Neuregulin-1/ErbB4 signaling controls the migration of oligodendrocyte precursor cells during development

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ABSTRACT

During embryonic development, the oligodendrocyte precursors (OPCs) are generated in specific oligoden-drogliogenic sites within the neural tube and migrate to colonize the entire CNS. Different factors have been shown to influence the OPC migration and differentiation, including morphogens, growth factors, chemotropic molecules, and extracellular matrix proteins. Neuregulins have been shown to influence the migration of neuronal precursors as well as the movement and differentiation of Schwann cells for peripheral myelination, but their role in the motility of OPCs has not been explored. In the present study, we have used the optic nerve as an experimental model to examine the function of Nrg1 and its ErbB4 receptor in the migration of OPCs in the developing embryo. *In vitro* experiments revealed that Nrg1 is a potent chemoattractant for the first wave of OPCs, and that this effect is mediated *via* ErbB4 receptor. In contrast, OPCs colonizing the optic nerve at postnatal stages (PDGFRα$^+$–OPCs) does not respond to Nrg1-chemoattraction. We also found that mouse embryos lacking *ErbB4* display deficits in early OPC migration away from different oligodendrogliogenic regions *in vivo*. The present findings reveal a new role for Nrg1/ErbB4 signaling in regulating OPC migration selectively during early stages of CNS development.

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Introduction

Myelination is a tightly controlled developmental process that regulates the conduction of action potentials in the nervous system. Two different classes of specialized glial cells, Schwann cells and oligodendrocytes, are responsible for the myelination of axons in the peripheral (PNS) and central nervous system (CNS), respectively. Although previous works have described that multiple factors in-fluence the process of OPCs specification and migration, including specifically the optic nerve (de Castro and Bribián, 2005; Le Bras et al., 2005; Richardson et al., 2006; Rowitch, 2004; Tsai and Miller, 2002), EGF-related neuregulin ligands and their ErbB receptors are emerging as key regulators of glial cells (Adlkofer and Lai, 2000). In the PNS, different members of the Neuregulin family have been implicated in almost every step in the development of Schwann cells, including cell specification, proliferation, survival, migration and myelination

(Nave and Salzer, 2006). In contrast, the functions of neuregulins in oligodendrocyte development are far less understood (Calaora et al., 2001; Sussman et al., 2005).

Neuregulins are a family of polypeptides that contains a single epidermal growth factor (EGF)-like domain, which binds and activates different members of the ErbB family of tyrosine kinase receptors (Esper et al., 2006). Neuregulins serve as ligands for ErbB3 and ErbB4, and can function as activators of ErbB2 and the EGF receptor (EGFR or ErbB1) through heterodimerization (Adlkofer and Lai, 2000; Carraway and Cantley, 1994; Pinkas-Kramarski et al., 1998). Four genes encoding neuregulins have been identified so far, of which *NRG1* is the best characterized. In humans, *NRG1* may generate up to six different types of protein through alternative splicing and differential use of 5′ regulatory elements. Among these, type I/II and type III Nrg1 isoforms are the most studied. Type III Nrg1 contains an extracellular cysteine-rich domain (CRD) and is not released from the membrane, whereas types I/II isoforms contain an immunoglobulin (Ig)-like domain and are released to the extracellular space through protease cleavage (Esper et al., 2006; Falls, 2003; Nave and Salzer, 2006).

Nrg1 has been linked to many diverse functions in glial and neuronal development. In particular, different isoforms of Nrg1 have been shown to play critical roles at multiple stages in Schwann cell development and different classes of neurons through the activation of different ErbB receptors (Anton et al., 1997; Esper et al., 2006;

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Flames et al., 2004; Grinspan et al., 1996; Meyer and Birchmeier, 1995; Syroid et al., 1996). Previous studies have suggested that Nrg1 may control the proliferation, survival and maturation of oligodendrocytes (Corfas et al., 1995; Fernandez et al., 2000; Sussman et al., 2005; Vartanian et al., 1999), and that these functions are primarily mediated by the ErbB4 receptor (Kim et al., 2003; Vartanian et al., 1997). In contrast, it is presently unclear whether this signaling system also influences their mobility.

Oligodendrocyte precursor cells (OPCs) are generated at restricted sites in the CNS, from where they migrate to colonize the entire brain and spinal cord to become mature myelinating cells (Le Bras et al., 2005; Lu et al., 2002; Spassky et al., 1998; Yu et al., 1994; Zhou et al., 2000). Multiple chemotropic or chemotactic molecules, such as platelet-derived growth factor A (PDGF-A), Sema3A, Sema3F, Netrin1 and CXCL1, control the migration of OPCs (de Castro and Bribián, 2005). In addition, several morphogens and multiple extracellular matrix proteins and their receptors have been shown to influence OPC migration (Bradl and Lassmann, 2010; Bribián et al., 2006, 2008; García-López and Martínez, 2010; Merchán et al., 2007; Miller, 2002; Olivier et al., 2001). Here we have combined experimental embryology experiments with the analysis of ErbB4 mutant mice to study the possible role of Nrg1/ErbB4 signaling in the migration of OPCs. Our results suggest that Nrg1 signaling *via* its ErbB4 receptor regulates specifically the migration of the first wave of OPCs (*plp-dm20*$^+$, non-expressing PDGFRα^+) in the embryonic optic nerve (ON). Intriguingly, this effect disappeared in the second wave, when OPCs begin to express PDGF receptor alpha (PDGFRα).

Material and methods

Mice

In these experiments CD1, plp-GFP (Spassky et al., 2001), *ErbB4*$^{+/+}$; HER4^{heart} and *ErbB4*$^{-/-}$;HER4^{heart} mouse embryos were used. *HER4*^{heart} transgenic mice, which express a human ErbB4 (*HER4*) cDNA under the control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter, were mated to ErbB4 heterozygous mice to generate the desired genotypes as described previously. *ErbB4*$^{-/-}$;HER4^{heart} mouse line (Tidcombe et al., 2003) was generated from the K.O. line *ErbB4*$^{-/-}$ (Gassmann et al., 1995), which has midembryonic lethality because of cardiac malformation. In order to overcome/bypass this problem, Tidcombe et al. created this transgenic line by re-inserting the human ErbB4 gene HER4 under a cardiac specific promoter, α-MHC, so ErbB4 in these mice is only present in the heart.

The day in which the vaginal plug was detected was considered as embryonic day 0.5 (E0.5). Animals were maintained and used in accordance with Spanish (RD223/88) and European (86/609/ECC) regulations, following protocols approved by the Experimental Review Boards at the host institutions.

ON explants and cultures

ON explants were cultured with aggregates of COS-7 cells transfected with either a mock plasmid or a plasmid encoding for type I Nrg1 (*Ig-Nrg1*, accession number AY648976). In brief, the ONs from E16.5 wild type or *ErbB4*$^{-/-}$;HER4^{heart} mouse embryos were dissected and placed in three-dimensional collagen gels confronting COS cell aggregates and co-cultured at 37 °C, 5% CO$_2$ and 95% humidity in Bottenstein–Sato medium (BS) supplemented with 1% L-glutamine, 1% fetal calf serum, and 20 ng/ml FGF-2 (R&D Systems), as described previously (Bribián et al., 2006; Spassky et al., 2002) . The size of explants used was similar in all conditions (Control, 282 ± 8 μm; Ig-Nrg1, 277 ± 5 μm; P = 0.577, t-test). After 3 days in vitro (DIV), the cultures were fixed with 4% PFA. For each explant, we counted the number of OPCs in quadrants facing and opposing the COS cell aggregates, the maximum distance migrated by OPCs, and the diameter of the explant.

