

The erbB2 gene is required for the development of terminally differentiated spinal cord oligodendrocytes

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Development of oligodendrocytes and the generation of myelin internodes within the spinal cord depends on regional signals derived from the notochord and axonally derived signals. Neuregulin 1 (NRG)-1, localized in the floor plate as well as in motor and sensory neurons, is necessary for normal oligodendrocyte development. Oligodendrocytes respond to NRGs by activating members of the erbB receptor tyrosine kinase family. Here, we show that erbB2 is not necessary for the early stages of oligodendrocyte precursor development, but is essential for proligerodendroblasts to differentiate into galactosylcerebroside-positive (GalC+) oligodendrocytes. In the presence of erbB2, oligodendrocyte development is normal. In the absence of

erbB2 (erbB2^{-/-}), however, oligodendrocyte development is halted at the proligerodendroblast stage with a >10-fold reduction in the number of GalC+ oligodendrocytes. ErbB2 appears to function in the transition of proligerodendroblast to oligodendrocyte by transducing a terminal differentiation signal, since there is no evidence of increased oligodendrocyte death in the absence of erbB2. Furthermore, known survival signals for oligodendrocytes increase oligodendrocyte numbers in the presence of erbB2, but fail to do so in the absence of erbB2. Of the erbB2^{-/-} oligodendrocytes that do differentiate, all fail to ensheath neurites. These data suggest that erbB2 is required for the terminal differentiation of oligodendrocytes and for development of myelin.

Introduction

Development of oligodendrocytes capable of forming myelin internodes requires several distinct environmental cues. These include early, regional, and later axonally derived signals. The initial specification of spinal cord oligodendrocyte precursor cells (OPCs)* is dependent on signals from ventral structures, such as the notochord and floor plate (Warf et al., 1991; Noll and Miller, 1993; Yu et al., 1994; Ono et al., 1995; Timsit et al., 1995). For example, notochord ablation delays OPC appearance, and notochord transplantation induces adjacent ectopic OPCs (Pringle et al., 1996; Orentas et al., 1999). One critical signaling molecule in early OPC specification appears to be Sonic hedgehog derived from floor plate and notochord (Pringle et al., 1996; Orentas et

al., 1999), which induces the basic helix-loop-helix molecules *olig-1* and *olig-2* (Lu et al., 2000; Zhou et al., 2000). After their initial appearance in the ventral ventricular zone (Noll and Miller, 1993; Timsit et al., 1995), OPCs migrate widely throughout the neuraxis (Warrington et al., 1993), mature through antigenically and morphologically distinct stages, and ultimately form myelin internodes. An early stage of OPC is an mAb A2B5 immunoreactive, bipolar (Raff et al., 1983), motile cell (Small et al., 1987; Noble et al., 1988; Armstrong et al., 1991; Osterhout et al., 1997; Simpson and Armstrong, 1999) with a mitogenic response to PGDF-AA and bFGF (Noble et al., 1988; Raff et al., 1988; Bogler et al., 1990; Gard and Pfeiffer, 1990; McKinnon et al., 1990). These A2B5+ cells mature into proligerodendroblasts, less-motile cells (Warrington et al., 1993) (characterized by surface labeling with the O4 mAb, but not antigalactosylcerebroside antibodies; Gard and Pfeiffer, 1990), and a fine arbor of processes. Proligerodendroblasts proliferate in response to a different spectrum of mitogens from A2B5+/O4- cells (Gard and Pfeiffer, 1990). Differentiation of proligerodendroblasts is accompanied by exit from the cell cycle (Hart et al., 1989; Gard and Pfeiffer, 1990) and acquisition of galactosylcerebroside expression (Raff et al., 1978; Sommer and Schachner, 1981), identified

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*Abbreviations use in this paper: CNS, central nervous system; dpc, days post conception; E, embryonic day; ES, embryonic stem; GalC+, galactosylcerebroside-positive; GFAP, glial fibrillary acidic protein; LIF, leukemia inhibitory factor; MBP, myelin basic protein; NF, neurofilament; NRG, neuregulin; OPC, oligodendrocyte precursor cell; PNS, peripheral nervous system; RT, reverse transcriptase.

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by mAb O1. Before axonal ensheathment and myelination, oligodendrocytes mature and express myelin genes such as myelin basic protein (MBP) (Sternberger et al., 1978). Maturing oligodendrocytes undergo progressive remodeling of their process arbor (Hardy and Friedrich, 1996) during a dramatic but poorly understood metamorphosis from premyelinating to myelinating cells. The multiple distinct steps in oligodendrocyte and myelin formation are regulated by distinct signaling systems. Although some of these signaling systems are beginning to be understood, control of the later stages in myelin formation is still poorly understood.

Axonally derived signals appear to be required for several aspects of oligodendrocyte and myelin formation (for review see Barres and Raff, 1999). One potential axonally derived signaling molecule critical for oligodendrocyte and myelin formation is neuregulin (NRG)-1 (for review see Carraway and Burden, 1995; Pinkas-Kramarski et al., 1998; Riese and Stern, 1998; Adlkofer and Lai, 2000). In serially passaged OPCs, NRG is mitogenic (Canoll et al., 1996, 1999). Likewise, in conjunction with cAMP and/or PDGF, NRG promotes proliferation of optic nerve OPCs (Shi et al., 1998). In long-term cultures of OPCs ("aged OPCs"), NRG transduces a potent survival signal (Fernandez et al., 2000; Flores et al., 2000) mediated through the Akt pathway (Flores et al., 2000), whereas *in vivo* inhibitors of NRG significantly reduce the number of optic nerve OPCs (Fernandez et al., 2000). By contrast, in low-density cultures of primary forebrain oligodendrocytes, NRG promotes cell spreading and process extension (Vartanian et al., 1994; Raabe et al., 1997). In spinal cord explants bearing a loss-of-function mutation in the NRG-1 gene, O4⁺ prolifodendroblasts completely fail to develop (Vartanian et al., 1999). This early defect in oligodendrocyte development in NRG-1 mutants can be reversed by the addition of recombinant NRG (Vartanian et al., 1999). Consistent with a role for NRGs during early development of the oligodendrocyte lineage, early ventral structures such as the ventral ventricular zone and floor plate of the spinal cord (Vartanian et al., 1999) and the subventricular zone of the forebrain (Vartanian et al., 1994; Corfas et al., 1995) express NRG at the time that OPCs initially arise.

Multiple factors dictate the cellular effects of NRG. These include the specific ligand, levels and repertoire of receptor expression, and the cellular context. The large number of NRG ligands arise from four known genes with multiple splice and promoter variants (for review see Fischbach and Rosen, 1997; Adlkofer and Lai, 2000). In some cases, splice variants such as the cysteine-rich domain isoform have been shown to be required for specific aspects of nervous system development (Wolpowitz et al., 2000). Receptor usage is relatively restricted for the ligands EGF (to erbB1) and NRG-4 (to erbB4) and relatively broad for NRG-1 (erbB2, erbB3, and erbB4) (for review see Yarden and Slivkowsky, 2001). Signal transduction through erbB receptors occurs as the consequence of essential ligand-induced receptor dimerizations. For example, erbB2 lacks a binding site for NRG, whereas erbB3 lacks intrinsic tyrosine kinase activity, and neither can transduce NRG signals in isolation (Riese and Stern, 1998; Yarden and Slivkowsky, 2001). By contrast, erbB4 is both capable of binding ligand and possesses an intact tyrosine kinase domain (Riese and Stern, 1998; Yarden and Slivkowsky, 2001). Although erbB3 or erbB4 will bind ligand alone, heterodimerization with erbB2

increases the affinity of the receptor complex for its ligand ~14-fold (Slivkowsky et al., 1994). ErbB2 appears to be the preferred receptor partner in most, if not all, erbB heterodimers (Graus-Porta et al., 1997), and it increases the complexity of signal transduction after NRG stimulation (Pinkas-Kramarski et al., 1997; Riese and Stern, 1998; Yarden and Slivkowsky, 2001). Furthermore, unique and overlapping docking sites for adapter proteins and cytosolic enzymes exist for individual erbBs, making them capable of transducing both common and distinct signals (Plowman et al., 1993; Wang et al., 1998a; Jones et al., 1999). The cellular context of NRG-receptor interactions is also critical for determining cellular responses. For example, neural crest cells, Schwann cell precursors, and Schwann cells all express erbB2 and erbB3 receptors for NRG. In multipotent crest cells, NRG-1 specifies a glial fate (Shah et al., 1994) and in Schwann cell precursors, NRG-1 specifies survival (Dong et al., 1995), while in Schwann cells NRG-1 specifies proliferation (Dong et al., 1995; Morrissey et al., 1995).

