyelination and retrovirus labeling in the SVZ, we showed that, as soon as 4 days after induction of the focal lesion by LPC injection, actively dividing NG2⁺Olig2⁺Mash1⁺ cells from the SVZ migrate out of the SVZ into the CC (Aguirre et al., 2007). These migratory NG2⁺ progenitor cells differentiated into mature myelinating oligodendrocytes between 10–14 days after LPC injection. Interestingly, this endogenous regenerative response was enhanced in a transgenic mouse in which the hEGFR was overexpressed in progenitor cells expressing the CNP gene, including NG2⁺ cells. Consistent with our findings, Cantarella et al. (2007) demonstrated the importance of EGFR signaling in remyelination, particularly with regard to activation of SVZ progenitors. Intranasal administration of heparin binding-EGF (HB-EGF) after focal demyelination increased proliferation and mobilization of neural progenitors into the demyelinated CC (Cantarella et al., 2007).

In the present study, we further confirm the importance of EGFR signaling in myelination and remyelination by analyzing SVZ progenitor cells in the wa2 mouse, which is characterized by hypomorphic EGFR signaling (Luetteke et al., 1994). In a previous study (Aguirre et al., 2007), we demonstrated that SVZ cell expansion after focal demyelination of the CC is significantly reduced in the wa2 mouse. Consistent with these findings, in the present study we show that: i) the NG2⁺Olig2⁺Mash1⁺ cell population is significantly reduced in the adult wa2 SVZ; ii) after retroviral infection of SVZ progenitor cells in the wa2 mouse, the majority of GFP⁺ infected cells are found around the ventricles and only a very small percentage reaches the demyelinated CC, and iii) this is due to a reduction in NG2⁺Olig2⁺Mash1⁺ cell proliferation and migration (see also Aguirre et al., 2005 and 2007). GFP⁺ infected cells found around the ventricles are viable and able to differentiate, however only a very small percentage differentiated into mature oligodendrocytes.

Our findings on the role of EGFR signaling in SVZ cell migration are consistent with previous studies (Caric et al., 2001; Ciccolini et al., 2005), including our own showing that overexpression of EGFR induced a migratory phenotype in non-migratory cortical NG2⁺ both in vitro and in vivo after transplantation (Aguirre et al., 2005). These results have been confirmed in an independent study, in which an EGFR-GFP fusion protein was constitutively expressed in white matter glial progenitors. Cell proliferation and migration were enhanced in EGFR-GFP⁺ cells (Ivkovic et al., 2008). Therefore, the reduction of GFP⁺ infected cells in the demyelinated CC of the wa2 mouse is likely to be due to a reduction of both SVZ cell proliferation and migration. Different reports indicate that regeneration of demyelinated lesion occurs primarily from local proliferating neural progenitors (Gensert and Goldman, 1997), but also from migratory SVZ cells (Aguirre et al., 2007; Nait-Oumesmar et al., 2007). In the wa2 mouse, spontaneous remyelination occurs even when NG2⁺ cell proliferation and migration are attenuated, although at a significantly slower rate than in WT and CNP-hEGFR (Aguirre et al., 2007) mice. This could be due to the presence of local neural progenitors in the CC that are responsive to PDGF and FGF2 signaling (Baron et al., 2000; Simpson and Armstrong, 1999; Murtie et al., 2005).

Mobilization of neural progenitor cells to areas of oligodendrocyte death is important for strategies of cell based therapies, including myelin repair. Growth factors that are secreted by endothelial cells (Gama Sosa et al., 2007; Rosenstein and Krum, 2006) as well as by other glial

