Aberrant development of motor axons and neuromuscular synapses in erbB2-deficient mice

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Receptor tyrosine kinase erbB2, which is activated by neuregulin, is expressed in Schwann and muscle cells in the developing neuromuscular junction (NMJ). In vitro studies have shown that neuregulin promotes the survival and migration of Schwann cells and stimulates acetylcholine receptor gene transcription in cultured muscle cells. These findings suggest an important role for erbB2 in the development of the NMJ. Here we examine erbB2-deficient mice to determine whether erbB2 is required for NMJ development in vivo. Our analysis shows that there are pre- and postsynaptic defects of developing NMJ in erbB2-deficient embryos. The presynaptic defects include defasciculation and degeneration of the motor nerves, and an absence of Schwann cells. The postsynaptic defect features an impairment of junctional folds at the neuromuscular synapse in the mutants. These results demonstrate that erbB2 is essential for in vivo development of the NMJ.

Neuromuscular junction (NMJ) development is regulated by reciprocal interactions between presynaptic (nerve terminal and Schwann cell) and postsynaptic (muscle cell) components. Two of the hallmarks of NMJ development are clustering of acetylcholine receptors (AChRs) on muscle cell membranes and selective transcriptional activation of the AChR genes at synaptic sites. Agrin plays an essential role in mediating the clustering of AChRs through its activation of muscle-specific kinase (MuSK) receptor complexes, as demonstrated by a lack of AChR clustering at normal synaptic sites in mice lacking either agrin or MuSK, although some ectopic AChR clusters are detected in agrin mutant mice (1, 2). Several studies demonstrated that AChR gene expression is induced in culture by acetylcholine receptor-inducing activity in the absence of innervation (3). However, there is no in vivo evidence as to whether acetylcholine receptor-inducing activity affects embryonic AChR gene activation. Acetylcholine receptor-inducing activity belongs to a family of growth factors that are translated from alternatively spliced transcripts of the neuregulin-1 (NRG-1) gene (4, 5). Recently, three additional NRG genes (NRG-2, NRG-3, and NRG-4) have been cloned, but their role in AChR gene activation has not yet been established (6–10). In addition to activation of the AChR gene, NRG-1 has been shown to promote the survival and migration of Schwann cells (11, 12).

The biological effects of NRGs are mediated through erbB receptors, including erbB2, erbB3, and erbB4, all of which are members of the epidermal growth factor receptor family. Expression patterns of NRG isoforms and their receptors are complex at developing NMJ. Motor neurons express sensory and motor neuron-derived factor or cysteine-rich domain-NRG isoforms, which do not contain the Ig-domain (13, 14), whereas muscle cells express the Ig-NRGi isoform that lacks the cysteine-rich domain (15). erbB2, erbB3, and erbB4 receptors are expressed in muscle cells and concentrated in the synaptic sites of both the mature and developing NMJ (16–19), whereas only erbB2 and erbB3 receptors are expressed in Schwann cells. These results suggest that erbB receptors and NRGs are involved in mediating the development of NMJ through reciprocal interactions between pre- and postsynaptic components (20). Here we performed detailed developmental analysis on motor axons and synapses in these erbB2-deficient mice. We find that the phrenic nerve is dramatically defasciculated and projects aberrantly across the entire diaphragmatic surface. No Schwann cells are associated with motor axons. At synaptic sites, nerve terminals are not capped with Schwann cells. In addition, junctional folds are rarely observed at the NMJ of the intercostal muscle in the mutants. Our finding suggests that erbB2 plays essential roles in the development of the NMJ.

Materials and Methods

Animals. erbB2-deficient animals were generated and genotyped as described (21). Embryos at various stages [embryonic day 12 (E12), E12.5, E13.5, E14, E14.5, E15.5, E16.5, and E18.5] were collected, and three to eight embryos of the same genotype from each stage were analyzed.

