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Erbb2 regulates neuromuscular synapse formation and is essential for muscle spindle development

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SUMMARY

Neuregulins and their Erbb receptors have been implicated in neuromuscular synapse formation by regulating gene expression in subsynaptic nuclei. To analyze the function of Erbb2 in this process, we have inactivated the *Erbb2* gene in developing muscle fibers by Cre/Lox-mediated gene ablation. Neuromuscular synapses form in the mutant mice, but the synapses are less efficient and contain reduced levels of acetylcholine receptors. Surprisingly, the mutant mice also show proprioceptive defects caused by abnormal muscle spindle development. Sensory Ia afferent neurons establish initial contact with Erbb2-deficient myotubes. However, functional spindles never develop. Taken together, our data suggest that Erbb2 signaling regulates the formation of both neuromuscular synapses and muscle spindles.

Key words: Erbb2, Nrg1, Muscle spindle, Synapse, Neuromuscular junction, Mouse

INTRODUCTION

The development of striated muscle is dependent on the exchange of signals between the nerve terminals of sensory and motoneurons with developing muscle fibers. Motoneuron derived signals regulate the development of neuromuscular junctions (NMJs) (Burden, 1998; Sanes and Lichtman, 1999). Sensory neuron derived signals are essential for the development of muscle spindles (Maier, 1997; Walro and Kucera, 1999).

During formation of the NMJ, synaptic proteins such as acetylcholine receptors (AChRs), rapsyn, Musk, Nrg1 and its Erbb receptors become clustered in the postsynaptic muscle membrane. Furthermore, the expression of genes that encode postsynaptic proteins becomes restricted to subsynaptic nuclei (Burden, 1998; Sanes and Lichtman, 1999). Clustering of proteins and synaptic gene expression are dependent on agrin that is released from motor nerve terminals and activates the Musk receptor tyrosine kinase in muscle. Accordingly, in mice that lack agrin or Musk, the formation of stable synaptic AChR clusters and synaptic gene expression are defective (DeChiara et al., 1996; Gautam et al., 1996). Agrin/Musk is not only necessary, but also sufficient for postsynaptic differentiation. Ectopic expression of agrin or of constitutively active Musk in muscle induces the formation of a postsynaptic apparatus and the upregulation of genes normally restricted to subsynaptic nuclei (Cohen et al., 1997; Jones et al., 1997; Jones et al., 1999; Meier et al., 1997; Moore et al., 2001).

Nrg1 and its Erbb receptors have also been implicated in the regulation of gene expression in subsynaptic nuclei. Several Nrg1 isoforms are generated from the *Nrg1* gene by alternative splicing. Isoforms containing an EGF domain induce AChR gene expression in cultured myotubes in vitro (Buonanno and Fischbach, 2001; Schaeffer et al., 2001). Mice heterozygous for a targeted mutation ablating an Ig-domain encoding exon of the *Nrg1* gene have decreased numbers of synaptic AChRs, suggesting that Ig-domain containing Nrg1 isoforms are important in vivo (Sandrock et al., 1997). The cellular source of Nrg1 that regulates synaptic gene expression is at present unclear. Mice that lack Nrg1 specifically in motoneurons still develop NMJs (Schaeffer et al., 2001; Yang et al., 2001), suggesting that Nrg1 may be provided by another cellular source, e.g. by muscle fibers. Consistent with this interpretation, Nrg1/Erbb receptors are clustered at postsynaptic sites induced by ectopic expression of agrin in muscle fibers in the absence of nerve terminals (Jones et al., 1996; Jones et al., 1999; Meier et al., 1998; Moore et al., 2001). Interestingly, expression of synapse specific genes in ectopically induced postsynaptic membranes is decreased when the function of Erbb2 or Erbb4 is blocked in muscle (Jones et al., 1996; Jones et al., 1999; Meier et al., 1998; Moore et al., 2001). Nrg1/Erbb are also concentrated at endogenous NMJs, but fail to cluster in mice that lack agrin or Musk (DeChiara et al., 1996; Gautam et al., 1996). Taken together, these data suggest that muscle derived Nrg1 acts downstream of nerve-derived agrin.

Genetic evidence implicating Erbb receptors in NMJ formation is still missing. Synapse formation and synaptic gene expression are initiated in mice that carry null mutations in the genes encoding *Erbb2* and *Erbb3*, but the synaptic band is broadened (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Riethmacher et al., 1997). It is at present unclear whether the changes in the synaptic band arise due to defects in muscle fibers or in Schwann cells that are also affected in the mutants (Lin et al., 2000; Morris et al., 1999; Riethmacher et al., 1997; Woldeyesus et al., 1999; Wolpowitz et al., 2000). Furthermore, Erbb4 that is expressed in muscle (Zhu et al., 1995) may compensate for the loss of Erbb2/b3, and account for the activation of synapse-specific gene expression.

Little is known about the signaling mechanisms that lead to muscle spindle differentiation. Muscle spindles consist of multiple fibers such as nuclear bag_1 and bag_2 fibers and multiple nuclear chain fibers. Nuclear bag_2 fibers differentiate first, followed by the formation of nuclear bag_1 fibers and multiple nuclear chain fibers. The timing of ingrowths of nerve fibers relative to the time when spindle development is initiated suggests that muscle fibers are recruited to develop into spindle-forming intrafusal fibers by signals derived from the innervating sensory Ia afferent neurons (Maier, 1997; Walro and Kucera, 1999). Mice with a targeted mutation in the genes for neurotrophin 3 (*Ntf3*) and its *TrkC* (*Ntrk3* – Mouse Genome Informatics) receptor lack Ia afferents and muscle spindles, providing genetic evidence that contact between sensory neurons and developing muscle fibers is required for muscle spindle development (Ernfors et al., 1994; Farinas et al., 1994; Klein et al., 1994; Tessarollo et al., 1994). Interestingly, NT3 regulates the survival of proprioceptive sensory neurons prior to innervation of muscle spindles, but is also required for the survival of Ia afferent neurons once they reach their target (ElShamy and Ernfors, 1996; ElShamy et al., 1998; Farinas et al., 1996; Kucera et al., 1995; Oakley et al., 1995; Oakley et al., 1997; Ockel et al., 1996; Ringstedt et al., 1997; Wright et al., 1997). NT3 is expressed in spindles, and maintenance of NT3 expression is dependent on nerve contact (Chen et al., 2002; Copray and Brouwer, 1994). These data suggest the existence of a regulatory loop, where yet to be defined neuronal signals induce muscle spindle formation and NT3 expression, while NT3 serves to maintain Ia afferent neurons.

To investigate the function of Erbb2 receptors in muscle, we have generated a mouse line carrying a floxed allele of the *Erbb2* gene, and a second mouse line expressing Cre in developing skeletal muscle. Using these mice, we have inactivated *Erbb2* expression in muscle. We demonstrate that Erbb2 is not essential for the development of NMJs, although the Erbb2-deficient synapses contain reduced numbers of AChRs and are less efficient. Unexpectedly, the mutant mice also have proprioceptive defects. Consistent with this finding, we demonstrate that Erbb2 is essential for muscle spindle development.