Here we examine the requirement for erbB2 signaling during development of the spinal cord oligodendrocyte lineage. Through a targeted disruption of the murine erbB2 gene, we demonstrate that, in striking contrast to the absence of NRG-1, the absence of erbB2 has no effect on the formation of the early oligodendrocyte lineage. However, ErbB2 does appear to be essential for normal development of later stages of the oligodendrocyte lineage. In the absence of erbB2, the development of differentiated O1⁺ oligoden-

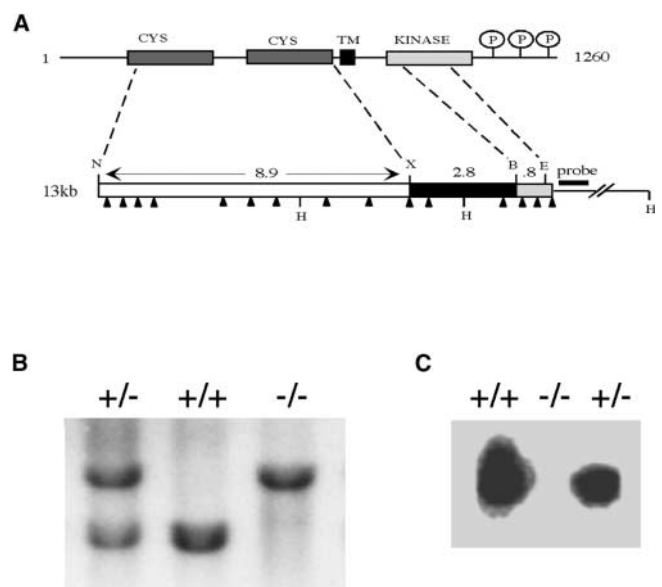


Figure 1. Targeted disruption of the murine erbB2 gene. (A) Schematic representation of the 1,260 amino acid erbB2/neu protein (top) and the 13-kb genomic fragment (bottom) used to generate the targeting construct. The shaded 2.8-kb region represents the genomic fragment containing the exon encoding the essential transmembrane domain of the erbB2 receptor protein, as well as flanking sequences encoding parts of the extracellular and cytoplasmic domains, replaced by the neo^r cassette. (B) Southern blot analysis of HindIII-restricted genomic DNA probed with a cDNA fragment found outside the targeting construct. (C) Western blot analysis of 10.5 dpc embryos homogenized in SDS-PAGE buffer, resolved by 5% SDS-PAGE, and probed for erbB2.

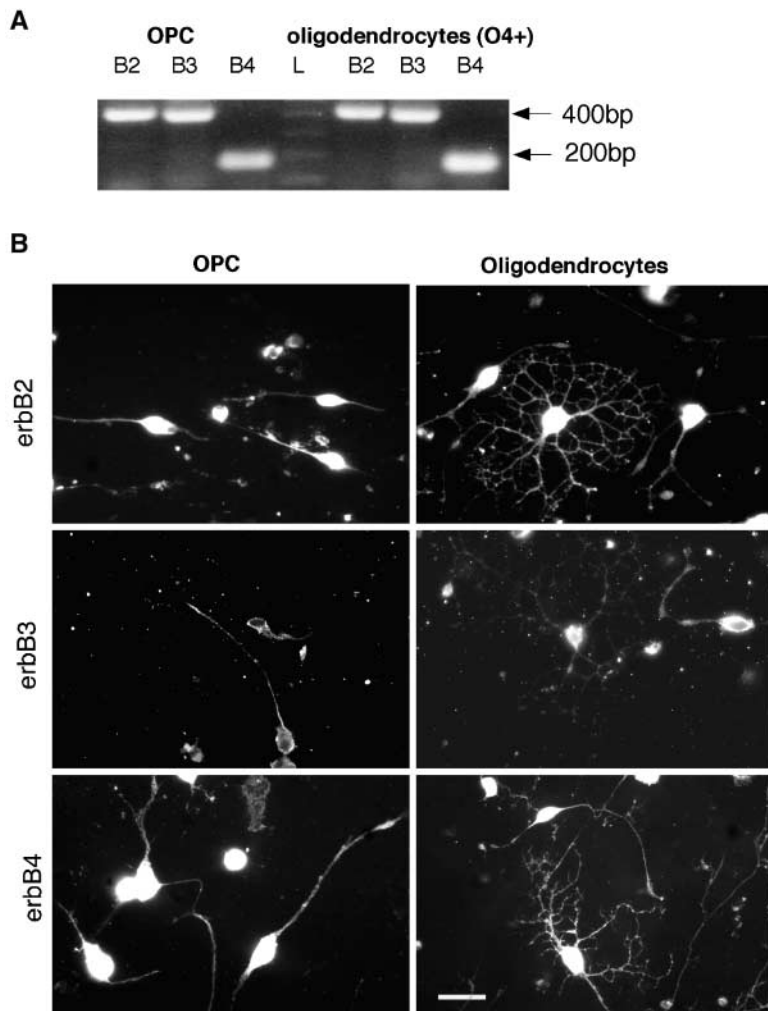


Figure 2. Developing spinal cord oligodendrocytes express the NRG receptors erbB2, erbB3, and erbB4. (A) RT-PCR of erbB2 (B2), erbB3 (B3), and erbB4 (B4) fragments from immunopanned OPC and oligodendrocyte total RNA. A2B5+/O4- OPCs or O4+ oligodendrocytes were immunopanned from newborn rat spinal cord using cell surface-specific antibodies, and then used directly for isolation of total RNA. Using receptor-specific primer pairs, mRNAs encoding each receptor were identified. (B) Immunostaining of OPCs and oligodendrocytes for erbB2, erbB3, and erbB4. OPCs and oligodendrocytes purified from rat forebrain mixed glia were cultured for 2 d in PDGF and bFGF to study OPCs, or for an additional 2 d in the absence of these growth factors (in the presence of N2 additives) to study O4+ oligodendrocytes. Both stages in the lineage express the NRG receptors erbB2, erbB3, and erbB4 throughout their soma and processes, although levels of erbB3 expression are less than erbB2 and erbB4. Bar, 30 μ m.

drocytes was dramatically deficient. In addition, the limited O1+ oligodendrocytes that develop in the absence of erbB2 signaling failed to ensheath neurites and form myelin in long-term cultures. These findings are analogous to those in the peripheral nervous system (PNS), in which an erbB2/Schwann cell conditional mutant (*Krox20-cre/+; erbB2^{fllox}/-*) shows reduced numbers of Schwann cell precursors and deficient PNS myelination (Garratt et al., 2000). The inhibition of oligodendrocyte differentiation in the absence of erbB2 appears lineage-specific, since the lack of erbB2 did not quantitatively effect astrocyte development or the extent of neurite outgrowth. Our data suggest that distinct receptor combinations are used by cells of the oligodendrocyte lineage to transduce NRG signals at distinct stages in development. Interestingly, Canoll et al. (1996, 1999) observed an increase in erbB2 expression as oligodendrocytes differentiate from OPCs. Although erbB2 signaling is not required during early OPC development, our findings suggest it is essential for later oligodendrocyte differentiation and the effective genesis of the myelin internode.

Results

Targeted disruption of the murine erbB2 gene

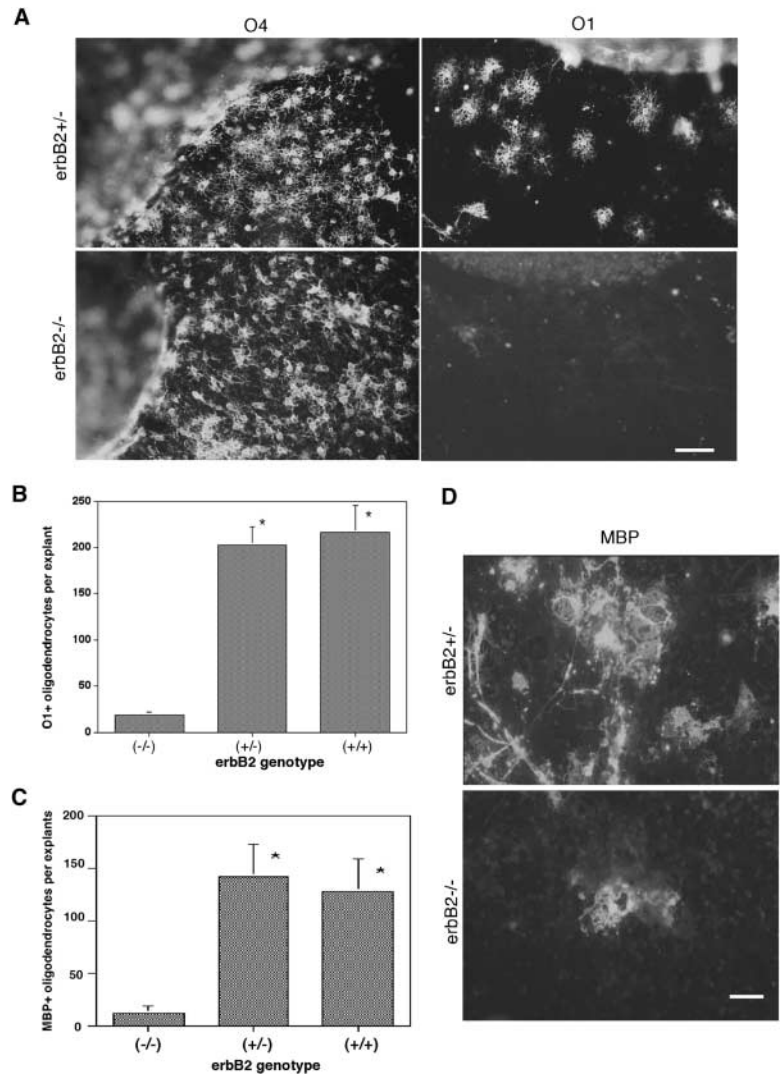
To determine the importance of erbB2 in oligodendrocyte development, a loss-of-function mutation in the erbB2 gene

was generated. To construct a targeting vector, the mouse erbB2 gene was cloned from a 129/SvJ genomic library and the PGK-1-driven neomycin resistance cassette was inserted to replace a 2–3-kb fragment containing the exon encoding the essential transmembrane domain of erbB2, as well as flanking sequences encoding portions of the extracellular and cytosolic domains (Fig. 1 A). After transfection and selection of embryonic stem (ES) cells, ES cell clones bearing the disrupted erbB2 gene were identified by Southern analysis and injected into C57BL/6 blastocysts. Chimeric founder mice were able to pass the disrupted allele through the germ line (Fig. 1 B). Homozygous null mutants failed to develop past 11.5 d postconception (dpc), as reported previously by Lee et al. (1995), and apparently express no full-length erbB2 (Fig. 1 C), whereas heterozygous animals developed normally and expressed approximately half the wild-type levels of erbB2 (Fig. 1 C).