Immunohistochemistry and Whole-Mount Immunochemistry. The muscles were dissected, rinsed with PBS, pH 7.3, and incubated with 0.1 M glycine in PBS for 1 h, and then with 0.5% Triton X-100 in PBS. The muscles were blocked in dilution buffer (150 mM NaCl/0.01 M phosphate buffer/3% BSA/0.01% thimerasol) overnight at 4°C, and then incubated with rabbit antibodies against neurofilament (NF150, 1:500, Chemicon), or synaptophysin (1:1,000, kindly provided by R. Jahn, Yale University) in dilution buffer overnight at 4°C. After washing three times for 1 h each in 0.5% Triton X-100 in PBS, the muscles were then incubated with fluorescein-conjugated goat anti-rabbit IgG (1:400, Cappel) and Texas Red conjugated α-bungarotoxin (α-BTX) (10 μg/ml, Molecular Probes) overnight at 4°C. The muscles were then washed three times for 1 h each with 0.5% Triton X-100 in PBS, once with PBS, and flat-mounted in 90% glycerol, polyvinyl alcohol, and N-propyl gallate.

Frozen sections (12 μm) were prepared from unfixed diaphragm, leg, and intercostal muscle. Immunofluorescence staining was performed according to conventional procedures. The following primary antibodies were used in the analysis: rabbit anti-agrin (1:1,000), rabbit anti-MuSK (1:500), rabbit anti-rapsyn (1:100), and mouse anti-heparan sulfate proteoglycan (1:100, NovoCastra, Newcastle, U.K.).

Whole-Mount in Situ Hybridization. E18.5 embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight. The diaphragm and intercostal muscles were dissected out as described above. Digoxigenin-labeled cRNA probe specific for the α-subunit of mouse AChR (kindly provided by S. Burden, Skirball Institute, New York University, New York) was transcribed in vitro. Hybridization was performed at 70°C overnight in the hybridization buffer containing 50% formamide, 1.3× SSC, 5 mM EDTA, 50 μg/ml yeast tRNA, 0.2% Tween-20, 0.5% NBT-34 solely to indicate this fact.

Abbreviations: NRG, neuregulin; NMJ, neuromuscular junction; AChR, acetylcholine receptor; MuSK, muscle-specific kinase; BTX, bungarotoxin.

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3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 100 μg/ml heparin. After hybridization, the diaphragm and intercostal muscles were washed with Tris-buffered saline containing 1% Tween-20 three times for 1 h each. The muscles were then blocked with 5% goat serum in antibody dilution buffer and incubated with alkaline phosphatase-conjugated anti-digoxigenin (1:1,000) overnight at 4°C. Detection was performed in 100 mM Tris, pH 9.5, containing nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Electron Microscopy. Pregnant females were sacrificed by cervical dislocation and the embryos (E18) were removed. Embryo tails were collected for PCR analysis, and the remainder of the body was placed in a solution of 4% formaldehyde and 4% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The intercostal muscles were removed and placed in the same solution for a total of 2 h on ice. The tissue was then rinsed with buffer and postfixed in 2% osmium tetroxide in buffer for 1 h on ice. The tissue was then rinsed in H2O, dehydrated in a graded series of ethanol, infiltrated, and polymerized in Durcupan resin (EM Science). Ultrathin sections were stained with Sato lead, and electron micrographs were recorded by using either a JEOL 100CX or 2000FX electron microscope operated at 80 keV.

Results

erbB2-Deficient Mice Die at Birth and Display Defects in Presynaptic Development. erbB2-deficient mice die at birth (21). Histological analysis of hematoxylin/eosin-stained lung sections showed that the alveoli of mutants were tightly compressed (data not shown), suggesting that the inability for erbB2 mutants to breathe is likely...
due to a lack of functional NMJs. To study this possibility, we analyzed the innervation of the diaphragm, intercostal, and limb muscles.

