Maturation and Maintenance of the Neuromuscular Synapse: Genetic Evidence for Roles of the Dystrophin–Glycoprotein Complex

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Summary

The dystrophin–glycoprotein complex (DGC) links the cytoskeleton of muscle fibers to their extracellular matrix. Using knockout mice, we show that a cytoplasmic DGC component, α-dystrobrevin (α-DB), is dispensable for formation of the neuromuscular junction (NMJ) but required for maturation of its postsynaptic apparatus. We also analyzed double and triple mutants lacking other cytoskeletal DGC components (utrophin and dystrophin) and myotubes lacking a α-DB or a transmembrane DGC component (dystroglycan). Our results suggest that α-DB acts via its linkage to the DGC to enhance the stability of postsynaptic specializations following their DGC-independent formation; dystroglycan may play additional roles in assembling synaptic basal lamina. Together, these results demonstrate involvement of distinct protein complexes in the formation and maintenance of the synapse and implicate the DGC in the latter process.

Introduction

Synaptic development is sometimes viewed as an embryonic event, but in fact it is a lifelong process. For example, mammalian skeletal neuromuscular junctions (NMJs) are functional by birth but undergo dramatic changes in structure and function during the first postnatal weeks. Thereafter, most NMJs persist for the life of the animal, but their apparent stability masks a dynamic equilibrium that permits rapid remodeling in response to alterations in activity during adulthood and gradual disassembly in old age (reviewed by Sanes and Lichtman, 1999). Recent studies have led to elucidation of some of the signaling mechanisms that organize the initial stages of NMJ formation (Burden, 1998). Less is known, however, about the molecules required for later developmental steps at this or any other synapse (Sanes and Lichtman, 1999). Here, we implicate the dystrophin–glycoprotein complex (DGC) in the maturation and maintenance of the NMJ.

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Dystrophin was identified as the cytoskeletal protein product of the gene mutated in Duchenne and Becker muscular dystrophies (Hoffman et al., 1987). Subsequently, dystrophin was shown to be a major component of a multimolecular membrane-associated complex, the DGC (Ervasti and Campbell, 1991). The DGC includes dystrophin or its homolog, utrophin; three groups of transmembrane proteins (dystroglycans [DGs], sarcoglycans, and sarcospan); and two groups of soluble proteins, the dystrobrevins and syntrophins. The DGC links the cytoskeleton to the extracellular matrix; cytoskeletal actin binds to dystrophin, which binds to β-DG in the membrane, and the extracellular domain of β-DG interacts with α-DG, which in turn binds to laminin $\alpha2$ in the basal lamina. This complex is required for muscle stability, as demonstrated by the findings that mutations in dystrophin, laminin $\alpha2$, or any of four sarcoglycan genes all lead to muscular dystrophies (reviewed by Tinsley et al., 1994; Straub and Campbell, 1997; Ozawa et al., 1998).

The DGC is found at synaptic as well as extrasynaptic regions of the muscle fiber, and several results have suggested that it is important for synaptic development as well as for muscle stability. First, the DGC at the postsynaptic membrane is molecularly specialized (Figure 1a). Whereas dystrophin, syntrophins $\alpha1$ and $\beta1$, and laminin $\alpha2$ are present throughout the muscle fiber surface, utrophin, syntrophin $\beta2$, and laminins $\alpha4$ and $\alpha5$ are highly concentrated at synaptic sites (Ohlendieck et al., 1991; Peters et al., 1994; Patton et al., 1997). Second, agrin, a nerve-derived signal that triggers postsynaptic differentiation, binds tightly and specifically to α-DG (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). Third, the cytoplasmic protein rapsyn, which is required for clustering of acetylcholine receptors (AChRs), can interact with DG (Apel et al., 1995; Cartaud et al., 1998). Fourth, expression of a presumptive dominant-negative form of utrophin (Namba and Scheller, 1996) or of DG antisense sequences (Jacobson et al., 1998) attenuates AChR clustering in cultured muscle cells.

Despite these data, there is little direct evidence that the DGC is important for synapse formation in vivo. Mice lacking utrophin ($utrn^{-/-}$) display only subtle defects in neuromuscular structure (Deconinck et al., 1997a; Grady et al., 1997a). Dystrophin mutants (mdx mice) display some defects in synaptic geometry, but these appear to reflect muscle fiber degeneration and regeneration rather than lack of dystrophin per se (Lyons and Slater, 1991). Likewise, mutant mice lacking α- or γ-sarcoglycan have qualitatively normal NMJs (Duclos et al., 1998; Hack et al., 1998).