Oligodendrocytes express erbB2, erbB3, and erbB4

Our previous studies suggested oligodendrocytes expressed erbB2 and erbB4 but not erbB3 receptors, whereas Schwann cells expressed erbB2 and erbB3 and astrocytes expressed all three receptors (Vartanian et al., 1997). However, others have shown that oligodendrocytes express erbB3 as well as erbB2 and erbB4 (Canoll et al., 1996, 1999). To reconcile these differences in the re-

Figure 3. Severe loss of O1+ oligodendrocytes in the absence of erbB2. (A) Development of O4+ oligodendrocytes is normal in the presence of erbB2 (erbB2^{+/-}), as well as in its absence (erbB2^{-/-}). The absence of erbB2 results in a severe loss of O1+ oligodendrocytes. Spinal cord explants from E9.5 mouse embryos were cultured for 10 d before fixation and assessment of oligodendrocyte development. (B) Quantitative analysis of explant data reveals a >10-fold reduction in the number of O1+ oligodendrocytes in the absence of erbB2 compared with erbB2^{+/-} and erbB2^{+/+} explants. Results are presented as \pm SEM and comparisons by ANOVA are significant at $P < 0.0001$ (indicated by asterisks). (C) Quantitative analysis of MBP+ oligodendrocytes shows results similar to those for O1. Spinal cord explants from E9.5 embryos were cultured for >30 d before fixation. In the absence of erbB2, the number of MBP+ oligodendrocytes is reduced by an order of magnitude. (D) The defect in erbB2 loss-of-function mutants is sustained throughout late stages of oligodendrocyte development. Similar to results for O1, there is a severe loss of MBP+ oligodendrocytes in the absence of erbB2. Bars, 100 μ m.



ported repertoire of erbB receptor expression in oligodendrocytes, purified cells from different stages of the rat spinal cord oligodendrocyte lineage were assayed by reverse transcriptase (RT)-PCR and immunostaining using improved antibodies to erbB3. Immunopan-purified OPCs, as well as oligodendrocytes, expressed erbB2, erbB3, and erbB4 transcripts by RT-PCR, and the levels appeared equivalent between these two stages of development (Fig. 2 A). To determine whether mouse OPCs and oligodendrocytes expressed detectable levels of erbB receptor protein, purified forebrain OPCs and oligodendrocytes were double-labeled with erbB and oligodendrocyte-specific antibodies. ErbB2 and erbB4 were strongly detected on OPCs, whereas the expression levels of erbB3 were lower (Fig. 2 B). These data, in contrast to our earlier studies, suggest that both OPCs and oligodendrocytes express mRNA and protein for erbB2, erbB3, and erbB4, all of which may contribute to NRG signaling.

A specific requirement of erbB2 for the transition from prolifodendroblasts to oligodendrocytes

To determine if erbB2 plays a specific role in oligodendrocyte development, spinal cord explant cultures from embryonic day (E)9.5 mouse embryos, which were the prod-

uct of erbB2^{+/-} matings, were prepared. E9.5 was selected for obtaining embryos, as it was 2 d before any observable loss of viability in erbB2^{-/-} embryos. Explants from individual spinal cords were cultured in single wells on a polylysine/laminin substrate. Genotyping of the embryos was carried out concurrently, but results were kept blinded until the scoring of oligodendrocyte development in the explant cultures was completed. Spinal cord explants grown for 10 d were labeled with either mAb O4 to detect prolifodendroblasts and oligodendrocytes or O1 to detect differentiated oligodendrocytes. This explant culture paradigm has been shown previously to faithfully replicate oligodendrocyte development in vivo with the appearance of O4+/O1- prolifodendroblasts at the equivalent of E15, and O4+/O1+ oligodendrocytes by E17 (Fok and Miller, 1994). In spinal cord explants from erbB2^{+/+}, erbB2^{+/-}, and erbB2^{-/-} embryos cultured for 10 d (equivalent to E20), development of the oligodendrocyte lineage to the O4+ stage appears normal and essentially indistinguishable in all three genotypes. By contrast, the number of O1+-differentiated oligodendrocytes was dramatically reduced in explants derived from erbB2^{-/-} spinal cords compared with wild-type and erbB2^{+/-}-derived explants (Fig. 3 A). Quantitative analyses of O1+ oligodendrocytes dem-

onstrated that wild-type and *erbB2*^{+/-} spinal cord explants contained a similar number of differentiated oligodendrocytes after 10 d in vitro. Wild-type-derived explants contained ~216 O1+ cells (*n* = 50 explants), whereas heterozygote-derived explants contained ~202 (*n* = 60) O1+ cells/explant (Fig. 3 B). In parallel spinal cord explants derived from *erbB2*^{-/-} littermates, the number of O1+ oligodendrocytes was dramatically diminished. Homozygote (*erbB2*^{-/-})-derived explants contained ~18 O1+ cells/explant (*n* = 46) (Fig. 3 B). The small number of O1+ oligodendrocytes in *erbB2*^{-/-}-derived explants appeared viable and were retained when the explants were maintained for >30 d (see below). These data suggest that *erbB2* transduces a signal that is not required for the development of the oligodendrocyte lineage before and including O4+ precursors, but is necessary for the subsequent appearance of O1+-differentiated oligodendrocytes.

Before myelination, oligodendrocytes undergo extensive remodeling of their process arbor (Hardy and Friedrich, 1996) and begin to express genes of myelination such as MBP. If *erbB2* signaling was required for progression from O4+/O1- progenitor cells to O4+/O1+ oligodendrocytes, the number of cells expressing myelination genes such as MBP should also be significantly diminished in the absence of *erbB2*. To test this hypothesis, spinal cord explants from E9.5 wild-type, *erbB2*^{+/-}, and *erbB2*^{-/-} embryos were grown for 30 d, (to the equivalent of approximately P20) and the number of MBP+ oligodendrocytes were compared. Explants derived from wild-type and *erbB2*^{+/-} embryos contained 10–12 times the number of MBP+ oligodendrocytes compared with *erbB2*^{-/-} explants (Fig. 3, C and D). This result is consistent with the hypothesis that *erbB2* is required for development of differentiated O1+ oligodendrocytes.

A predominant role for ErbB2 in oligodendrocyte development: neurite outgrowth and astrocyte development appear normal in the absence of *erbB2*

Other neural cell types express *erbB* receptors (Pinkas-Kramarski et al., 1994; Corfas et al., 1995). To test whether *erbB2* was required for the differentiation of all neural cell types, spinal cord explants from E9.5 wild-type, *erbB2*^{+/-}, and *erbB2*^{-/-} embryos were assayed for neurite outgrowth and astrocyte development after 10 d in vitro. Explants were fixed and stained with antibodies to neurofilament (NF) to detect neurites, or to glial fibrillary acidic protein (GFAP) to detect astrocytes. Neurite outgrowth was extensive and comparable in explants from wild-type, *erbB2*^{+/-}, and *erbB2*^{-/-} embryos (Fig. 4). Astrocytes express the NRG receptors *erbB2*, *erbB3*, and *erbB4* (Pinkas-Kramarski et al., 1994; Vartanian et al., 1997); however, in contrast to the effects on the oligodendrocyte lineage, the number and morphology of GFAP+ astrocytes appeared similar between wild-type-, *erbB2*^{+/-}-, and *erbB2*^{-/-}-derived explants (Fig. 4). These data indicate that *erbB2* signaling is not essential for astrocyte development, although the presence of astrocyte mitogens in culture serum supplements may compensate for the absence of *erbB2* signaling. These data suggest that development of the oligodendrocyte lineage is most dependent on *erbB2* signaling.