cells (Aschner, 1996; Suzumura et al., 2006; Sen and Levison, 2006) during development and under pathological conditions (Ferrer et al., 1996; Lisovoski et al., 1997; Jin et al., 2002; Covey and Levison, 2007) are crucial regulators of progenitor cell mobilization. Previous reports demonstrated upregulation of several growth factors in the CC after demyelination, including HB-EGF and TGF- α (Ferrer et al., 1996; Lisovoski et al., 1997; Aguirre et al., 2007). In the present study, we extended the analysis of these growth factors under pathological conditions to include the SVZ itself, which is a neurogenic brain region outside of the area of demyelinated lesion itself, and is a rich source of NG2⁺ cells. Interestingly, we further demonstrate not only that neural cells outside of the focal lesion could also contribute to the remyelination process, but also that a defined group of growth factors are upregulated in the SVZ after focal demyelination. We observed upregulation of HB-EGF, TGF- α , VEGF, PDGFA and B and IGF-1 in the SVZ, at 5 and 10 days after focal demyelination. This time-course of growth factor upregulation is consistent with our findings demonstrating the highest rate of SVZ cell proliferation and migration toward the demyelinated CC at 5dpl (Aguirre et al., 2007). Although the presence of cytokines and other growth factors in the lesion itself is important to regulate the process of local progenitor proliferation, differentiation and survival, our data point to a similar role for a set of growth factors in the regulation of progenitor cell proliferation and mobilization at the source of progenitor expansion (SVZ).

In the present study, we further confirm the direct participation of EGFR signaling in oligodendrogenesis and myelination. We show that differentiation of WT SVZ neural progenitor cells to oligodendrocytes is promoted by EGFR ligands, and that this effect involves functional EGFR signaling. We observed a higher number of mature CNP⁺ cells and higher levels of MBP protein expression in WT SVZ progenitor cells stimulated with EGF and TGF- α than in cells cultured with PDGF, likely due to the mitogenic effects of PDGF, which might partially prevent oligodendrocyte progenitor maturation in vitro (Armstrong et al., 1990). Conversely, in SVZ progenitor cells isolated from the wa2 mouse, only a small percentage of CNP⁺ oligodendrocytes and low levels of MBP expression were found after stimulation either with EGF, PDGF or T3.

Although direct effects of EGF on oligodendrocyte development in vitro are demonstrated by our results obtained in cultured cells (Aguirre et al., 2007, and present study) and in vivo (Aguirre et al., 2007), EGFR ligands could also activate astrocytes and increase expression of growth factors in these cells, which in turn could modulate oligodendrogenesis and myelination. This potential indirect pathway of EGFR ligand action via astrocytes could be particularly important in a pathological setting.

In conclusion, our results obtained in a mouse in which EGFR signaling are reduced show that EGFR signaling is important in the processes of oligodendrocyte regeneration and remyelination. Our data also suggest that activation of SVZ progenitors that are able to remyelinate areas of demyelination depends on a variety of growth factors upregulated by demyelination stimulus. Although the mechanism that links demyelination of CC with upregulation of these growth factors in the SVZ is still undefined, our findings point to a significant endogenous regenerative potential of SVZ progenitors after white matter injury.

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Figure 1. NG2-expressing cells are present in the SVZ and migrate out to the CC after focal demyelination

(a) Confocal images of immunostaining in the CNP-hEGFR mouse brain at P60 with anti-NG2. A large percentage of NG2⁺ cells are present in the ependymal layer of the SVZ (a1, epl-SVZ), anterior subventricular zone (a2, aSVZ), corpus callosum (a3, CC) and cortex (a4, Ctx), displaying different morphologies. (a5) NIT-GFP retrovirus was injected into the adult SVZ 2 days prior to LPC injection. GFP⁺ cells were detected migrating out of the SVZ, and a significant number of GFP⁺ cells were observed in the lesioned area of the CC of the CNP-hEGFR mouse at 5dpl (a5). (b1–b4) At 2dpl, a significant percentage of infected GFP⁺ cells in the SVZ express NG2 (red, b2) and none of the GFP⁺ cells was GFAP⁺ (blue, b3). (c1–c4) At 5dpl, a significant number of virus-infected GFP⁺ cells were found in CC and a large percentage still expressed NG2 (c2 and c4, red). (d–e) At 10 dpl, the majority of GFP⁺ cells found in the lesioned CC express markers for mature glia, including CC1 and S100b. However, in CNP-hEGFR mice, the percentage of CC1⁺ oligodendrocytes generated from virus-infected

cells was higher than in WT mice. Arrows indicate triple-labeled cells. Inserts show immunostaining for the individual antigens for cells indicated by arrows. Scale bar = 50 μ m

