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Complete Deletion of All α-Dystrobrevin Isoforms Does Not Reveal New Neuromuscular Junction Phenotype

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The dystrophin glycoprotein complex (DGC) is critical for muscle stability, and mutations in DGC proteins lead to muscular dystrophy. The DGC also contributes to the maturation and maintenance of the neuromuscular junction (NMJ). The gene encoding the DGC protein α-dystrobrevin undergoes alternative splicing to produce at least five known isoforms. Isoform-specific antibody staining and reverse transcription PCR in mutant mice with a deletion of exon 3 of the α-dystrobrevin gene suggested the existence of a remaining synaptic isoform, which might be compensating for α-dystrobrevin function. To test this possibility and to more completely understand the synaptic function of α-dystrobrevin, we used a two-step homologous recombination strategy combined with in vivo Cre-mediated excision to generate mice with a large deletion of the α-dystrobrevin gene to disrupt all isoforms. However, these mice did not exhibit a more severe NMJ phenotype than that observed in the exon 3-deleted mice. Nonetheless, these mice not only eliminate possible compensation by remaining isoforms of α-dystrobrevin, but also offer a conditional allele that could be used to identify tissue-specific and developmental functions of α-dystrobrevin. This work also demonstrates a successful strategy to achieve deletion of a large genomic sequence, which can be a valuable tool for functional studies of genes encoding multiple isoforms that span a large genomic region.

Key words: Dystrophin glycoprotein complex; Neuromuscular junction; Isoform diversity; Alternative splicing; Homologous recombination

INTRODUCTION

The dystrophin glycoprotein complex (DGC) is a large protein complex first characterized based on one of its major components, dystrophin, a cytoskeletal protein originally identified as the gene product mutated in Duchenne and Becker muscular dystrophies (11). Although the DGC is classically known for its critical role in maintaining muscle stability by linking the actin cytoskeleton to the extracellular matrix, many of the DGC proteins are also found at the synaptic site of the muscle membrane, the neuromuscular junction (NMJ) (7,29,32,35). The proteins in the DGC have been implicated in the agrin-stimulated clustering of nicotinic acetylcholine receptors (nAChRs) and in the postsynaptic development of the NMJ, most likely playing a role in the maturation and maintenance of the synapse (1,2,5,13–16,18,19,21, 22,24,27,28,31).

The DGC consists of dystrophin or its related protein utrophin; three groups of transmembrane proteins, the dystroglycans, the sarcoglycans and sarcospan; and two groups of cytoplasmic proteins, the dystrobrevins and the syntrophins. In addition to its

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structural role, the DGC may have a signaling function, in particular through two cytoplasmic components, the syntrophins and the dystrobrevins (6,12,20, 25,39,40). α-Dystrobrevin can bind to both dystrophin and the syntrophins (35,38), and the syntrophins in turn interact with both neuronal nitric oxide synthase and voltage-gated sodium channels (12,20,39). Thus, α-dystrobrevin may serve as a link between the structural and signal transduction functions of the DGC (6,23–25,35,38,40).

The α-dystrobrevins in mammals consist of at least five known possible isoforms produced by one gene through alternative splicing (4,8,32,35–37) (Fig. 1A). The regulation of different isoforms, either constitutively or in response to developmental or physiological cues, can be a powerful mechanism to generate related proteins with unique functional features and distinct temporal and spatial expression patterns, greatly expanding the functional diversity that can be produced by a comparatively small total number of genes (30). This feature of many genes can pose a challenge in the generation of loss-of-function genetic mouse models. In some cases, traditional homologous recombination strategies cannot span a large enough region of the genomic sequence to delete all isoforms of a gene. Remaining splice variants may compensate for the function of deleted isoforms, therefore complicating the interpretation of functional studies.

Mutant mice in which α-dystrobrevin was disrupted by the deletion of exon 3 are mildly dystrophic and have defects in the maturation of the postsynaptic apparatus at the NMJ (1,3,23,24). Interestingly, immunostaining with an antibody specific for α-dystrobrevin isoforms 2 and 5 (35) (anti-DB2/5) persisted at the NMJ of the exon 3-deleted mutant mice, although extrasynaptic muscle membrane staining was lost (24). This persistent staining and the amplification of α-dystrobrevin transcripts by reverse transcription-PCR reported in this study suggest that there may be a synaptic α-dystrobrevin isoform that remains in the exon 3-deleted mutant mice and that could be masking a more severe NMJ phenotype.

To investigate this possibility and to gain a more complete understanding of the synaptic function of α-dystrobrevin, we used a double homologous recombination strategy combined with Cre-mediated excision in vivo to produce a large genomic deletion resulting in the loss of all potential α-dystrobrevin isoforms. Surprisingly, staining with the α-dystrobrevin isoform-specific antibody persisted at the NMJ in these complete-deletion mice, despite the deletion of the gene sequence encoding the antigen against which the antibody was made. This suggests that the anti-DB2/5 antibody cross-reacts with an unidentified synapse-specific protein and thus may not be a reliable marker for α-dystrobrevin-2 and -5. Mice with this large genomic deletion show a NMJ phenotype very similar to that seen in the exon 3-deleted mutant mice (23,24). In addition to eliminating the possibility of compensation by a remaining α-dystrobrevin isoform, this new line of mice also provides a useful tool for further study of the function of α-dystrobrevin because it offers a conditional deletion of all α-dystrobrevin isoforms. In addition, the use of sequential homologous recombination combined with in vivo Cre-mediated excision to generate the large genomic deletion in these complete-deletion α-dystrobrevin mice demonstrates a valuable strategy for designing functional studies of genes that encode multiple isoforms spanning a large region of the genome.