The generation of α-dystrobrevin (α-DB) mutant mice ($adbn^{-/-}$; Grady et al., 1999) has provided an opportunity to reexamine the role of the DGC at the NMJ. α-DB is a cytoplasmic DGC protein that is present throughout the sarcolemma but concentrated at the NMJ. α-DB binds directly to dystrophin, utrophin, and syntrophins and is subject to phosphorylation by tyrosine kinases (Carr et al., 1989; Wagner et al., 1993; Peters et al., 1997a, 1998; Sadoulet-Puccio et al., 1997). The $adbn^{-/-}$
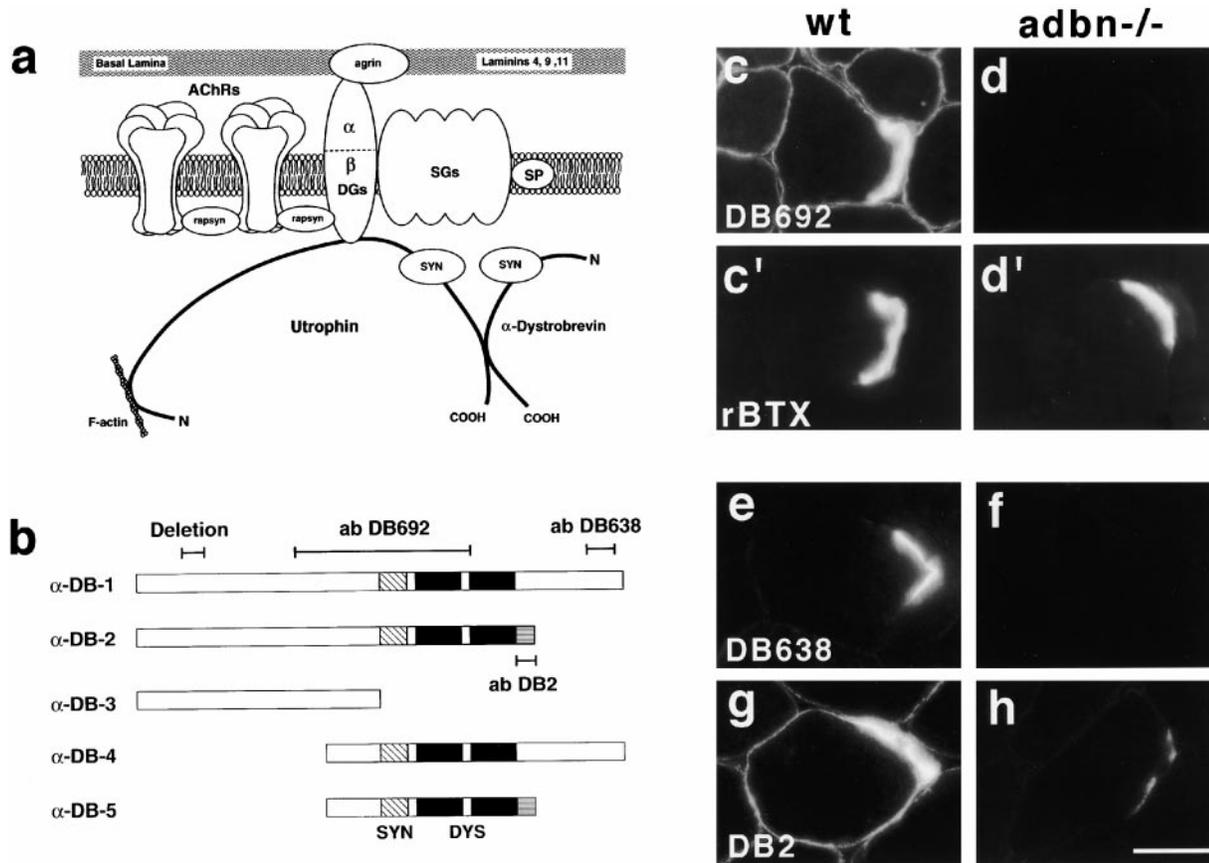
Figure 1. Isoforms of α-DB at the NMJ

(a) Schematic of the DGC at the NMJ. Its components include the sarcoglycans (SGs), DGs, sarcospan (SP), utrophin, α-DB, and syntrophins (SYN). DGs interact with agrin, laminins, and rapsyn.