Figure 2. Reduced NG2⁺Mash1⁺Olig2⁺ progenitor cells in the adult SVZ of the wa2 mouse (a and b) Triple labeling with anti-Olig2 (green), anti-Mash1 (red), and BrdU (blue) in the SVZ of WT (a) and Wa2 (b) mice at P60. (c and d) Triple labeling with anti-NG2 (green), anti-Mash1 (red), and BrdU (blue) in the SVZ of WT (c) and wa2 (d) mice at P60. Scale bar = 50μm. (e) Total Olig2⁺, Mash1⁺, NG2⁺, and BrdU⁺ cells in the SVZ of WT and wa2 mice at P60. (f) Total Olig2⁺Mash1⁺BrdU⁺ and NG2⁺Mash1⁺BrdU⁺ cells in the SVZ of WT and wa2 mice at P60. Data are shown as means ± S.E.M. (n=2–3 brains for each phenotype). ***p<0.001, ** p<0.002 and *p<0.01 paired t-test wa2 vs. WT.

Figure 3. Defect in migration of neural progenitors from the SVZ to the CC of the wa2 mouse after focal demyelination

NIT-GFP retrovirus was injected into the adult SVZ of wa2 (a1), WT (a2), and CNP-hEGFR (a3) mice 2 days prior to LPC injection. At 2dpl, a large percentage of GFP⁺ infected cells were observed in the WT and CNP-hEGFR mice, but not in the SVZ of the wa2 mouse. (b) At 10dpl, GFP⁺ infected cells were still found around the SVZ/LV in the wa2 mouse (b1–b4), and a very small percentage reached the lesioned CC (c1–c4 and d1). (d1) At 5 and 10dpl, the total number of virus-infected GFP⁺ cells found in CC was higher in WT than in wa2 mice. (d2) At 10 dpl, CC1⁺ oligodendrocytes and GFAP⁺ astrocytes were observed both in WT and wa2 mice, however the number of differentiated cells was significantly higher in WT than in wa2 mice. Insert panels show immunostaining for the individual antigens for cells indicated by arrows. NI = not identified - cells that were not CC1⁺, S100⁺ or GFAP⁺. *p<0.01; **p<0.05. Scale bar = 50μm

Figure 4. EGFR ligand expression is induced in the SVZ after focal demyelination of the CC (a) RT-PCR analysis using specific primers for mouse TGF α , HB-EGF, VEGF, PDGFA, PDGFB, IGF1 and Actin. Growth factors expression levels were detected using total RNA from the SVZ after demyelination of the CC at 2dpi, 5dpi and 10dpi. Controls were from NaCl-injected brains at 5dpi. (b) Histograms were obtained by expressing results as percentage of the saline-injected control values.

Figure 5. EGF promotes oligodendrogenesis from SVZ neural progenitor cells in culture, and its effect is attenuated in wa2 cells

P15 SVZ tissue was dissociated into single cells and plated onto glass coverslips. Cells were cultured for 4 days in vitro (DIV) in stem cell medium with daily additions of T3, PDGF or EGF, as indicated. After 4DIV, cells were fixed and processed for immunocytochemistry, or were harvested for biochemical analysis. (a and b) Representative images of differentiated cells in WT (a) and wa2 (b) cultures. Cells were stained with anti-CNP (red), anti-GFAP (green) and DAPI (blue). Scale bar = 50 μ m. (c) Percentage of differentiated glial phenotypes derived from WT and wa2 cells. Data are expressed as percentage of total DAPI⁺ cells. Approximately 300 total cells were counted for each antigen. * $p < 0.05$; ** $p < 0.03$. (d) Western blot analysis of MBP expression in SVZ cells from WT and wa2 mice cultured in T3, PDGF or EGF for 4 days. EGF enhances MBP expression in WT, but not in wa2 cells, and is more effective than T3 or PDGF. * $p < 0.05$; ** $p < 0.001$.