

# Rescue of the Cardiac Defect in ErbB2 Mutant Mice Reveals Essential Roles of ErbB2 in Peripheral Nervous System Development

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## Summary

ErbB2 receptor tyrosine kinase plays a role in neuregulin signaling and is expressed in the developing nervous system. We genetically rescued the cardiac defect of *erbB2* null mutant embryos, which otherwise died at E11. These rescued *erbB2* mutant mice die at birth and display a severe loss of both motor and sensory neurons. Motor and sensory axons are severely defasciculated and aberrantly projected within their final target tissues. Schwann cells are completely absent in the peripheral nerves. Schwann cell precursors are present within the DRG and proliferate normally, but their ability to migrate is decreased. Acetylcholine receptors cluster within the central band of the mutant diaphragm muscle. However, these clusters are dispersed and morphologically different from those in control muscle. Our results reveal an important role for *erbB2* during normal peripheral nervous system development.

## Introduction

Neuregulins (NRGs) are a family of growth factors that include NRG-1, NRG-2, and NRG-3 (Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Fischbach and Rosen, 1997; Zhang et al., 1997). Multiple isoforms for each NRG are translated from alternatively spliced mRNAs. NRG-1 was initially characterized by its distinct biological activities, including glial growth factor (GGF) (Marchionni et al., 1993), neu differentiation factor (Wen et al., 1992) (Peles et al., 1992), heregulin (Holmes et al., 1992), and acetylcholine receptor inducing activity (ARIA) (Falls et al., 1993). Other isoforms were later characterized, such as sensorimotor-derived factor (SMDF) (Ho et al., 1995) (or neuregulin containing cysteine-rich domain [CRD-NRG] [Yang et al., 1998]). NRGs exert diverse bioactivities in multiple cell lineages (Burden and Yarden, 1997). For example, NRG-1 can induce *in vitro* cultures of neural crest (NC) cells to a glial (Schwann) cell fate (Shah et al., 1994). NRG-1 also promotes the survival of Schwann cell precursors (Dong et al., 1995) and proliferation (Marchionni et al., 1993) and migration of neonatal Schwann cells (Mahanthappa et al., 1996; Trachtenberg and Thompson, 1997).

The activities of these diverse NRG isoforms are mediated through members of the *erbB* receptor tyrosine kinase family, which includes *erbB2*, *erbB3*, and *erbB4*.

*In vitro* studies have demonstrated that ligand-induced heterodimerization of *erbB* receptors is required for NRG-mediated signaling (Carraway and Cantley, 1994). The developmental role of NRGs and their receptors has been studied in mice carrying targeted mutations in genes that encode NRG-1 or its receptors. Deletion of *erbB2*, *erbB4*, NRG-1, and the Ig or intracellular domain of NRG-1 resulted in embryonic lethality before embryonic day (E) 11 due to improper formation of cardiac trabeculae (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Kramer et al., 1996; Erickson et al., 1997; Britsch et al., 1998; Liu et al., 1998). *ErbB2* and *erbB4* are expressed in cardiac myocytes while NRG-1 is expressed in the adjacent endocardium; thus, these results were consistent with a model of NRG-1 activation of *erbB2/erbB4* heterodimers via a paracrine mechanism in the developing heart. Additionally, NC-derived cranial sensory ganglia were absent in NRG-1, *erbB2*, or *erbB3* null mutant embryos (Lee et al., 1995; Meyer and Birchmeier, 1995; Kramer et al., 1996; Erickson et al., 1997; Riethmacher et al., 1997; Britsch et al., 1998; Liu et al., 1998), suggesting that *erbB2/erbB3* heterodimers are required for the formation of cranial ganglia. Examination of *erbB3* mutant embryos, which survived beyond E13.5, demonstrated the absence of Schwann cells in the peripheral nerves (Erickson et al., 1997; Riethmacher et al., 1997).

Several lines of evidence suggest that NRGs and their *erbB* receptors are important in mediating the development of the NMJ through reciprocal interactions between presynaptic (nerve and Schwann cells) and postsynaptic (muscle) components (Burden, 1998). SMDF or CRD-NRG isoforms are expressed in motor neurons (Ho et al., 1995; Yang et al., 1998), and the Ig-NRG isoform is expressed in muscle (Meier et al., 1998). *ErbB2*, *erbB3*, and *erbB4* are expressed in muscle cells and are concentrated in the synaptic sites of both mature and developing NMJ (Altiok et al., 1995; Jo et al., 1995; Moscoso et al., 1995; Zhu et al., 1995), whereas only *erbB2* and *erbB3* are expressed in Schwann cells. ARIA, a NRG isoform, induced acetylcholine receptor (AChR) gene expression in noninnervated primary skeletal myocyte cultures (Falls et al., 1993). Deletion of one allele of the Ig-NRG-1 isoform in adult mice demonstrated a significant loss of AChRs at the NMJ (Sandrock et al., 1997). This result suggests that the concentration of the Ig-NRG-1 isoform is critical to the synthesis and/or maintenance of AChRs in the NMJ. However, it is still not apparent which *erbB* receptors and NRG isoforms are required for development and/or maintenance of the NMJ.

Since *erbB2* mutant embryos died at E11 of a trabeculation defect before gliogenesis in the peripheral nervous system (PNS) at E11 (Lawson and Biscoe, 1979) and before onset of NMJ at E12 (Noakes et al., 1993; Piette et al., 1993), the role of *erbB2* in neural and NMJ development at later stages has not been studied. Therefore, we genetically rescued the cardiac defect in *erbB2* null mutants by creating transgenic mice that

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expressed the rat *erbB2* under the control of an  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) promoter. When the transgenic mice were crossed into an *erbB2* null background, the rescued *erbB2* null embryos formed cardiac trabeculae and survived to birth. However, due to the loss of innervation within the diaphragm muscle, the rescued mice were stillborn. Examination of the PNS in the rescued mutant embryos demonstrated a severe loss of both sensory and motor neurons and a complete absence of Schwann cells precursors in the peripheral nerves. These results demonstrate that *erbB2* is required for normal development of the peripheral nervous system.

## Results

### Rescue of Cardiac Defect

Several lines of transgenic mice carrying the rat *erbB2* cDNA under the control of a 5.5 kb cardiac-specific  $\alpha$ -myosin heavy chain promoter (Subramaniam et al., 1991) were established (Figure 1A). RNase protection assays showed low levels of transgene expression detected in line #924, but not in line #922 (Figure 1B). Transgenic mice transmitted the transgene in a Mendelian fashion and exhibited no overt phenotype. Mice from line #924 were bred with *erbB2* heterozygote mutants to produce heterozygous *erbB2* mutant mice carrying the transgene, which were then intercrossed. As shown in Table 1, only homozygote mutant animals, which also contained the transgene, survived to birth. Western blot analyses showed that rat *erbB2* protein expression in the heart was increased in rescued mutant embryos between E10.5 and E18.5. However, overall levels of *erbB2* protein were lower in mutant cardiac muscle compared to those in control embryos (Figure 1C). Moreover, the rat *erbB2* protein was expressed only in the heart of the rescued mutant embryos (Figure 1D). Consistent with breeding data, histological analysis of H and E stained sections revealed that the morphology of the trabeculae of E14.5 rescued embryos was indistinguishable from that of control embryos (compare Figures 1E and 1F). The rescued mutant embryos died at birth due to a loss of motor axons within the diaphragm muscle (see below).