(b) Structures of α-DB1–5, produced from the *adbn* gene by alternative splicing. Epitopes recognized by antibodies used in this study and sequences deleted in the *adbn*$^{-/-}$ mutant are indicated. Also indicated are binding sites for syntrophin and dystrophin (DYS).

(c–h) Distribution of α-DB at synaptic sites in wild-type (wt) (c, e, and g) and *adbn*$^{-/-}$ (d, f, and h) muscles. Sections were stained with antibodies that recognize α-DB1–3 (DB692) (c and d), α-DB1 and -4 (DB638) (e and f), or α-DB2 and -5 (DB2) (g and h). Sections were doubly stained with rBTX (c′ and d′). Scale bar, 20 μm.

mice exhibit a mild muscular dystrophy but are outwardly healthy. We show here that NMJs form normally in these mice but that the postsynaptic apparatus matures abnormally. Using myotubes cultured from *adbn*$^{-/-}$ mice, we show that α-DB is dispensable for agrin-stimulated AChR clustering but required for maintenance of clusters following removal of agrin. By analyzing mutants lacking combinations of dystrophin, utrophin, and α-DB, and myotubes lacking DG, we provide evidence that α-DB acts via its linkage to the DGC. Comparison of mutant phenotypes suggests that a major role of the synaptic DGC is to anchor α-DB but that DG plays additional roles in synaptic maturation. Together, these results demonstrate that distinct mechanisms regulate formation and maintenance of the postsynaptic apparatus, implicate the DGC in the latter process, and reveal that α-DB is a key component of the synaptic DGC.

Results

DB at the NMJ

The α-*DB* gene is subject to alternative splicing and generates mRNAs that encode at least five proteins, called α-DB1–5 (Figure 1b; Blake et al., 1996; Sadoulet-Puccio et al., 1996; Peters et al., 1998). RNA and protein analyses have shown that α-DB1–3, but not α-DB4 and -5, are present in muscle. Within muscle, α-DB1 is concentrated in the postsynaptic membrane, α-DB2 is present both synaptically and extrasynaptically, and the localization of α-DB3 is uncertain (Peters et al., 1998). In the *adbn*$^{-/-}$ mutant allele, sequences shared by α-DB1, -2, and -3 were deleted, and no α-DB-like immunoreactivity was detected in extrasynaptic portions of muscle fibers (Grady et al., 1999).

We used isoform-specific antibodies to determine whether any α-DB persisted at the NMJ in *adbn*$^{-/-}$ mice. Sections were double labeled with fluorescein-tagged antibody plus rhodamine-α-bungarotoxin (rBTX), which binds to AChRs and marks synaptic sites. Neither antibody DB638 (to α-DB1 and -4) nor antibody DB692 (to α-DB1 and -2) detectably stained the sarcolemma in *adbn*$^{-/-}$ muscle (Figures 1c–1f). Thus, staining confirmed loss of isoforms α-DB1 and -2. On the other hand, an antibody to sequences shared by α-DB2 and -5 did weakly stain synaptic sites in *adbn*$^{-/-}$ muscle (Figures 1g and 1h). This immunoreactivity may reflect upregula-

tion of α-DB5 at mutant synapses or cross reaction of the antiserum with a related protein, such as β-DB (Peters et al., 1997b; Blake et al., 1998).

Distribution of AChRs in the Absence of α-DB

To assess the geometry of the postsynaptic apparatus, we stained longitudinal sections of sternomastoid muscle with rBTX. In control mice, NMJs consist of multiple AChR-rich branches (Figure 2a). AChRs are fairly evenly distributed within each branch, although faint striations are visible at high power (Figure 2c). The striations reflect the fact that AChRs are concentrated at the crests of the junctional folds that indent the postsynaptic membrane (see below; Fertuck and Salpeter, 1976). In addition, AChRs appeared enriched along the borders of most of the branches, forming a relatively smooth outline that may reflect the curvature of the muscle membrane at the nerve terminal's edge.