E16.5 and P0 dissociated ON cells were also cultured in 12 mm coverslips, as described before (Bribián et al., 2008). Coverslips were pre-coated with poly-L-lysine (10 μg/ml in 0.1 M borate buffer; pH 8.5, Sigma-Aldrich), rinsed in distilled water, and re-coated for 2 h at 37 °C with laminin (10 μg/ml; Sigma-Aldrich). Cells were placed at a density of 3×10^4 cells/coverslip in BS medium and fixed after 1DIV. OPCs were labeled with: goat anti-PDGFRα (R&D; 1:200), rabbit anti-ErbB4 (0618, a gift from C. Lai; 1:200) and goat anti-Olig2 (R&D; 1:200). The number of ErbB4$^+$–PDGFRα^+ and ErbB4$^+$–Olig2$^+$ cells was counted from 10 randomly selected fields in each condition.

Cultures of plp-GFP derived ONs were also fixed after 1DIV and label with: rabbit anti-PDGFRα (Santa Cruz Biotechnology, 1:200), goat anti-GFP (Abcam, 1:200) and anti-Olig2 (Millipore; 1:200).

Differentiation assays

For differentiation assays we first performed E16.5 dissociated ON cells cultures as described above and adding: the purified EGF domain of human NRG1-β1 (NRG1-β1; 1 μg/ml; Peprotech), or GST-purified SMDF–Nrg1 (SMDF; 1 μg/ml). Cultures were fixed after 2DIV. Immunocytochemistry procedures were performed using mouse monoclonal anti-A2B5 (clone 105, ATCC, 1:10). The number of contact points, branches and the length of branches of OPCs were measured. To analyze the differentiation of OPCs in the presence of the proteins described above, we performed similar cultures, but we fixed them at 5DIV. The number of mature oligodendrocytes was identified as APC$^+$/vimentin$^-$. The following antibodies were used: goat anti-vimentin (Santa Cruz; 1:200) and mouse monoclonal anti-APC (Calbiochem; 1:100).

Transwell chamber assays

Nrg1-induced chemotaxis of OPCs was analyzed in Transwell chambers divided by a polycarbonate membrane (pore size: 8 μm; Corning Costar). Optic chiasms and ONs at E16.5 or P0 were dissociated in DMEM (GibCo) containing 1.14 U/ml Papain (Worthington), 12% Collagenase (Sigma-Aldrich), and 0.48 mg/ml Cysteine (Sigma-Aldrich). In each transwell, 5×10^4 cells were seeded in BS medium supplemented with PDGF-AA, NRG1-β1 or SMDF, or combination of them, were added at different concentrations (0.1, 0.5 and 1 μg/ml) to the lower chamber. After 18 h in culture, non-migratory cells were removed with a cotton swab from the upper membrane surface and the membranes fixed with 4% PFA. To discard the possible effect of the BS containing serum with growth factors (Singh et al., 1982), we repeated the experiments with BS medium without serum. To identify cells belonging to the oligodendroglial lineage, the cells that had transmigrated to the lower part of the filter were stained using standard immunocytochemistry protocols (Bribián et al., 2008) with mouse anti-A2B5 (clone 105, ATCC; 1:10) and rabbit anti-Olig2 (Millipore; 1:200). For quantification, 10 random fields were selected from each membrane using a 20× objective in a Leica microscope, and the number of A2B5$^+$/Olig2$^+$ cells quantified for each condition in at least three independent experiments.

Immunohistochemistry and in situ hybridization

Cryostat brain sections were stained using standard immunohistochemistry procedures with the following primary antibodies: rabbit anti-Olig2 (Millipore, 1:500), goat anti-Olig2 (R&D; 1:200), rabbit anti-PH3 (Millipore; 1:200) and rabbit anti-PDGFRα (Santa Cruz; 1:200). Appropriate fluorescent-tagged (1:1000, Invitrogen) or biotinylated (1:200; Vector Laboratories) secondary antibodies were used. Cell nuclei were stained with Hoechst 33342 (10 μg/ml). In situ hybridization was carried out in 20 μm frozen sections using digoxigenin-labeled probes as described before (Flames et al., 2004). Bright field digital

images were obtained with a Leica DFC480 digital camera coupled to a Leica DM5000B microscope.

Western blot

Cells or tissue were homogenized in TBS-T (50 mM Tris pH 7.4, 150 mM NaCl, 10% Glycerol, 5 mM EDTA, 1% Triton X-100, and a mixture of proteases inhibitors). Proteins were resolved by SDS-PAGE on 10% or 12.5% gels and blotted to 0.2 mm nitrocellulose in transfer buffer, pH 8.3 (25 mM Tris, 192 mM glycine, and 20% v/v methanol). Immunoblots were blocked for 1 h in TBS-T (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.2% Tween 20) containing 5% BSA and incubated overnight at 4 °C with primary antibodies diluted in TBS-T containing 1% BSA powder. The following primary antibodies were used: rabbit anti-ErbB4 (0618, a gift from C. Lai; 1:200) and rabbit anti-Olig2 (Millipore; 1:2000). Membranes were then washed in TBS-T, incubated for 1 h with secondary antibodies (goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG; 31,444 and 31,460, Pierce, 1:25,000) diluted in TBS-T, washed and incubated for 1 min with ECL chemiluminescence reagents (Immobilon Western, Millipore).

RT-PCR

Total RNA was extracted from E16.5 ONs with Trizol® according to the manufacturer's instructions (Invitrogen). RNA (150 ng) was treated with DNaseI RNase-free (Fermentas) for 30 min at 37 °C prior to reverse transcription into single-stranded cDNA using SuperScriptII Reverse Transcriptase and Oligo(dT)12–18 primers (Invitrogen) for 1 h at 42 °C. PCR reactions were prepared with 2 μl of cDNA, the appropriate primers, and recombinant Taq DNA polymerase (Invitrogen). The sequences of the primers used were: *EGFR* forward, 5′GCAGATCAGGATCGGGAGT3′; *EGFR* reverse, 5′CCGTGCCACTGGGAGTTAAGG; *ErbB2* forward, 5′GGCCCTCAT-CACCTACAACA3′; *ErbB2* reverse, 5′GCCCAGACCATAGCATACTCC3′; *ErbB3* forward, 5′CGCCAGCTCCGGTTC3′; *ErbB3* reverse, 5′CCGAAGCAGC-GACCGTTACAC3′; *ErbB4* forward, 5′CCCCCAGGCTTTCAACATA3′; *ErbB4* reverse, 5′GCCGTTGCACCCTGAG3′. ErbB4 primers amplify two alternative exons corresponding to Cyt1 and Cyt2 isoforms, respectively. PCR products were analyzed by electrophoresis on a 2% agarose gel.

Statistics

Data were analyzed with the Sigmastat statistics software package (Jandel Scientific, Germany) applying the Student's *t*-test, ANOVA, and/or the corresponding rank tests. All results are expressed as mean ± S.E.M.

