Brief Communication

The ErbB4 Neuregulin Receptor Mediates Suppression of Oligodendrocyte Maturation

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Neuregulin is required for proper oligodendrocyte development, but which receptors are involved and whether neuregulin promotes or inhibits maturation remain controversial. To assess the roles of the neuregulin receptor ErbB4 in oligodendrocyte development, we examined oligodendrocyte initiation and maturation in cultures derived from erbB4 knock-out mice and rat spinal cord in the presence of neutralizing erbB4 antibodies. No differences in the development of O4$^+$ oligodendrocytes were detected in the presence or absence of erbB4 signaling. All four epidermal growth factor receptor family members were detected in the ventral neural tube at approximately the time of initial oligodendrocyte development, consistent with redundancy in neuregulin receptor signaling at the onset of oligodendrocyte development. In contrast, greater numbers of differentiated (monoclonal antibody O1$^+$) oligodendrocytes developed in neural tube explants from *erbB4*$^{-/-}$ mice than either *erbB4*$^{+/+}$ or *erbB4*$^{+/-}$ littermates as well as in cultures treated with anti-erbB4. These data indicate that ErbB4 is not required for oligodendrocyte development and, in fact, inhibits oligodendrocyte lineage maturation. Together with previous studies, these data suggest a model in which early oligodendrocyte lineage development is regulated by promiscuous neuregulin receptor signaling, but subsequent lineage progression occurs through a balance of receptor-specific promotion or inhibition of maturation.

Key words: differentiation; neural tube; oligodendrocyte; receptor; growth factor; embryo; neuregulin; erbB4; spinal cord; CNS; immunohistochemistry; knock-out

Introduction

The neuregulin family of growth factors has profound effects on the development of oligodendrocytes, the myelinating cells of the CNS. Among them is the lack of O4$^+$ oligodendrocytes in the absence of neuregulin-1 *in vitro* (Vartanian et al., 1999). Neuregulin-1 knock-out mice die during midgestation before the first oligodendrocytes arise; however, oligodendrocyte development occurs in neural tube explants from midgestation wild-type embryos on a schedule similar to the schedule that occurs *in vivo* (Warf et al., 1991), allowing analysis of the effect of an absence of neuregulin. In contrast, the earliest committed oligodendrocytes fail to arise in parallel cultures from neuregulin-1 knock-out mice, and this effect can be rescued by addition of recombinant neuregulin to explant culture medium from embryonic day 10 (E10) onward. The loss of O4$^+$ oligodendrocytes can be phenocopied by addition of a soluble neuregulin inhibitor to wild-type explant cultures, suggesting a direct effect of neuregulin on neural tube tissue.

Neuregulins have pleiotropic effects on oligodendrocyte de-velopment. Neuregulin increases cell numbers, survival, or proliferation (Vartanian et al., 1994; Canoll et al., 1996; Raabe et al., 1997; Cannella et al., 1998; Fernandez et al., 2000; Flores et al., 2000; Sussman et al., 2000; Colognato et al., 2002; Lai and Feng, 2004). Effects of neuregulin appear to depend on environmental influences. Several studies have demonstrated either no effect or a negative effect of neuregulin on oligodendrocyte cell numbers (Vartanian et al., 1994; Raabe et al., 1997; Canoll et al., 1999; Calaora et al., 2001), and these contrasting data may reflect the conditions under which the cells were grown. Neuregulins also influence the maturation of oligodendrocyte lineage cells, either promoting or inhibiting process formation and expression of mature oligodendrocyte markers (O1 and MBP) (Vartanian et al., 1994; Canoll et al., 1996, 1999; Raabe et al., 1997). Consistent with environmental influences on neuregulin responses, the differential effects of neuregulins may reflect interaction with other signaling systems such as the integrins because contact with laminin alters neuregulin signaling and effects on oligodendrocyte survival and differentiation (Colognato et al., 2002).