The size, number, and arrangement of branches appeared normal at most synapses in adult $adbn^{-/-}$ mice, but the distribution of AChRs within branches was strikingly abnormal (Figures 2b, 2d, and 2e). Within branches, AChRs exhibited a patchy or granular distribution, and when striations were visible, they were more widely and less evenly spaced than those in controls. Branch borders were often ragged or frayed rather than smooth and sharply delineated, with AChR-rich spicules radiating outward. The degree of abnormality varied among junctions, but >95% of the postsynaptic sites examined were aberrant in all 16 sternomastoid muscles examined from eight mice >1 month of age. To ask whether these defects were secondary consequences of myopathy or muscle fiber degeneration and regeneration, we sectioned two adult sternomastoid muscles to score both dystrophy (by central nucleation) and synaptic structure (with rBTX). Only ~40% (1422/3434) of the fibers were dystrophic (similar to the ~50% seen in other muscles; Grady et al., 1999), but all 707 synapses scored were clearly abnormal. Conversely, dystrophin-deficient muscles, in which >90% of fibers are dystrophic, did not show these defects (see below).

To determine whether α-DB affected the initial formation of the NMJ, we examined muscles from young mice. In controls, AChRs aggregate into a diffuse plaque by birth. During the first 2 postnatal weeks, some portions of the plaque lose AChRs, while other portions expand, until the postsynaptic apparatus evolves an elaborate branched morphology (Steinbach, 1981; Slater, 1982). No significant defects were detected in mutant NMJs at postnatal day 7 (P7; Figures 2f and 2g), but by 1 month of age, the abnormalities described above were evident. The severity of the defects increased only slightly over time, up to at least 14 months of age (data not shown). Thus, α-DB is dispensable for the formation of the NMJ but is required for its maturation. Despite these abnormalities, movement was not obviously impaired in $adbn^{-/-}$ mice at any age.

Synaptic Structure in the Absence of α-DB

To test whether α-DB is required to maintain cytoplasmic as well as membranous components of the postsynaptic apparatus, we stained muscle sections with antibodies to utrophin and rapsyn, which are concentrated beneath the postsynaptic membrane. Both were present in a uniform pattern throughout each branch of control NMJs but were distributed in a patchy fashion at $adbn^{-/-}$ synapses (Figures 2h and 2i; data not shown). Double staining revealed that both proteins precisely colocalized with AChRs in both mutant and control muscles (Figures 2h' and 2i'; data not shown). Thus, α-DB regulates the arrangement of proteins in the postsynaptic cytoskeleton as well as in the postsynaptic membrane.

In contrast, the molecular architecture of the synaptic basal lamina was not detectably affected by loss of α-DB. Three components of synaptic basal lamina had similar distributions at control and mutant synapses: acetylcholinesterase, N-acetylgalactosamine-terminated glycoconjugates (recognized by the plant lectin VVA-B$_4$; Scott et al., 1988), and agrin (Figures 2j and 2k; data not shown). Likewise, in $adbn^{-/-}$ muscle as in controls, branches of the motor nerve terminal appeared intact and were directly apposed to each postsynaptic branch (Figures 2l and 2m). Thus, the profound derangement of the postsynaptic membrane in $adbn^{-/-}$ mice had surprisingly little effect on the organization of the synaptic cleft and nerve terminal.

We next examined $adbn^{-/-}$ muscles in the electron microscope to seek structural correlates of the altered distributions of postsynaptic components detected immunohistochemically. In both mutants and controls, vesicle-laden nerve terminals were separated from an infolded postsynaptic membrane by a layer of basal lamina (Figures 3a and 3b). The structure of $adbn^{-/-}$ synapses was, however, abnormal in two respects. First, the density of junctional folds was reduced by almost 50% in the postsynaptic apparatus of mutants as compared with that of controls (0.93 ± 0.05 folds/μm of primary synaptic cleft [mean ± SEM] in 147 synaptic sites from three adult wild-type mice versus 0.51 ± 0.05 in 104 sites from two adult $adbn^{-/-}$ mice, p < 0.0001, Student's t test). This reduction is similar to that observed in $utrn^{-/-}$ mice, in which the distribution of AChRs is normal (Deconinck et al., 1997a; Grady et al., 1997a), and it is therefore unlikely to account for the fragmentation of AChRs seen with light microscopy. Second, the distribution of electron-dense material associated with the postsynaptic membrane was altered. In control muscles, this material thickens the membrane at the crests and partway down the sides of junctional folds, precisely mirroring the distribution of AChRs; at the base of the folds, the membrane appears as a simple thin bilayer (Figures 3a and 3c). The transition from a "thick" to "thin" membrane occurs abruptly about halfway down the sides of the folds. In $adbn^{-/-}$ muscle, in contrast, the electron-dense material was broken up into small patches. Moreover, thickened membrane was present both at the tops and in the depths of junctional folds (Figures 3b and 3d–3f). As a result, the ultrastructural distinction between the tops and depths of folds was blurred in mutants.