Results

Oligodendrocyte precursor cells express the neuregulin receptor ErbB4

To study the possible role of Nrg1/ErbB4 signaling in the migration of OPCs we focused on the developing ON, as this structure has been frequently used as a model to characterize molecules controlling the movement of these cells. In the mouse, the ON is colonized by OPCs from E14.5 onwards, in a gradient from the chiasm to the retina (Spassky et al., 2002). Postmitotic OPCs emanate from the ventricular zone of the third ventricle immediately adjacent to the chiasm (Fig. 1A). We found that this region of the developing diencephalon expressed high levels of *ErbB4*, and that cells entering the ON also express this neuregulin receptor (Fig. 1B). Furthermore, double-labeling experiments demonstrated that Olig2$^+$ cells also contain *ErbB4* mRNA in this area (Fig. 1C). Consistent with these observations, RT-PCR and biochemical experiments revealed that the developing ON contains cells that express different isoforms of *ErbB4*, as well as other members of this family of receptors (Fig. 1D–E and Fig. S1). Analysis of the expression of ErbB4 in longitudinal sections of the ON revealed slightly higher expression proximal to the optic chiasm than to the retina, which is consistent with the gradient of colonization of the ON by OPCs (Fig. S1). In addition, we found that Nrg1 was also strongly expressed by retinal ganglion cells (Fig. 1F–G). The expression of both
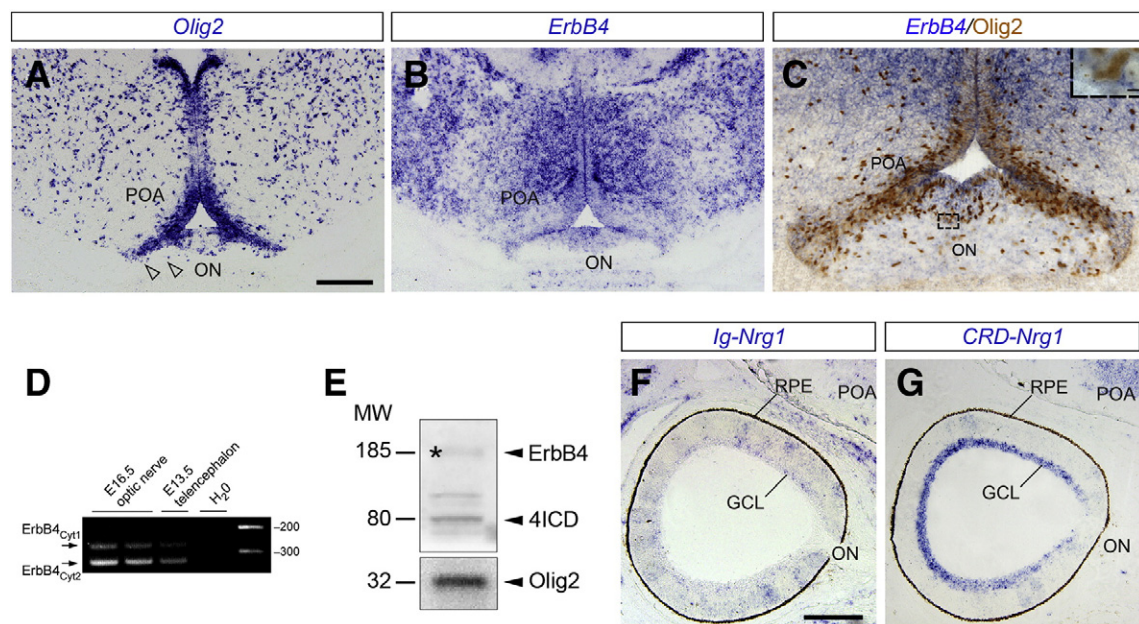

Fig. 1. ErbB4 and NrG-1 are expressed in the optic nerve. (A, B) Coronal sections through the optic chiasm of an E16.5 mouse embryo showing mRNA expression for *Olig2* (A) and *ErbB4* (B). (C) Double staining of the optic chiasm showing Olig2 immunoreactivity (brown) with mRNA expression of ErbB4 (blue). The inset showed Olig2$^+$ cells expressing ErbB4. (D) Semiquantitative RT-PCR analysis of the expression of different ErbB4 transcripts in cells isolated from E16.5 ON and E13.5 whole telencephalon. (E) Immunoblot of ErbB4 protein in E16.5 ONs. Asterisk points the ErbB4 band. 4ICD points to the intracellular domain of ErbB4, cleaved after activation. Olig2 was used as a positive control. (F, G) Coronal sections through the retina of an E16.5 mouse embryo showing mRNA expression for *Ig-Nrg1* (F) and *CRD-Nrg1* (G). GCL, ganglion cell layer; ON, optic nerve; POA, preoptic area; RPE, retina pigmented epithelium. Scale bars equal 300 μm (A, B), 130 μm (C), 5 μm (inset in C) and 300 μm (F, G).

Nrg1 domains (*CRD-Nrg1* and *Ig-Nrg1*) in the retina was maintained also at P0 (Fig. S1).

Thus, we focused on determine the expression of different markers during the first wave of OPCs (*plp-dm20*[+]) that colonize the optic nerve, while Nrg1 is simultaneously being produced by ON axons. Taking advantage from the plp-GFP strain (Spassky et al., 2001), we observed that at E16.5 *plp-GFP* expressing cells are oligodendrocytes, by double labeling of Olig2 and GFP (Fig. 2A–C). OPCs *plp-GFP*[+] also
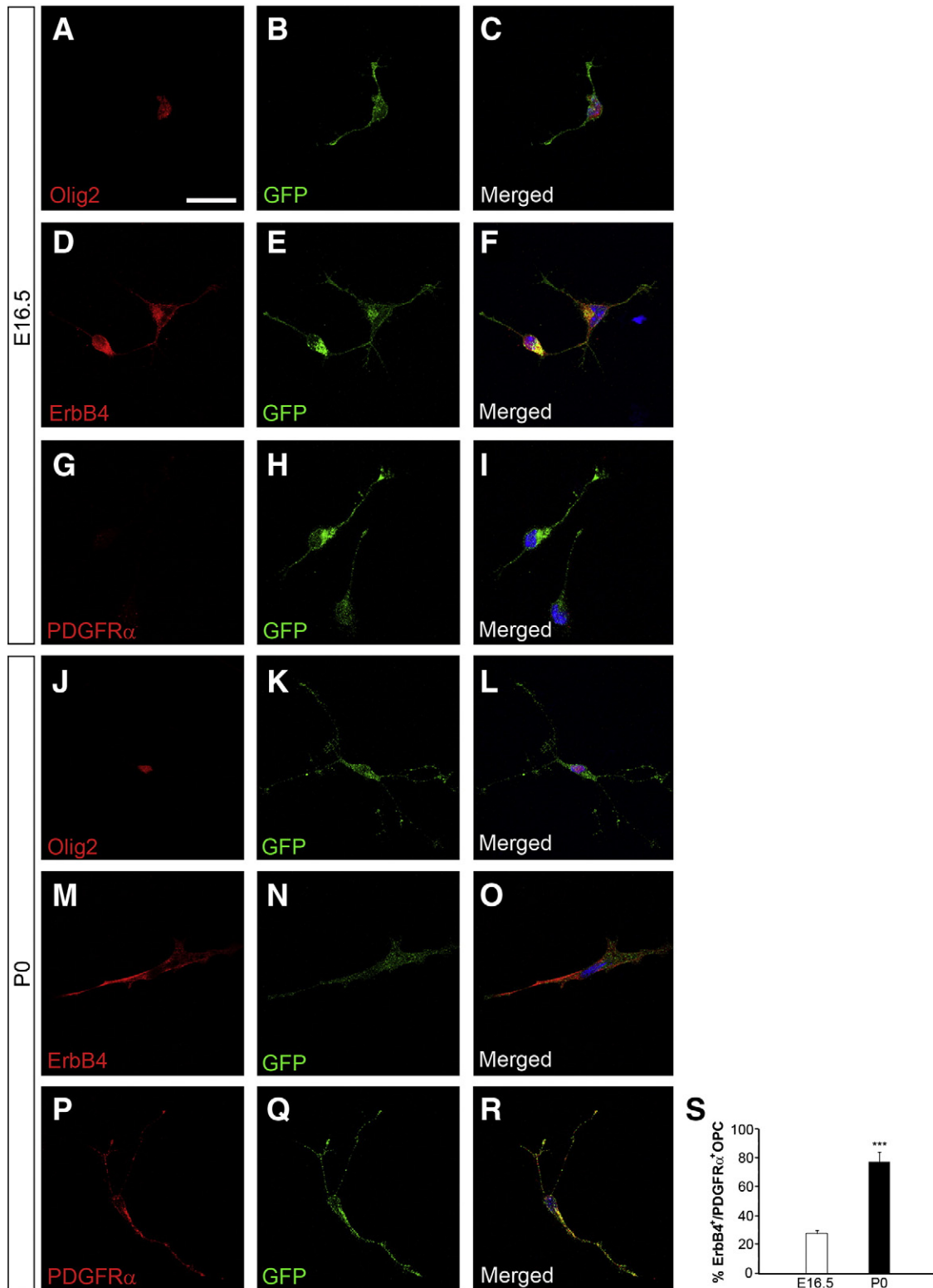
Fig. 2. Characterization of ON OPCs. (A–C) Photomicrographs showed OPCs derived from E16.5 ONs from plp-GFP mice. Plp-GFP cells are identified as oligodendrocytes by the expression of Olig2. At this stage plp-GFP OPCs expressed also ErbB4 (D–F). Thus, they not expressed PDGFRα (G–I). (J–L) Photomicrographs shown OPCs derived from P0 ONs from plp-GFP mice. At this stage plp-GFP expressing cells are still identified as oligodendrocytes by the expression of Olig-2. (M–R) Images showed ErbB4 (M–O) and PDGFRα (P–R) expression by plp-GFP positive cells. (S) The graph represented the percentage of OPCs expressing ErbB4/PDGFRα at E16.5 and P0. Student's *t*-test, ***$P<0.001$. Scale bars equal 20 μm (A–I), 35 μm (J–O) and 25 μm (P–R).