### Loss of Both Sensory and Motor Neurons

Initial histological examination of the mutant embryos at E18.5 revealed a marked reduction in size of the DRG in the rescued mutant embryos (Figures 2A and 2B). To determine whether the reduced size in the DRG was due to increased cell death, E14.5 sections were labeled using a TUNEL assay and counterstained with anti-neurofilament 150 ( $\alpha$ -NF150) antibodies. A significant number of apoptotic cells were detected in the mutant DRG (Figures 2C and 2D), yet not all TUNEL-positive cells were NF150 immunoreactive, suggesting that both neuronal and nonneuronal (glial) cells might be lost through apoptosis.

Additionally, we determined the number of motor neurons present within the ventral horn of the spinal cord at E14.5 and E18.5 following in situ hybridization with a choline acetyltransferase (ChAT) RNA probe (Figures 2E and 2F). The number of motor neurons present at E14.5 was not significantly different between the wild-type and

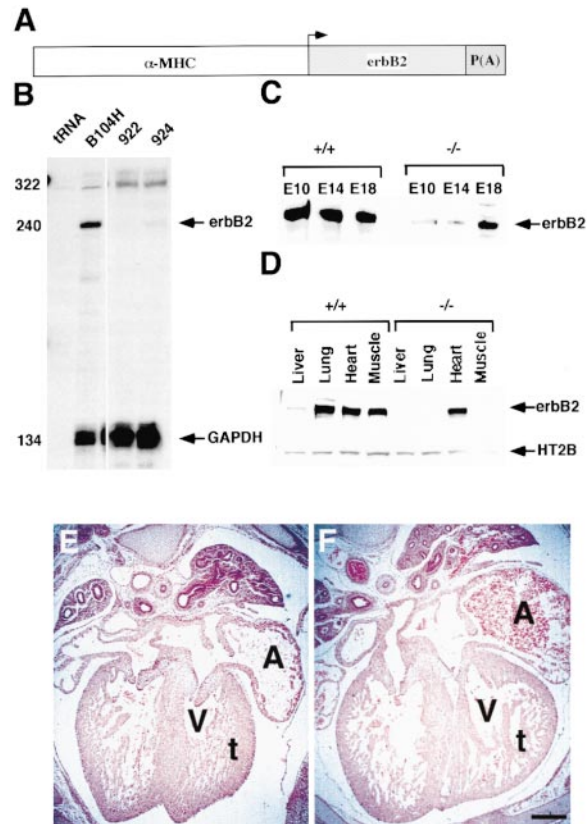


Figure 1. Genetic Rescue of the Cardiac Phenotype in *ErbB2* Null Mutant Mice

(A) The 5.5 kb region of the  $\alpha$ -MHC promoter was ligated to the rat *erbB2* cDNA followed by a bovine poly(A) sequence. (B) Expression of the transgene in the E10.5 embryonic hearts was determined. Ten micrograms of RNA isolated from B104-H (NIH 3T3 cell overexpressing the rat *erbB2* gene) used a positive control, and 100  $\mu$ g of RNA from transgenic lines #922 and #924 were used in RNase protection assay. Probe for rat *erbB2*, 322 nucleotides (nt); probe for GAPDH, 164 nt. (C) Western blot of protein isolated from the control and rescued mutant embryo hearts was probed with anti-*erbB2*. (D) *ErbB2* protein was detected in the liver, lung, heart, and muscle of control embryos at E18.5. In contrast, transgene expression *erbB2* protein was only detected in the heart of the rescued mutant embryos. Similar levels of serotonin receptor HT2B were detected in all tissues examined in both control and mutant embryos. (E–F) In H and E stained sections from E14.5 embryos, cardiac trabeculation was indistinguishable between control (E) and mutant (F) embryos. A, atrium; V, ventricle; t, trabeculae. Scale bars: (E and F), 100  $\mu$ m.

mutant spinal cords (Figure 2G). However, at E18.5 the number of motor neurons in mutant spinal cord was 70% less than those counted in the wild-type spinal cord (Figure 2G). Thus, a severe loss of both sensory and motor neurons was observed in the absence of *erbB2*.

**Absence of Schwann Cells in the Peripheral Nerves**  
*ErbB2* is expressed in glial cells, but not sensory neurons, during embryonic development (Jin et al., 1993). Furthermore, NRG induces the differentiation of NC cells to glial or Schwann cell precursors in vitro (Shah et al., 1994; Dong et al., 1995). Therefore, in the absence of

Table 1. Breeding of Heterozygous ErbB2 Mutant Mice Carrying the Transgene

Stage	Transgene	Number of Animals per ErbB2 Genotype		
		+/+	+/-	-/-
E10	YES	8	17	3
E12-E13.5	YES	22	40	12
E14-E15.5	YES	20	36	30
E16-E18	YES	17	30	16
P0	YES	8	27	5
E10	NO	4	10	3
E12-E13.5	NO	10	12	0
E14-E15	NO	13	32	0
E16-E18	NO	18	11	0
P0	NO	7	11	0

erbB2 embryonic Schwann cell development may be affected. Analysis of H and E stained sections of E14.5 embryos revealed that embryonic Schwann cells were absent in the spinal nerves of rescued mutants (compare Figures 3A and 3B). In addition, the absence of Schwann

cells in the peripheral nerve of E14.5 and E18.5 mutant embryos was confirmed by immunological staining with S100 and p75 (data not shown). Thus, these studies demonstrated a requirement for erbB2 during Schwann cell development.

**Schwann Cell Precursors within the DRG Were Present and Proliferated Normally**

As gliogenesis of the PNS in mouse begins at E11 (Lawson and Biscoe, 1979), we determined whether Schwann cell precursors were present at E11 and E12. Immunostaining with the early glial cell marker, erbB3, demonstrated that Schwann cell precursors were absent in the spinal nerve of erbB2 mutant embryos at E12 (Figures 3E and 3F, white arrows). In contrast to the absence of erbB3-positive cells in the peripheral nerve, erbB3-positive glial cells, which are characterized by elongated nuclei and longitudinal shape, were present within the DRG of rescued mutant embryos (Figures 3C and 3D, white arrows, and Figures 3E and 3F, black arrows). Additionally, embryos were analyzed using early glial cell markers, Sox10 (Kuhlbrodt et al., 1998) (Figures 3G-