MATERIALS AND METHODS

Mice

Mice with a deletion of exon 3 of the α-dystrobrevin gene (exon 3-deleted mutant mice) were obtained from Dr. Joshua Sanes (Harvard University, Cambridge, MA) (23). Mice with a deletion of exons 5–18 of the α-dystrobrevin gene were generated as described below. Wild-type mice were either C57BL/6J (Jackson Laboratories, Bar Harbor, ME) or wild-type littermates from the two mutant mouse lines.

Antibodies

The rabbit polyclonal antibody specific for α-dystrobrevin-2 and -5 (anti-DB2/5) was previously described (35). It was prepared against the peptide GVSYVPYCRS, corresponding to the unique 10 amino acid C-terminal sequence shared by α-dystrobrevin-2 and -5. The mouse monoclonal antibody that recognizes α-dystrobrevin isoforms 1 and 2 (anti-DB1/2) was from BD Biosciences (San Jose, CA). Alexa-488-conjugated bungarotoxin was from Molecular Probes (Invitrogen, Carlsbad, CA). The Cy3 goat anti-rabbit and Cy3 goat anti-mouse IgG1 antibodies were from Jackson ImmunoResearch (West Grove, PA).

Immunohistochemistry

Cryosections of adult mouse tibialis anterior muscle were blocked for at least 1 h with 5% NGS and 2% BSA in PBS. Following blocking, sections were incubated with primary antibodies in blocking solution for 2–4 h at room temperature or overnight at 4°C at the following dilutions: anti-DB2/5 at 1:900, anti-DB1/2 at 1:100. Sections were then washed five times with PBS for 5 min each, and incubated for 2 h

with Alexa-488-conjugated bungarotoxin in blocking solution at 1:2000 and Cy3-conjugated secondary antibodies in blocking solution at the following dilutions: Cy3 goat anti-rabbit at 1:2000, Cy3 goat anti-mouse IgG1 at 1:2000. Following secondary antibody incubation, sections were washed five times with PBS for 5 min each and mounted for fluorescent imaging under coverslips in 90% glycerol with 0.1% *p*-phenylenediamine.

Cryosections of sternomastoid muscle from mice that had been perfused transcardially with 4% PFA in PBS were blocked overnight with 5% NGS, 2% BSA, and 0.2% Triton in PBS. Following blocking, sections were incubated with Alexa-488-conjugated bungarotoxin at 1:2000 in blocking solution for 4 h at room temperature. Sections were then washed five times with PBS for 10 min each and mounted for confocal imaging under coverslips in 90% glycerol with 0.1% *p*-phenylenediamine.

Reverse Transcription-PCR

Total RNA was isolated from adult wild-type, exon 3-deleted mutant or complete-deletion mouse muscle and brain using the RNAqueous-4PCR Kit according to the manufacturer's instructions (Ambion, Austin, TX). Total RNA was used for reverse transcription with Stratascript Reverse Transcriptase (Stratagene, La Jolla, CA) and the resulting cDNA was used as a template for amplification by PCR. Primers used were F1 (CTGAGATTTGTGCAGAA GAAATGC) in exon 2 of the α-dystrobrevin gene, R1 (CTGGAGTCAGAGATCATTGAG) in exon 5, F2 (GCTAAGAAGCTAACGAATGC) in exon 8, and R2 (AGACCTGCAGTAGGGGACATAAC) in exon 18 (Fig. 2A). Control primers were from the RNAqueous-4PCR Kit (Ambion) and amplified a region of the constitutively expressed small ribosomal subunit gene rig/S15.

Northern Analysis

Total RNA was isolated from adult wild-type or complete-deletion mouse muscle using TRI reagent and protocol from Ambion. Total RNA (25 μg) was loaded in each lane, separated on a 0.8% agarose gel, and transferred to a nylon membrane. The membrane was probed with a 1.6-kb DIG-labeled dystrobrevin cDNA containing exons 1–13, using High Prime DNA Labeling Kit and protocol (Roche, Indianapolis, IN). A probe for EF-1α was used as a RNA quality and loading control.

Dystrobrevin Targeting Vector

Two targeting vectors were designed to insert loxP sites between exons 4 and 5 and between exons 18 and 19 of the α-dystrobrevin gene. Targeting Vector A contained a single loxP site and a phosphoglycerate kinase-neo gene cassette (Neo) for positive selection through antibiotic resistance flanked by frt sites. The targeting vector comprised 4 kb of 5′ homology sequence and 1.3 kb of 3′ homology sequence flanking 40 bp within intron 4. The targeting vector also contained the gene sequence for thymidine kinase (TK) for negative selection through FIAU incorporation. Targeting Vector B contained a phosphoglycerate kinase-neo gene cassette (Neo) for positive selection through antibiotic resistance flanked by loxP sites. The targeting vector comprised 4 kb of 5′ homology sequence and 1.2 kb of 3′ homology flanking 35 bp of intron 18. The targeting vector also contained the gene sequence for TK for negative selection through FIAU incorporation (Fig. 3A).