express ErbB4 (Fig. 2D–F), but they did not PDGFRα (Fig. 2G–I). At P0 OPCs continued expressing plp-GFP (Fig. 2J–L) and ErbB4 (Fig. 2M–O), but at this stage OPCs also expressed PDGFRα (Fig. 2P–R), as described before (Spassky et al., 2002). In agreement with these data, we found that most OPCs at E16.5 expressed ErbB4 and only the $27.7\% \pm 2.2$ of them expressed PDGFRα (Fig. 2S). The ErbB4^{+}/PDGFRα^{+} co-expression increased up to $77.2\% \pm 7$ by P0 (Fig. 2S).

Nrg1 is a chemoattractant for OPCs via ErbB4 receptors

Since different isoforms of the ErbB4 ligand *Nrg1* were expressed by retinal ganglion cells at the time of OPC migration (Fig. 1F–G), it is conceivable that Nrg1 may stimulate OPC migration *via* the ErbB4 receptor. To test this hypothesis, we first carried out experiments using chemotaxis chambers designed to measure chemotactic/chemotropic effects on dissociated cells. In these assays, dissociated cells from E16.5 ONs were exposed to control medium or to different concentrations of the purified EGF domain of human NRG1-β1 (NRG1-β1) or GST-purified SMDF–Nrg1 (SMDF). We found that both forms of Nrg1 significantly increased in a dose-dependent manner the number of OPCs that transmigrate to the lower chamber compared to control experiments (Fig. 3A). To confirm these results in a more physiologically relevant context, we performed confrontation assays by co-culturing E16.5 ON explants with aggregates of COS cells expressing a diffusible form of neuregulin, *Ig-Nrg1* (Fig. 3B). We observed that the

ratio of OPCs migrating towards the *Ig-Nrg1*-COS cell aggregates significantly increased (Fig. 3C–D, G; Control, $n = 55$ explants; *Ig-Nrg1*, $n = 123$ explants; $P<0.01$, *t*-test). The average distance migrated by OPCs facing COS cell aggregates expressing *Ig-Nrg1* was also significantly larger than in controls (*Control*, 173 ± 9 μm; *Ig-Nrg1*, 231 ± 6 μm; $P<0.001$, *t*-test), while no differences were observed in distal quadrants between both experimental conditions (*Control*, 144 ± 7 μm; *Ig-Nrg1*, 145 ± 6 μm; $P = 0.287$, Student's *t*-test). In addition, we found no differences in the total number of migrating OPCs between both experimental groups (*Control*: 200 ± 10 cells; *Ig-Nrg1*: 216 ± 8 cells; $P = 0.278$, paired *t*-test), which suggested that Nrg1 does not influence the survival and/or proliferation of OPCs, at least in these conditions. Thus, Nrg1 acts as a potent chemoattractant of OPCs derived from the embryonic ON.