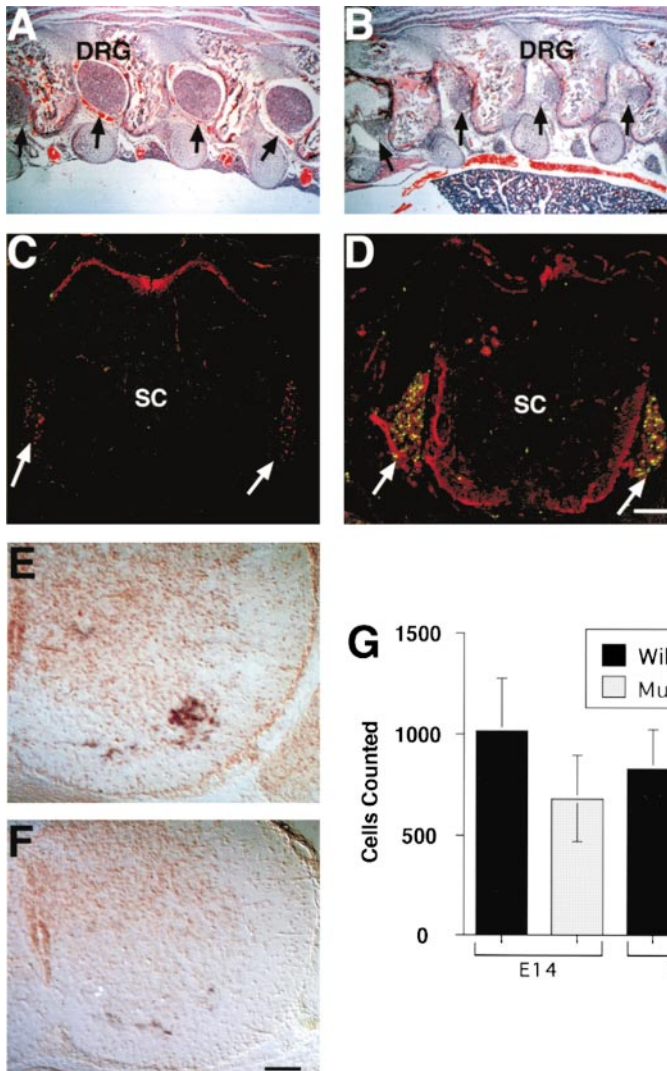
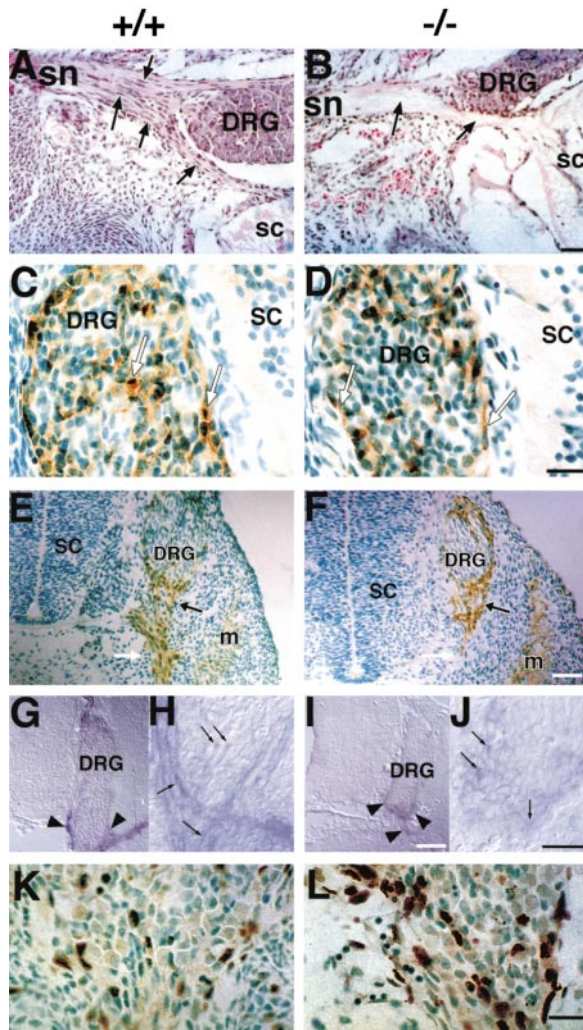


Figure 2. Severe Loss of Sensory and Motor Neurons

(A and B) Dorsal root ganglion (DRG, arrows) in the rescued mutants (B) were smaller in size than those of controls (A) at E18.5. (C and D) Sections labeled with a TUNEL assay and counterstained with anti-NF150 antibodies demonstrated increased cell death within the DRG (arrows) of rescued mutant embryos (D) at E14.5, as compared to controls (C). However, not all TUNEL-positive cells were NF150 immunoreactive, suggesting that both neuronal and nonneuronal cells were dying. (E-G) The number of motor neurons labeled with a ChAT in situ probe within the ventral horn of the spinal cord was reduced in the mutant embryos (F), as compared to the wild-type embryos (E). (G) The number of motor neurons was counted in every eighth section of the C1-C6 region of the spinal cord and was expressed as the mean ± SD. \*\* designates P < 0.01; Scale bars: (A-D), 100 μm; (E and F), 50 μm.



**Figure 3.** Absence of Schwann Cell Precursors in the Peripheral Nerve (A and B) Schwann cells (arrows) were absent in the spinal nerve (sn) of the rescued mutant embryos (B) at E14.5. (C and D) ErbB3-immunoreactive glial cells (white arrows) were present within the DRG of wild-type (C) and rescued mutant embryos (D) at E12.5. (E and F) ErbB3-positive cells were detected predominantly in the nerve roots (black arrows) at E11.5 immediately adjacent to DRG as the glial cells migrated out of the DRG in both control (E) and mutant (F) embryos. ErbB3-immunoreactive cells were also detected in the spinal nerve (white arrows) of control but not mutant embryos. Note that muscle (m) were also immunoreactive for erbB3. (G–J) E12.5 embryo sections were hybridized with a Sox10 in situ probe. Sox10 was expressed in Schwann cell precursors present in the spinal nerve and DRG of the wild-type embryos (G) and mutant embryos (I). Most Sox10-positive cells within the mutant embryo appeared to cluster in the base of the DRG (II, arrowheads). Higher magnification demonstrated that Sox10-positive glial cells were present in both wild-type (IH, arrows) and mutant DRG (IJ, arrows). (K and L) BrdU labeling demonstrated normal proliferation in the DRGs of mutant E12.5 embryos (L) when compared to wild type (K). DRG, dorsal root ganglion; SC, spinal cord; sn, spinal nerve; m, muscle. Scale bars: (A, B, E, F, G, and I), 100  $\mu$ m; (C, D, K, and L), 10  $\mu$ m; (H and J), 50  $\mu$ m.

3J) and Notch1 (Weinmaster et al., 1991) (data not shown) in situ probes. Sox10 mRNA expression was observed in the glial cells present within the nerve root

of both the wild-type and mutant embryos at E12.5 (compare Figures 3G and 3I, arrowheads), but not in the peripheral nerves of the mutant embryos. Higher magnification demonstrated that Sox10 was expressed in glial cells that were associated with the nerve root in both controls and mutants (compare Figures 3H and 3J, arrows).

It is not clear whether Schwann cells in the peripheral nerve and glial cells within the DRG (both Schwann cells and satellite cells) are derived from distinct neural crest cell lineages (see Discussion). However, the presence of erbB3-, Sox10-, and Notch1-positive glial cells within the DRG at E11 and E12 raises the possibility that the absence of Schwann cell precursors in the periphery may be due to an inability of these precursor cells to proliferate and/or migrate into the peripheral nerve. In vivo BrdU labeling at E12.5 revealed that qualitative differences in proliferation were not observed within the DRG (compare Figures 3K and 3L, controls and mutants, respectively). These results demonstrate that glial cells were present within the DRG of erbB2 mutant embryos and the glial cells proliferated normally.