**Phrenic nerve.** We examined phrenic nerve innervation at various stages of development, ranging from E12 to E16.5. As shown in Fig. 1, the phrenic nerve reached the dorsal portion of the diaphragm at E12 in both control and mutant mice (Fig. 1 A and D, respectively). In the controls, the main nerve trunk remained bundled after reaching the muscle surface (arrow in Fig. 1A). In contrast, the phrenic nerve of the mutants was bundled before reaching the muscle surface (arrow in Fig. 1D). At E12.5, the phrenic nerve in the controls extended toward the dorsal and ventral diaphragm, with intramuscular branches emanating perpendicularly from the nerve trunk (Fig. 1B). In contrast, the phrenic nerve of the mutants became markedly defasciculated and diffusely projected to the entire dorsal surface of the muscle (arrowheads in Fig. 1E). As development proceeded, the phrenic nerve of control embryos extended toward the dorsal and ventral diaphragm, and elongated progressively along the central region of the diaphragm from E13.5 to E16.5 (Fig. 1C and G–I). In contrast, in the mutants, the nerves remained diffusely projected across the dorsal portion of the diaphragm at E13.5 (Fig. 1F), and then migrated across the entire muscle surface by E14 (data not shown). At E14.5, the nerves began to withdraw (Fig. 1F), leaving only a few nerve branches by E15.5 (Fig. 1K). At E16.5, the entire diaphragm was devoid of phrenic innervation (Fig. 1L). Thus, in erbB2-deficient embryos, the phrenic nerves appeared defas-culated and projected diffusely across the diaphragmatic surface for 2 days (E12–E14), and withdrew during the following 2 days (E14.5–E16.5).

**Transient targeting of phrenic nerve terminals to the muscle at E14.** The above observations indicated that the phrenic nerve in the erbB2 mutant projected diffusely across the diaphragm muscle up to E14 and then started to withdraw. To visualize the presynaptic nerve terminals, whole-mount diaphragm was stained with antibodies against synaptophysin. In the control diaphragm, the staining was confined within a narrow band in the middle of the diaphragm muscle (Fig. 2 A and B). In the mutants, nerve terminals were also intensely labeled by synap-
nerves (contrast, there were no Schwann cells detectable in the mutants in the phrenic (SC). Axons were separated apart by extracellular matrix (ec). In the mutant and overall axon diameters are smaller. [Bars extracellular matrix. Note that the density of axons in the mutants is higher, also found in the intercostal nerve in the controls (arrowheads in E).]

Figs. 2 A–F Whole-mount preparations from controls (A, C, and E) and erbB2 mutants (B, D, and F) were labeled with antibodies against S100. In the control preparations, S-100 labeled Schwann cells delineated the entire phrenic nerves as early as E13.5 (A), and were readily detectable at E15.5 (C). S100-positive cells were also found in the intercostal nerve in the controls (arrowheads in E). In contrast, there were no Schwann cells detectable in the mutants in the phrenic nerves (B and D), or in the intercostal nerves (F). Ribs (r) also stained positively for S100. (G and H) Electron micrographs of the intercostal nerves at E18.5. In the control (G), every single axon (ax) was wrapped by Schwann cell processes (SC). Axons were separated apart by extracellular matrix (ec). In the mutant (H), axons were tightly packed together. There were no Schwann cells or extracellular matrix. Note that the density of axons in the mutants is higher, and overall axon diameters are smaller. [Bars ~ 50 μm (A, B, E, and F), 100 μm (C and D), and 1 μm (G and H).]

Fig. 4. Absence of Schwann cells in erbB2-deficient embryos. (A–F) Whole-mount preparations from controls (A, C, and E) and erbB2 mutants (B, D, and F) were labeled with antibodies against S100. In the control preparations, S-100 labeled Schwann cells delineated the entire phrenic nerves as early as E13.5 (A), and were readily detectable at E15.5 (C). S100-positive cells were also found in the intercostal nerve in the controls (arrowheads in E). In contrast, there were no Schwann cells detectable in the mutants in the phrenic nerves (B and D), or in the intercostal nerves (F). Ribs (r) also stained positively for S100. (G and H) Electron micrographs of the intercostal nerves at E18.5. In the control (G), every single axon (ax) was wrapped by Schwann cell processes (SC). Axons were separated apart by extracellular matrix (ec). In the mutant (H), axons were tightly packed together. There were no Schwann cells or extracellular matrix. Note that the density of axons in the mutants is higher, and overall axon diameters are smaller. [Bars ~ 50 μm (A, B, E, and F), 100 μm (C and D), and 1 μm (G and H).]