Since it has been proposed that at least two different waves of oligodendrogenesis exist (Spassky et al., 2002), we next tested the effect of Nrg1 on OPC migration at different developmental stages. Intriguingly, we found significant differences in the migratory behavior of OPCs depending on their age. We observed that at E16.5 OPCs did not respond to PDGF-AA (20 ng/ml), a well characterized chemoattractant for OPCs isolated from the cerebral cortex of newborn rats (Zhang et al., 2004), whereas they were attracted by Nrg1 (both NRG1-β1 and SMDF; Fig. 2H). This data are coincident with the expression of the receptors for both molecules at E16.5 (Fig. 2). The combined effect of PDGF-AA and Nrg1 did not differ from the effect of neuregulin alone (Fig. 3H). To
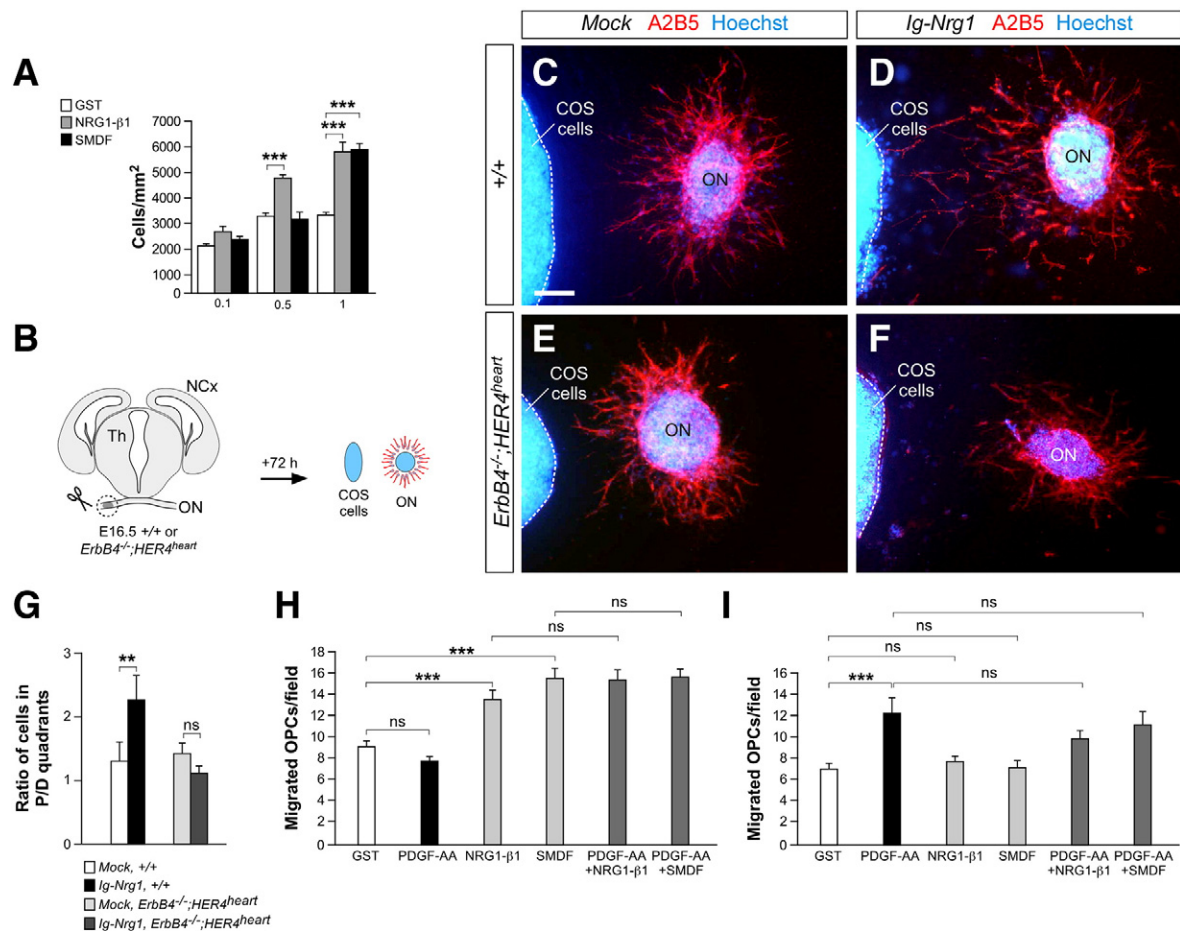
Fig. 3. Nrg1 is a chemoattractant for embryonic OPCs. (A) Quantification of the number of transmigrated OPCs in chemotaxis chambers in which control medium or different forms of Nrg1 were added to the lower chamber. $n = 10$ membranes, from 5 independent experiments. (B) Schematic diagram of experimental design. (C–F) Migration of ON-derived OPCs (stained with antibodies against the A2B5 epitope) in response to mock transfected (C, E) or *Nrg1* transfected (D, E) COS cells aggregates obtained from wild type (C, D) or *ErbB4$^{-/-}$* mutant (E, F) E16.5 embryos cultured in collagen matrices for 72 h. The dotted lines indicate the limits of the COS cell aggregates. (G) Quantification of the ratio of cells located in proximal (P) and distal (D) quadrants in each of the experimental conditions, $n = 36$. (H–I) Quantification of the number of transmigrated OPCs at E16.5 (H) and P0 (I) in chemotaxis chambers in which control medium, different forms of Nrg1, PDGF-AA and combination of both, were added to the lower chamber. Student's *t*-test, **$P<0.0$; ***$P<0.001$; *ns*, not significant. NCx, neocortex; Th, Thalamus. Scale bar equals 200 μm.

avoid the possibility that traces of growth factors which would be present in the serum of the culture medium were affecting OPCs migration, we performed the chemotaxis assays without serum. The behavior of the embryonic OPCs was completely similar to that shown in the presence of serum (Fig. S2).

In contrast, we found that at P0, when most ON OPCs expressed PDGFRα$^+$, these cells responded vigorously to PDGF-AA, while the different isoforms of Nrg1 fail to trigger any response (Fig. 3I). Consistently, the combination of PDGF-AA and Nrg1 attracted OPCs in the same proportion as PDGF-AA alone (Fig. 3I). These changes, from chemoattractant to silent (Nrg1) or *vice-versa* (PDGF-AA), might reflect an evolution in the expression of receptors by developing OPCs. To examine whether ErbB4 mediates the function of Nrg1 in OPC migration *in vitro*, we used mice that selectively lack ErbB4 expression in the brain (Gassmann et al., 1995; Tidcombe et al., 2003). Co-culture experiments with tissue obtained from *ErbB4* mutant mice revealed that the chemoattractive effect of Nrg1 on ON explants is entirely dependent on ErbB4 function (Fig. 3E–G; Control, $n = 24$ explants; *Ig-Nrg1*, $n = 20$ explants; **$P < 0.01$, t-test). The absence of ErbB4 did not influence the size of the explants or the number of migrated cells, which reinforced the view that ErbB4 mediates Nrg1-induced chemoattraction in these cells.

Nrg1 modifies the morphology of migrating OPCs

We observed that the morphology of migrating OPCs confronted to COS cell aggregates expressing *Ig-Nrg1* was different than that of control cells (Fig. 4A–B). In particular, the length of the leading process was found to be twice as long in OPCs exposed to Ig-Nrg1 than in control cells (Fig. 4C). In addition, the trailing process of OPCs confronted to COS cells expressing *Ig-Nrg1* was also significantly longer than in control OPCs (Fig. 4C). To extend these observations, we next cultured ON cells in coverslips coated with recombinant Nrg1 (either SMDF or NRG1-β1). We found that OPCs migrating in the presence of Nrg1 developed many more processes than control OPCs (Fig. 4D–G). Moreover, the number of processes was significantly higher in OPCs migrating on Nrg1-coated coverslips than in controls (Fig. 4D–G). No differences were observed between those experiments performed in the presence of full-length Nrg1 or its EGF-like domain (Fig. 4E), suggesting that this later domain of the protein is largely responsible for the morphological modifications induced by Nrg1. Altogether, these data suggest that Nrg1 increase the number and the length of OPC processes.