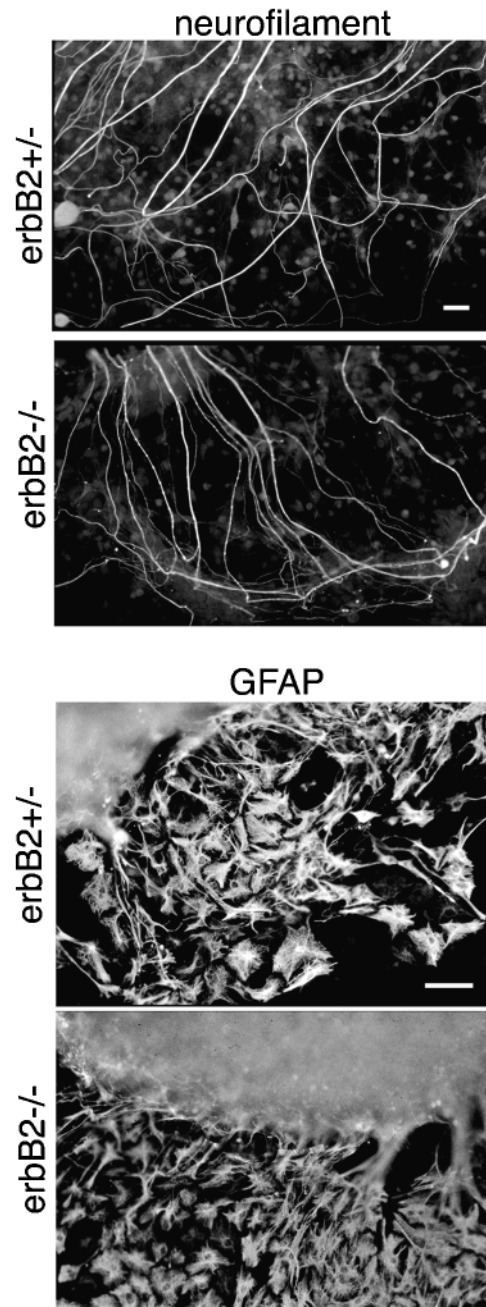


Figure 4. **The absence of *erbB2* does not impact the extent of neurite outgrowth or astrocyte development.** Spinal cord explants from E9.5 embryos were cultured for 10 d in vitro and then studied for neurite outgrowth and astrocyte development. Similar results were obtained for *erbB2*^{+/-} and *erbB2*^{+/+} explants and for simplicity *erbB2*^{+/-} are shown. Neurite outgrowth in *erbB2*^{-/-} spinal cord explants, assessed by NF staining, was not apparently different from *erbB2*^{+/-} explants. The quantity and morphology of astrocytes assessed by GFAP immunostaining in *erbB2*^{-/-} spinal cord explants is similar to that of *erbB2*^{+/-} explants. Bars: (neurofilament) 20 μ m; (GFAP) 100 μ m.

Excess NRG does not rescue development of oligodendrocytes in the absence of *erbB2*

The *erbB2* receptor is required for the generation of high affinity NRG binding sites in *erbB2/3* or *erbB2/4* heterodimers. Although *erbB2* is required for high affinity ligand binding, *erbB4* homodimers as well as *erbB3/erbB4*

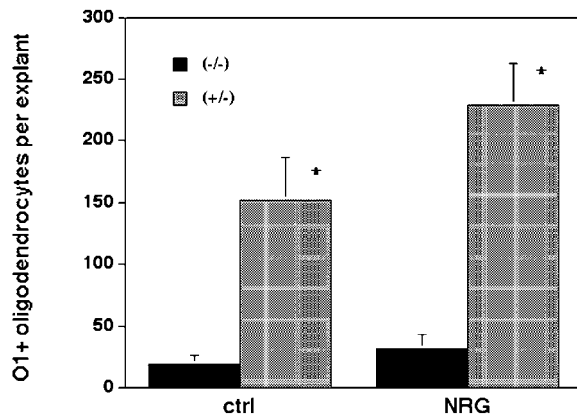


Figure 5. Failed rescue of O1+ oligodendrocytes by addition of recombinant NRG. Spinal cord explants from E9.5 embryos were cultured for 10 d in the persistent presence or absence of 20 nM recombinant NRG-1. Supramaximal concentrations of NRG-1 are not sufficient to promote progression from prolignodendrocyte to O1+ oligodendrocyte in *erbB2*^{-/-} mutants. Comparisons by ANOVA showed statistical differences between *erbB2*^{-/-} and *erbB2*^{+/-} in both control (ctrl) and NRG treatment groups; $P < 0.0001$ (indicated by asterisks). There was no statistical difference in *erbB2*^{-/-} explants between control and NRG treatment.

heterodimers are capable of transducing NRG-mediated signals (Plowman et al., 1993; Riese et al., 1995; Wang et al., 1998a). Thus, the effect of the *erbB2* loss-of-function mutation on oligodendrocyte development may reflect either a reduction in the affinity of the NRG receptor complex for NRG-1, or a lack of specific intracellular signaling events dictated by phosphorylation of the *erbB2* cytosolic domain. To determine whether the failure of oligodendrocyte differentiation simply reflected a reduction in the affinity of ligand binding, spinal cord explants were grown in the presence of high concentrations of exogenous NRG. Alternating spinal cord segments from embryos of each genotype were divided into two groups, one of which received 20 nM NRG (four times the reported K_D of NRG for *erbB4* alone; Culouscou et al., 1993) for the entire culture period, whereas the other received control vehicle alone. After 10 d, exogenous NRG-1 significantly increased the number of O1+ oligodendrocytes in wild-type and *erbB2*^{+/-} explants (Fig. 5). By contrast, exogenous NRG at supramaximal concentrations did not increase the numbers of O1+ oligodendrocytes in explants from *erbB2*^{-/-} embryos (Fig. 5). The NRG-induced increase in oligodendrocytes in *erbB2*-containing explants suggests that endogenous NRG concentrations are limiting in normal embryos (Fig. 5) and, in conjunction with our previous work in the NRG-1 null mutant (Vartanian et al., 1999), indicates that exogenous NRG has access to explant cells. The absence of response in explants lacking *erbB2* indicates that activation of *erbB3/erbB4* alone is not sufficient for production of O1+ oligodendrocytes. This finding supports the concept that *erbB2* generates a specific signal necessary for the appearance of O1+ oligodendrocytes.

Lack of *erbB2* signaling does not enhance cell death in the oligodendrocyte lineage

The lack of differentiated, galactosylcerebroside-positive (O1+) oligodendrocytes in explants derived from animals

Table I. Percentage of cells with pyknotic nuclei for O4+ and O1+ oligodendrocytes

	O4+	O1+
	%	%
<i>ErbB2</i> ^{+/+}	1.7	3.5
<i>ErbB2</i> ^{+/-}	1.5	2.7
<i>ErbB2</i> ^{-/-}	1.5	2.7

The number of O4+ or O1+ oligodendrocytes with pyknotic nuclei were counted and expressed as the percentage of the total number of O4+ or O1+ oligodendrocytes per explant. ANOVA revealed no statistical differences between genotypes.

lacking *erbB2* may reflect either the requirement for an *erbB2*-specific signal for differentiation or a reduction in a survival signal. To determine whether *erbB2* mediated a specific survival signal for newly generated oligodendrocytes, the levels of cell death at different developmental stages of the oligodendrocyte lineage were compared in explants derived from embryos of different genotypes. In each case, the total number of oligodendrocyte lineage cells and the proportion of apoptotic cells identified by nuclear pyknosis using propidium iodide labeling (Fig. 6 C) were quantified. Explants derived from *erbB2*^{-/-} embryos showed no increase in the number of apoptotic O4+ prolignodendroblasts or differentiated O1+ oligodendrocytes compared with those derived from *erbB2*^{+/-} and *erbB2*^{+/+} embryos (Fig. 6). Furthermore, even though *erbB2*^{-/-}-derived explants contained far fewer oligodendrocytes, the ratio of apoptotic oligodendrocytes to total oligodendrocytes did not vary between explants derived from different *erbB* genotypes (Table I).

To determine whether known oligodendrocyte lineage survival factors could compensate for the lack of *erbB2* signaling, explants were grown in the presence of exogenous survival factors not requiring *erbB2* for signal transduction. The best characterized mitogens and survival factors for newly formed oligodendrocytes are PDGF and leukemia inhibitory factor (LIF), each of which activate distinct signaling pathways (Barres et al., 1992a,b; Mayer et al., 1994). PDGF acts through the PDGF- α receptor in oligodendrocyte lineage cells, whereas LIF signals are mediated through the gp130 cytokine receptor. Explants of embryonic spinal cords derived from *erbB2*^{+/-} matings were grown in the presence of exogenous PDGF or LIF. In the presence of PDGF-AA, there was an ~30% increase in the number of O1+ oligodendrocytes in *erbB2*-containing explants compared with controls (Fig. 7 A). These findings are consistent with the hypothesis that survival and mitogenic factors are at submaximal concentrations both in vitro and in vivo (Raff et al., 1998). In explants derived from *erbB2*^{-/-} embryos, there was also a small increase in the number of O1+ oligodendrocytes in explants grown in the presence of PDGF compared with controls (Fig. 7 A). Even in the presence of exogenous PDGF, however, explants derived from *erbB2*^{-/-} embryos had fewer than one tenth the number of O1+ oligodendrocytes than *erbB2*^{+/-} or *erbB2*^{+/+} explants, indicating that PDGF was unable to compensate for the lack of *erbB2* signaling in the development of spinal cord oligodendrocytes. Similar results were obtained with LIF. In the presence of LIF, the number of O1+ oligodendrocytes nearly doubled in spinal cord explants from *erbB2*^{+/+} or *erbB2*^{+/-} mice. In explants derived from *erbB2*^{-/-} embryos there was