MATERIALS AND METHODS

Generation of *Erbb2^{flox}* mice

A 5.8 kb *Eco*RI/*Bam*HI fragment of genomic DNA containing the exon of the *Erbb2* gene encoding its signal sequence was used for gene targeting (Fig. 1A). A double-stranded oligonucleotide containing a *Bam*HI restriction site and a loxP site was inserted into an *Xba*I site 5′ of the first coding exon of the *Erbb2* gene. A loxP-neo-IRES-tk-loxP cassette was inserted into a *Cla*I site 3′ of the same exon. ES cell transfection, selection and screening was carried out as described (Graus-Porta et al., 2001; Müller et al., 1997), using the probe indicated in Fig. 1A. One positive clone was retransfected with a Cre-expressing plasmid (Gu et al., 1993) and counterselected in ganciclovir, and clones that had retained two loxP sites flanking the first coding exon were identified by Southern blot (Fig. 1B). Germline-transmitting chimeras were generated as described (Müller et al., 1997).

Generation of *HSA-Cre* mice

A 2.2 kb fragment of the human skeletal α-actin (HSA) promoter was used to drive skeletal muscle specific gene expression of Cre-recombinase. A cDNA encoding a nuclear localization signal and Cre (kindly provided by M. Lewandowski) was inserted into the *Eco*RI/*Eco*RV sites of HSA-pA4 (Tinsley et al., 1996). Preparation of DNA for pronuclear injection was carried out as described (Aigner et al., 1995). Founder mice were crossed with C57Bl/6 mice, and F1 offspring genotyped by PCR and Southern blot to identify germline-transmitting founder mice. Positive F1 offspring were further crossed with C57Bl/6 mice to obtain F2 offspring. F2 mice and mice from subsequent generations were used to analyze Cre activity and to inactivate the *Erbb2* gene.

Analysis of Cre-mediated recombination

DNA was isolated and analyzed by PCR or Southern blot as described (Graus-Porta et al., 2001; Muller et al., 1997). The sequence of the PCR primers for the *Erbb2*$^{-}$ allele are as follows: 5′-CTCCC-AAGTCTGGGCTCTTTCTC-3′, 5′GCGTGTTTTGCCTGTGTGTA-TGTC-3′ and 5′-CCTTGGGAAAAGCGCCTCCCCTAC-3′. Primers that amplify Cre were used to genotype *HSA-Cre* mice: 5′-GACATGTTCAGGGATCGCCAGGCG-3′ and 5′-GACGGAAATC-CATCGCTCGACCAG-3′. Primers to identify the recombined *Erbb2^{flox}* allele were as follows: 5′-CTGTTGCAAACAAATGCC-TGC-3′ and 5′-CAGAATGGCTAAATCTGGGATC-3′.

PCR conditions

Erbb2^{-} allele: 5 minutes at 95°C followed by 35 amplification cycles (30 seconds at 95°C; 30 seconds at 62°C; 30 seconds at 72°C) and 10 minutes final extension at 72°C.

Cre transgene: 10 minutes at 94°C followed by 30 amplification cycles (1 minute at 94°C; 1 minute at 65°C; 1 minute at 72°C) and 10 minutes extension at 72°C.

Recombined *Erbb2^{flox}* allele: as for *Cre*, but 35 amplification cycles.

PCR reaction products were 520 bp (*Erbb2*$^{+}$), 330 bp (*Erbb2*$^{-}$), 170 bp (recombined *Erbb2^{flox}*) and 600 bp (*Cre*). For Southern blot analysis of *Cre* transgenic animals, the full-length cDNA encoding Cre was used.

Histology and electron microscopy

Tissues were dissected, fixed overnight at 4°C in 4% PFA in PBS without Mg^{2+} and Ca^{2+} (PBS^{-}), and incubated overnight at 4°C in 30% sucrose in PBS^{-} prior to freezing in OTC. Alternatively, fresh tissue was embedded in OTC. Cryosections were stained with Hematoxylin and Eosin as described (Graus-Porta et al., 2001; Muller et al., 1997). *lacZ* staining was performed as described (Farinas et al., 1996), either on whole-mount embryos or on 20-30 μm cryosections of isolated soleus muscle, after fixation in 2% PFA in PBS^{-}. For electron microscopy, embryos were collected at different developmental stages and immersion fixed in 4% PFA/2%glutaraldehyde in PBS^{-} at 4°C overnight. Hindlimbs were osmicated in 2% osmium tetroxide for 2 hours, stained with 2% uranyl acetate in 70% ethanol en bloc, dehydrated and embedded in araldite (Durcupan, Fluka). Complete serial of transverse 2 μm sections

through the thigh were stained with 1% Toluidine Blue for light microscopic inspection. Those sections containing nerve bundles of recognizable muscle spindles were sectioned into 70 nm sections for EM analysis after lead citrate staining.

Immunohistochemistry

Immunohistochemistry with antibodies to Erbb2 (Santa Cruz, sc-284) on histological sections of soleus muscle or with primary muscle cells in culture was carried out as described (Trinidad et al., 2000). For staining with antibodies to α-dystroglycan and utrophin (antibodies kindly provided by M. Ruegg) 30 μm cryosections of freshly embedded soleus muscle were fixed for 10 minutes with 2% PFA in PBS^-, washed three times for 5 minutes with PBS^- and permeabilized for 10 minutes with 1% Triton X-100 in PBS^-. Sections were incubated in 100 mM glycine in PBS^- for 10 minutes at room temperature, and in 3% normal goat serum (NGS), 0.3% Triton X-100 in PBS^- for 1 hour at room temperature. Sections were incubated overnight at 4ºC with primary antibody in 3% NGS, 0.3% Triton X-100 in PBS^-. For immunofluorescence detection, sections were washed in PBS^- and incubated for 1 hour at room temperature with FITC-, or TRITC-labeled secondary antibodies (Jackson ImmunoResearch), or FITC- or TRITC-labeled α-bungarotoxin (Molecular Probes). Sections were washed in PBS^- and mounted in Mowiol (Calbiochem).

In experiments using an antibody against parvalbumin (Swant), detection was performed with peroxidase-coupled secondary antibodies and the Vectastain ABC kit (Vector Laboratories). Embryonic muscles were dissected, fixed in 4% PFA for 2 hours at 4°C and incubated overnight at 4°C in 30% sucrose in PBS^-. Tissue was embedded in OTC and sections were cut at various thickness. Prior to staining, sections were fixed again in 4% PFA for 10 minutes at room temperature, washed three times in PBS^- and endogenous peroxidase was inactivated in 10% methanol, 3% H_2O_2 in PBS^- for 30 minutes. Blocking was performed in 0.4% Triton X-100, 10% NGS, 3% bovine serum albumin (BSA) in PBS^- for 1 hour at room temperature. Primary antibody was diluted in blocking solution and incubated on sections at 4°C overnight. Samples were further processed following the instructions in the Vectastain ABC kit. Digital images were collected on a Deltavision microscope (Applied Precision) and processed by deconvolution.

Primary muscle cell cultures

Hindlimb muscles from P2 animals were dissected, minced and incubated at 37°C for 15 minutes in HBSS-CMF (Gibco-BRL) containing collagenase type IV (100 U/ml, Sigma) and dispase type II (2.4 U/ml, Roche Molecular Diagnostics). The solution was triturated to obtain a single cell suspension, and cell clumps were allowed to settle. The supernatant was collected and supplemented with 1/10 of the volume with fetal calf serum (FCS). The solution was filtered through a cell strainer (Becton Dickinson), cells were harvested by centrifugation at room temperature and resuspended in DMEM supplemented with 25% 199 medium (Gibco-BRL), 10% FCS and antibiotic-antimycotic (Gibco-BRL). Cells were plated onto plastic dishes coated with 1% gelatine (Sigma) and cultured in a humidified atmosphere at 37°C/5% CO_2. To induce differentiation into myotubes, medium was changed to DMEM supplemented with 25% 199 medium, 2% horse serum, antibiotic-antimycotic and 10 μg/ml insulin (Sigma) when myoblast cultures had reached subconfluency.