These results suggested that α-DB is required to maintain the crests and depths of folds as molecularly distinct domains. To test this possibility, we used horseradish peroxidase– (HRP-) coupled BTX to localize AChRs. In control muscle, as noted above, AChRs are
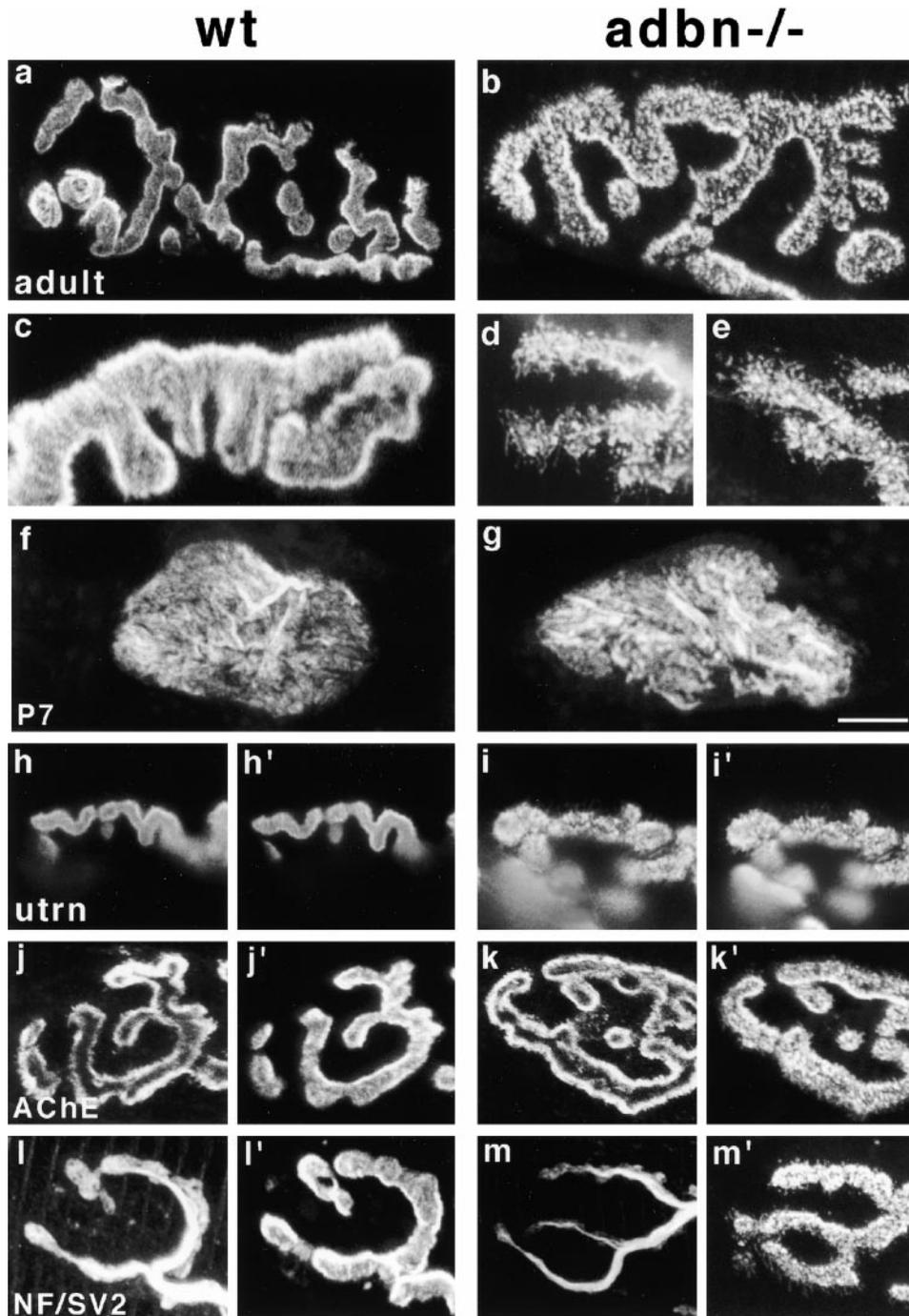
Figure 2. Architecture of NMJs of *adbn*$^{-/-}$ Mice