The finding that the length of OPC processes was increased by Nrg1 suggested a possible effect of this molecule in the differentiation of oligodendrocytes. We tested this by culturing dissociated cells from E16.5 ONs under pro-differentiation conditions (see Methods). After 5 DIV, we found that the proportion of APC$^+$/vimentin$^-$ cells grown on laminin substrate was significantly higher in the presence of Nrg1 substrates than in controls (Fig. 4H). In sum, our data suggested that Nrg1 seem to act as a pro-differentiation cue for the oligodendroglial lineage. In early, undifferentiated OPCs, Nrg1 enhances the length of the leading and trailing processes, thereby facilitating the migratory
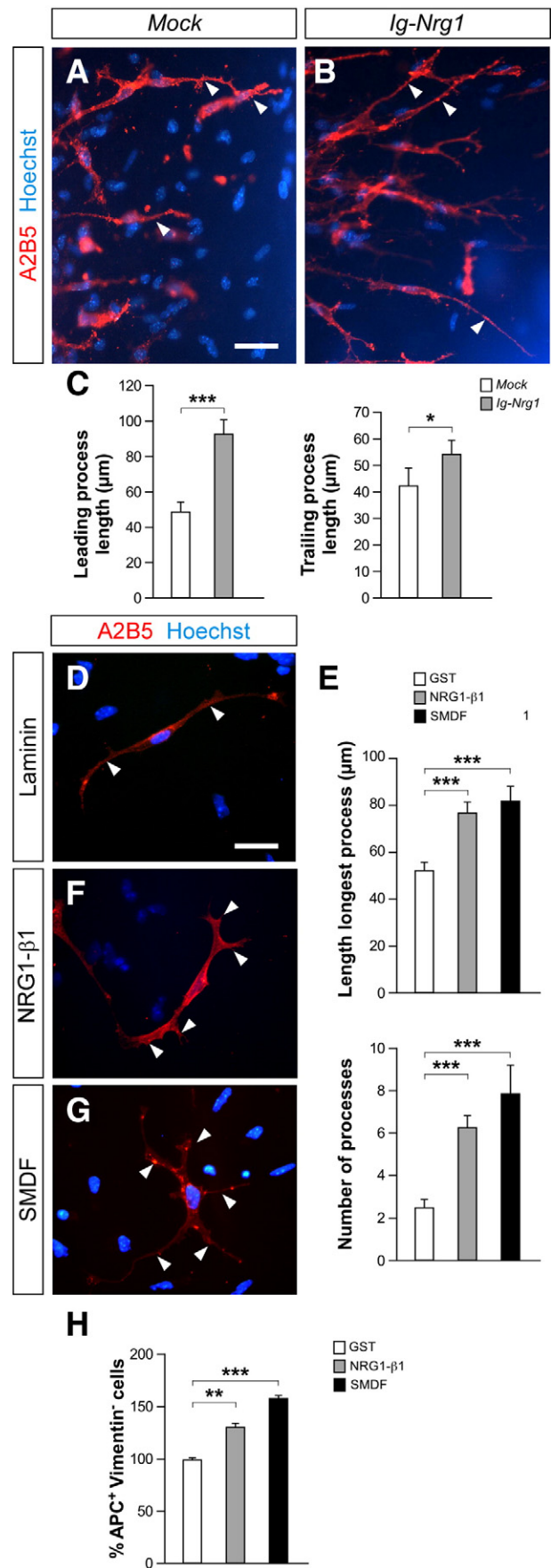
Fig. 4. Nrg1 modifies the morphology of migrating OPCs. (A, B) Representative images of migrating OPCs confronted to mock transfected (A) or *Nrg1*-transfected (B) COS cell aggregates. (C) Quantification of the length of the leading and trailing process in migrating OPCs. Arrowheads point to leading processes. $n = 20$ cells from 10 different explants. (D, F, G) Representative images of OPCs dissociated from ONs and cultured in the presence of different substrates: laminin as control (D), Nrg1-β1 (F), or SMDF (G). Arrowheads point to cell processes. (E) Quantification of the length of the longest process and the number of processes in OPCs. $n = 20$ cells from 10 independent experiments. OPCs were stained with antibodies against the A2B5 epitope. (F) Quantification of the percentage of APC$^+$/vimentin$^-$ cells over GST, NRG-β1 and SMDF–Nrg1 coating. Student's t-test: *$P < 0.05$; ***$P < 0.001$. Scale bars equal 100 μm (A, B) and 20 μm (D, F, G).

capabilities of OPCs. This may also contribute to promote cell differentiation towards pre-mature oligodendrocytes.

ErbB4 regulates OPC migration in vivo

We next examined the role of *ErbB4* in the migration of OPCs by analyzing *ErbB4*$^{-/-}$ mutant mice. We found that deletion of *ErbB4* significantly reduced the number of OPCs observed in the diencephalic ventral surface, the optic chiasm and even more entering the ON at E16.5 (Fig. 5A–C). This defect is likely caused by delayed migration because the number of Olig2-expressing cells found in the preoptic/suprachiasmatic region, which gives rise to ON OPCs, was similar in control and *ErbB4* mutant embryos (Fig. 5A–C). Consistently, no differences were observed in the number of proliferating (Fig. S3A) or
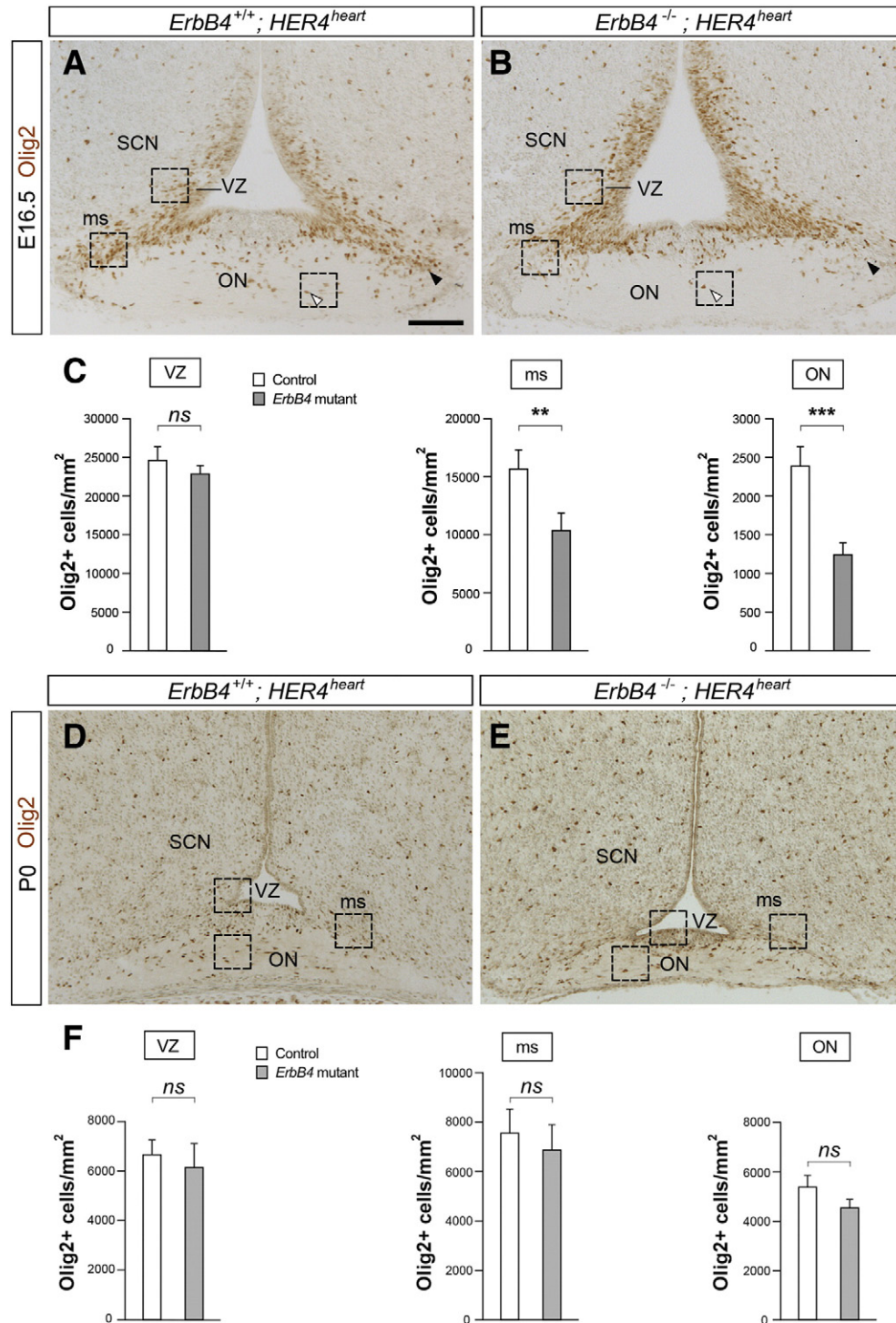
Fig. 5. Defective OPC migration in *ErbB4* mutant embryos in the ON. (A, B) Coronal sections through the E16.5 optic chiasm showing immunohistochemistry against Olig2 in wild type (A) and *ErbB4* mutant (B) embryos. Arrowheads point to cells leaving the oligodendrogliogenic progenitor regions through the lateral migratory streams (ms) reaching the ON. Open arrowheads point to cells within the ON. SCN, suprachiasmatic region. (C) Quantification of the number of Olig2-expressing cells in the ventricular zone (VZ) of the suprachiasmatic region, ms and ON. *n* = 3 control and *n* = 4 mutant mice. (D, E) Coronal sections through the P0 optic chiasm showing immunohistochemistry against Olig2 in wild type (D) and *ErbB4* mutant (E) animals. (F) Quantification of the number of Olig2-expressing cells in the ventricular zone (VZ) of the suprachiasmatic region, ms and ON. *n* = 5 control and *n* = 3 mutant mice. Student's *t*-test, **P<0.01, ***P<0.001; ns, not significant. Scale bar equals 100 μm in A–E.