#### Decreased Migration of Schwann Cell Precursors

At E11.5, numerous erbB3-positive cells accumulated at the nerve root immediately adjacent to the DRG in mutant embryos (Figure 3F, black arrow), suggesting that migration of these cells beyond the nerve root may be attenuated. To determine the ability of these precursors to migrate, an in vitro migration assay was used to quantitate their migration as shown in Figure 4A (Mahanthappa et al., 1996). Schwann cell precursors were identified with vital dye and verified with anti-erbB3 antibodies (Figure 4D). A significant decrease in the distance Schwann cell precursors of rescued mutants migrated (Figure 4B; mutants,  $0.87 \pm 0.1$  mm,  $n = 12$ , versus controls,  $1.68 \pm 0.1$  mm,  $n = 12$ ;  $P < 0.0001$ ) in the presence of bovine serum albumin (BSA) was observed. Unexpectedly, NRG did not significantly augment migration of Schwann cell precursors in control DRG (BSA,  $1.68 \pm 0.1$  mm,  $n = 12$ , versus NRG,  $1.58 \pm 0.10$  mm,  $n = 12$ ;  $P < 0.32$ ) nor mutant DRG (BSA,  $0.87 \pm 0.1$  mm,  $n = 12$ , versus NRG,  $1.01 \pm 0.1$  mm,  $n = 8$ ;  $P < 0.38$ ). Since these results contrasted with those in rat neonatal Schwann cells (Mahanthappa et al., 1996) and to exclude that our culture conditions did not support NRG-induced cell migration, neonatal DRGs from control mice were used in the migration assays. The results show that NRG significantly induced migration of neonatal mouse Schwann cells (BSA,  $1.68 \pm 0.62$  mm,  $n = 14$ , versus NRG,  $2.1 \pm 0.65$  mm,  $n = 14$ ;  $P < 0.05$ ) (Figure 4C). These data demonstrate that the absence of Schwann cells in the peripheral nerve of rescued erbB2 mutant embryos may be due to the decreased ability of Schwann cell precursors within the DRG to migrate into peripheral nerves. Since NRG did not directly induce migration of Schwann cell precursors, it may be required to induce a differentiation state that is required for migration of these precursors.

#### Axonal Defasciculation and Degeneration in the ErbB2 Mutants

Neurotrophins are produced by axonal targets and are required for the survival of developing neurons (Lewin

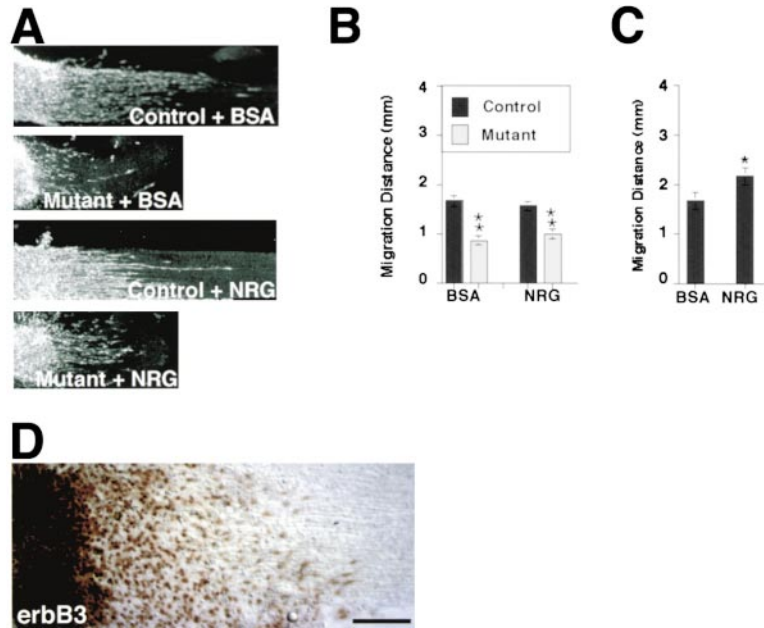


Figure 4. Decreased Migration of Mutant Schwann Cell Precursors in an In Vitro Migration Assay

(A) E12.5 DRGs were isolated and cultured on sciatic nerve sections in the presence or absence of NRG as described in the Experimental Procedures. Schwann cells were identified with vital dye and verified by immunostaining using anti-erbB3 antibodies (D). (B) The distance between the edge of the DRG and the leading Schwann cell was measured and expressed as the mean  $\pm$  SEM (mm). NRG did not promote Schwann cell precursor migration as compared to bovine serum albumin (BSA) ( $P < 0.32$ ). (C) NRG induced significant migration of P1 Schwann cells ( $P < 0.05$ ). (D) Schwann cell precursors in migration cultures were verified with anti-erbB3 staining. Scale bar, 100  $\mu$ m.

and Barde, 1996). Thus, the severe loss of both sensory and motor neurons in erbB2 mutants (Figure 2) may be due to alterations in their axonal projections. E11.5 embryos were serially sectioned and immunostained with anti-neurofilament antibodies. As illustrated in Figures 5A and 5B, both sensory and motor nerve branches exited correctly from the DRG and the spinal cord, respectively. In the immediate target field, mutant axons continued to project normally and maintained fasciculation. However, as the mutant nerves entered their final targets, the nerves did not fasciculate and were aberrantly projected (arrows in Figures 5B and 5D). In addition, whole-mount immunohistochemistry illustrated in the forelimbs at E12.5 that the axonal branches from branchial plexus were severely scattered, and individual axons navigated aberrantly before entering the limb buds in the rescued mutants (Figure 5F, arrows) as compared to controls (Figure 5E). Some axonal branches failed to reach the base of limb buds in the rescued mutants (Figures 5E and 5F, arrowheads). In the hindlimbs, the sciatic axons of mutant embryos (Figure 5H, arrows) were not bundled as compared to those of controls (Figure 5G, arrows). Alterations in axonal trajectory of cutaneous nerves were also observed in the hindlimbs (compare Figures 5G and 5H, arrowheads). Consistent with whole-mount immunohistochemistry results, immunostaining of E12.5 embryo sections revealed that some nerves were severely defasciculated and misrouted to the middle of forelimbs (compare Figures 5C and 5D). These results demonstrated that most nerves were not properly fasciculated and appeared to aberrantly project in the regions of their final targets. Therefore, proper neurotrophic support may not be received by the motor or sensory neurons, thereby leading to markedly increased cell death as shown in Figure 2.

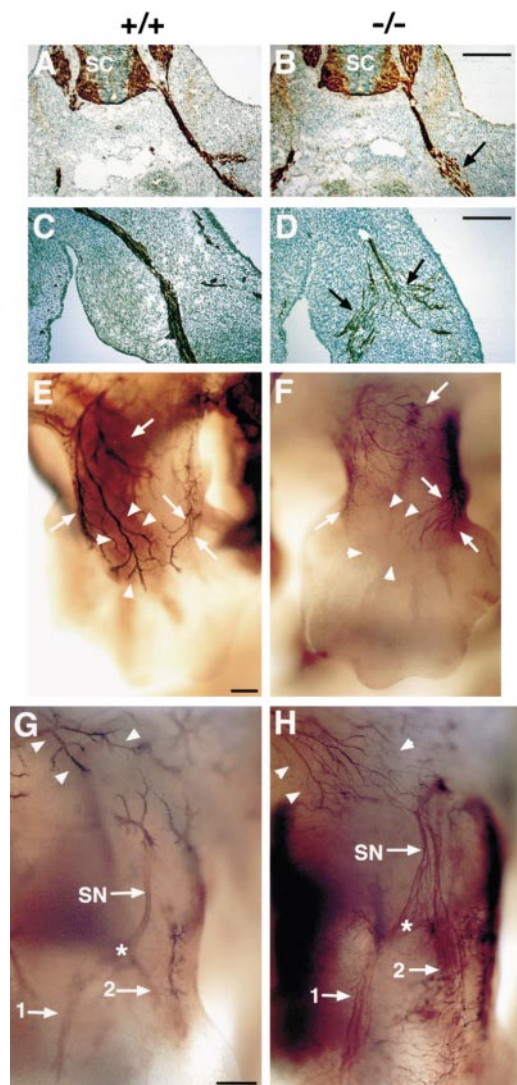
#### Decreased NGF-Elicited Neurite Outgrowth