The synergistic interactions of neuregulin with other ligand–receptor systems are likely to result from activation of different signal transduction pathways. Neuregulin receptors activate multiple signaling cascades, and their signaling is altered depending on receptor composition (Carpenter, 2003). Neuregulins signal through homodimerized or heterodimerized receptors of the epidermal growth factor receptor (EGFR) family, EGFR, ErbB2, ErbB3, and ErbB4 (Yarden and Sliwkowski, 2001; Murphy et al.,

2002; Carpenter, 2003; Citri et al., 2003). Studies investigating ErbB2 and ErbB3 demonstrate that neither is required for oligodendrocyte progenitor development (Park et al., 2001; Stolt et al., 2002; Kim et al., 2003; Schmucker et al., 2003). Although ErbB2 appears important in the transition from progenitor to differentiated oligodendrocyte, ErbB3 is dispensable for oligodendrocyte maturation. Because ErbB2 requires a neuregulin-binding dimerization partner to exert its effects, these results imply that ErbB4 is involved in oligodendrocyte progenitor maturation.

Materials and Methods

Neural tube explant cultures. Embryos from *erbB4* $^{+/-}$ mice (Gassmann et al., 1995) were removed at E9.5 (day of plug = day 0.5). The neural tube between the lateral ventricles and the posterior limb buds was removed in calcium- and magnesium-free MEM (Sigma, St. Louis, MO), sliced in cross sections to obtain approximately seven explants per embryo, and grown in Matrigel (BD Biosciences, Franklin Lakes, NJ) for the indicated time. Rat spinal cord cultures (E14 + 7) were grown as described previously (Sussman et al., 2002) in the presence of 10 μg/ml anti-erbB4 (antibody 72; Neomarkers, Fremont, CA) or control IgG. Cell proliferation was assayed after a 15 h pulse of 10 μM 5-bromo-2-deoxyuridine (BrdU) (Sigma). Cultures were grown in DMEM (Cellgro, Herndon, VA) supplemented with 1% FBS (HyClone, Logan, UT), 1% ITS supplement (Sigma), and 10 ng/ml PDGF-AA (Sigma). Cells were maintained at 37°C (5% CO_2 and 95% air) in a humidified incubator.

Genotyping. Genomic DNA was extracted from embryos using the DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was used to distinguish genotypes using the following primers: 5′-CTG CAC GAG ACT AGT GAG AC, 5′-TGT GCG CAG GAA CAG AGA AC, and 5′-CCG CAG GAA GGA GAG GTC. Primers, DNA, and Ex *Taq* (Takara, Fisher Scientific, Hampton, NH) were cycled on a DNA engine thermal cycler at 94°C for 2 min, 35 times at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. This yielded bands of 155 kb (wild-type) and 320 kb (knock-out).

Immunocytochemistry. Explants were fixed in 4% paraformaldehyde, labeled with monoclonal antibody (mAb) O4 or O1 hybridoma supernatant (1:5–1:2) overnight, and visualized with goat anti-mouse IgM conjugated to fluorescein isothiocyanate (MP Biomedicals, Irvine, CA). Cell cultures were labeled for 30 min. Cell death was assessed with anti-caspase 3 labeling and proliferation with anti-BrdU labeling as described previously (Sussman et al., 2002). Labeled cells were visualized and photographed under epifluorescence with a Zeiss (Thornwood, NY) microscope.

Reverse transcription-PCR. Neural tubes from mouse (CD-1; Harlan Sprague Dawley, Indianapolis, IN) or rat (Sprague Dawley; Zivic Laboratories, Pittsburgh, PA) embryos were dissected at indicated ages in calcium- and magnesium-free MEM (Sigma), meninges were removed, and dorsal ventral regions were separated along the sulcus limitans. mRNA was extracted from ventral neural tubes using Oligotex beads (Qiagen) according to the manufacturer's instructions, reverse-transcribed using Superscript (Invitrogen, San Diego, CA), and used for PCR (as above). EGFR, erbB2 (Sundaresan et al., 1998), and erbB3 (Britsch et al., 2001) were amplified as described previously. ErbB4 was amplified as described above. The identities of the amplified products were confirmed by sequencing or restriction digest using four different restriction enzymes on each product.

Data analysis. Neural tube explants were cultured individually from each embryo. The data are expressed as mean ± SEM. Statistical significance was assessed using a two-tailed *t* test.