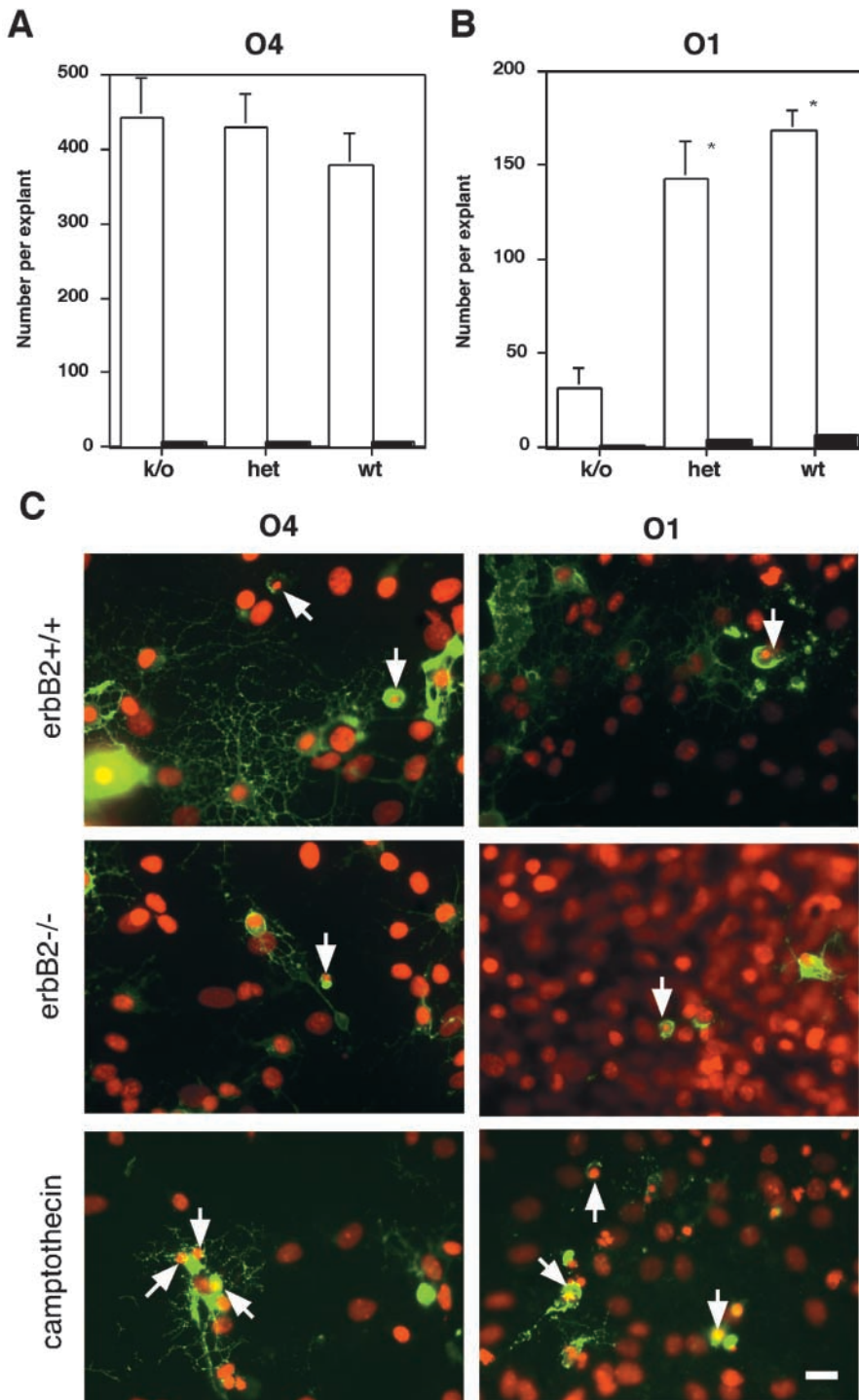


Figure 6. Lack of erbB2 signaling does not result in increased cell death of OPCs or oligodendrocytes in spinal cord explants. Spinal cord explants from erbB2^{-/-}, erbB2^{+/-}, and erbB2^{+/+} E9.5 mouse embryos were cultured for 9 d and double-labeled with propidium iodide, and the stage-specific markers O4 (A) or O1 (B) and the number of apoptotic cells identified by nuclear pyknosis. (A) Explants from all three genotypes contain similar numbers of O4+ cells, and a similar proportion of labeled cells had pyknotic nuclei. (B) Explants derived from knockout animals contain dramatically decreased numbers of O1+ oligodendrocytes compared with hets and wild-type-derived explants. The proportion of O1+ cells with pyknotic nuclei is not different between explants derived from embryos of different genotypes. (C) Examples of O4+ and O1+ cells with characteristic pyknotic nuclei (arrows) in explant cultures derived from wild-type and erbB2^{-/-} embryos. These fields were selected because they contained pyknotic cells of the oligodendrocyte lineage and are not representative of the whole culture. Most fields show no apoptotic oligodendrocytes. As a positive control for detecting changes in nuclear morphology consistent with cell death, wild-type cultures were treated overnight with camptothecin (10 μ M) as described in Materials and methods. In camptothecin-treated cultures, numerous oligodendrocytes with pyknotic nuclei can be identified (arrows). Bar, 20 μ m.

no significant increase in the number of O1+ oligodendrocytes in explants grown in the presence of LIF compared with controls. Together, the absence of increase in cell death and the failure of known survival factors to compensate for the lack of erbB2 signaling suggest that erbB2 transduces an essential signal for terminal differentiation of oligodendrocytes rather than for their survival.

Deficient development of O1+ oligodendrocytes in low density cultures from erbB2^{-/-} spinal cord explants

Although the above data are consistent with a cell autonomous function of erbB2 in oligodendrocyte development,

they do not distinguish cell-autonomous from cell-extrinsic effects. To reduce the relative contribution of factors secreted by other cell types that might influence oligodendrocyte development, we generated low-density cell cultures (final density ~ 120 cells/cm²) from spinal cord explants that were enriched for oligodendrocytes (see Materials and methods). Cultures were then studied for development of O1+ oligodendrocytes. Morphologically, cultures contained ~ 60 – 70% cells of the oligodendrocyte lineage. In the presence of erbB2 (erbB2^{+/+} and erbB2^{+/-}) there were ~ 5 O1+ oligodendrocytes per well. In contrast, in the absence of erbB2, O1+ oligodendrocytes averaged less than 1 cell/

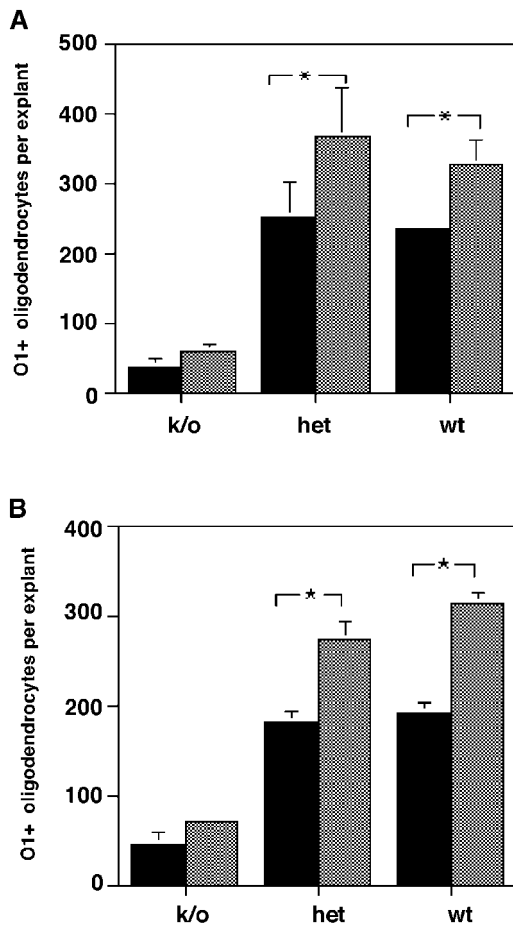


Figure 7. Ligands that transduce survival signals to oligodendrocytes through distinct transmembrane receptors do not rescue the *erbB2*^{-/-} phenotype. (A) PDGF-AA does not rescue GalC⁺ oligodendrocytes in *erbB2* loss-of-function mutants. Similar to the results with exogenous NRG, addition of recombinant PDGF was unable to induce GalC⁺ oligodendrocytes in *erbB2*^{-/-} explants. By ANOVA, there was a statistical difference between *erbB2*^{-/-} and *erbB2*^{+/-}, $P < 0.0001$ (indicated by asterisks), but not between control and PDGF treatment for *erbB2*^{-/-} explants. (B) LIF does not rescue the GalC⁺ oligodendrocytes in the *erbB2* loss-of-function mutants. By ANOVA, there was a statistical difference ($P < 0.0001$) between control and LIF treatment groups for the *erbB2*^{+/-} and the *erbB2*^{+/+} genotypes. In addition there was a statistical difference in the number of GalC⁺ (O1⁺) oligodendrocytes between *erbB2*^{-/-} and the *erbB2*^{+/-} or *erbB2*^{+/+} genotypes regardless of LIF treatment. There was no statistical difference between control and LIF treatment in the *erbB2*^{-/-} genotype group ($P = 0.4$).

well (Fig. 8). Although these results do not prove a cell autonomous effect of *erbB2* on oligodendrocyte development, they reduce the likelihood that *erbB2* is influencing oligodendrocyte development by an extrinsic mechanism.