Postsynaptic differentiation was induced by adding a C-terminal fragment of the neuronal isoform of chick agrin (200 pM-2 nM) (Ruegg, 1996) to myotubes in culture. Myotubes were analyzed 24 hours after induction by immunohistochemistry as described above.

Electrophysiology

Muscle fibers of the diaphragm were voltage clamped to –70 mV with a conventional two-electrode clamp system at endplates identified visually, and miniature endplate currents (mepcs) were recorded at room temperature in Tyrode solution. Amplitudes and decay phases of 20-50 mepcs per synapse were analyzed individually, assuming a single exponential decay. Amplitudes and decay time constants (reflecting mean burst durations of the AChR channels) from 20-50 mepcs per synapse were averaged. Only synapses with mepcs with rise times of less than 700 μs were included in the analysis.

Quantitative immunofluorescence measurements

In order to measure AChR density in mutant versus wild-type endplates, confocal image stacks were recorded with calibrated photomultiplier settings. Images were truncated at a lower intensity threshold of 10 because preliminary analysis showed that all voxels displaying intensity above 10 were associated with endplates. The remaining α-bungarotoxin Texas Red-positive voxels were processed as follows: the number of voxels at each intensity was multiplied by this intensity and the products were summed. This sum was divided by the total number of voxels remaining after truncation, thus yielding an estimate of average voxel intensity.

RESULTS

Erbb2^{flox} and HSA-Cre mice

To test for a function of *Erbb2* in the development of striated muscle, we established a mouse line carrying an *Erbb2* allele where the first coding exon was flanked by two loxP sites (*Erbb2^{flox}*; Fig. 1A,B). Mice that were homozygous for the *Erbb2^{flox}* allele were indistinguishable from wild-type mice, confirming that the loxP sites did not interfere with Erbb2 function prior to Cre-mediated recombination (data not shown). We also generated transgenic mouse lines expressing Cre recombinase under the control of the human skeletal alpha-actin (HSA) promoter (Fig. 1C; M.S., M.L. and U.M., unpublished). This promoter directs transgene expression to developing striated muscle (Brennan and Hardeman, 1993; Muscat and Kedes, 1987). To test for Cre activity, we crossed mice from several independent *HSA-Cre* transgenic lines with mice from a reporter line that carries a *Rosa26-lacZ^{flox}* gene. In this line, *lacZ* expression is induced by Cre-mediated recombination (Mao et al., 1999). Whole-mount *lacZ* staining confirmed widespread recombination in developing striated muscle with one Cre line. *lacZ* expression was detectable at embryonic day (E) 9.5 in the developing somites, and at subsequent ages in developing striated muscle (Fig. 1D; M.S., M.L. and U.M, unpublished). In sections of forelimbs, *lacZ* expression was detected throughout developing premuscle masses (Fig. 1E). In muscle cross-sections, *lacZ* expression was detected in essentially all nuclei of muscle fibers, as well as in the nuclear bag and chain fibers of muscle spindles (Fig. 1F). We conclude that in the *HSA-Cre* line, Cre-mediated recombination is induced efficiently in developing muscle fibers of striated muscle starting at or around E9.5, including intrafusal and extrafusal muscle fibers. Ectopic Cre expression was also detected in single cells scattered throughout the skin, heart and lung, and in the trigeminal ganglion (Fig. 1D,E, and data not shown).

Inactivation of *Erbb2* in skeletal muscle

We next crossed the *HSA-Cre* transgene on an *Erbb2$^{+/-}$* background. These mice were crossed with *Erbb2$^{flox/flox}$* mice. Offspring that inherited one *Erbb2^{-}* allele, one *Erbb2^{flox}* allele and the *HSA-Cre* transgene were born with the expected Mendelian frequency and were viable. DNA analysis