Results

Absence of erbB4 did not affect the development of mAb O4 $^+$ oligodendrocytes

Erbb4 knock-out mice die between 10 and 12 d of gestation before the first committed oligodendrocytes are easily detectable. *In vitro,* however, embryonic neural tubes grown in explant culture
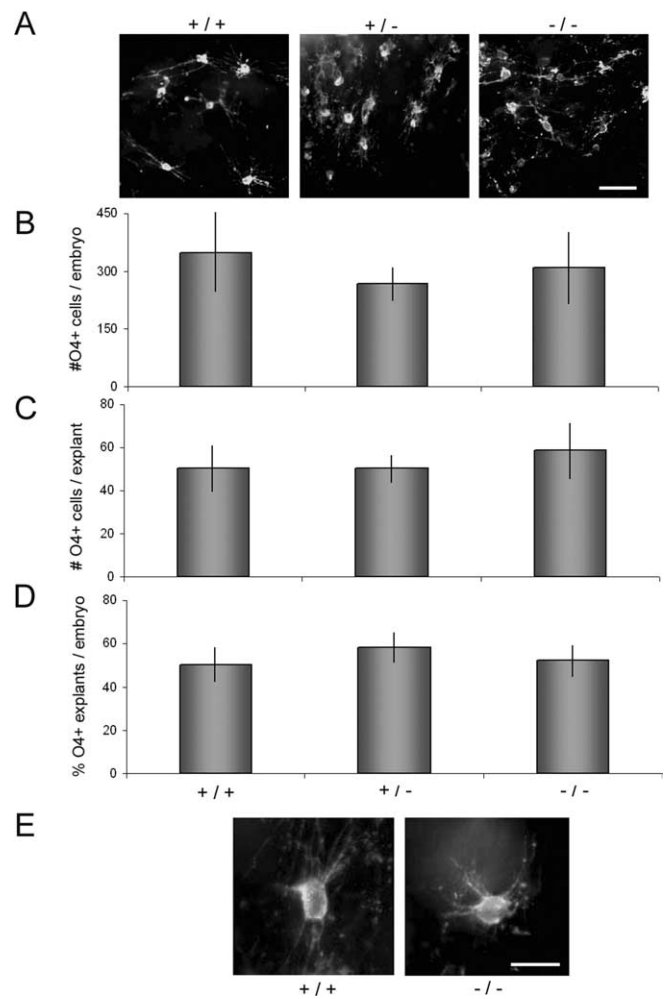

Figure 1. Oligodendrocyte development in erbB4 knock-out mice was indistinguishable from control or heterozygous littermates. ***A***, Explants were grown for 9 –10 d, fixed, and labeled with mAb O4. ***B–D***, The total number of O4 $^+$ cells per embryo (***B***) and per explant (***C***) and the percentage of positive explants per embryo (***D***) are shown as mean ± SE. ***E***, Higher-magnification view showing indistinguishable morphology (*n* = 7–16 embryos each). Scale bars: ***A***, 50 μm; ***E***, 20 μm.

develop O4 $^+$ and O1 $^+$ oligodendrocytes at approximately the same time that these cells first appear *in vivo* (Warf et al., 1991). To determine whether the oligodendrocyte lineage developed normally in the absence of ErbB4 signaling, oligodendrocyte development was assessed in explant cultures derived from E9.5 *erbB4* $^{+/+}$, *erbB4* $^{+/-}$, and *erbB4* $^{-/-}$ mice and E14 + 7 rat cultures. Similar numbers of the same-size explants were generated from mice of each genotype, and neither the total number of O4 $^+$ cells nor their distribution among explants was significantly different after 9 d (Fig. 1). That is, both the number of O4 $^+$ cells per explant and the proportion of explants containing O4 $^+$ cells were similar. Likewise, in both control and anti-ErbB4 rat cultures, oligodendrocytes developed in 20–25 clusters containing similar numbers of O4 $^+$ cells (control, 98 ± 39; anti-ErbB4, 116 ± 36; *n* = 3). The overall morphologies of O4 $^+$ cells were unaffected by anti-Erbb4 antibodies. There were no significant differences in either the number of primary processes per cell (control, 8.1 ± 0.3; anti-ErbB4, 7.9 ± 0.3; *p* = 0.6; *n* = 20 cells from three experiments) or the length of processes measured as cell diameters including processes (control, 71.6 ± 4 μm; anti-Erbb4, 70.4 ± 4 μm; *p* > 0.8; *n* = 20 cells) (Fig. 2).
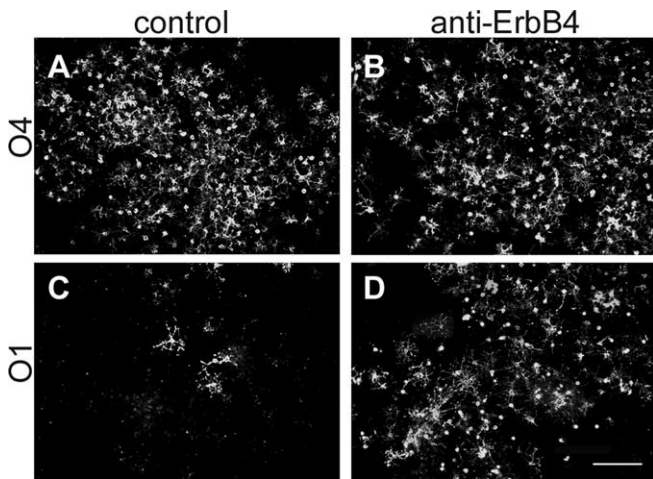
control | anti-ErbB4