apoptotic cells (data not shown) found in this region in control and *ErbB4* mutant embryos. Moreover, quantification of the number of OPCs in the ON of neonatal mice revealed no differences between control and *ErbB4* mutants (Fig. 5D–F), reinforcing the view that loss of ErbB4 causes a delay in the ON invasion by OPCs. This observation is also consistent with the analysis of the distribution of OPCs found at P0. *In vivo*, while *ErbB4$^{-/-}$* and wild type mice showed a very low number of PDGFRα^+–OPCs within the ON and preoptic area by E16.5 (Fig. 6A–B, E), they were more abundant in these areas by P0 (Fig. 6C–E). Interestingly no differences in the amount of PDGFRα^+–OPCs were shown between both genotypes at P0 (Fig. 6E), indicating that the absence of ErbB4 does not affected the PDGFRα colonization by OPCs. These results reinforced the view that Nrg1/ErbB4 signaling is required for the migration of early OPCs, but not for subsequent waves of oligodendrogenesis. Finally, we studied the distribution of OPCs in another oligodendrogliogenic region of the forebrain, the telencephalic subventricular zone (SVZ). The SVZ also contains many cells that express the Nrg1 receptor ErbB4 (Fig. S4), which suggests that this signaling system may also influence the migration of OPCs in this region. We found that loss of *ErbB4* leads to a significant accumulation of OPCs in the SVZ region at E16.5, with many OPCs failing to disperse throughout the basal telencephalon (Fig. 7A–C). In the absence of proliferation defects (Fig. S3B), these results suggested that ErbB4 is required for the outward migration of OPCs from oligodendrogliogenic sites in the forebrain towards their final targets of myelination.

Discussion

Using the mouse ON as an experimental system to study the migration of OPCs, we have found that the EGF-related ligand Nrg1 is a potent chemoattractant for these cells, and that this function is mediated by the ErbB4 receptor. Different isoforms of the *Nrg1* gene are expressed by retinal ganglion cells, which thereby may contribute to promote the centrifugal invasion of the ON by migrating OPCs. This effect is restricted to the first wave of OPCs colonizing the optic nerve (embryonic stages), because OPCs from the second wave (PDGFRα^+) do not respond to Nrg1 and are instead attracted by PDGF-AA (Bradl and Lassmann, 2010; Richardson et al., 2006; Spassky et al., 2002). The chronologic changes in the expression of receptors by OPCs seem to explain the different effects of both cues. Similar changes in the expression of other receptors and changes in the effects of other cues, such Netrin-1 and Sonic Hedgehog, have also been reported previously (Merchán et al., 2007; Spassky et al., 2002; Sugimoto et al., 2001). Together with the present results, these observations suggest that the early colonization of the optic nerve by OPCs is under a specific guidance program (Netrin-1, Sonic Hedgehog and Nrg1 attraction, no effect for PDGF-AA) that is different from that used by the second wave (Netrin-1 repulsion/stop signal, PDGF-AA attraction and no effect for Nrg1). It remains to be studied if secreted semaphorins influence early OPCs (Spassky et al., 2002), while the effects of FGF-2 and Anosmin-1 *via* FGFR1 seem rather constant during embryonic and postnatal stages (Bribián et al., 2006; Clemente et al., 2011). Different studies *in vivo* seem to reinforce, at least partially, this scenario (Fruttiger et al., 1999; Taveggia et al., 2008).