Because some nerve branches did not completely extend to their targets (Figures 5G and 5H, arrowheads),

it is possible that the absence of Schwann cells compromised neurotrophin-induced axonal outgrowth in the erbB2 mutant embryos. To test this possibility, we used an in vitro neurite outgrowth assay in the presence of NGF at a saturating concentration (50 ng/ml). Figure 6 shows that NGF-induced neurite outgrowth was significantly decreased in the erbB2 mutant embryos (Figure 6B; controls,  $3.38 \pm 0.14$  mm,  $n = 14$ , versus mutants,  $2.24 \pm 0.2$ ,  $n = 14$ ;  $P < 0.001$ ). Interactions between axons and Schwann cells have been shown to be bidirectional during axonal growth (Jessen and Mirsky, 1991). Axons can regulate migration of Schwann cell precursors (Bhattacharyya et al., 1994), which in turn may promote outgrowth of axons to their targets. Thus, addition of exogenous wild-type Schwann cells to the assay may enhance neurite outgrowth from the mutant DRGs. As shown in Figure 6A (arrows), the neurites of mutant DRG neurons that were associated with exogenous Schwann cells were markedly extended (Figure 6B; NGF,  $2.24 \pm 0.2$  mm,  $n = 14$ , versus NGF + Schwann cells,  $3.51 \pm 0.4$  mm,  $n = 9$ ;  $P < 0.02$ ). In contrast, the region of sciatic nerve substratum that did not contain exogenous Schwann cells displayed no additional neurite extension (Figure 6A, arrowheads). These results demonstrated that sensory neurite extension was decreased in the erbB2 mutant embryos and that addition of wild-type Schwann cells enhanced the neurite outgrowth. Therefore, the role of Schwann cells during development of the peripheral nervous system may be to enhance the ability of axons to reach peripheral targets.

#### Abnormal NMJ Development in ErbB2 Mutants

Since erbB2 is expressed in the muscle and Schwann cells are absent in the erbB2 mutants, neuromuscular junction development may be compromised. Neuromuscular junctions (NMJ) were examined by labeling presynaptic nerves with neurofilament antibodies and postsynaptic AChRs with  $\alpha$ -bungarotoxin (BTX) in



**Figure 5.** Aberrant Projection and Defasciculation of Axons within Final Target Fields

(A and B) Sensory and motor axons exited at correct positions from the DRG and the spinal cord (SC) at E11.5 in both mutant (B) and wild-type embryos (A).

(C and D) The nerves entering the forelimbs of the rescued mutant embryos (D) became severely defasciculated (arrows) and navigated to improper targets as they moved distally from cell bodies.

(E–H) Whole-mount immunohistochemistry using anti-NF150 antibodies in E12.5 mutant (F and H) and control (E and H) embryos demonstrated that some nerve branches innervating the forelimb buds (E and F) became diffuse and scattered (arrows) in the mutants (F), while others appeared missing (arrowheads). In the hindlimbs (G and H), two main branches (1 and 2) of the sciatic nerves (SN, arrows) and cutaneous nerves (arrowheads) in the mutants were also defasciculated. \*, branching points of the sciatic nerves. Scale bars: (A, B, G, and H), 100  $\mu\text{m}$ ; (C–F), 50  $\mu\text{m}$ .

whole-mount diaphragm preparation. As shown in Figure 7, the phrenic nerve innervated the central band of the diaphragm muscle in the control embryos at E18.5 (Figure 7C). Intramuscular branches of the phrenic nerve, which emanated from the main nerve trunk, terminated at synaptic sites, where AChRs were clustered (Figure 7C'). In contrast, the mutant diaphragm was completely

devoid of innervation at E18.5 (Figure 7D), which led to respiratory failure at birth. However, a distinct band of AChR clusters was confined to the central region of the diaphragm muscle (Figure 7D'), although individual AChR clusters were dispersed compared to controls. The shape of individual clusters was elongated in the control diaphragms (arrows in Figure 7E), while the shape of most AChR clusters was round or ovoid in mutant diaphragms (arrowheads in Figure 7F). Examination of the innervation of the diaphragm muscle at earlier stages (E12.5–E16.5) revealed that motor axons were scattered, defasciculated, and gradually degenerated (data not shown) as in the limbs shown in Figure 5.

Further examination of postsynaptic differentiation within E18.5 diaphragm muscle was done with several postsynaptic markers, including rapsyn and MuSK. Rapsyn, a 43 kDa cytoskeletal protein, is expressed at the onset of AChR clustering (Noakes et al., 1983) and is required for clustering AChRs at postsynaptic sites (Gautam et al., 1995; Apel et al., 1997). MuSK, a skeletal muscle-specific tyrosine kinase receptor, is important for agrin-induced AChR clustering (DeChiara et al., 1996). Double labeling with BTX and antibodies specific for rapsyn, or MuSK, demonstrated that both rapsyn and MuSK colocalized with BTX staining in control, as well as mutant, diaphragm muscle (Figures 8A–8D and 8A'–8D'). Similar results were obtained with antibodies specific to  $\beta$ -dystroglycan, laminin, and heparan sulfate proteoglycan (data not shown). In addition, acetylcholine esterase (AChE) clusters were normal in the erbB2 mutant embryos (Figures 8E and 8F, controls and mutants, respectively). Consistent with AChRs clustered in the central band of the diaphragm muscle, AChR $\alpha$  subunit transcripts were also concentrated in this region in both control and mutant diaphragms (data not shown). These results suggested that erbB2 is either not essential in AChR $\alpha$  gene activation or it is compensated for by other erbB receptors, such as erbB4, which is expressed in both mutant and control muscle (data not shown). These results demonstrated that in erbB2 mutant mice presynaptic development of NMJ is abnormal, whereas postsynaptic development is largely unaffected.

## Discussion

Cardiac rescue of the erbB2 null mutant embryo provides a valuable tool with which to study the role of erbB2 in the developing peripheral nervous system. The erbB2 null mutant embryos are unable to breathe and die at birth because of the degeneration of peripheral nerves and improperly formed neuromuscular junctions in the diaphragm. In addition, Schwann cell precursors are absent in the peripheral nerve, likely due to the precursors' inability to properly differentiate and migrate out of the DRG. The peripheral nerves are defasciculated and are aberrantly projected within their final target fields in the mutant animals, leading to degeneration of the nerves. NMJ presynaptic development in the erbB2 mutant mice is abnormal, whereas postsynaptic development is largely unaffected. Rescue of the erbB2 mutant embryos provides important evidence that erbB2 is required for proper development of the peripheral nervous system.