Tophysin antibody, along the middle of the diaphragm muscle (Fig. 2 D and E), whereas the preterminal axons were distributed randomly across the entire diaphragmatic surface (arrowhead in Fig. 2E). Although nerve terminals contacted the central band of muscle, the nerve arborization appeared disorganized in the mutants as compared with controls (compare Fig. 2 B and E, controls and mutants, respectively). Thus, although the phrenic nerve projected diffusely across the diaphragmatic surface, nerve terminals only contacted the muscle in a small region near the center part of muscle, a location similar to that in the wild-type diaphragm. However, the nerve terminals were unstable in the mutants and gradually withdrew. At E15.5, synaptophtysin-positive nerve terminals were not detectable in the erbB2 mutants (Fig. 2F). By E16.5, nerve terminals were not observed in the whole diaphragm of the mutants (data not shown).

Intercostal and limb motor nerves. Similar to the phrenic nerve, the intercostal nerves were also defasciculated at E12.5–E18.5 (Fig. 3) in the mutants. Terminal arborization was disorganized and diffuse in the mutants (Fig. 3 F and G). In contrast to a complete loss of the phrenic nerve at E16.5 (Fig. 1), the intercostal nerves were still present at E18.5, although they remain defasciculated (Fig. 3G). We have also examined the NMJ of forelimb and hindlimb muscles. Only a small number of motor nerves remained at E18.5 (Fig. 3I).

The absence of Schwann cells in motor nerves. Schwann cells have been shown to affect the development of axons in culture, including myelination and axon diameter (22). Immunological examination of developing embryos (E13.5, E14.5, and E15.5) with antibodies against S100 showed that Schwann cells were absent in both the phrenic and intercostal nerves (Fig. 4 B, D, and F). The absence of Schwann cells was then further confirmed by electron microscopy. As shown in Fig. 4G, every single axon of control intercostal nerves was wrapped by Schwann cell processes. Axons were separated by extracellular matrix (Fig. 4F). In contrast, axons of the mutant intercostal nerves were tightly packed together. There were no Schwann cells or extracellular matrix. Note that the density of axons in the mutants is higher, and overall axon diameters are smaller. [Bars ~ 50 μm (A, B, E, and F), 100 μm (C and D), and 1 μm (G and H).]

Impairment of Postsynaptic Development. Impairment of postsynaptic junctional folds. The ultrastructure of the NMJ synapse was examined with electron microscopy. As shown in Fig. 5, nerve terminals in control embryos were capped by Schwann cell processes (Fig. 5A), whereas mutant embryo nerve terminals were devoid of Schwann cells (Fig. 5 B and C). Furthermore, postsynaptic junctional folds developed at synaptic sites in controls (Fig. 5A), whereas the postsynaptic membrane in the mutants was lacking junctional folds (Fig. 5 B and C). We analyzed more than 10 neuromuscular synapses in the mutants and we rarely observed any junctional folds. The basal lamina, however, was present and normally arranged at the synaptic site in the mutants, similar to those in the controls. We also labeled...
muscle demonstrated that several other molecules, including laminin, heparin-binding agrin to be deposited at the basal lamina at synaptic sites to the muscle for about 2 days, this may be sufficient for neural embryos, the phrenic nerve terminals only transiently contacted necessary for inducing AChR clustering. The muscle isoform of through the activation of the MuSK receptor complex, is nec-
no neurotransmitters were released to activate AChRs on the Because the nerves completely degenerated in the diaphragm, AChRs were clustered along the central band of muscles. E12 to E14, yet nerve terminals transiently contacted the diaphragm muscle at similar locations as those in the controls. 

Transcriptional activation of the AChRα gene. We then determined whether transcriptional activation of the AChR gene is affected in erbB2 mutants. In situ hybridization experiments demonstrated that α-subunit transcripts were concentrated in the central band of the muscle in both the control (Fig. 6A) and erbB2-deficient diaphragm (Fig. 6C). Similar results were ob-
served in the intercostal muscles (Fig. 6B, controls; Fig. 6D, mutants). These results suggest that erbB2 is not essential in AChRα gene activation at synaptic sites.

Discussion
In the present study, we showed that erbB2-deficient mice died at birth, probably due to abnormal NMJ development at the diaphragm. The phrenic nerve was severely defasciculated from E12 to E14, yet nerve terminals transiently contacted the diaphragm muscle at similar locations as those in the controls. Motor nerves degenerated at later stages and completely disappeared after E16.5. In contrast to the phrenic nerve, the intercostal nerve did not degenerate at birth. Interestingly, AChRs were clustered along the central band of muscles. Because the nerves completely degenerated in the diaphragm, no neurotransmitters were released to activate AChRs on the diaphragm, thereby leading to a respiratory failure. 