(a–e) Longitudinal sections of adult wild-type (wt) (a and c) and *adbn*$^{-/-}$ (b, d, and e) sternomastoid muscles stained with rBTX are shown at low (a and b) and high (c–e) power. Number and arrangement of branches are similar in control and mutant synapses. However, the distribution of AChRs within branches differs between genotypes. In wild-type branches, AChRs are evenly distributed or faintly striated with sharp borders, whereas in *adbn*$^{-/-}$ branches, AChRs are patchily distributed, and borders are fragmented.

(f and g) At P7, AChR distribution was similar between control and mutant synapses.

(h–m) Longitudinal sections of adult wild-type (wt) (h, j, and l) or *adbn*$^{-/-}$ (i, k, and m) muscle, doubly stained with antibodies to utrophin (utrn) (h and i), acetylcholinesterase (AChE) (j and k), or neurofilaments and SV2 (NF/SV2) (l and m) plus rBTX (h′–m′). Utrophin is redistributed in mutant muscles so that it remains precisely colocalized with AChRs, whereas distribution of agrin is unaltered in mutants. Nerve terminals are apposed to each AChR-rich branch of postsynaptic membrane in both controls and mutants.

Scale bar, 10 μm (a and b); 4 μm (c–f); and 7 μm (g and h).
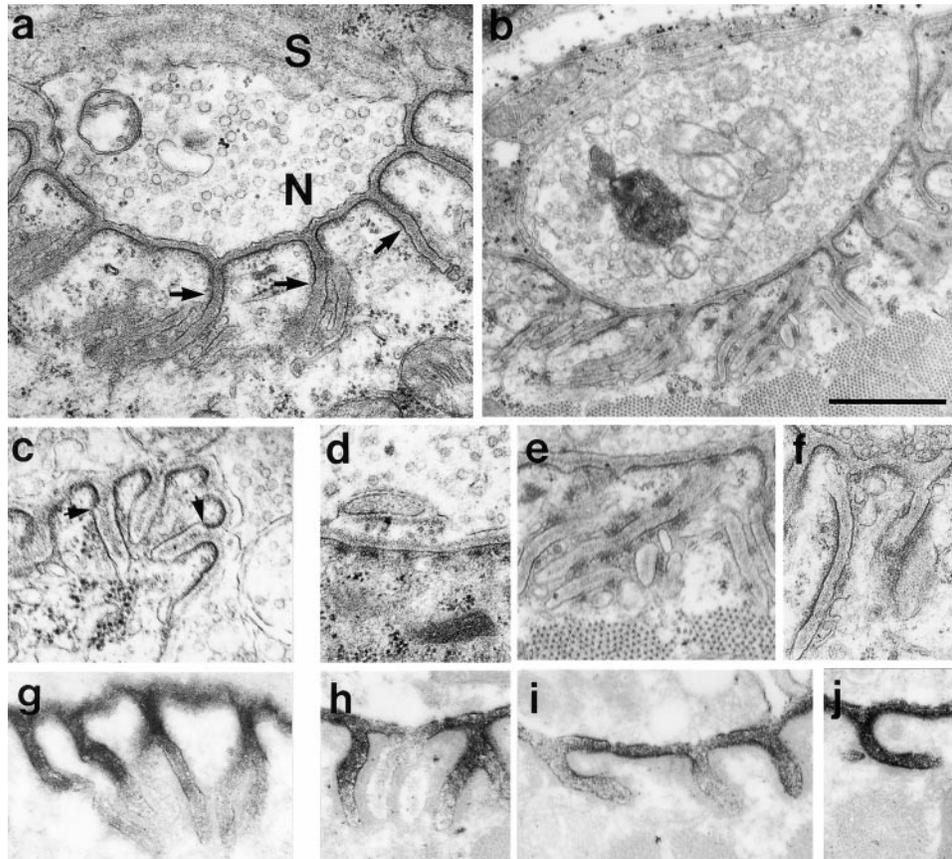
Figure 3. Ultrastructure of NMJs in *adbn*⁻/⁻ Mice

(a–f) Electron micrographs of control (a and c) and *adbn*⁻/⁻ (b and d–f) junctions are shown at low (a and b) and high (c–f) power. In controls, the postsynaptic membrane is electron dense (thick) at the crests of folds and partway down their sides, whereas the membrane is relatively electron lucent (thin) in the depths of folds. Arrows mark transition from thick to thin membranes. In *adbn*⁻/⁻ synapses, in contrast, both crests and sides of folds contain interspersed patches of dense and lucent membrane.