O4

O1

Figure 2. Greater numbers of O1$^+$ oligodendrocytes developed in the presence of anti-erbB4 antibody, whereas the number of O4$^+$ cells was not different. Rat cultures were grown in the presence of control Ig (**A, C**) or similar concentrations of anti-erbB4 (**B, D**) and labeled with mAb O4 (**A, B**) or O1 (**C, D**). Scale bar, 75 μm.

mouse | rat
E12 | E14 | E16
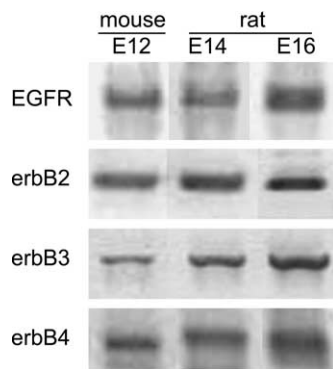
EGFR

erbB2

erbB3

erbB4

Figure 3. RT-PCR shows that all EGFR family members were expressed in the rodent ventral neural tube at the time of initial oligodendrocyte development.

All EGFR family members were present during oligodendrocyte progenitor appearance

The normal development of oligodendrocytes in the absence of ErbB4 signaling was surprising because neuregulin-1 is required for oligodendrocyte development (Vartanian et al., 1999), and this effect is independent of ErbB2 and ErbB3 (Park et al., 2001; Stolt et al., 2002; Schmucker et al., 2003). Together, these results suggest that there is functional redundancy among neuregulin receptors during early oligodendrocyte development. To assess this possibility, we examined the expression of all EGFR family members during spinal cord development.

Oligodendrocyte progenitors first arise in the ventral region of rodent embryonic neural tube at approximately E12 (mouse) or approximately E14 (rat). Reverse transcription (RT)-PCR of E12, E14, and E16 rodent ventral neural tube showed clear expression of all four EGFR family members (Fig. 3). Identities of PCR products were confirmed by sequencing or by restriction digests with four different enzymes.