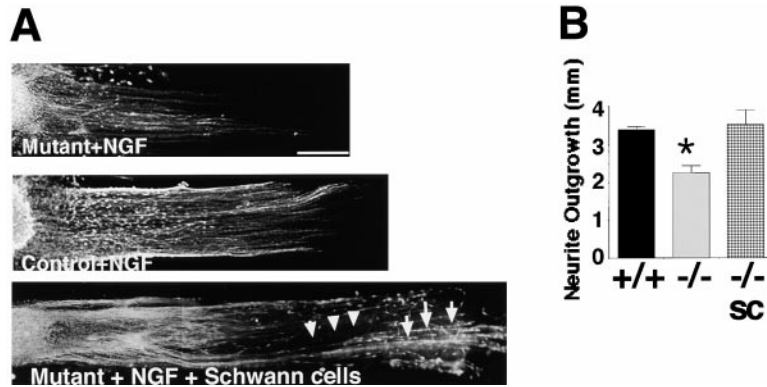


Figure 6. Decreased Sensory Neurite Outgrowth

(A) NGF-induced neurite outgrowth of mutant DRGs was significantly decreased as compared to control DRG ( $P < 0.002$ ). Note that neurite outgrowth was enhanced in the rescued mutants in the region containing exogenous Schwann cells (arrows) but not in the region absent of Schwann cells (arrowheads). (B) The distance from the edge of the DRG to the leading nerve process was measured and expressed as the mean  $\pm$  SEM (mm). Scale bars, 100  $\mu$ m.

The absence of Schwann cells in the peripheral nerves, observed in both these erbB2 rescue mice (present study) and previously reported erbB3 mutant embryos (Erickson et al., 1997; Riethmacher et al., 1997), provides *in vivo* evidence that both erbB2 and erbB3 are required for Schwann cell development. This study has demonstrated that Schwann cell precursors were present within the DRG in the erbB2 mutant embryos, but their ability to migrate into the peripheral nerve was compromised. Riethmacher et al. (1997) suggested that the loss of Schwann cells in the erbB3 mutant embryos was due to the inability of NC cells to differentiate into Schwann cell precursors. This conclusion was based on experiments to isolate Schwann cell precursors from chimeric E12.5 DRG explants after 12 days in culture (Riethmacher, et al., 1997). Since NRG-1 has been shown to promote the survival of Schwann cell precursors *in vitro* (Dong et al., 1995), it is important to consider that this method depended on the ability of Schwann cell precursors to survive in culture. Therefore, the loss of Schwann cells in peripheral nerve of both the erbB2 and erbB3 mutant embryos may be due to an inability of the precursors to survive and migrate. Consistent with this possibility, our results showed that not all TUNEL-positive cells within the DRG are NF immunoreactive, suggesting that some apoptotic cells are glial cells.

Alternatively, erbB2 and erbB3 may have different roles in Schwann cell development. Previous lineage studies in which NC cells were labeled with retroviruses expressing a  $\beta$ -gal marker prior to their migration out of the neural tube demonstrated that Schwann cells within the DRG and in the peripheral nerve could be derived from the same NC precursor cell (Frank and Sanes, 1991). However, it is not known whether after migrating out of the neural tube, NC cells differentiate into two separate cell lineages that eventually give rise to Schwann cells within the DRG and those in the peripheral nerves, respectively. Therefore, it may be that glial precursor cells within the DRG at E11–E12 give rise to Schwann cells in the periphery, and based on our data these precursors were unable to migrate out of the DRG in the absence of erbB2. Alternatively, glial cells within the DRG and in the periphery may be derived from two distinct committed NC precursors, and in the absence of either erbB2 or erbB3 these two sublineages never develop. It is also possible that erbB2 and erbB3 play sequential yet distinct roles in Schwann cell development at different stages. For example, erbB3 might be

important for induction of NC cells into Schwann cell precursors, while erbB2 might control the aspects of differentiation (e.g., cell migration). Therefore, targeted mutation of either gene would lead to a loss of Schwann cells in the periphery.

Furthermore, Qiu et al. (1998) recently demonstrated that erbB2 is required for IL-6-induced signaling via an interaction with IL-6 receptor gp130 in prostate cancer cells. It is possible that erbB2 and erbB3 could form complexes with other types of receptors to mediate these differential effects on Schwann cell precursor formation and differentiation. Finally, Sliwkowski et al. (1994) found that NRG weakly induced erbB3 phosphorylation in COS cells in the absence of erbB2. These results suggest that erbB3/erbB3, but not erbB2/erbB2, homodimers may elicit NRG signaling. It is possible that alternative erbB2 and erbB3 signaling pathways contributed to the different phenotypes of the erbB2 and erbB3 mutant embryos.

The severe loss of sensory and motor neurons in these embryos may be due to a variety of cellular defects. First, the neurons may not receive proper trophic support from their target tissues due to aberrant projections of axons to incorrect targets and attenuated neurotrophin-induced axonal growth as demonstrated in this study. Aberrant projection of axons in the null mutation of Krox20 also led to high levels of neuronal cell death (Schneider-Maunoury et al., 1997). Second, several lines of evidence suggest that neonatal Schwann cells in culture produce neurotrophic factors that promote the survival of sensory and motor neurons (Bunge, 1993). Because Schwann cell precursors were absent in the erbB2 mutants, these neurotrophic factors may also be absent, and this may lead to a loss of neurons through apoptosis. Finally, erbB2 is expressed in some target tissues (i.e., skin and muscle) and may be required for the induction and/or release of neurotrophic factors from these targets. Thus, the absence of erbB2 in the target tissues may also contribute to the phenotype.

Improper axonal projection in the final target fields shown in this study may be due to defasciculation of nerves in their final target fields (Figure 5). Defasciculation may be caused by the absence of Schwann cells in the peripheral nerve. It has been established that Schwann cells are important to guide axons during nerve regeneration and sprouting in postnatal rats (Son et al., 1996), but the role Schwann cells play during normal neural development is controversial (Keynes, 1987). Noakes

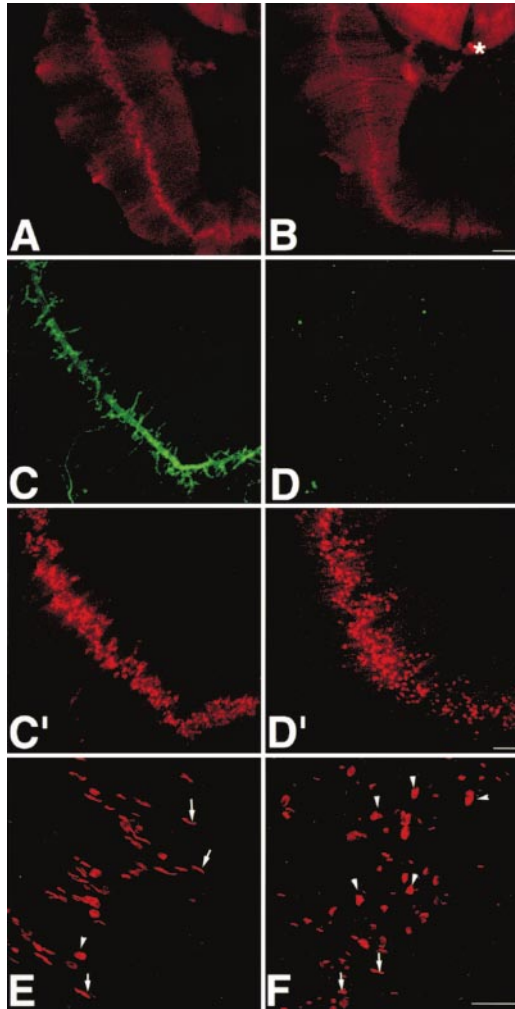


Figure 7. Abnormal NMJ Development in ErbB2 Mutant Embryos at E18.5

(A and B) Whole-mount diaphragm muscle was double stained with neurofilament antibodies to label the presynaptic axons and with Texas Red-conjugated  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) to label AChRs on the postsynaptic muscle membrane. Low power view of semidiaphragm showed that AChRs were clustered along a central band in both wild-type (A) and mutant (B) embryos.