Generation of Mice With Deletion of Exons 5–18 of the α-Dystrobrevin Gene

To place a loxP site close to the end of the α-dystrobrevin coding sequence, R1 mouse blastocyst-derived embryonic stem (ES) cells (17) were first transfected with Targeting Vector B (Fig. 3A) by electroporation and grown with positive selection using 300 μg/ml G418 (Promega, Fitchburg, WI) and negative selection using 0.27 μM FIAU (Moravek Biochemicals, Brea, CA). Surviving clones were screened by PCR to identify homologous recombination, which was confirmed by genomic Southern blot after digestion with *Bam*HI (Fig. 3B, left). Positive clones were expanded and cells were transiently transfected by electroporation with a vector expressing Cre recombinase. Cells were screened by PCR to identify clones with successful excision of the Neo gene, allowing for use of antibiotic selection in the subsequent homologous recombination. To place a loxP site close to the beginning of the α-dystrobrevin coding sequence, one of the Neo-excised clones was then expanded and cells were transfected with Targeting Vector A (Fig. 3A) by electroporation and grown with positive selection using 300 μg/ml G418 (Promega) and negative selection using 0.27 μM FIAU (Moravek Biochemicals). Surviving clones were screened by PCR to identify homologous recombination. To determine if Targeting Vector A had been inserted on the same copy of the chromosome as Targeting Vector B, four positive clones were transiently transfected by electroporation with a vector expressing Cre recombinase. Surviving clones were screened by PCR to identify successful recombination between the two loxP sites, excising 50 kb of the gene sequence. Cre-mediated excision was confirmed by genomic Southern blot after digestion with *Xba*I

(Fig. 3B, right). To generate the complete-deletion α-dystrobrevin mice, ES cells derived from one of the clonal lines that had been shown to produce successful excision in vitro were injected into blastocysts. High percentage male chimeras were crossed to C57BL/6J females. The resulting F1 generation was screened by PCR to identify mice heterozygous for the double loxP insertion, which were then bred to produce a line of homozygous conditional mice. These mice were then crossed to females expressing Cre recombinase under the β-actin promoter to excise the 50 kb of α-dystrobrevin gene sequence spanning exons 5 through 18. These matings produced a line of complete-deletion α-dystrobrevin mice, as confirmed by PCR analysis of genomic tail DNA (Fig. 3C). All subsequent experiments were conducted using these complete-deletion α-dystrobrevin mice.

RESULTS

Antibody Staining for α-Dystrobrevin-2/5 Persists in Mice With Deletion of Exon 3

α-Dystrobrevin is localized at the NMJ as part of the DGC and is thought to have a role in synapse maturation and maintenance, possibly through cytoplasmic signaling (6,23–25,35,38,40). Knockout mice generated with a deletion of exon 3 of the α-dystrobrevin gene are mildly dystrophic and have NMJ defects, suggesting an important role for α-dystrobrevin in the maturation of the NMJ (23,24). However, immunohistochemical staining with an antibody raised against a 10-amino acid sequence that is unique to the C-terminus of α-dystrobrevin isoforms 2 and 5 (35) (anti-DB2/5) (Fig. 1A) persisted at the NMJ in these exon 3-deleted mutant mice (24) (Fig. 1B, C).

These results suggest the presence of a remaining synaptic isoform of α-dystrobrevin that may provide functional compensation and thus mask a more severe synaptic phenotype. Although α-dystrobrevins 1 and 2 are the predominant isoforms in the DGC at the wild-type mouse NMJ (8–10,32–35), α-dystrobrevin-5, which does not normally include exon 3, could be upregulated at cholinergic synapses in the exon 3-deleted mutant mouse, thus accounting for the persistent antibody staining. Alternatively, with the deletion of exon 3 there might be new alternative splicing isoforms generated that skip exon 3; thus, the antibody could be recognizing a previously unidentified splicing isoform of α-dystrobrevin-2 that excludes exon 3 and is preserved in the exon 3-deleted mutant mice.

α-Dystrobrevin mRNA Persists in Mice With Deletion of Exon 3

To determine whether an mRNA isoform could be detected in vivo in the exon 3-deleted mutant mice, we isolated mRNA from muscle (Fig. 2B) and brain (Fig. 2C), where α-dystrobrevin is also expressed (10). We used cDNA generated by reverse transcription as a PCR template. In exon 3-deleted mutant mice, PCR using primer pairs spanning the region of exon 3 generated a product consistent with an isoform of α-dystrobrevin-2 that is shorter than the wild-type cDNA by approximately 200 bp, the length of exon 3 (Fig. 2A, primer pair F1-R1 and primer pair F1-R2, which is specific for α-dystrobrevin-2). PCR using primers not spanning exon 3 generated products of equal size in both wild-type and exon 3-deleted mutant mice (primer pair F2-R2), which could be consistent with either α-dystrobrevin-2 or -5. This result suggests that there is a novel splicing isoform of α-dystrobrevin-2 that can be generated in the absence of exon 3, and this novel isoform as well as α-dystrobrevin-5 may be expressed in the exon 3 mutant mice and recognized by the anti-DB2/5 antibody.