(g–j) Electron micrographs of junctional folds from control (g) and *adbn*⁻/⁻ (h–j) muscles that had been stained with biotin-bungarotoxin plus HRP-avidin. AChRs are concentrated at the crests of junctional folds and partway down their sides in controls but are more evenly distributed through the depths of most mutant folds.

Abbreviations: N, nerve terminal, and S, Schwann cell. Scale bar, 1 μm (a and b) and 0.5 μm (c–j).

concentrated at the crests of all junction folds and partway down their sides (Figure 3g). In *adbn*⁻/⁻ muscles, in contrast, AChRs extended to the depths of many folds, whereas other folds were AChR poor (Figures 3h–3j). Thus, molecular as well as structural distinctions between two postsynaptic domains are diminished in the absence of α-DB.

Structure of the Synaptic DGC in the Absence of α-DB

The observation that α-DB is required for maturation of the NMJ prompted us to reexamine the role of the DGC in synaptic structure. First, we asked whether α-DB is required for the stability of the synaptic DGC. To this end, we assessed the distribution of several DGC components (see Figure 1a) at synaptic sites in *adbn*⁻/⁻ muscles. Two cytoskeletal DGC components (dystrophin and utrophin), as well as two transmembrane DGC components (β-DG and β-sarcoglycan), appeared to be as concentrated at synaptic sites in *adbn*⁻/⁻ muscles as in controls; that is, utrophin was present only at synaptic sites, while the other three proteins were present at

higher levels in synaptic than in extrasynaptic regions (Figures 4a–4h). These results imply that α-DB is not required for the integrity of the DGC at the synapse.

On the other hand, levels of syntrophins, soluble DGC components that bind directly to α-DB as well as to dystrophin and utrophin (Peters et al., 1997a), were decreased at *adbn*⁻/⁻ synapses. In control muscles, α1 and β1 syntrophins were detected throughout the muscle fiber, but at substantially higher levels in synaptic than in extrasynaptic regions. Levels of these two proteins were slightly decreased in extrasynaptic regions of *adbn*⁻/⁻ muscle fibers (see also Grady et al., 1999), but synaptic levels were markedly decreased. In addition, β2 syntrophin, largely confined to synaptic sites in control muscles (Peters et al., 1994), was barely detectable in *adbn*⁻/⁻ muscle (Figures 4i–4l; data not shown). Levels of the DGC-associated signaling protein, nNOS, which binds directly to syntrophin (Brenman et al., 1996), were also reduced both in extrasynaptic regions of *adbn*⁻/⁻ muscle (Grady et al., 1999) and at synaptic sites (Figures 4m and 4n). Immunoblotting confirmed that levels of α1 syntrophin and nNOS (but not of dystrophin) were
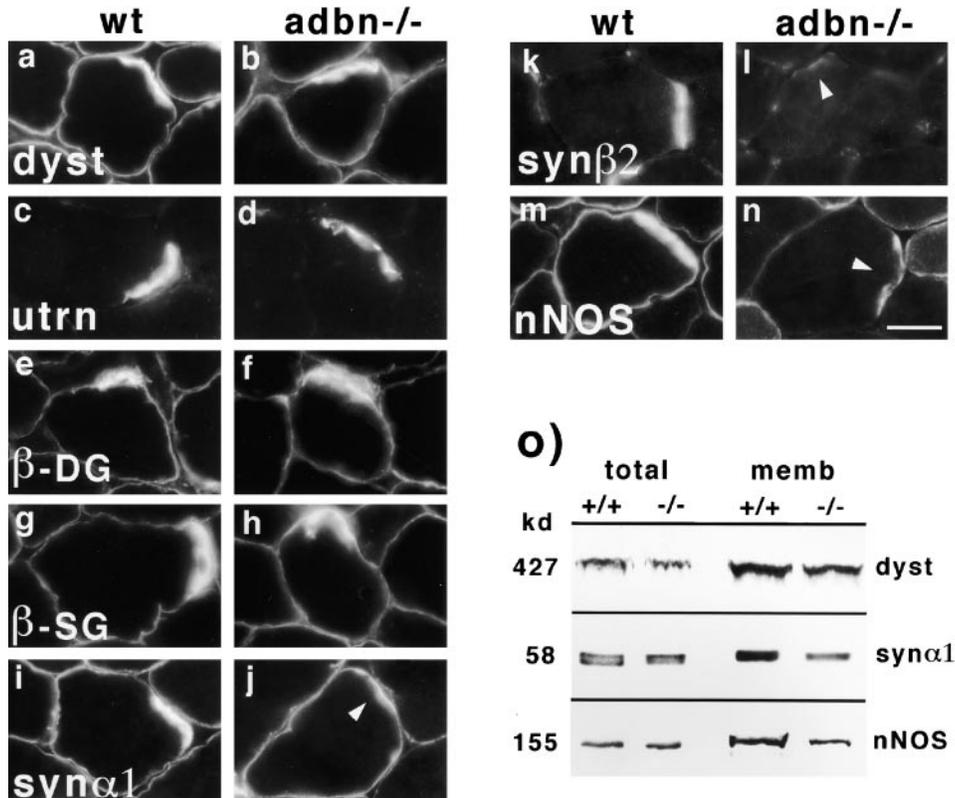
Figure 4. Components of the DGC at *adbn*$^{-/-}$ Synapses