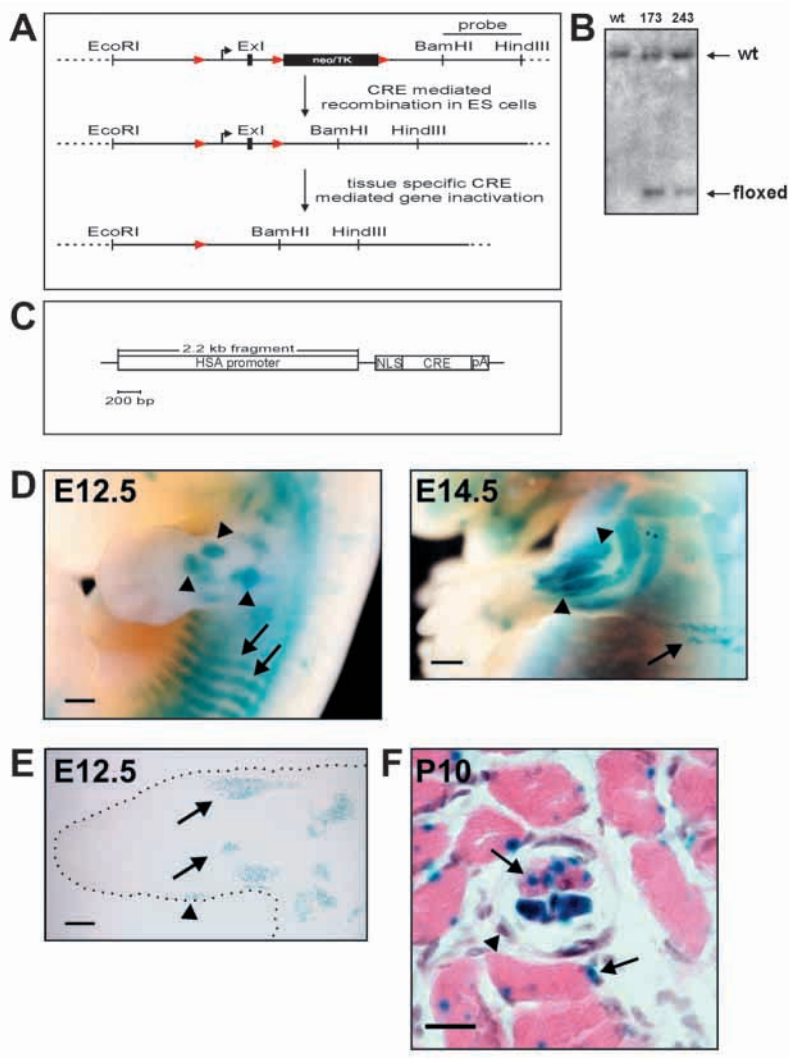

Fig. 1. *Erbb2*^{flox} and *HSA-CRE* mice. (A) The *Erbb2*^{flox} locus and the gene targeting strategy. A loxP-neo/tk-loxP cassette was inserted into the intron 3′ of the first coding exon of the *Erbb2* gene, and additional loxP-site was inserted into the intron 5′ to the exon (loxP-sites are shown as red triangles; the start site of transcription by an arrow). ES cells were transfected with a DNA fragment extending from the *Eco*RI site to the *Bam*HI site. Targeted ES clones were identified by Southern blot, using a probe located immediately 3′ of the targeting vector. One positive clone was retransfected with a Cre-expressing plasmid, and clones that had lost the neo-tk cassette, but retained two loxP sites flanking the first coding exon of the *Erbb2* gene were identified by Southern blot using the same probe as described above. One positive clone was used to generate germ line transmitting chimeric mice. (B) Southern blot analysis of targeted ES cells containing two loxP sites; wt, no targeting; clones 173, 243, properly targeted ES cell clones. (C) The HSA-Cre construct used to generate the transgenic mouse lines. HAS, human skeletal α-actin; NLS, nuclear localization signal; pA, polyadenylation signal. (D) Whole-mount *lacZ* staining of E12.5 (left panel) and E14.5 (right panel) embryos obtained from intercrosses between a *Rosa26-lacZ*^{flox} tester mouse with a *HSA-Cre* mouse. Arrows in the left panel indicate intercostal muscles, arrowheads highlight muscle groups in the forelimb; the arrow in the right panel marks recombined cells in the skin, the arrowheads mark muscle groups in forelimb. (E) Cross-section through forelimb of a E12.5 mouse embryo stained for *lacZ*. All muscle groups within the forelimb were *lacZ* positive (arrows), as well as some cells in the skin (arrowhead). (F) Extra- and intrafusal muscle fibers show strong nuclear *lacZ* staining (arrows), whereas perineural cells forming the spindle capsule are *lacZ* negative (arrowhead). Scale bars: 200 μm in D (left panel) and E; 500 μm in D (right panel); 50 μm in F.

confirmed that Cre induced efficient recombination of the *Erbb2*^{flox} allele in muscle (data not shown). To confirm that Erbb2 protein was absent from muscle, we carried out immunohistochemistry with antibodies against Erbb2. In wild-type mice, Erbb2 immunoreactivity was concentrated at NMJs that were visualized by co-staining with α-bungarotoxin, to label AChR clusters (Fig. 2A). In the mutants, AChR clusters still formed, but Erbb2 was not expressed at the synapse (Fig. 2B). Low levels of immunoreactivity for Erbb2 were still detectable adjacent to synaptic sites, probably reflecting Erbb2 expression in terminal Schwann cells that surround the synapses, but where the HSA promoter used to drive Cre expression was not active.

To confirm further that Erbb2 expression was absent in the muscle fibers of mutant mice, we cultured myoblasts from P2 hindlimb muscle and differentiated them in vitro into myotubes. We next induced AChR clusters by adding soluble agrin to the cultures. AChR clusters readily formed in myotubes derived from myoblasts of both wild-type and mutant mice (Fig. 2C,D), and the AChR clusters contained additional synaptic proteins such as utrophin (Fig. 2E). However, while Erbb2 was concentrated at AChR clusters in wild-type mice, it was completely absent in the mutants (Fig. 2C,D).

In summary, we conclude that we had effectively inactivated Erbb2 expression in developing muscle fibers. Furthermore, the data demonstrate that agrin can induce AChR clusters in myotubes in vitro in the absence of Erbb2 expression.

Reduced synaptic transmission at Erbb2-deficient NMJs

We next analyzed by immunohistochemistry and electrophysiology whether the structure and function of NMJs was affected in vivo in the absence of Erbb2. Staining with α-bungarotoxin revealed that AChR clusters had formed in the mutants, and that additional postsynaptic proteins, such as β-dystroglycan and utrophin were appropriately localized to synaptic sites. In addition, no defects were apparent in the characteristic pretzel-shape of the NMJs (Fig. 3A-C).

To test whether the NMJs in Erbb2-deficient mice functioned normally, we analyzed their electrophysiological properties (Fig. 3D,E). For this purpose, mepcs were recorded from NMJs of the diaphragm. Quantitative analysis showed that mepc amplitudes in mutant compared with wild-type muscles were reduced by about 15% (37 wild-type and 39 mutant endplates, from five wild-type and six mutant diaphragms). Decay time constants were 1-2 mseconds in both wild-type and mutant muscles, consistent with the induction of

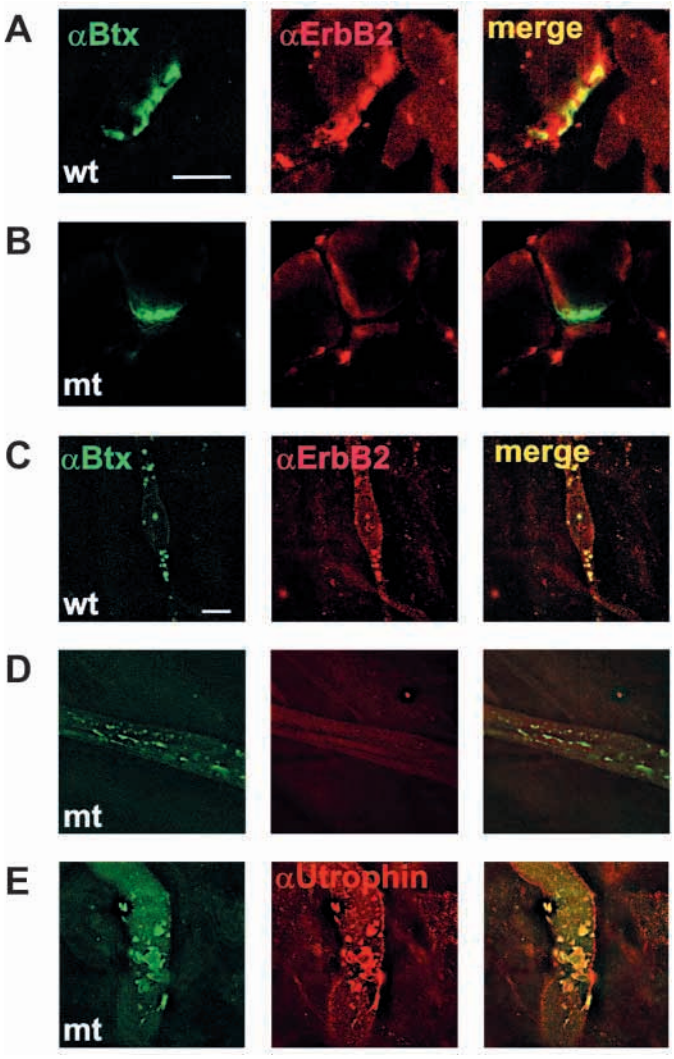

Fig. 2. Inactivation of the *Erbb2* gene. (A) At the NMJ of soleus muscle in wild-type mice Erbb2 (red) formed co-clusters with AChR (green) as identified by α-bungarotoxin staining (α-Btx). (B) No Erbb2 protein was detected at the NMJ of Erbb2-deficient mice. Diffuse staining of Erbb2 was seen in terminal Schwann cells. (C-E) Myoblasts from wild-type and mutant mice were isolated from P2 mice and induced to form myotubes in culture. Agrin was added to induce AChR clusters. In wild-type mice, Erbb2 (red) co-localized with AChR clusters (green) (C). In the mutants, agrin induced AChR clusters, but Erbb2 was absent from the clusters (D). Utrophin (red) still co-clustered with AChR (green) in the mutant muscle fibers (E). Scale bars: 15 μm in A,B; 10 μm in C-E.

rapidly gating 'adult type' AChR channels (Mishina et al., 1986) in the absence of Erbb2.

The reduced efficiency of NMJs in Erbb2-deficient mice could be due to reduced AChR levels at synaptic sites. We therefore measured AChR levels by quantitative immunofluorescence. Clusters of AChR at NMJs in wild-type and mutant mice were stained with Texas Red-labeled α-bungarotoxin, and serial optical sections were taken and quantified. Integration of fluorescent intensities revealed a decrease by about 20% in AChR numbers at endplates of mutant compared with wild-type muscles (Fig. 3F; 27 wild-type and 27 mutant endplates, from five wild-type and five mutant diaphragms), consistent with the electrophysiological data. Taken together, the data show that Erbb2 is not essential for NMJ formation. However, in the absence of Erbb2, the synapses are less efficient and contain reduced numbers of AChRs.