Ensheathment of neurites by oligodendrocytes is deficient in the absence of *erbB2*

The final stages of oligodendrocyte maturation involve spiraling of the leading edge of an oligodendrocyte process around an axon to form the myelin internode. Since *erbB2* appears important in the later stages of oligodendrocyte lineage development, its requirement in the ensheathment of neurites was examined. The small number of oligodendro-

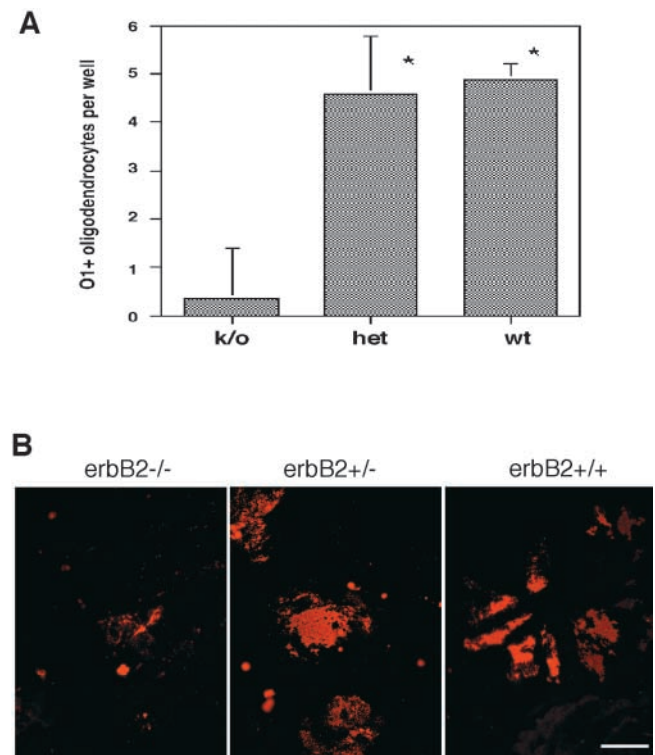


Figure 8. Low-density cultures from *erbB2*^{-/-} explants have significantly decreased numbers of O1⁺ oligodendrocytes compared with cells expressing *erbB2*. Spinal cord explants from E9.5 mouse embryos (*erbB2*^{+/+}, $n = 8$; *erbB2*^{+/-}, $n = 17$; *erbB2*^{-/-}, $n = 6$) were cultured for 8 d in 24-well plates then rotated overnight at 200 rpm. Supernatants containing dislodged cells were plated in 8-well chamber slides and cultured for 4 d in the presence of PDGF and bFGF. The medium was then changed to DME with N2 additives, PDGF (0.5 ng/ml), and 1% FBS for an additional 36 h before immunostaining with the O1 mAb. (A) There is a significant ($P = 0.047$ for *erbB2*^{+/+}, *erbB2*^{-/-} comparison, and $P = 0.036$ for *erbB2*^{+/-}, *erbB2*^{-/-} comparison) reduction in the number of O1⁺ oligodendrocytes in the absence of *erbB2*. (B) Morphology of O1⁺ oligodendrocytes in low density culture. O1⁺ oligodendrocytes expressing *erbB2* have large lamellipodia, characteristic of differentiated oligodendrocytes. In contrast, O1⁺ oligodendrocytes from *erbB2*^{-/-} explants have a reduced process extension. Bar, 100 μ m.

cytes that differentiated to maturity in *erbB2*^{-/-} mutant explants allowed analyses of myelination in long-term explant cultures. Explants from E9.5 wild-type, *erbB2*^{+/-}, and *erbB2*^{-/-} embryos were grown for 27–30 d under myelinating conditions and segments resembling internodes were identified morphologically by labeling for MBP. Since only small numbers of oligodendrocytes differentiate in *erbB2*^{-/-} explants, the number of separate experiments was increased to enhance data significance. In wild-type and *erbB2*^{+/-} explant cultures, numerous MBP⁺ segments surrounding neurites (Fig. 9) developed in all explants. By contrast, although some MBP⁺ oligodendrocytes were detectable in explants derived from embryos lacking *erbB2*, and these cells did extend processes that contacted neurites, they failed to form MBP⁺ segments surrounding neurites (Fig. 9). The absence of MBP⁺ segments in the *erbB2*^{-/-} explants was not simply related to the reduction in terminally differentiated oligodendrocytes, since wild-type and *erbB2*^{+/-} explants generate ~2–3 internode-like segments per oligoden-

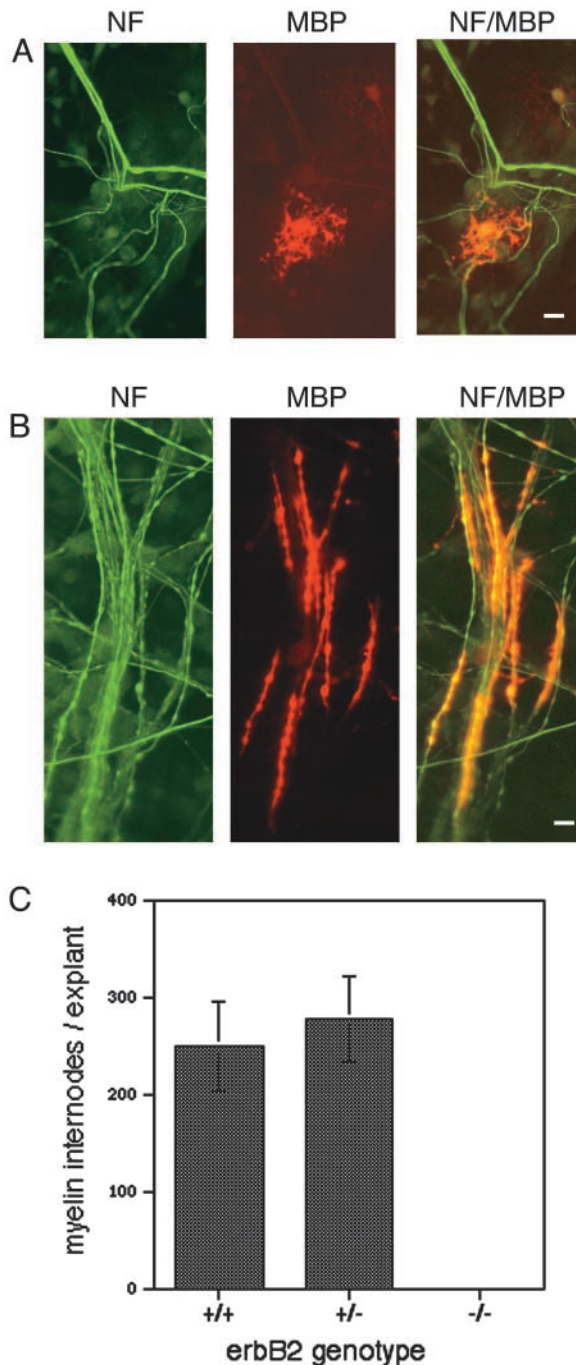


Figure 9. Deficient axonal ensheathment in erbB2 loss-of-function mutants. Spinal cord explants derived from $erbB2^{+/-}$ and $erbB2^{-/-}$ mice at E9.5 were cultured for 30 d and then double-labeled for NF and MBP. Single exposures for NF (green), MBP (red), and double exposures for NF/MBP are shown. (A) In spinal cord explants from $erbB2^{-/-}$ mice, some oligodendrocytes mature to the MBP+ stage. However, even when surrounded by neurites, these cells fail to ensheath neurites or form internodes morphologically. (B) Spinal cord explants from $erbB2^{+/-}$ mice show numerous myelin internodes morphologically. The cell body of the oligodendrocyte from which this cluster of internodes is derived is only faintly visible, since immunoreactivity to MBP after myelination is diminished in the soma and restricted to internodes. (C) Quantitation of the number of myelin internodes per explant from $erbB2^{+/+}$, $erbB2^{+/-}$, and $erbB2^{-/-}$ mice. In the presence of erbB2, numerous myelin internodes are identified, whereas in the absence of erbB2, none were observed. Bars, 20 μ m.

drocyte, and in $erbB2^{-/-}$ explants, based on the number of differentiated oligodendrocytes, 40–60 MBP+ segments per explant would have been expected.

Together, these data suggest that signals transduced through the erbB2 receptor are required at distinct stages during the development of the spinal cord oligodendrocyte lineage. In the absence of erbB2 there is a dramatic reduction in the number of terminally differentiated oligodendrocytes. $erbB2^{-/-}$ oligodendrocytes that do develop fail to ensheath neurites, consistent with the notion that myelination in the central nervous system (CNS) requires a signal, presumably axonal in origin, that activates erbB2 in oligodendrocytes.