Absence of erbB4 modified oligodendrocyte lineage maturation

To assess the effect of ErbB4 on maturation of oligodendrocyte progenitors, the appearance of mature (O1$^+$) oligodendrocytes
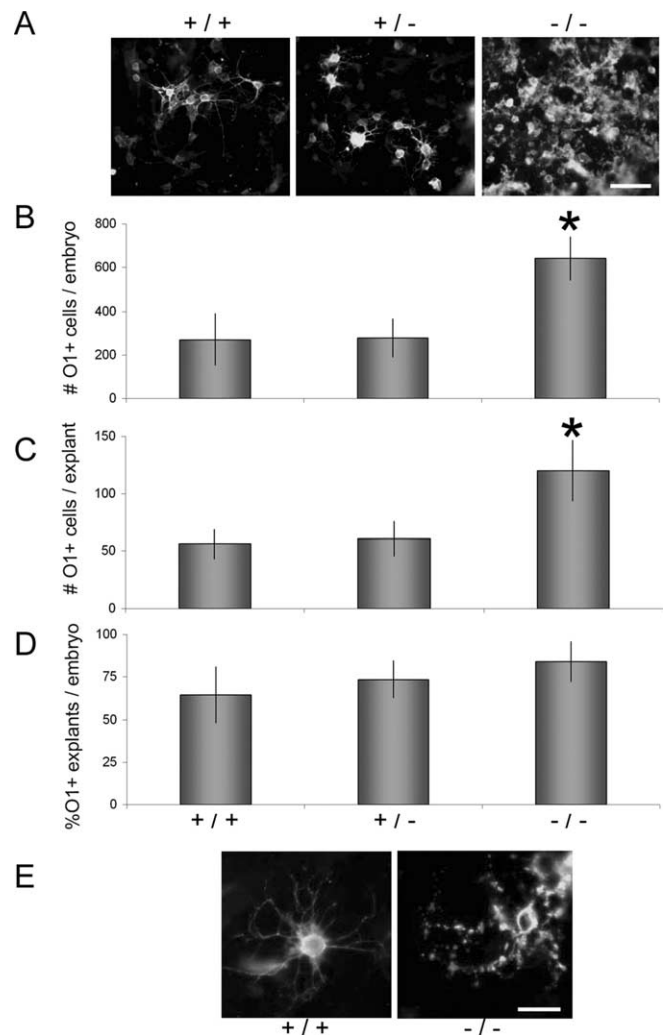

A | +/+ | +/− | −/−

Figure 4. Homozygous erbB4 knock-outs produced more O1$^+$ oligodendrocytes. **A**, Explants were grown for 12–14 d, fixed, and labeled with mAb O1. **B–D**, The total number of O1$^+$ cells per embryo (**B**) and per explant (**C**) and the percentage of positive explants per embryo (**D**) are shown as mean $\pm$ SE. **E**, Higher-magnification view showing characteristic disrupted membranous labeling in cells from erbB4$^{-/-}$ mice. *Significantly different from erbB4$^{+/+}$ or erbB4$^{+/-}$ by t test (t < 0.05; n = 3–7 embryos). Scale bars: **A**, 50 μm; **E**, 20 μm.

was examined from erbB4 knock-out mice. The first O1$^+$ cells appeared in E9.5-derived neural tube explants from mice of all erbB4 genotypes between 10 and 12 d *in vitro* (data not shown), suggesting that the overall timing of oligodendrocyte maturation was not affected by the absence of ErbB signaling. Quantitative analyses, however, revealed a significant difference in the number of mature oligodendrocytes that developed in the absence of erbB4 (Fig. 4A,B). After 12–14 d *in vitro*, the number of O1$^+$ oligodendrocytes was dramatically higher in the erbB4$^{-/-}$-derived explants (642 $\pm$ 101) compared with control animals (270 $\pm$ 119) or heterozygous animals (277 $\pm$ 89). This increase in number reflected local cell maturation. For example, the number of O1$^+$ cells per explant increased significantly, although the proportion of O1$^+$ explants was unchanged, suggesting that the rostrocaudal distribution of oligodendrocyte progenitors in the developing neural tube was not different (Fig. 4C,D). Comparable results were obtained in rat cultures. The number of O1$^+$ cells per cluster increased from 23 $\pm$ 17 in controls to 108 $\pm$ 31 in anti-ErbB4-treated cultures (n = 3).

BrdU and caspase 3 labeling indicated that neither an increase in proliferation nor a decrease in cell death explained the increase in $O1^+$ cell number. Inhibition of ErbB4 signaling did not alter BrdU or caspase 3 labeling of $O4^+$ cells, whereas the proportion of BrdU-labeled $O1^+$ cells decreased by almost 50% (14 ± 3 to $8 \pm 3\%$; $p = 0.01$; $n = 3$), and that of caspase 3-labeled $O1^+$ cells increased by 35% (19 ± 4 to $54 \pm 14\%$; $p = 0.0006$; $n = 3$) in the presence of anti-ErbB4. In addition to affecting cell number, the absence of ErbB4 signaling clearly altered the morphology of $O1^+$ cells (Fig. 4E). Together, these data indicate a critical role of ErbB4 in normal oligodendrocyte maturation.