(C–D') In wild-type diaphragm (C), phrenic innervation was confined to a narrow band through the central region of the diaphragm muscle, at which AChR clusters were localized (C'). In erbB2 mutant diaphragm, although the phrenic nerve was completely absent (D), a distinct band of AChR clusters (D') localized to the central band of the diaphragm muscle.

(E and F) However, AChR clusters were more dispersed in the mutants (F) as compared to the controls (E). Furthermore, most individual clusters were round or ovoid in shape (arrowheads) in the mutants, whereas most clusters were elongated (arrowheads) in the controls. Scale bars: (A and B), 200  $\mu$ m; (C, D, C', and D'), 100  $\mu$ m; (E and F), 50  $\mu$ m. \* designates background staining from a piece of esophagus.

et al. (1988) demonstrated that motor axons did not fasciculate and failed to reach their proper targets in the chick following NC cell ablation, which would remove only sensory neurons and Schwann cells. These data demonstrated an important role for Schwann cells in motor axon bundling and guidance. In contrast, Harrison

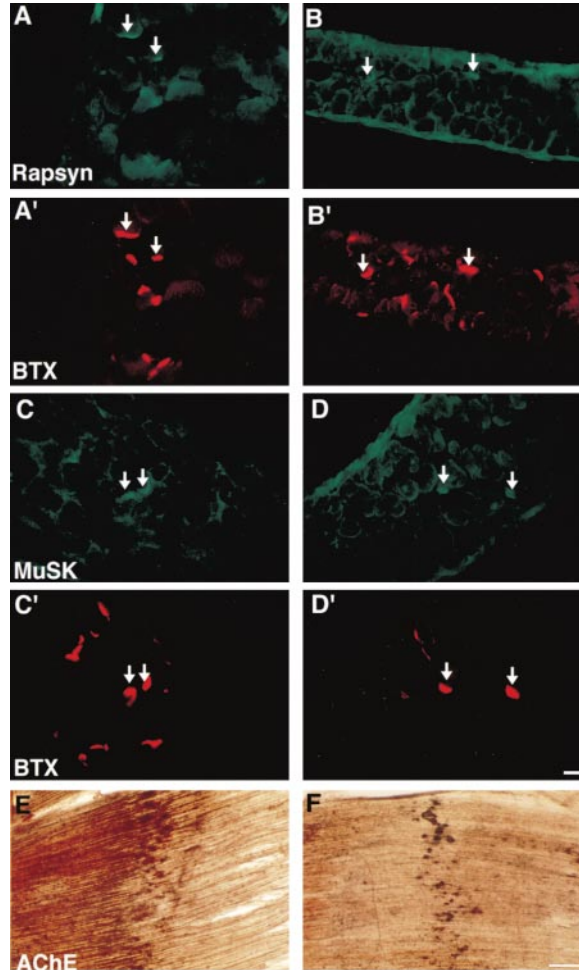


Figure 8. Normal Postsynaptic Differentiation in the Neuromuscular Junctions

(A–D) Frozen sections of the diaphragm muscle were double labeled with Texas Red-conjugated  $\alpha$ -bungarotoxin ( $\alpha$ -BTX) (A'–D') and antibodies against rapsyn (A and B) or MuSK (C and D). Rapsyn (A and B) and MuSK (C and D) staining in the erbB2 mutant (B and D) and control (A and C) embryos colocalized with  $\alpha$ -BTX labeling (A'–D').

(E and F) Acetylcholine esterase (AChE) was clustered at normal locations in the mutants (F) as compared to controls (E). Scale bars: (A–D'), 10  $\mu$ m; (E and F), 100  $\mu$ m.

showed that amphibian NC ablation does not prevent motor axons from reaching their targets (Harrison, 1924). Analysis of the erbB2 mutant provided additional data to support a role for Schwann cells in nerve fasciculation. One study has shown that Schwann cells migrate ahead of growing axons (Noakes and Bennet, 1987), while another study demonstrated that Schwann cells only closely associated with growing axons (Carpenter and Hollyday, 1992). Thus, whether or not Schwann cells may migrate ahead of growing axons, their ability to closely associate with growing axons raises the possibility that Schwann cells influence axonal bundling and growth. Consistent with this notion, our *in vitro* culture data showed that addition of wild-type Schwann cells increased neurotrophin-induced neurite outgrowth in mutant DRG (Figure 6). Our present study demonstrated

that when Schwann cells, but not neurons, are specifically eliminated, axons failed to bundle and some nerves were aberrantly projected. These results support the hypothesis that Schwann cells play a role in axonal fasciculation and guidance. ErbB2 is expressed in some target tissues of the sensory (e.g., skin) and motor (e.g., muscle) neurons. ErbB2 may be required for the target tissue to release cues that induce fasciculation of the axons and guide axonal projections. These cues may be absent in erbB2 mutants, which may contribute to the defect in axonal fasciculation and targeting.

In the erbB2 mutants, development of the NMJ was abnormal. Several lines of evidence demonstrated that neural agrin and MuSK receptor complex are required in AChR clustering (DeChiara et al., 1996; Gautam et al., 1996). Clustering of AChRs at the central band of the diaphragm muscle in the mutants may be due to transient contact of motor nerves with the muscle. This contact may be sufficient for the neural form of agrin to be deposited in the basal lamina at synaptic sites in order to initiate AChR clustering. However, the clusters were more dispersed, and most of the individual clusters were ovoid rather than elongated in shape. These results suggest that essential signals to induce or maintain the shape of clusters are lost in the absence of erbB2. Specific signals may come from nerve terminals, Schwann cells, or muscle. Schwann cells in the NMJ may play a role to maintain the tight adhesion between pre- and postsynaptic elements, as Schwann cells express molecules such as N and E cadherins, which are implicated in the stability of synapses (Colman, 1997). NMJ in the erbB2 mutants was unstable, which may be due to the absence of Schwann cells. The mutant synapses may not convey synaptic transmission, which could lead to axonal retraction. Previous studies have shown that functional synapses are formed only in cocultures with CNS neurons and oligodendrocytes (Kaplan et al., 1997; Pflieger and Barres, 1997). These findings suggest that glial cells in the peripheral and central nervous systems play a general role in promoting synapse formation and in maintaining and modulating functional synapses (Pflieger and Barres, 1996). However, it cannot be ruled out that NRG released by the nerve requires erbB2 signaling to create a proper NMJ and that in the absence of erbB2 these synapses are not maintained.

In summary, a genetic rescue strategy was used to restore cardiac trabeculation in erbB2 null mutant embryos. The rescued erbB2 null mutants displayed severe loss of both motor and sensory neurons and demonstrated improper axonal targeting and fasciculation. In addition, Schwann cell precursors were not present in the peripheral nerves but were present within the DRG. These rescued mice provide a model to further elucidate mechanisms by which neuregulins and their receptors control neuronal and glial development in the peripheral nervous system.