Several lines of evidence indicate that the neural form of agrin, through the activation of the MuSK receptor complex, is necessary for inducing AChR clustering. The muscle isoform of agrin fails to induce clustering (23). Although in erbB2 mutant embryos, the phrenic nerve terminals only transiently contacted the muscle for about 2 days, this may be sufficient for neural agrin to be deposited at the basal lamina at synaptic sites to initiate AChR clustering. Alternatively, in addition to agrin, several other molecules, including laminin, heparin-binding growth-associated molecule, midkine, and basic fibroblast growth factor have been shown to induce AChR clustering in cultured muscle cells (24–28). These results raise the possibility that in the context of the erbB2 mutation, these neural agrin-independent pathways induce clustering of AChRs. Our results showed that α-subunit transcripts were concentrated in a central band of the muscle in both the control and erbB2-deficient diaphragm, suggesting that erbB2 is not essential for embryonic AChR gene activation. Alternatively, other erbB receptors might compensate for the loss of erbB2 in synapse-specific activation of the AChR gene. Indeed, both erbB3 and erbB4 receptors are expressed in the diaphragm and other skeletal muscles of erbB2 mutants. If there are no nerves, where do NRGs come from? Muscle cells have been shown to express the Ig-domain containing the NRG isoform (15, 18). Thus, the muscle form of NRGs may activate erbB receptor via an autocrine mechanism.

Several lines of evidence suggest that ligand-induced erbB receptor heterodimerization is the key step in the activation of NRG-mediated signaling pathways. Our present results demonstrated that Schwann cells are absent in the peripheral nerve in erbB2 mutant embryos. Similarly, Schwann cells in peripheral nerve are also absent in erbB3-deficient embryos (29, 30). Taken together, these studies support the idea that both erbB2 and erbB3 are required for normal development of Schwann cells.

In erbB2 mutant mice, the phrenic nerve became defasci-
culated and subsequently degenerated. Because erbB2 is expressed in both Schwann and muscle cells, the loss of Schwann cells and/or a defect in the muscle might lead to axonal defasciculation and degeneration. Schwann cells have been shown to promote the growth of motor axons to their muscle targets during development and nerve regeneration. In the absence of Schwann cells in the chick following neural crest cell ablation, motor axons become defasciculated and fail to enter the limb bud to innervate limb muscles (31). These results suggest that glial cells play an essential role in axonal fasciculation and growth during development. In addition, the muscle may also provide important signals for axonal fasciculation and growth. Because erbB2 is expressed in muscle cells, this lack of erbB2 may result in the loss of these important signals.

Despite the dramatic defasciculation and disorganization, synaptic connections were established transiently at normal locations in the diaphragm of mutant embryos at E14, whereas synaptic connections persisted in the intercostal muscle at least until birth. However, terminal arborization was reduced and less elaborate. These synapses in the diaphragm were not stable and the nerve withdrew shortly afterwards. Although synapses were formed in the intercostal muscle, normal junctional folding was not observed in these mutants. Schwann cells have been shown to affect terminal arborization (32) and axon caliber (22) in vitro. The absence of Schwann cells in erbB2-deficient embryos may contribute to these axonal and synaptic defects. Why are the synapses not stable? Several studies show that Schwann cells produce trophic factors that promote the survival of neurons and the integrity of axons. We cannot rule out the possibility that erbB2-deficient muscle also play a role in this phenotype. Additional trophic factors may be produced in the intercostal muscle but not in the diaphragm muscle, which may explain why intercostal nerve remained in the mutants at birth. In addition, Schwann cells may maintain the tight adhesion between pre- and postsynaptic elements and promote synaptic transmission. Schwann cells express molecules such as N- and E-cadherins, which are implicated in the stability of synapses (33). Schwann cells have been shown to modulate synaptic efficacy and synaptic depression at the NMJ (34). Thus, the synapses may be unstable and may not convey synaptic transmission properly in the absence of Schwann cells, and therefore, result in retraction of the axons because of the lack of activity. Finally, because erbB2 is expressed in both Schwann and muscle cells, the Schwann and synaptic defects could be separate consequences of the erbB2 deficiency.
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