Discussion

ErbB4 was not required for development of $O4^+$ oligodendrocyte lineage cells, suggesting involvement of promiscuous neuregulin and receptor signaling

The similar distribution of $O4^+$ cells in E9.5 $erbB4^{+/+}$, $erbB4^{+/-}$, and $erbB4^{-/-}$ mice indicates that neither the number of initial progenitor cells nor their subsequent proliferation was altered in the absence of ErbB4 signaling. The fact that both the number of $O4^+$ cells per explant and the proportion of explants that contained $O4^+$ cells were similar among genotypes implies that the founder cells of the oligodendrocyte lineage are distributed similarly along the rostrocaudal axis in the neural tubes of each genotype. Rat cultures in the presence and absence of anti-ErbB4 supported these observations and directly demonstrated the lack of an effect on $O4^+$ cell proliferation and death. These data are in contrast to the results of similar experiments on neuregulin-1-null animals in which the appearance of $O4^+$ cells was dramatically inhibited (Vartanian et al., 1999), suggesting that this effect does not require ErbB4 signaling.

Neuregulin has been proposed to regulate the morphology of maturing oligodendrocytes (Vartanian et al., 1994; Canoll et al., 1996; Raabe et al., 1997; Colognato et al., 2002); however, our data indicated no obvious differences in the number or length of cell processes or in the size of the cell bodies associated with the erbB4 genotype. The data therefore suggested that ErbB4 was expendable for normal neural tube oligodendrocyte development in terms of commitment to the lineage, survival, proliferation, and morphological development.

The finding that $O4^+$ oligodendrocytes developed in the absence of ErbB4 was surprising because neuregulin is critical for their development (Vartanian et al., 1999), and yet oligodendrocyte progenitors arise normally in the absence of the other neuregulin receptors, ErbB2 and ErbB3 (Park et al., 2001; Stolt et al., 2002; Kim et al., 2003; Schmucker et al., 2003). These observations suggest that there is redundancy among neuregulin receptors during early stages of the oligodendrocyte lineage, a possibility supported by the data presented here showing expression of all EGFR family members at the time and place of oligodendrocyte progenitor appearance in the spinal cord. Thus, expression of EGFR family members is consistent with a model of promiscuous neuregulin receptor signaling regulating early oligodendrocyte lineage development.

Absence of erbB4 modified oligodendrocyte lineage maturation

Most studies of neuregulin and receptor effects on oligodendrocytes do not distinguish between early and later effects on the lineage, although a requirement for ErbB2 specifically in oligodendrocyte maturation has been demonstrated (Park et al., 2001; Kim et al., 2003). Similarly, our data indicated a role for ErbB4 specifically in oligodendrocyte maturation; however, although

ErbB2 is required for maturation, absence of ErbB4 promoted maturation, implying that ErbB4 inhibits oligodendrocyte maturation.

Several environmental factors are known modulators of oligodendrocyte maturation and myelination. For example, galactolipids are influential in oligodendrocyte development and maturation (Bansal and Pfeiffer, 1989; Marcus et al., 2000). Similarly, fibroblast growth factor-2 potently inhibits oligodendrocyte maturation by stimulating proliferation and preventing terminal differentiation (Bansal, 2002). Whether there is any relationship between ErbB4 activity and the effects on oligodendrocyte maturation by these factors is unknown.

There are several possible mechanisms that might account for the increase in $O1^+$ cells in $erbB4^{-/-}$ neural tube explants. Enhanced progenitor proliferation would lead to elevated numbers of $O1^+$ cells. This is unlikely, however, because there was not a dramatic increase in $O4^+$ cells, cell proliferation is relatively low in $O1^+$ cells, and the BrdU incorporation studies did not demonstrate a positive effect of anti-ErbB4 on the proliferation of either cell type. During transition from progenitors to differentiated oligodendrocytes, oligodendrocyte progenitors are particularly susceptible to cell death (Barres et al., 1992). A reduction in cell death would result in increased numbers of $O1^+$ cells in the erbB4-null animals; however, this also seems unlikely because significant cell death of oligodendrocytes was not seen in wild-type- or heterozygous-derived explants or in control rat cultures. Furthermore, in $erbB4^{-/-}$ animals, many of the $O1^+$ cells had an altered morphology (Fig. 4) characteristic of dying cells (Trapp et al., 1997), suggesting that, instead of decreased cell death, there was increased cell death in the absence of erbB4. Consistent with this hypothesis, cell death of $O1^+$ cells increased in the anti-ErbB4-treated cultures >30% compared with controls. This increased cell death may result from competition among the additional cells for survival factors that become limiting as a result of increased cell number. Although the effect may not be completely autonomous to the oligodendrocyte lineage, a direct effect is likely because oligodendrocyte progenitors express all three ErbB receptors (Canoll et al., 1996; Raabe et al., 1997; Flores et al., 2000; Park et al., 2001).