Generation of a Conditional Complete-Deletion α-Dystrobrevin Mouse

Based on these results, we hypothesized that this novel isoform of α-dystrobrevin-2 and/or α-dystrobrevin-5 could account for the persistent NMJ staining in the exon 3-deleted α-dystrobrevin mutant mice. Functional compensation by this novel isoform and/or α-dystrobrevin-5 may be masking a more severe synaptic phenotype in these mice. Therefore, we used a two-step homologous recombination strategy (41) to generate a mouse with a conditional deletion of 50 kb of the α-dystrobrevin gene that should result in a loss of all isoforms of α-dystrobrevin. Two targeting vectors were designed to sequentially insert loxP sites first between exons 18 and 19 and then between exons 4 and 5 of the α-dystrobrevin gene such that Cre-mediated excision of the sequence between the two sites would result in a deletion of exons 5–18, including the sequence against which the anti-DB2/5 antibody was raised (Fig. 3A). The appropriate homologous recombination in embryonic stem cells was confirmed by Southern blot (Fig. 3B). ES cells from the confirmed recombinant cell line were injected into blastocyst stage embryos, and the recombinant allele was transmitted to the germline. Crossing these conditional mice with mice expressing Cre recombinase resulted in the germline excision of 50 kb of the α-dystrobrevin gene, as confirmed by tail PCR (Fig. 3C).

We isolated mRNA from the muscle (Fig. 3D) and brain (Fig. 3E) of these complete-deletion α-dystrobrevin mice and generated cDNA through reverse transcription. No α-dystrobrevin transcript was seen in the complete-deletion α-dystrobrevin mice. Fur-

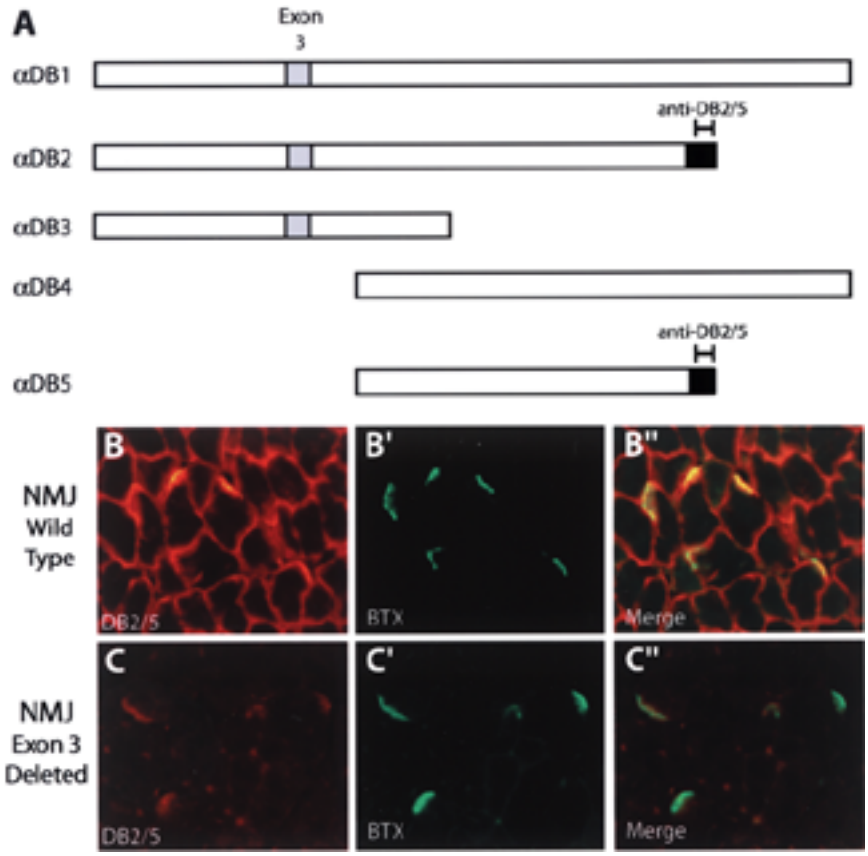

Figure 1. Antibody staining for α-dystrobrevin-2/5 persists in mice with deletion of exon 3. (A) Schematic of α-dystrobrevin isoforms 1–5 (4,8,32,35–37) (GenBank Accession X95226 for α-dystrobrevin-1 and X95227 for α-dystrobrevin-2, the predominant forms in mouse muscle) (8–10,32–35). The anti-DB2/5 antigenic region is indicated. (B, C) Cryosections of adult wild-type and exon 3-deleted mutant mouse anterior tibialis muscle were costained with the anti-DB2/5 antibody (red), which is specific for α-dystrobrevin-2 and -5, and bungarotoxin (BTX) (green).

thermore, Northern blot analysis using a probe containing exons 1–13 of the α-dystrobrevin gene, which recognizes all known splicing forms, revealed that the five major previously reported splicing forms (8) were not detected in the muscle of the complete-deletion α-dystrobrevin mice (Fig. 3F). Both heterozygous and homozygous mice were viable and fertile. There were no immediately apparent phenotypic differences compared to α-dystrobrevin-exon 3-deleted mutant mice.