Muscle spindle development

Mice that lacked Erbb2 in muscle showed abnormal hindlimb extension reflexes and ataxia (Fig. 4A, panels 1-3). When positioned on a slippery surface, the mutant mice were unable to position their limbs properly (Fig. 4A, panel 4). They also showed spastic movements (data not shown). This behavior is consistent with proprioceptive defects, suggesting that muscle spindle development or function may be defective in the mutants.

To analyze whether Erbb2-deficient muscle was defective in proprioceptive sense organs, we examined serial sections through limb muscles of P9 and adult mice stained with Hematoxylin and Eosin. Although muscle spindles could readily be detected in the soleus muscle of wild-type mice, as encapsulated bundles of intrafusal fibers with multiple nuclei at the equatorial plane (8±1; n=4), they were completely absent in the mutants (0±0; n=10) (Fig. 4B). No spindles were present in all other muscles analyzed, including gastrocnemius and gluteus (data not shown). These data suggest that Erbb2 is essential for muscle spindle development or maintenance.

To test whether Erbb2 was expressed in muscle spindles, we stained transverse sections through skeletal muscle with antibodies to Erbb2. Unfortunately, Erbb2 expression was difficult to detect in early developing muscle spindles, presumably because it was expressed at low levels and distributed diffusely (data not shown). However, we could detect Erbb2 protein in mature muscle spindles, where it became concentrated at synaptic sites where γ-motoneurons innervate muscle spindles (Fig. 4C). These data suggest, that Erbb2 is already expressed in early developing muscle spindles, and becomes clustered upon synapse formation.

We next analyzed the embryonic development and differentiation of muscle spindles in wild-type and Erbb2-deficient muscles using electron microscopy (EM) to determine whether Erbb2 signaling in muscle was required for muscle spindle induction, differentiation and/or maintenance (Fig. 5). In wild-type mice, muscle spindle morphogenesis in hindlimb muscle is initiated around E15.5-16.5, when sensory Ia afferents reaching the muscle first contact myotubes. At this developmental time, most nascent spindles are formed by a single myotube that is approached by one or more sensory afferents arising from a neighboring nerve bundle (Kucera and Walro, 1995). EM examination of E16.5 muscles revealed Ia afferent nerve fiber-myotube contact in both wild-type and mutant muscles, suggesting that initial contact is not dependent of Erbb2 expression in myotubes (Fig. 5A,B). These contacts between sensory Ia afferent axons and muscle fibers can readily be distinguished from putative contacts between motoneurons and muscle fibers because they lack a basal lamina between the axon terminal and the muscle fiber (Landon, 1972). Overall, mutant muscle did not appear to have fewer sensory terminals-myotube contacts. The contacted myotubes represented early nuclear bag_2 intrafusal fibers and those present in the mutant muscle appeared less differentiated than in wild types, with smaller size and less developed packages of myofibrils (data not

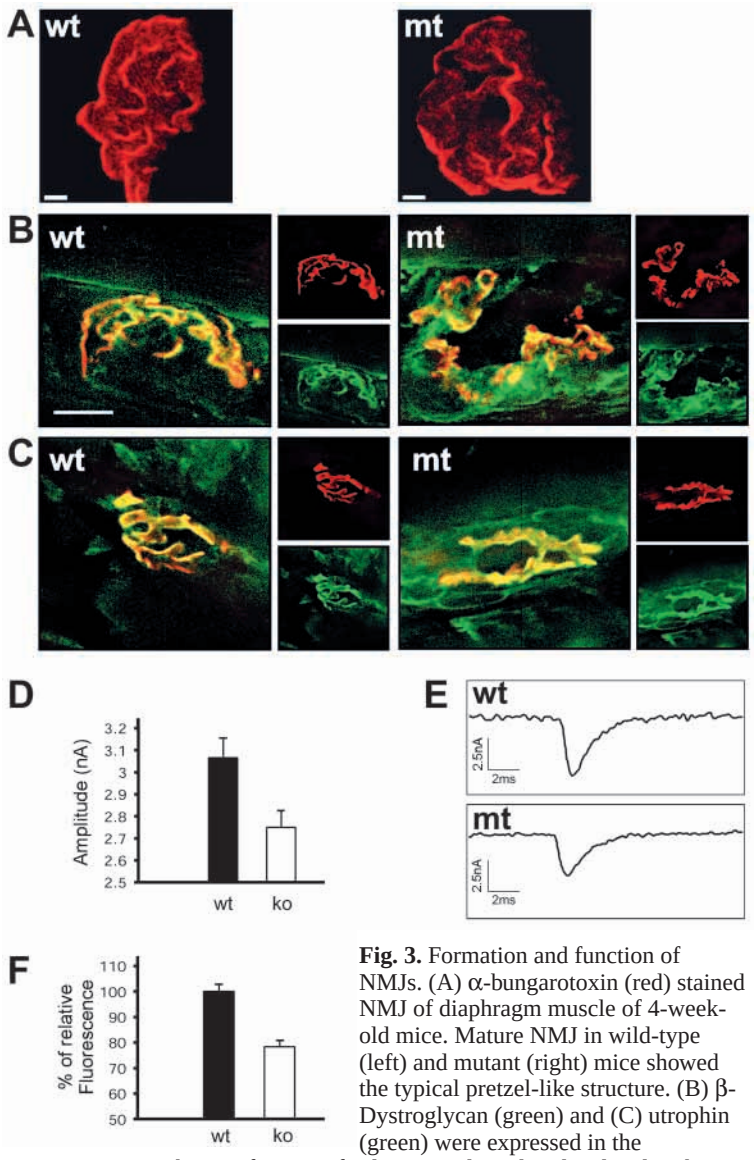

Fig. 3. Formation and function of NMJs. (A) α-bungarotoxin (red) stained NMJ of diaphragm muscle of 4-week-old mice. Mature NMJ in wild-type (left) and mutant (right) mice showed the typical pretzel-like structure. (B) β-Dystroglycan (green) and (C) utrophin (green) were expressed in the postsynaptic membrane of NMJs of soleus muscle and co-localized with α-bungarotoxin (red) in both wild-type (left) and mutant (right) mice. (D) Miniature endplate currents were decreased in amplitude by ~15% in mutant mice when compared with wild-type mice, while decay time constants (E) were similar (means±s.e.; data from 37 wild-type and 39 mutant endplates, from five wild-type and six mutant diaphragms), as shown in these representative recordings. (F) Quantitative immunofluorescence of α-bungarotoxin-labeled AChRs at NMJs of diaphragm muscle reveals decrease in AChR density by about 20% in mutant mice (means±s.e.; data from 27 wild-type and 27 mutant endplates, from five wild-type and five mutant diaphragms), consistent with the reduction in mepc. amplitude observed in electrophysiological recordings. Scale bars: 3 μm in A-C.