The observation that ErbB4 and ErbB2 deletion results in opposite effects on oligodendrocyte development suggests divergent downstream signaling from these two receptors. In other cell types, evidence indicates that signaling downstream of ErbB2 differs from that of ErbB4, and even signaling of ErbB4 homodimers differs from that of ErbB4–ErbB2 heterodimers (Carpenter, 2003; Citri et al., 2003). Arrays of downstream signaling cascades are activated by both ErbB2 and ErbB4, including phosphatidylinositol-3-kinase/Akt, Janus kinase/signal transducer and activator of transcription, and Ras/extracellular signal-regulated kinase. The specific signaling events induced vary depending on dimer composition, a potentially highly variable parameter in the developing CNS. All EGFR members are present in the developing CNS, including the regions that generate oligodendrocytes (Fig. 3) and within cells spanning the oligodendrocyte lineage (Canoll et al., 1996; Pinkas-Kramarski et al., 1997; Raabe et al., 1997, 2004; Vartanian et al., 1997; Deadwyler et al., 2000; Flores et al., 2000; Kornblum et al., 2000; Calaora et al., 2001; Park et al., 2001; Schmucker et al., 2003). ErbB2 acts only as a heterodimer and is the preferred dimerization partner for all EGFR family members, although dimer composition is ultimately a function of both affinity and levels of expression of receptor monomers (Tzahar et al., 1996; Graus-Porta et al.,

1997). Thus, the potential for differential signaling in the oligodendrocyte lineage is extensive.

The differential responses of the oligodendrocyte lineage to selective deletion of ErbB receptors provide a potential explanation for the diversity of effects seen in response to neuregulin *in vitro*. For example, exposure to neuregulin results in diminished process formation and expression of mature markers (Canoll et al., 1996, 1999; Colognato et al., 2002). In contrast, other studies have shown that neuregulin increases oligodendrocyte maturation as assayed by process formation (Vartanian et al., 1994; Raabe et al., 1997). These apparently contradictory results may be reconciled if the conditions under which the cells are grown influence the expression of distinct ErbB receptors. Relatively high expression of ErbB2 and low expression of ErbB4 would tend to induce differentiation in response to neuregulin, whereas low expression of ErbB2 and high expression of ErbB4 would result in inhibition of differentiation. This model is consistent with the observation that neuregulin and ErbB4 expression decreases with time in culture and maturation of oligodendrocyte progenitors, whereas ErbB2 and ErbB3 levels rise or remain constant (Canoll et al., 1996; Calaora et al., 2001). In addition, this model could explain the variable effects of neuregulin depending on the availability of laminin-2 (Colognato et al., 2002).

In summary, our results demonstrate an inhibitory effect of ErbB4 on oligodendrocyte maturation and may explain the numerous dedifferentiating effects of neuregulin that have been reported. Combined with the results of others, our data suggest a model in which neuregulin has pleiotropic effects on oligodendrocyte lineage development, depending on the composition of its receptors. Neuregulin may act via all receptor dimer combinations to promote oligodendrocyte lineage commitment, survival, and proliferation. Maturation, however, may occur only through ErbB2-containing dimers, whereas it may be suppressed by ErbB4-containing dimers. Downregulation of ErbB4 would then be required to allow maturation. Although this is the most straightforward model that accounts for both maturation-promoting and -inhibiting effects of neuregulin, additional complexity, including environmental factors and the involvement of nonoligodendrocyte lineage cells, may contribute to cellular responses.

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