Antibody Staining for α-Dystrobrevin-2/5 Persists in Complete-Deletion α-Dystrobrevin Mice

As the initial step in investigating the effects of deleting all α-dystrobrevin isoforms, we stained the NMJ of both homozygous complete-deletion α-dystrobrevin mice and wild-type littermates with the anti-DB2/5 antibody. Just as in the exon 3-deleted mutant mice (24) (Fig. 1B, C), there was no longer detectable extrasynaptic muscle membrane staining in the knockout muscle. Surprisingly, however, there was persistent, albeit weak, staining at the NMJ in the complete-deletion mice (Fig. 4A, B). In contrast, staining in the complete null α-dystrobrevin muscle using the anti-DB1/2 antibody was absent (Fig. 4C, D), as in the exon 3-deleted mutant mice (24).

The persistence of this antibody staining despite deletion of the antigenic sequence in exon 18 and excision of a large enough sequence of the α-dystrobrevin gene to disrupt all α-dystrobrevin isoforms suggests that the cholinergic synapse-specific staining seen with this antibody is in fact a cross-reaction with an unknown antigen. As a result, this antibody does not appear to be a specific marker for α-dystrobrevin isoforms 2 and 5, as previously reported (35).

Synaptic Phenotype in Complete-Deletion α-Dystrobrevin Mice Is Similar to Exon 3-Deleted Mutant Mice

α-Dystrobrevin exon 3-deleted mutant mice have an altered distribution of nAChR clusters at the NMJ (1,24). To determine if the neuromuscular synaptic phenotype is similar in the complete-deletion α-dystrobrevin mice, we stained longitudinal sections

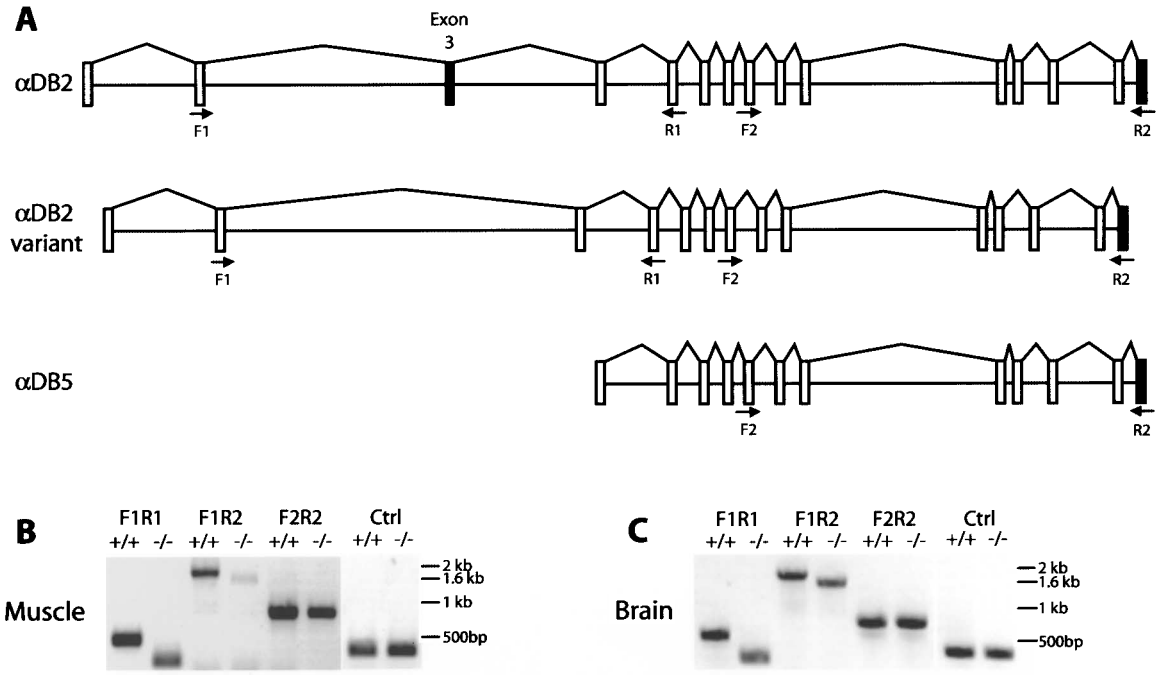

Figure 2. α-Dystrobrevin mRNA persists in mice with deletion of exon 3. (A) Schematic of exon–intron structure of α-dystrobrevin-2 (GenBank Accession X95227) as well as proposed exon–intron structures for the possible splice variant excluding exon 3 and for α-dystrobrevin-5. PCR primers are indicated: F1 (in exon 2), R1 (in exon 5), F2 (in exon 8), and R2 (in the α-dystrobrevin-2 and -5 unique terminal sequence within exon 18). PCR amplification with the indicated primers using cDNA templates derived by reverse transcription from total RNA isolated from wild-type and exon 3-deleted mutant mouse muscle (B) and brain (C).

of sternomastoid muscle with the nAChR marker α-bungarotoxin (Fig. 5). Compared to wild-type muscle, the NMJs from the complete-deletion α-dystrobrevin knockout mice exhibit a patchy, granular distribution of acetylcholine receptors with radiating spicules and a loss of smooth, clearly delineated borders; this phenotype is very similar to that of the exon 3-deleted mutant mice (24). Thus, excision of a large enough sequence of the α-dystrobrevin gene to disrupt all α-dystrobrevin isoforms did not reveal any obvious new synaptic phenotype at the NMJ.