shown). At E17, most spindles in wild-type muscles contained an extra myotube (nuclear bag_1) in addition to the initial nuclear bag_2 fiber (Fig. 5C). Normal morphogenesis of wild-type muscle spindles was also characterized by the lateral expansion of the axon-associated Schwann cells around the intrafusal bag fibers and by an increase in the myotube surface covered by the sensory axon terminals (Fig. 5C). By contrast, mutant spindles at E17.5 had the characteristic appearance of spindles at E16.5 (Fig. 5D). The initial contacts between sensory afferents and the primary myotube were still evident, but morphological differentiation was halted. Morphological analysis of serial sections indicated that in Erbb2-deficient muscle the initially contacted myotube did not become a nuclear bag fiber and that a second myotube was never recruited. Moreover, Schwann cells in the mutants extended laterally following the axon terminals but they failed to surround the muscle fiber that was contacted by the nerve ending (Fig. 5D). At E18.5, wild-type muscle spindles continue to develop normally but spindles in the Erbb2-deficient muscles still resembled the initial stages of muscle spindle formation (Fig. 5E,F). Despite the fact that normal morphological differentiation of muscle spindles was abrogate, sensory innervation became more extensive and numerous around mutant myotubes (Fig. 5F). At birth, when muscle spindles in wild-type muscle can be easily identified by their characteristic surrounding capsule and are composed of a few muscle bag fibers innervated in much of their surface (Fig. 5G), it was still possible to observe isolated muscle fibers completely surrounded by afferent fibers in Erbb2-deficient muscle (Fig. 5H). Some of the nerve terminals in the mutants appeared altered, with a swollen morphology. Mature muscle spindles were never observed in the Erbb2 conditional mutants. Taken together, the data suggest that lack of Erbb2 signaling in muscle fibers is not essential for the initial contact between sensory Ia afferent neurons and myotubes but is necessary for the subsequent development of the structure.

To confirm further that the neurons that contacted muscle fibers were Ia afferent neurons, we stained muscle sections with antibodies to parvalbumin that selectively labels Ia afferent neurons that contact muscle spindles (stretch receptors), but also Ib afferent neurons that innervate Golgi tendon organs (tension receptors) (Fig. 6) (Carr et al., 1989). Ia and Ib afferent neurons can readily be distinguished by their projection pattern as Golgi tendon organs are located towards the end of muscles, while spindles are distributed throughout muscle. Ia and Ib afferent projections were present in wild-type mice, and they formed connections with muscle spindles and Golgi tendon organs, respectively (Fig. 6A,C). In the mutants, both types of projections were visible (Fig. 6A-C), but contacts with muscle spindles appeared abnormal. Accordingly, the staining intensity was much reduced and highly irregular (Fig. 6D). Golgi tendon organs appeared unaffected.

Development of central projections of sensory Ia afferents

It is at present unclear whether normal spindle development is required for the development of central projections of Ia afferent neurons. Ia afferent neurons project to the ventral spinal cord where they establish synaptic contact with motoneurons. Ia and Ib afferents establish projections to the Clark columns in the medial aspect of the spinal cord, where they form synaptic contact with interneurons (Brown, 1981). Parvalbumin positive cell bodies of Ia and Ib afferent neurons could readily be detected distributed throughout the dorsal root ganglia of both wild-type and Erbb2-deficient mice (Fig. 7A). Projections to Clark columns and to the motoneuron

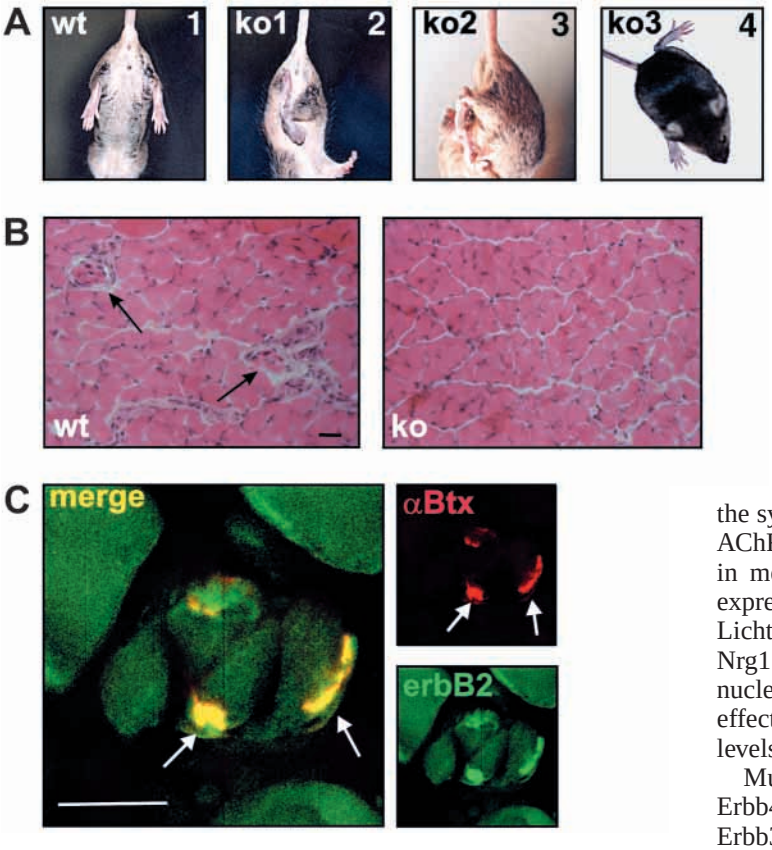

Fig. 4. Proprioceptive defects and absence of spindles. (A) Wild-type animals but not the mutants showed an abnormal hind-limb extension reflex (panels 1-3). They also had difficulties in coordinating limb movement when placed on a slippery platform (panel 4). (B) Hematoxylin/Eosin stained cross-sections of P9 soleus muscle. Two muscle spindles of a wild-type mouse are indicated by arrows. In the mutant mice no spindles could be identified. (C) Erbb2 (green) was expressed in intrafusal muscle fibers of muscle spindles. It co-localized with α-bungarotoxin labeled AChR clusters in postsynaptic membranes of γ-motoneuron contact sites (yellow, arrows). Scale bars: 50 μm in B; 15 μm in C.

pools in the ventral spinal cord were detected in both wild-type and mutant mice (Fig. 7B). We therefore conclude that in the absence of normal muscle spindle development Ia afferent neurons are still able to establish their normal central projection pattern.

DISCUSSION

We provide here genetic evidence that Erbb2 receptors in skeletal muscle fibers regulate the formation of neuromuscular synapses and are essential for muscle spindle development. Motoneurons contact Erbb2-deficient skeletal muscle fibers, and neuromuscular synapses develop. However, the synapses are less efficient and contain reduced levels of AChRs. Sensory Ia afferent neurons also establish contact with Erbb2-deficient muscle fibers, but functional muscle spindles never develop. Importantly, Ia afferent neurons still elaborate their projections within the spinal cord, demonstrating that normal muscle spindle development is not essential for the development of central projections of Ia afferent neurons.

Erbb receptors in NMJ formation

The function of Erbb receptors in NMJ formation has been studied previously. Skeletal muscle fibers express Erbb2, Erbb3 and Erbb4 receptors. NMJ formation and gene expression in subsynaptic nuclei was observed in mice that lack Erbb2 or Erbb3 (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Riethmacher et al., 1997). However, the previously described genetically modified mice died at birth, preventing a more detailed analysis of Erbb2/3 receptor function in NMJ development. We now show that mice genetically modified to lack Erbb2 specifically in muscle survive into adulthood and develop NMJs, but the synapses are less efficient and contain reduced numbers of AChRs. As the Erbb receptor ligand Nrg1 has been implicated in mediating its effect on muscle fibers by regulating gene expression in subsynaptic nuclei (Burden, 1998; Sanes and Lichtman, 1999), our data are consistent with a model where Nrg1 activates via Erbb2 gene expression in subsynaptic nuclei. In the absence of Erbb2, Nrg1 signaling may be less effective, leading to decreased gene expression and reduced levels of synaptic proteins.