#### Experimental Procedures

##### Generation of the $\alpha$ MHC-ErbB2 Transgenic Mice

A 5.5 kb  $\alpha$ -MHC promoter (gift of J. Robbins) was ligated with a rat erbB2 cDNA (gift of M.-C. Hung) followed by bovine growth hormone poly(A) sequences. Transgenic mice were generated by standard procedures.

##### RNAse Protection Assay

RNA was isolated by a modified guanidium isothiocyanate procedure (Ambicon). The radiolabeled erbB2 probe was synthesized with T3 RNA polymerase using a 400 bp BamHI fragment from the rat erbB2 cDNA as described (Hayes et al., 1992). The radiolabeled erbB2 and GAPDH probes, and either 10  $\mu$ g of mRNA isolated from B104-H cell line expressing rat erbB2 (gift of M. Hung), 100  $\mu$ g of tRNA, or 100  $\mu$ g of RNA isolated E10.5 embryonic heart were mixed in hybridization buffer (40 mM PIPES [pH 6.4], 400 mM NaCl, 1 mM EDTA, 80% formamide). The hybridization reactions were denatured at 92°C for 5 min and hybridized overnight at 42°C. RNase A/RNase T1 resistant transcripts were separated on a 6% denaturing acrylamide gel and analyzed by autoradiography.

##### Western Analysis

Whole cell lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blocked in 5% milk in Tris-buffered saline. The membrane was incubated with anti-erbB2 (Santa Cruz; 1:1000) or HT2B (1:1000; gift of L. Maroteaux) antibodies, followed by peroxidase-conjugated goat anti-rabbit secondary antibodies (1:2,000), and visualized with enhanced ECL reagents.

##### Histology, Immunohistochemistry, and Whole-Mount Immunohistochemistry

Embryos were fixed overnight in 4% PFA, washed in 0.1 M phosphate buffer, dehydrated, and embedded in paraffin. Embryo sections (7  $\mu$ m) were collected onto vectorbond-coated slides and stained with hematoxylin and eosin (H and E). For immunohistochemistry, embryos were fixed with 4% PFA and equilibrated with 30% sucrose. Sections were washed three times with TBS and blocked in dilution buffer (Lee et al., 1995) with 10% normal goat serum. Antibodies used were neurofilament 150 (1:500; Chemicon) and erbB3 (1:300; Santa Cruz). The antibodies were visualized using the ABC kit (Vector Laboratories) and DAB. For whole-mount immunohistochemistry, E12.5 embryos were fixed in 4% PFA and then postfixed in Dent's fixative (4 parts methanol: 1 part DMSO) plus 5% H<sub>2</sub>O<sub>2</sub> overnight at 4°C. After several washes in TBS, the embryos were blocked in 5% goat serum with 1% DMSO overnight at room temperature and then incubated with anti-NF150 antibodies (1:500; Chemicon) containing 1% DMSO for 2 days at room temperature. The embryos were washed with TBS containing 1% Tween 20 and 1% DMSO and then incubated with peroxidase conjugated secondary antibodies (1:1000; Vector Laboratories). Texas Red-conjugated  $\alpha$ -bungarotoxin (10<sup>-8</sup> M; Molecular Probes) was used to label AChR clusters.

##### AChE Staining

Acetylcholine esterase was visualized based upon the "direct method" (Karnovsky, 1964). Briefly, the E18.5 diaphragm was fixed in 1% PFA in 0.1 M phosphate buffer (pH 6.0) overnight at 4°C. The diaphragm was rinsed with 0.1 M phosphate buffer (pH 6.0) three times at 10 min each and then incubated for 3–5 hr at room temperature in solution (10% sucrose, 65 mM sodium hydrogen maleate buffer [pH 6.0], 5 mM sodium citrate, 3 mM cupric sulfate, 0.5 mM potassium ferricyanide, and 1.7 mM acetylthiocholine iodide [Sigma]).

##### In Situ Hybridization and Cell Counts

In situ hybridization was carried out as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). Embryos were fixed in 4% PFA in 0.1 M phosphate buffer and sectioned at 20  $\mu$ m thickness. Sections were acetylated, digested with proteinase K (1  $\mu$ g/ml), and hybridized at 68°C overnight in hybridization buffer containing digoxigenin-labeled cRNA probe, 50% formamide, 5 $\times$  SSC, 5 $\times$  Denhardt's, and 250  $\mu$ g/ml yeast tRNA. After hybridization, sections were washed with 0.2 $\times$  SSC at 68°C for 1–3 hr, followed by TBS at room temperature for 5 min. After blocking with TBS containing 10% heat inactivated goat serum (HINGS), sections were incubated with alkaline phosphatase conjugated anti-digoxigenin (1:5000, Boehringer Mannheim) in TBS and 1% HINGS overnight at 4°C. Detection was carried out in 100 mM Tris (pH 9.5) containing NBT/BCIP (Vector Laboratories). Motor neurons (n = 3) were counted through the C1–C6 region in every eighth section. The

total number of cell counts was not corrected for split nuclei, and statistical analysis was done using a Student's *t* test.

#### TUNEL Assay

Frozen sections (10  $\mu$ m) were collected onto poly-L-lysine coated subbed slides. Sections were postfixed in 10% neutral buffered formalin. After washing three times in PBS, sections were incubated with ethanol:acetic acid (1:1) for 5 min at  $-20^{\circ}\text{C}$ . Sections were digested with proteinase K (2.5  $\mu\text{g}/\text{ml}$ ) for 5 min at room temperature. After washing in PBS three times, they were incubated with labeling reagents from the Apoptag kit (Oncor) as described by the manufacturer. The images were visualized and collected with a Bio-Rad confocal microscopy.

#### BrdU Labeling

Time-plugged females were injected with BrdU (50 mg/kg body weight) 2 hr before harvest. Embryos were fixed with 4% PFA and sectioned at 7  $\mu$ m thickness. For detection of BrdU incorporation, sections were treated in 50% formamide/2 $\times$  SSC at  $65^{\circ}\text{C}$ , washed in 2 $\times$  SSC, deproteinated in 2 N HCl in TBS for 30 min at room temperature, neutralized with 0.1 M borate buffer (pH 8.0), and treated with 2%  $\text{H}_2\text{O}_2$  in TBS. After blocking with buffer containing 3% normal donkey serum, 0.1% Triton X-100 in TBS, sections were incubated with rat anti-BrdU antibodies (1:500; Accurate) overnight at  $4^{\circ}\text{C}$ . After washing with TBS, sections were developed with the ABC kit (Vector Laboratories).

#### Schwann Cell Migration and Neurite Outgrowth Assays

Migration assays were carried out as described previously (Mahanthappa et al., 1996). P1 or E12.5 DRGs were isolated and explanted onto 20  $\mu$ m thick cryostat sections of the sciatic nerves of control adult animals in the presence or absence of NRG (or NDF-EGF $\beta$  as described previously [Dong et al., 1995] at 0.1 ng/ml = 16 pM). For neurite outgrowth assay, NGF (50 ng/ml) was used. After 72 hr, cultures were stained with 10 mM vital dye 5(6)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes) and examined in a fluorescence microscope. In some experiment, exogenous Schwann cells were prepared and added to the cultures at  $2 \times 10^5$  cells per 35 mm dish. The distance between the edges of the DRG and the leading Schwann cells or neurites was measured and expressed as mean  $\pm$  SEM. Statistical significance of differences was tested by Student's *t* test.

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