DISCUSSION

The DGC is classically known to play a critical role in maintaining muscle stability, and mutations in the genes encoding proteins in this complex lead to muscular dystrophy in humans (11). Genetic studies in mice have also shown a role for the DGC in the maturation and maintenance of the NMJ (1,2,13–16,21,22,24,27,31). One component of the DGC is α-dystrobrevin, which is found both at the extrasynaptic muscle membrane and at the NMJ and may serve as a link between the structural and signal transduction functions of the DGC (6,23–25,35,38,40).

In mammals, at least five possible isoforms of α-dystrobrevin have been reported, all produced by one gene through alternative splicing; these different isoforms have distinct expression patterns and may serve both overlapping and unique functions (4,8,35–37). The α-dystrobrevin-2 and -5 isoforms share a unique C-terminal sequence, and staining with an antibody raised against this sequence persisted at the NMJ of knockout mice with a deletion of exon 3 of the α-dystrobrevin gene (24) (Fig. 1). This suggested that a synapse-specific isoform of α-dystrobrevin may remain in the exon 3-deleted mutant mice.

Based on this staining and the presence of α-dystrobrevin transcript in the exon 3-deleted mutant mice, we hypothesized that an alternative splicing isoform of α-dystrobrevin-2 that excludes exon 3 and/or α-dystrobrevin-5, which does not normally include exon 3, may be upregulated in the exon 3-deleted α-dystrobrevin mutant mice and may compensate for normal α-dystrobrevin function at cholinergic synapses. To investigate this possibility and to further understand the synaptic function of α-dystrobrevin, we generated mice with a large deletion encompassing exons 5 through 18 of the α-dystrobrevin gene, eliminating all known isoforms. Deletion of all splicing isoforms of α-dystrobrevin did not reveal any obvious new synaptic phenotype at the NMJ, confirming that the original exon 3-deleted mice are functionally null at the NMJ and validating the origi-