Muscle fibers express not only Erbb2, but also Erbb3 and Erbb4 (Zhu et al., 1995). It is therefore possible that Erbb3/Erbb4 may have partially compensated for a loss of Erbb2. Unfortunately, mice that carry a null mutation in the *Erbb4* gene die during embryogenesis (Gassmann et al., 1995), preventing an analysis of its function in NMJ formation by conventional genetic approaches. However, our recent data implicate Erbb4 in NMJ formation. When postsynaptic differentiation is induced in Erbb2-deficient muscle fibers by ectopic expression of agrin, clustering of proteins in the postsynaptic membrane is less efficient. This defect can be rescued by overexpressing Erbb4 (Moore et al., 2001), suggesting that both Erbb2 and Erbb4 are sufficient to induce gene expression in subsynaptic nuclei. As we observed in vivo decreased levels of AChRs in muscle that only lacked Erbb2, the data also suggest that in the more physiological setting where Erbb2 and Erbb4 are expressed at lower levels than achieved by ectopic overexpression, both receptors may be essential for maximal expression of AChR subunit genes. To test for a partially redundant function of Erbb2 and Erbb4 in muscle fibers, it will be important to inactivate both receptors simultaneously in muscle fibers.

Erbb2 functions in muscle spindle development

Our data show that Erbb2 expression in skeletal muscle fibers is essential for muscle spindle development. Previous studies have provided strong evidence that muscle spindle development is initiated by a signal from sensory Ia afferent neurons. We show here that contact of Ia afferents with myotubes is initiated in the absence of Erbb2 expression in myotubes, but muscle spindle formation does not progress and the rudimentary spindles degenerate. This suggests that Erbb2 is not required for the establishment of the initial contact between sensory neurons and myotubes, but for the subsequent differentiation of muscle spindles. Interestingly,

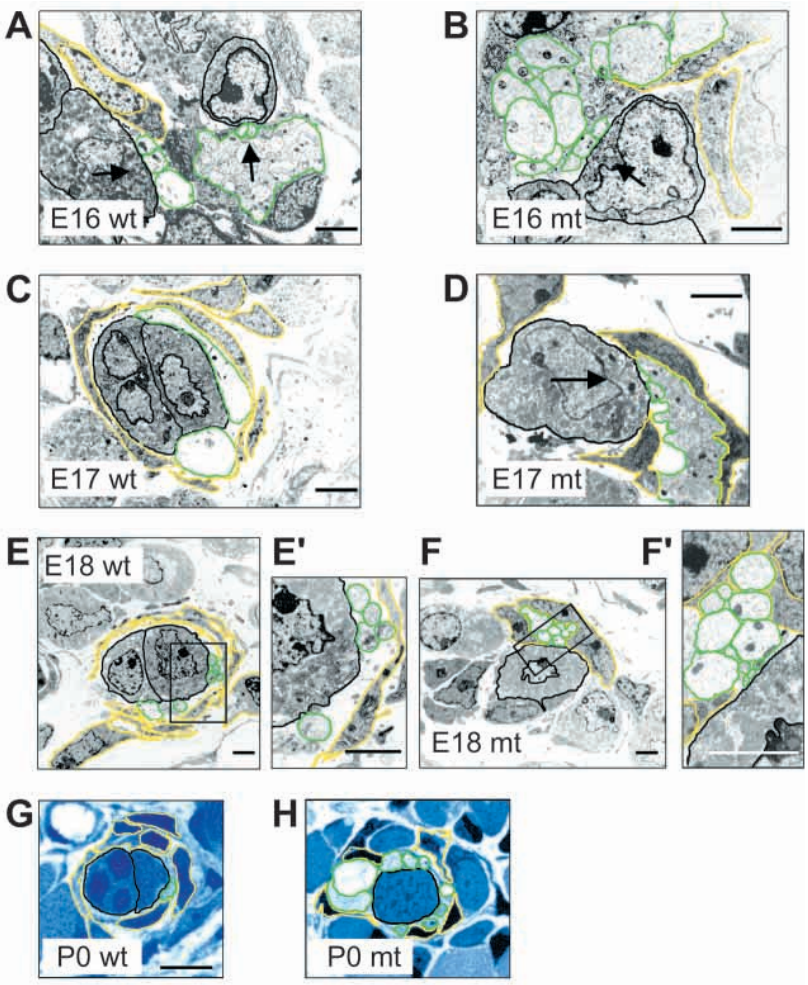

Fig. 5. Analysis of muscle spindle development. (A,B) EM pictures of a primary myotube being contacted by Ia afferent sensory fibers at E16 in a wild-type (A) and a mutant (B) embryo. Single myotubes (circled black) were contacted by nerve terminals (circled green) as indicated by the arrow. Schwann cells (circled yellow) accompanied the ingrowing afferent fiber. (C,D) At E17, many muscle spindles in wild-type embryos consisted of two intrafusual fibers (black) contacted by multiple nerve endings (green). Schwann cells (yellow) accompanying the spindle nerve bundle formed a rudimentary unilamelar capsule surrounding the developing spindle. In some cases two nuclei were observed at the equatorial plane of the myotube that was developing into a nuclear bag_2 fiber. In muscle in mutant mice (D), myotubes contacted by sensory nerve terminals were still frequently observed close to nerve bundles but muscle spindle differentiation appeared halted. Arrow in D indicates contact between myotube and nerve terminal. Rudimentary spindles were still formed by a single myotube contacted by unexpanded afferent nerve terminals and were not surrounded by Schwann cell processes. Innervated myotubes did not progress towards a nuclear bag phenotype and other myotubes were not recruited as intrafusal fibers. Nevertheless, synaptic contacts by sensory nerve terminals, as revealed by the lack of a basal lamina in the sypnaptic cleft still persisted. (E,F) By E18, spindles in wild-type mice were surrounded by a multi-lamellar spindle capsule, and spindle fibers were innervated by a Ia afferent (boxed contact zone enlarged in E′). Spindles in the mutant mice (F) did not differentiate further. Only one spindle fiber was present, and no capsule had formed. However, contacts between the muscle fiber and Ia-afferent was maintained (boxed area enlarged in F′). (G,H) Plastic sections through P0 muscle of wild-type and mutant mice showed that although muscle spindles in wild-type mice (G) typically consisted of three or four encapsulated multinucleated intrafusal fibers innervated by multiple nerve terminals, spindles in the mutants (H) only consisted of one muscle fiber still surrounded by nerve terminals. Some of the afferents appeared abnormally enlarged and swollen. Scale bars: 2 μm in A-F; 5 μm in G,H.