Discussion

There is an absolute requirement for NRG-1 in the formation of O4+ proligerodendroblasts in cultures of murine spinal cord (Vartanian et al., 1999). Here we show that spinal cord oligodendrocytes and OPCs express erbB2, erbB3, and erbB4, and also examine the function of erbB2 in spinal cord oligodendrocyte development and myelination. In striking contrast to the lack of spinal cord oligodendrocyte precursor development in the absence of NRG-1 (Vartanian et al., 1999), O4+ proligerodendroblasts develop normally in the absence of erbB2. Wild-type and $erbB2^{+/-}$ spinal cord explants develop abundant numbers of differentiated O1+ oligodendrocytes, whereas in $erbB2^{-/-}$ spinal cord explants, development is halted at the proligerodendroblast stage and very few O1+ oligodendrocytes develop. Similarly, in wild-type and $erbB2^{+/-}$ explants MBP+ oligodendrocytes are abundant, whereas $erbB2^{-/-}$ explants contained relatively few mature MBP+ oligodendrocytes. Finally, wild-type or $erbB2^{+/-}$ oligodendrocytes generate numerous initiator processes and ensheathment tubes as described previously (Hardy and Friedrich, 1996). By contrast, although neurite and astrocyte development appear normal in $erbB2^{-/-}$ explants, myelination is completely absent in $erbB2^{-/-}$ explants. $erbB2^{-/-}$ oligodendrocytes contact neurites, but do not form initiator processes or ensheathment tubes, suggesting a critical role for erbB2 signaling in axonal ensheathment and myelination.

NRG-mediated signals transduced through diverse erbB receptors are required for distinct stages in oligodendrocyte development

Although the precise function of NRGs during the early development of OPCs is unclear, during later stages of oligodendrocyte development there is a clear role for NRG mediated signals. NRGs appear to be required for the survival of established OPCs (Fernandez et al., 2000; Flores et al., 2000). In spinal cord explants grown in the presence of a soluble inhibitor of NRG, O4+ proligerodendroblasts do not develop (Vartanian et al., 1999). Similarly, O4+ proligerodendroblasts do not develop in spinal cord explants from NRG-1-deficient mice, and this can be reversed by the addition of recombinant NRG-1 (Vartanian et al., 1999). It was thus surprising that in the erbB2 null mouse there is normal development of O4+ proligerodendroblasts in explants derived from erbB2 null embryos. These findings suggest that in oligodendrocyte lineage cells expressing erbB2,

erbB3, and erbB4, signals derived from the erbB2 cytosolic domain are not essential for the generation of the lineage before the appearance of O4+ proligerodendroblasts. Similarly, the normal development of O4+ proligerodendroblasts in erbB2^{-/-} explants suggests that NRG receptors other than erbB2 are capable of transducing the signals necessary for the formation of these cells. Signals transduced by erbB2 do appear to be essential for the appearance of differentiated O1+ oligodendrocytes, however, since these cells fail to develop in erbB2^{-/-} explants despite the presence of high concentrations of insulin, PDGF-AA, or LIF. These observations strongly suggest that erbB2 transduces a distinct signal required for the appearance of terminal-differentiated oligodendrocytes and myelination.

The ability of individual erbB receptors to transduce distinct intracellular signals is well established in transformed cell lines (for review see Pinkas-Kramarski et al., 1998; Riese and Stern, 1998; Yarden and Sliwkowski, 2001). For example, the cellular response, patterns of receptor phosphorylation, and recruitment of intracellular signaling proteins varies between erbB4 homodimers stimulated with different NRGs (Sweeney et al., 2000). Secondly, receptor usage is critically important to signal diversification (Pinkas-Kramarski et al., 1996). There is evidence that individual class I receptors transduce overlapping and nonoverlapping intracellular signals. For example, analyses of the potential SH2 docking sites on erbB receptors demonstrated both unique as well as homologous sites, indicating the potential for overlapping and nonoverlapping signaling through individual receptors (Carraway and Cantley, 1994). Furthermore, in addition to transducing signals common to other erbB receptors, erbB4 appears to activate distinct signaling pathways (Jones et al., 1999; Zhao et al., 1999; Huang et al., 2000). In conjunction with our previous results, the current data from the erbB2^{-/-} mouse shows that in primary cells, the NRG receptor erbB2 is likely transducing distinct intracellular signals necessary for the appearance of differentiated oligodendrocytes.

Several lines of evidence suggest that the signaling through the erbB2 receptor mediates a differentiation rather than survival signal for emerging oligodendrocytes. For example, there is no apparent increase in cell death in the oligodendrocyte lineage in the absence of erbB2 signaling and addition of known growth/survival factors fails to reverse this cellular phenotype. It remains possible that interactions between different classes of receptors are required for the differentiation of oligodendrocytes. The inability of LIF to enhance survival of erbB2^{-/-} oligodendrocytes may reflect interactions between erbB2 and the LIF receptor gp130. In prostate cancer cells overexpressing erbB2, IL-6 induces a ligand-dependent association of erbB2 with the gp130 component of the IL-6 receptor and is required for mitogen-activated protein kinase activation by IL-6 (Qiu et al., 1998). The current work does not exclude such a possibility. Furthermore, although it seems likely that the lack of differentiated oligodendrocytes in erbB2^{-/-}-derived explants reflects cell-intrinsic signaling in OPCs, astrocytes and neurons express NRG receptors and the effects on oligodendrocyte development may result from an erbB2-regulated synthesis of an inhibitor of oligodendrocyte differentiation in these other cell types.

Axonally derived signals are necessary for oligodendrocyte development and myelination

By the time OPCs populate white matter tracts axonogenesis is completed, and thus oligodendrocyte differentiation and myelination follow axonogenesis. This timing is consistent with the inhibitory influence of CNS myelin on axonal growth (Filbin et al., 1990; Bandtlow and Schwab, 2000; Chen et al., 2000; GrandPre et al., 2000). The critical importance of axonal-derived signaling at multiple stages of oligodendrocyte development is inferred from several experimental studies. During development, oligodendrocyte numbers are closely matched to the number of axons. Increasing axon numbers increases the number of OPCs in parallel (Burne et al., 1996). Removal of axons by neonatal enucleation or optic nerve transection (Fulcrand and Privat, 1977; Privat et al., 1981; David et al., 1984; Valat et al., 1988; Ueda et al., 1999) significantly reduces the number of optic nerve oligodendrocytes; although, some develop normally in the absence of axons (Ueda et al., 1999) as they do in the absence of erbB2. Thus, in neonatal mammals, axonal signals appear to induce proliferation of OPCs and survival of oligodendrocytes (Fulcrand and Privat, 1977; Privat et al., 1981; David et al., 1984; Valat et al., 1988; Barres et al., 1992a; Barres and Raff, 1993). In the postnatal and adult mammalian CNS, however, axonal signals appear to serve a different purpose. Specific signals from the axon are required for myelination since other elements such as dendrites do not undergo myelination (Lubetzki et al., 1993). In the adult, axotomy or neurectomy results in axonal degeneration and concomitant myelin degradation in the absence of significant oligodendrocyte apoptosis (Fulcrand and Privat, 1977; Stoll et al., 1989; Wisniewski, 1983; Butt and Kirvell, 1996). The physical presence of axons is sufficient to maintain myelin internodes. In the C57BL/Ola mouse, a spontaneously occurring mutant with abnormally prolonged Wallerian degeneration (Brown et al., 1991; Ludwin and Bisby, 1992), distal axonal segments, and oligodendrocyte-myelin units remain intact for up to 4 wk after axotomy or transection compared with 1–2 d for normal controls (Ludwin and Bisby, 1992). The ability of a distal, posttransection axonal segment to provide trophic influences is not limited to oligodendrocytes and Schwann cells. The neuromuscular junction, which also requires axonal-derived NRG for its development (Fischbach and Rosen, 1997; Sandrock et al., 1997; Fromm and Burden, 1998; Wolpowitz et al., 2000), is also maintained for prolonged periods of time after transection in the C57BL/Ola mouse (Ribchester et al., 1995).

At least two candidate molecules have been described that might mediate the axonally derived signals which influence oligodendrocyte development: Jagged1 and NRG. Jagged1 acting through Notch receptors on OPCs is a potent inhibitor of oligodendrocyte differentiation in the optic nerve (Wang et al., 1998b). Jagged1 is expressed on axons and its levels fall as myelination proceeds, suggesting it may be an important signal inhibiting differentiation or myelination until the appropriate developmental stage. Therefore, it is possible that the defect in terminal differentiation of oligodendrocytes in the absence of erbB2 could be the consequence of up-regulation of Jagged1 or related ligands in spinal cord neurons.

NRG is a candidate axonal signal for oligodendrocyte formation and myelination

In the developing nervous system, NRG is localized to neurons and axons (Corfas et al., 1995; Dong et al., 1995; Jo et al., 1995; Sandrock et al., 1995; Trachtenberg and Thompson, 1996; Loeb et al., 1998) as well as floor plate structures in early CNS development (Corfas et al., 1995; Vartanian et al., 1994, 1999). In vitro studies demonstrate that axonally derived NRG in its membrane-associated form is capable of activating erbB receptors (Vartanian et al., 1997), indicating that axonally associated NRG can mediate its effects as a consequence of direct axonal–glial interactions.