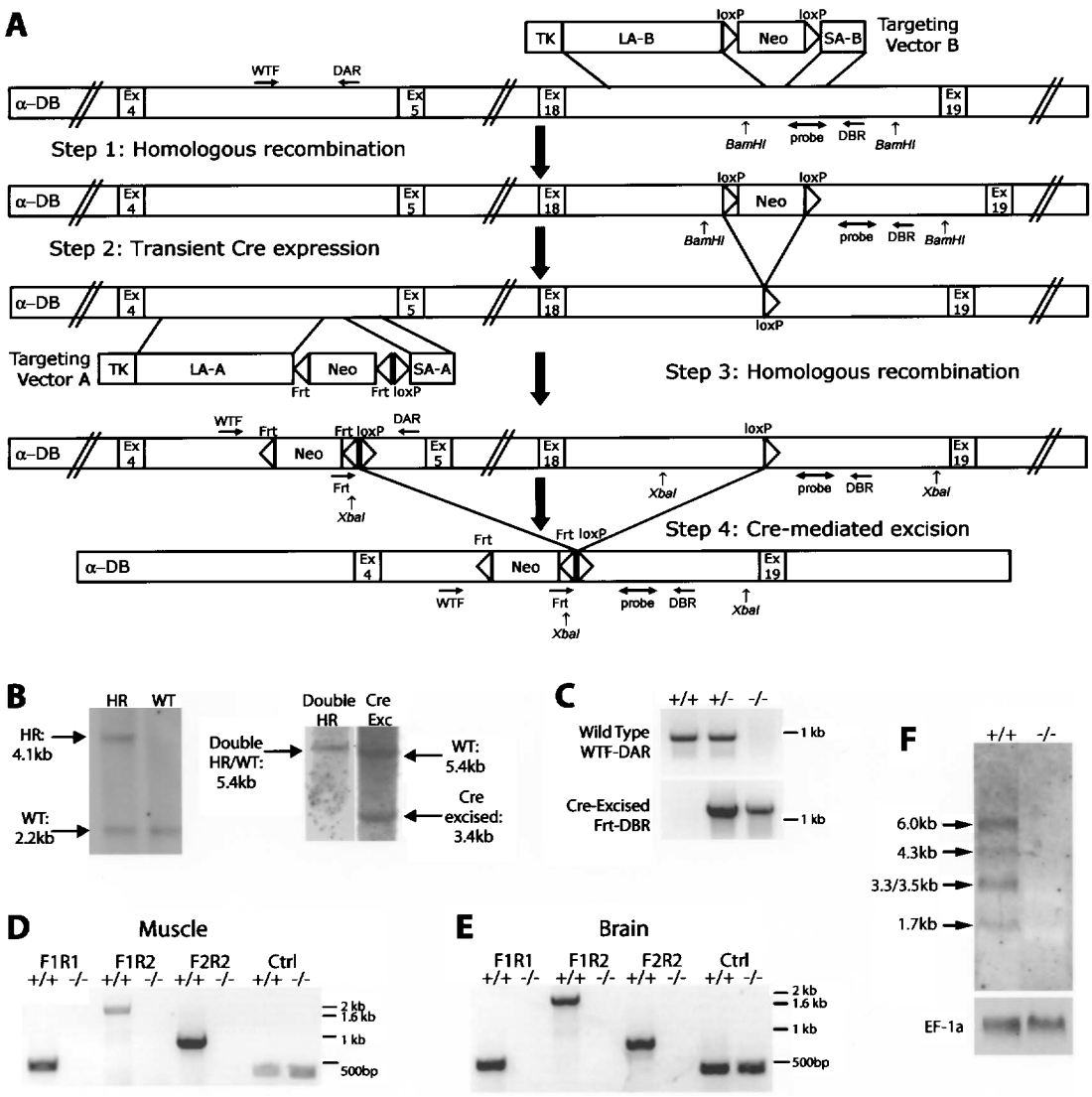

Figure 3. Generation of complete-deletion α-dystrobrevin mice. (A) Schematic of the region from exon 4 to exon 19 of the α-dystrobrevin gene demonstrating the targeting strategy used to generate complete-deletion α-dystrobrevin mice. Two loxP sites were sequentially inserted by homologous recombination between exons 4 and 5 and exons 18 and 19. Southern blot digestion sites, Southern blot probe, and PCR primers for genotyping are indicated. (B) Southern blot analysis of genomic DNA after digestion with *Bam*HI confirmed the first homologous recombination (left). Southern blot analysis of genomic DNA after digestion with *Xba*I confirmed the second homologous recombination and successful Cre-mediated excision (right). (HR, homologous recombinant; WT, wild type; Cre Exc, Cre excised). (C) PCR of mouse tail DNA confirmed the 50 kb excision of the α-dystrobrevin gene sequence. PCR amplification with the same primers indicated in Figure 2 using cDNA templates derived by reverse transcription from total RNA isolated from wild-type (+/+) and complete-deletion α-dystrobrevin mouse muscle (D) and brain (E). (F) Total RNA from wild-type (+/+) and complete-deletion α-dystrobrevin mouse muscle were probed with a 1.6-kb cDNA containing exons 1–13 of the α-dystrobrevin gene. All five major splicing forms previously reported (6.0, 4.3, 3.5/3.3, and 1.7 kb) were detected in the muscle of the wild-type but not the complete-deletion α-dystrobrevin mice. Transcription factor EF-1α was used as a loading control.

nal analysis of the loss of synaptic α-dystrobrevin (23,24).

Surprisingly, staining with the anti-DB2/5 antibody persisted in these complete-deletion mice, despite the deletion of the gene sequence in exon 18 that encodes the antigen against which the antibody was made. These results suggest that the persistent staining with the anti-DB2/5 antibody in the exon 3-deleted mutant mice is in fact unlikely due to an isoform of α-dystrobrevin, such as α-dystrobrevin-5 or a novel isoform of α-dystrobrevin-2. Instead, it suggests that this antibody cross-reacts with an unknown protein at cholinergic synapses and therefore is not a specific marker for α-dystrobrevin-2 and -5.

Although this new line of α-dystrobrevin mutant mice does not reveal a new synaptic phenotype, these