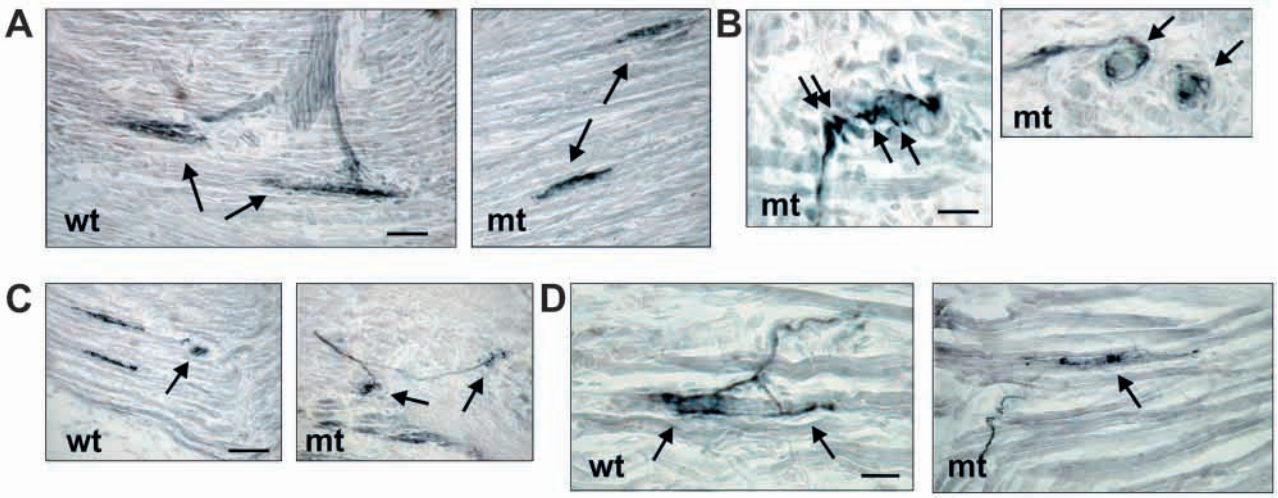

Fig. 6. Analysis of Ia and Ib afferent projections by parvalbumin staining. (A) Immunohistochemical analysis using an antibody against parvalbumin as a marker for Ia- and Ib afferents. At E18.5, spindles were innervated by sensory afferents in both wild-type and mutant mice (arrows). (B) Two enlarged innervated spindles in mutant mice (right) at E18.5 showing the Ia afferent spiraling around the intrafusal muscle fiber (arrows). Notice the presence of only one intrafusual fiber in mutant mice. Left panel, longitudinal; right panel, cross-section. (C) Golgi tendon organs formed and were properly innervated in wild-type and mutant animals at E18.5 (arrows). (D) At postnatal day 4, spindles in wild-type and mutant mice were innervated, but the nerve terminals in the mutants were weakly stained, appeared irregular and were less frequently found. Scale bars: 100 μm in A; 30 μm in B; 200 μm in C; 40 μm in D.

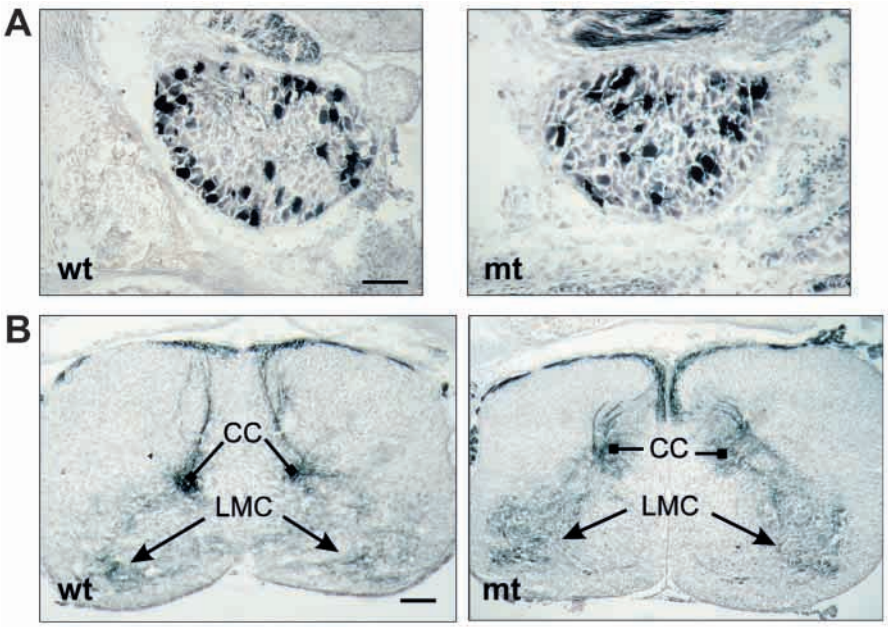

Fig. 7. Central projections of Ia and Ib afferent neurons. (A) Parvalbumin positive cell bodies of sensory neurons were present throughout the dorsal root ganglia at lumbar levels in both mutant and wild-type mice at P3. (B) Central projections of Ia and Ib neurons were still present at P3 in mutant mice (CC, Clark columns; LMC, lateral motor columns. Scale bars: 50 μm in A; 150 μm in B.

during development of sympathetic neurons, NT3 expression is activated by Nrg1 (Verdi et al., 1996). Likewise, muscle spindles express NT3 (Copray and Brouwer, 1994) and muscle spindles are absent in NT3-deficient mice (Farinas et al., 1994). This raises the possibility that NT3 is more generally a downstream effector of Nrg1/Erbb signaling, and that it is an essential component by which Erbb2 regulates muscle spindle development. In this model, activation of Erbb2 in muscle fibers may regulate expression of NT3. As NT3 promotes survival of Ia afferent neurons (ElShamy and Ernfors, 1996; ElShamy et al., 1998; Farinas et al., 1996; Oakley et al., 1995; Oakley et al., 1997; Ockel et al., 1996; Ringstedt et al., 1997; Wright et al., 1997), and because ectopic expression of Ntf3 induces muscle spindle development (Wright et al., 1997), Nrg1/Erbb signaling may be essential to maintain NT3 expression in muscle spindles during their development, which in turn may affect sensory Ia afferent neurons. As the zinc-finger transcription factor Egr3 appears to regulate spindle specific NT3 expression (Chen et al., 2002), and is essential for muscle spindle maintenance (Tourtellotte and Milbrandt, 1998), it is possible that Erbb2 may act at least in part upstream of Egr3 thereby regulating NT3 expression.

It has recently been reported that Erbb2 is required for survival of muscle spindles and myoblasts, and that regeneration of muscle fibers is impaired in the absence of Erbb2 (Andrechek et al., 2002). We cannot substantiate these findings. We show that muscle fiber development and maintenance is not impaired in the absence of Erbb2 in vivo. Primary myoblasts isolated from the genetically modified mice described here are not impaired in their ability to survive and replicate in vitro, and they fused to form muscle fibers. In addition, the regenerative capacity of Erbb2-deficient muscle is unaltered (H.R.B., M.L., and U.M., unpublished). Likewise, although our data do not exclude a function for Erbb2 in muscle spindle maintenance, they clearly show an earlier function in spindle development. In the previous study it was demonstrated that muscle spindles were absent in postnatal Erbb2-deficient muscle (Andrechek et al., 2002). As a likely explanation, the previously described defects were not caused by a defect in muscle spindle maintenance, but rather in development. Alternatively, differences in genetic background could have contributed to the different results.

Muscle spindle development and central projections of Ia afferent neurons

It has remained unclear whether spindle development is required for the development and maintenance of the monosynaptic reflex circuit. The data presented here provide evidence that large parts of the monosynaptic reflex circuit form in the absence of normal muscle spindle development. Sensory Ia afferent neurons not only develop their peripheral projections into muscle. They also develop central projections towards the ventral spinal cord to the motoneuron pools. These central projections are maintained even in early postnatal animals when no signs of muscle spindle development can be detected. However, although our studies demonstrate that sensory Ia afferent neurons develop normal projections, it will be important to establish whether they also develop functional synaptic connections. The availability of the genetically modified mice described here will allow testing this hypothesis.

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