In the PNS, NRG plays multiple distinct roles in Schwann cell development. Early in development, NRG induces a glial fate in neural crest precursors (Shah et al., 1994). In committed Schwann cell precursors axonally derived NRG serves as a survival factor (Dong et al., 1995). In mature Schwann cells axonally derived NRG is responsible for all or most of the survival and mitogenic effects of axons on the Schwann cell lineage. For example, antibodies to erbB2 inhibit axon-induced proliferation of Schwann cells (Morrissey et al., 1995). Similarly, addition of exogenous NRG markedly reduced Schwann cell apoptosis during normal development or as the consequence of axotomy (Grin-span et al., 1996; Trachtenberg and Thompson, 1996). Furthermore, in mice bearing mutations in NRG-1 or the NRG receptors erbB2 and erbB3, there is a profound reduction in the number of Schwann cells (Lee et al., 1995; Meyer and Birchmeier, 1995; Erickson et al., 1997; Riethmacher et al., 1997; Morris et al., 1999), as well as effects on axonal fasciculation and target finding (Morris et al., 1999; Wolpowitz et al., 2000). A conditional erbB2 mutation in promyelinating and myelinating Schwann cells results in diminished PNS myelination (Garratt et al., 2000).

Likewise, our data suggest that in the CNS, NRG plays multiple distinct roles in oligodendrocyte development which are mediated by distinct erbB receptors. NRG, possibly derived from ventral midline structures, but not the erbB2 receptor, is required for oligodendrocyte precursors to develop to O4+ cells (Vartanian et al., 1999). NRG, possibly derived from axons, and the erbB2 receptor are required for differentiation of O4+ precursors to oligodendrocytes and subsequent steps in myelination. Although the requirement of erbB2 in oligodendrocyte development and ensheathment of axons may be cell autonomous, it remains possible that mutations of erbB2 influence oligodendrocyte development indirectly through effects on neurons or astrocytes.

Materials and methods

Targeted disruption of the murine erbB2 gene

For targeted mutation of the murine erbB2 gene, a 129/SvJ genomic DNA library was screened with a full-length erbB2-specific probe. Two overlapping clones of ~15–16 kb in length were obtained (I13 and I21). A 9-kb NotI-XhoI fragment of clone I13 was inserted 5' of the neomycin resistance cassette of the pPNT vector, and an 800 bp BamHI-EcoRI fragment was placed on the 3' side of the neo^r cassette. This construct removes from the erbB2 gene a 2–3-kb genomic fragment containing the exon encoding the essential transmembrane domain of the erbB2 receptor protein as well as flanking sequences encoding parts of the extracellular and cytoplasmic domains. After electroporation of ES cells with the linearized construct and double selection with G418 and FIAU, resistant clones were studied by Southern analysis using a 500 bp 3' fragment of the genomic clone not in-

corporated in the targeting vector. A clone bearing the disrupted gene was injected into C57BL/6 blastocysts and several resulting chimeric animals were obtained. These animals carried the disrupted allele and were able to pass it through the germ line. Homozygous null mutants fail to develop past 11.5 dpc and express no full-length protein by Western blot analysis. Heterozygous animals develop normally and express about half the wild-type amount of erbB2 protein.

ErbB2 null mice and spinal cord explants

To generate erbB2^{+/+}, erbB2^{+/-}, and erbB2^{-/-} embryos, litters were obtained from timed pregnancies of erbB2^{+/-} female and erbB2^{+/-} male matings 9.5 dpc. Each embryo was given a code number and spinal cords were dissected out and cut transversely into 1–2-mm fragments for explant culture onto polylysine and laminin-coated coverslips in DME, N2 additives, and 2% FBS. The remainder of each embryo was used to isolate genomic DNA for Southern blot analysis. To study myelination in vitro, medium containing N2 additives, 0.5 ng/ml PDGF-AA, and 2% FBS was used. These are conditions that we have previously determined to be optimal for myelination in spinal cord cultures.

To generate purified populations of OPCs and oligodendrocytes, cells were immunopanned from newborn rat spinal cord using mAbs A2B5, O4, or O1, respectively, as described previously (Robinson et al., 1998). Purity of the cell population was assayed on a separate cell aliquot 12 h after panning, and in all cases was >98% pure panned cells. Samples were snap frozen in liquid nitrogen and stored at -70°C until use.

Low-density cultures of enriched oligodendrocytes were generated as follows. Spinal cord explants from E9.5 mice were cultured in poly-L-ornithine-coated 24-well plates with a single explant per well. After 8 d in vitro, the medium was changed to DME with N2 additives and 20% FBS and the plates were rotated for 16 h at 200 rpm at 37°C. Supernatants from individual explants were plated into individual wells of 8-well Permax chamber slides (Nunc). After 2 h, the majority of cells adhered and the medium was replaced with DME containing N2 additives, 0.5 ng/ml PDGF-AA, and 1% FBS. OPCs were briefly expanded with PDGF (10 ng/ml) and bFGF (10 ng/ml) for 4 d, then switched to medium containing N2 additives, 0.5 ng/ml PDGF, and 1% FBS for an additional 36 h, then immunostained for surface galactocerebroside with the mAb O1. After fixation the cell density was determined by cell counts and averaged ~120 cells/cm². Approximately 60% of the cells were morphologically within the oligodendrocyte lineage. The remaining cells were morphologically astrocytes and fibroblasts. No neurons were observed.

Immunofluorescence microscopy

For A2B5, O4, or O1 immunofluorescence, live explant cultures were incubated for 15 min with the relevant mAb, washed with PBS, then fixed in fresh 4% paraformaldehyde in PBS for 7 min at ambient temperature, washed with PBS, then incubated with the relevant secondary antibody (Jackson ImmunoResearch Laboratories) and visualized by epifluorescence. For NF and GFAP immunofluorescence, cells were treated with 0.125% Triton X-100 in PBS for 20 min before incubation with primary antibody. For MBP staining, after fixation with paraformaldehyde, cells were permeabilized with ice-cold methanol for 10 min before incubation with primary antibody. Primary antibodies used were against the following: A2B5, O4, and O1 (American Type Culture Collection), MBP SMI99 (Sternberger Monoclonals, Inc.), NF 200 kD (Roche and Sigma-Aldrich), GFAP (Roche), erbB2 and erbB3 (Upstate Biotechnology), erbB4 (Santa Cruz Biotechnology, Inc.). Immunofluorescent images were obtained using a Nikon Eclipse 660 microscope with 20, 40, and 60× objectives, exposing 400 ASA Kodak color 35-mm film, images were then digitized using a slide scanner.

Cell counts

In wild-type and erbB2^{+/-} explants (after unblinding), O4+ and O1+ oligodendrocytes were dense within the explant itself and thus difficult to count with any certainty. Thus, for wild-type and erbB2^{+/-} explants, the outgrowth was counted. In erbB2^{-/-} explants, there are so few O1+ oligodendrocytes that the occasional O1+ cell within the explant is easily identified and can be counted. Thus, we included O1+ cells within the explant for the erbB2^{-/-} explants, but excluded them in the erbB2^{+/-} and erbB2^{+/+} explants. Thus, the results were biased against the observed difference. There were so few O1+ oligodendrocytes within the erbB2^{-/-} explants (1–2 of 20–30) that the results did not statistically differ whether or not they were included in the analysis.

Viability studies

To assess cell death of oligodendrocytes (O1+ and O4+) in the presence or absence of erbB2, spinal cord explant cultures were immunolabeled

with O4 or O1 mAbs, fixed with 95% ethanol/5% glacial acetic acid for 1 min, incubated with FITC goat anti-mouse IgM (μ chain specific), and stained with propidium iodide (20 mg/ml) for 5 min. The total number of O4+ or O1+ oligodendrocytes and the number of O4+ or O1+ oligodendrocytes showing pyknotic nuclei were counted by epifluorescence microscopy. Camptothecin was used at the concentration of 10 μ M overnight to induce cell death. Camptothecin is a cytotoxic plant alkaloid that induces DNA damage by inhibiting the activity of DNA topoisomerase-I (Morris and Geller, 1996).

RT-PCR of messages encoding erbB receptors

Total RNA was isolated from rat OPCs (A2B5) and oligodendrocytes (O4+) by using Trizol Reagent (Life Technologies). Complementary DNA (cDNA) was synthesized from total RNA with oligo(dT) primers and SuperScript II reverse transcriptase (Life Technologies) according to the manufacturer's protocol. A specific cDNA fragment was amplified by Platinum TagPCRx DNA polymerase (Life Technologies) using specific primers for erbB2, erbB3, or erbB4 (36 cycles of 94°C for 2 min, 58°C for 1 min and 72°C for 2 min, and a final 10 min 72°C extension). Amplified sequences by PCR were the regions corresponding to nucleotides 1726–2133 (408 bp) of the rat erbB2 mRNA sequences (EMBL/GenBank/DBJ accession no. NM_017003), nucleotides 3606–4002 (397 bp) of the rat erbB3 mRNA sequences (EMBL/GenBank/DBJ accession no. NM_017218), and nucleotides 1891–2058 of the rat erbB4 mRNA sequences (EMBL/GenBank/DBJ accession no. AF041838). Primers for PCR were: erbB2 sense 5'-CGAGTGTGAGCCTCAAACA-3', antisense 5'-GTCAGCGGCTCCACTAACTC-3'; erbB3 sense 5'-CGTCATGCCAGATACACACC-3', antisense 5'-CTCCTCGTACCCTTGCTCAG-3'; and erbB4 sense 5'-AACTGCAC-CCAGGGGTGTA-3', antisense 5'-GACATAAACGGCAAATGTC-3'.

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