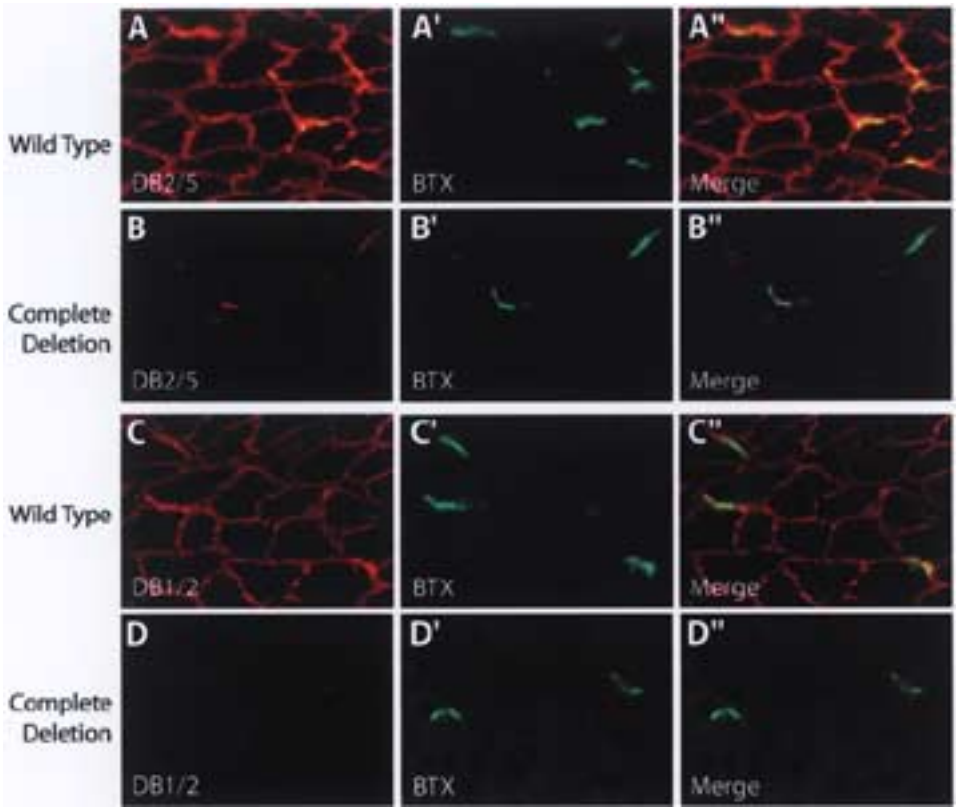

Figure 4. Antibody staining for α-dystrobrevin-2/5 persists in complete-deletion α-dystrobrevin mice. Cryosections of adult wild-type and complete-deletion α-dystrobrevin mouse anterior tibialis muscle were costained with bungarotoxin (green) and either the anti-DB2/5 antibody (red) (A, B) or the anti-DB1/2 antibody (red) (C, D).

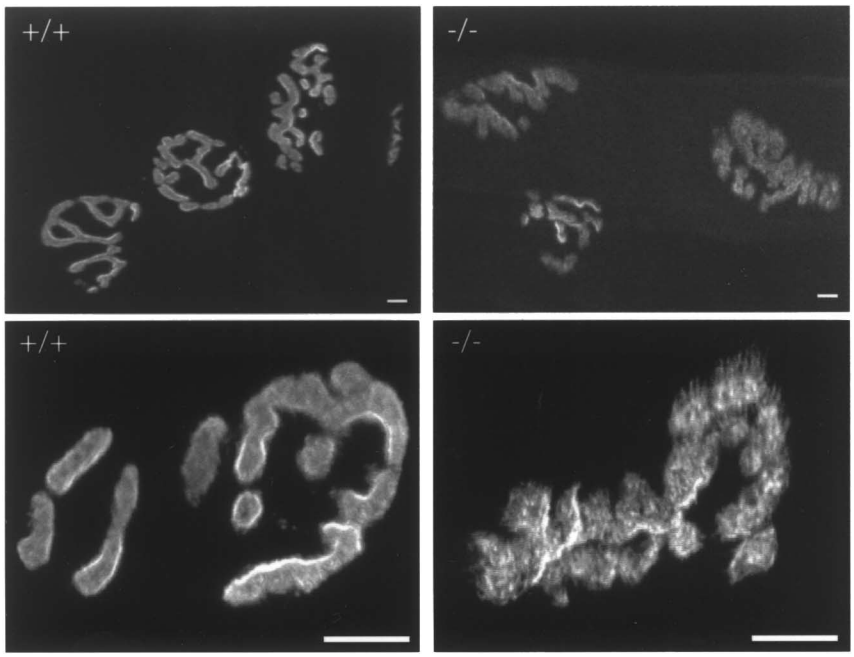

Figure 5. NMJ phenotype in complete-deletion α-dystrobrevin mice. Longitudinal sections of adult wild-type and complete-deletion mutant mouse sternomastoid muscle were stained with bungarotoxin to visualize the NMJ morphology. Scale bars: 10 μm.

mice are useful for further study of the function of α-dystrobrevin. First, the breadth of the deletion in the complete null mice does eliminate the lingering possibility that there may be compensation by alternate isoforms of α-dystrobrevin in the original exon 3-deleted mutant mice. Second, this line of mice offers a conditional deletion that is useful for further detailed study of the developmental or tissue-specific function of α-dystrobrevin. For example, although the initial phenotype characterized in α-dystrobrevin mutant mice focused on muscle and the NMJ (23,24), recent evidence also suggests a role for α-dystrobrevin at inhibitory synapses in the cerebellum (26). The extent to which the motor dysfunction in α-dystrobrevin mutant mice is due to peripheral effects in muscle versus central control of motor behavior in the cerebellum could be clarified by crossing these conditional mutant mice to tissue-specific Cre recombinase mouse lines. In addition, a deletion of the α-dystrobrevin gene via an inducible Cre recombinase would allow the role of α-dystrobrevin in development to be separated from its function in synaptic maintenance in the adult animal.

Many genes, like α-dystrobrevin, can produce isoforms from different promoter sequences and/or splice sites that lie within a range of genomic sequence that is too large to be spanned by a single targeted homologous recombination event. This presents a challenge in generating functionally null mice due to possible compensation by splice variants. This work demonstrates a successful strategy to overcome this challenge. Using sequential targeted homologous recombination events we inserted two loxP sites a large distance (50 kb) apart in the genome. Although this strategy requires prolonged passaging of ES cells in vitro compared to traditional single homologous recombination strategies, our results confirm that the pluripotency of these cells can be maintained and the ES cells can be used to successfully generate a viable mouse line. A similar approach has been used in the past to delete a large genomic sequence in ES cells in vitro and subsequently generate a knockout mouse line (41). We were able to demonstrate a successful strategy for a conditional deletion in which Cre-mediated recombination between loxP sites separated by as much as 50 kb can also be achieved successfully in vivo. This approach is a valuable tool for functional studies of the many genes that encode multiple isoforms spanning a large region of the genomic sequence.

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