The relevance of the first wave of OPCs in the final process of myelination remains to be understood. Although a change in the expression of markers (from PDGFRα^- to PDGFRα^+, for example) and their subsequent masking for the observer cannot be excluded, it has been suggested that this first wave of OPCs is an evolutionary relic (primitive source of OPCs) or reflects the turnover of oligodendrocytes due to environmental influences (Bradl and Lassmann, 2010; Richardson et al., 2006). In agreement with this later idea, the role of Nrg1 *via* ErbB4 in the myelination process seems different: while would be limited to the migration of the first wave of oligodendrogliogenesis (and thereby,
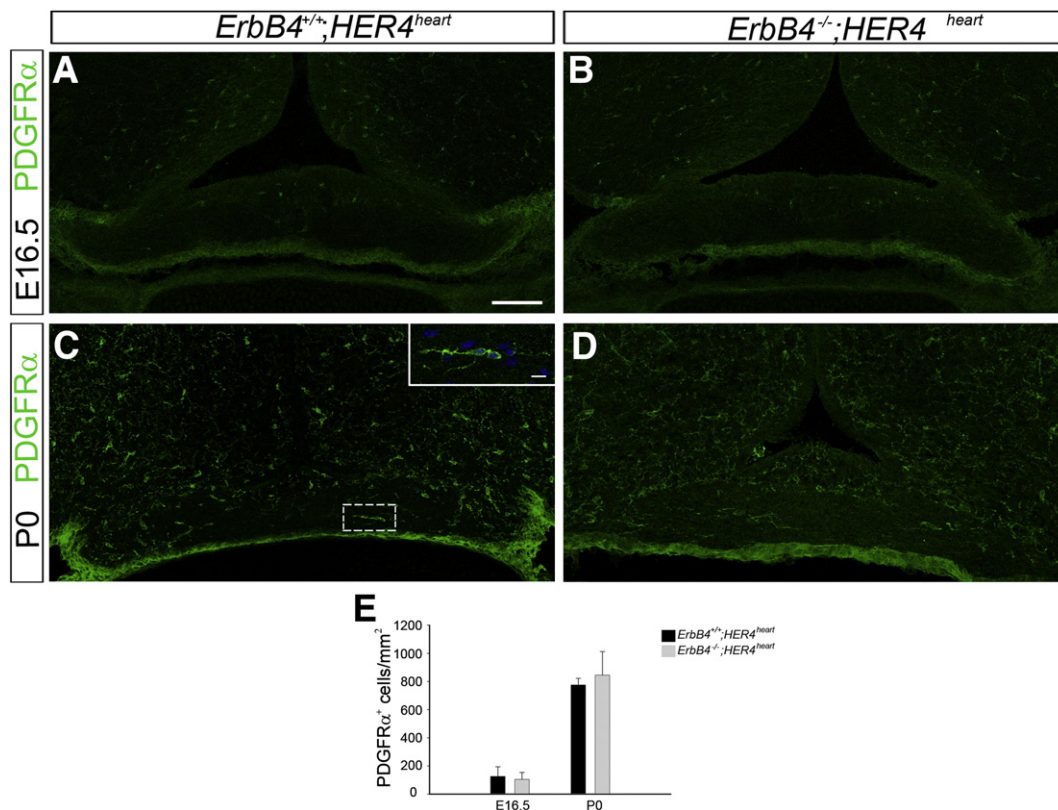
Fig. 6. PDGFRα expression in ErbB4 mutant mice (A, B) Coronal sections through ON and optic chiasm of E16.5 wild type (A) and *ErbB4* mutant (B) showing immunohistochemistry against PDGFRα. (C, D) Coronal sections through ON and optic chiasm of P0 wild type (C) and *ErbB4* mutant (D) showing immunohistochemistry against PDGFRα. Inset in C shows a PDGFRα positive cell within the ON. Note that at P0 PDGFR cells are significantly higher than at E16.5. There are no differences between both genotypes. (F) Histogram represents the density of PDGFRα^+ cells in the optic chiasm at E16.5 and P0 in the wild type and *ErbB4* mutant. Scale bar equals 100 μm in A–B, 85 μm in C–D, and 10 μm in the inset.
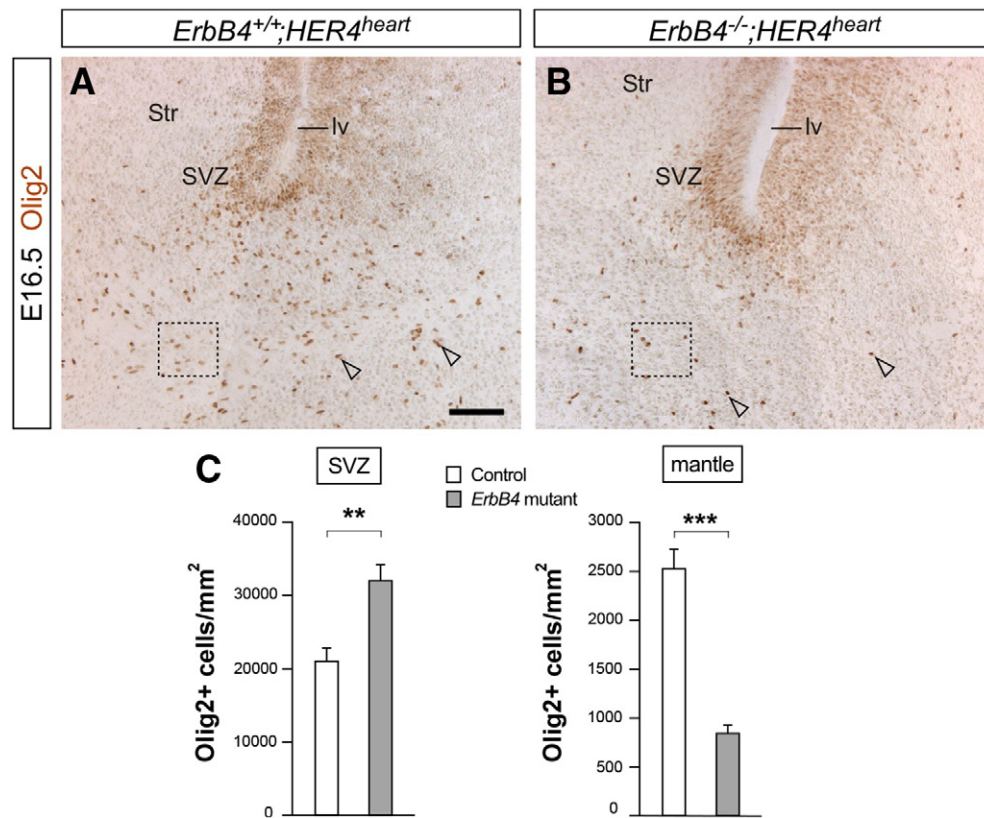
Fig. 7. Defective OPC migration in *ErbB4* mutant embryos in the SVZ. (A) Coronal sections through the E16.5 telencephalon showing immunohistochemistry against Olig2 in wild type (A) and *ErbB4* mutant (B) embryos. Open arrowheads point to cells leaving the progenitor regions. lv, lateral ventricle; Str, striatum; SVZ, subventricular zone. (C) Quantification of the number of Olig2-expressing cells in the basal telencephalon of control and *ErbB4* mutant embryos. $n = 3$ control and $n = 4$ mutant mice. Student's *t*-test, **$P < 0.01$, ***$P < 0.001$. Scale bars equal 100 μm in A–B.

with an undetermined relevance for the whole CNS myelination process, to date), it seems capital for Schwann cell-due myelination in the peripheral nervous system (Brinkmann et al., 2008; Sussman et al., 2005; Taveggia et al., 2008). A recent study shows higher levels of mature myelin proteins (MBP and CNPase) in the cerebral cortices of transgenic mice over-expressing Nrg1 (Kato et al., 2010). In the same sense, the loss of ErbB4 signaling by its dominant-negative form reduced oligodendrocyte number and myelination (Roy et al., 2007).

Nrg1 elicits a potent morphological adaptation of migrating OPCs, which extend more and longer processes in the presence of Nrg1. Considering that Nrg1 have been shown to cause a similar effect on migrating cortical interneurons (Martini et al., 2009), our findings indicate that this process may generally contribute to enhance chemotaxis. In addition, our results contribute to strengthen the notion that ErbB4 is the main receptor involved in mediating the chemotactic effects of Nrg1 in the CNS (Flames et al., 2004; Lopez-Bendito et al., 2006).

Several lines of evidence suggest that individuals with schizophrenia have white matter abnormalities, including reduced numbers of oligodendrocytes in the cerebral cortex (Corfas et al., 2004). There is a bulk of reports suggesting changes in Nrg1 expression/physiology in schizophrenia (Barakat et al., 2010; Chong et al., 2008; Nicodemus et al., 2010; Stefansson et al., 2002; Walsh et al., 2008). In addition, the absence of neuregulin in active multiple sclerosis lesions have been suggested to contribute to the paucity of remyelination in this disorder (Cannella et al., 1999; Viehover et al., 2001), which, in view of present results, should be carefully reinterpreted. The extent of this, as well as Nrg1 as putative target for efficient remyelination therapies remains to be elucidated.

These observations are consistent with our current findings, which suggest that Nrg1/ErbB4 signaling contributes to the early migration

of OPCs from their sites of origin. Of note, although it has been suggested that ErbB4 is required for CNS myelination (Cannella et al., 1999; Roy et al., 2007), current genetic evidence strongly suggest that oligodendrocytes have evolved an NRG/ErbB-independent mechanism of myelination control. Thus, Nrg1/ErbB4 signaling does not seem to be directly required for the process of myelinization, although based on our results it seems conceivable that defects in Nrg1/ErbB4 signaling might led to the abnormal colonization of specific brain areas. Considering the link between Nrg1/ErbB4 signaling and schizophrenia (Mei and Xiong, 2008), the possibility that defects in the first wave of oligodendrogliogenesis (as shown here) might contribute to the subtle anatomical changes observed in schizophrenia should be analyzed in future studies.

Conclusion

Using the optic nerve as model of OPC migration, we have shown that neuregulin 1 has a chemoattractant effect in OPC migration in embryonic stages. This effect is mediated by the ErbB4 receptor and is specific for *plp/dm20*[+] OPCs. Therefore does not persist in postnatal stages, coincidence with the second wave of the optic nerve colonization by the *PDGFRα*[+] OPCs. *In vivo*, the absence of ErbB4 derived in lower numbers of in different areas of the developing brain. This specific action in the first wave of OPCs population does not affect the posterior oligodendrocyte distribution and the subsequent myelination.

The interaction of ErbB4 and its ligand, neuregulin1 also triggers OPC differentiation. Therefore although alterations to the process of migration of the *plp/dm20*[+] population might play a role in brain development it is likely that other critical cues and the presence of the PDGFRα in OPCs in latter stages are major contributing factors to the correct disposition and posterior myelination.

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