(a–n) Sections were stained with antibodies to the indicated components. Synaptic concentrations of dystrophin (dyst) (a and b), β-DG (e and f), and β-sarcoglycan (β-SG) (g and h) were retained in *adbn*$^{-/-}$ muscle. Utrophin (utrn) was selectively associated with synaptic sites in both wild type (wt) and mutants (c and d). In contrast, synaptic levels of α1 syntrophin (synα1) (i and j), β2 syntrophin (synβ2) (k and l), and nNOS (m and n) were decreased in *adbn*$^{-/-}$ muscle. Synaptic sites were identified by double labeling with rBTX; arrows indicate sites in (j), (l), and (n). Scale bar, 15 μm.

(o) Total homogenates (left) and crude membrane fractions (right) of control and *adbn*$^{-/-}$ muscles were subjected to immunoblotting with antibodies to dystrophin, α1 syntrophin, or nNOS. Total levels of all three proteins were similar in control and mutant muscle, but levels of membrane-associated syntrophin and nNOS were reduced in *adbn*$^{-/-}$ muscle.

decreased in the membrane fraction of *adbn*$^{-/-}$ muscle compared with controls but showed that the total levels of these proteins were similar in control and mutant muscle (Figure 4o). Thus, α-DB appears to be required to anchor soluble DGC components to the cytoplasmic face of the cell surface, and release of these components could be responsible, in part, for the synaptic consequences of α-DB deficiency.

Synaptic Structure in the Absence of Utrophin and Dystrophin

As a second means of assessing the relationship of synaptic α-DB to the DGC, we compared synaptic structures in *adbn*$^{-/-}$ mutant mice with those in double mutants lacking both dystrophin and utrophin (*mdx:utrn*$^{-/-}$). In *mdx:utrn*$^{-/-}$ mice, synaptic and extrasynaptic DGCs are largely disassembled (Deconinck et al., 1997b; Grady et al., 1997b). Therefore, comparison of *mdx:utrn*$^{-/-}$ and *adbn*$^{-/-}$ synapses provides a test of whether a primary role of the synaptic DGC is to concentrate α-DB in the postsynaptic apparatus. If principal functions of the synaptic DGC are mediated by α-DB, the synaptic phenotypes of *mdx:utrn*$^{-/-}$ and *adbn*$^{-/-}$ mice should be similar. On the other hand, if the DGC plays roles that do not

require α-DB, one would expect NMJs to be more severely impaired in *mdx:utrn*$^{-/-}$ mice than in *adbn*$^{-/-}$ mice.

The most striking observations were made in 1-month-old mice. At this age, AChR distribution in both *mdx* and *utrn*$^{-/-}$ single mutant mice was indistinguishable from that measured in controls (Figure 5a; data not shown; see also Lyons and Slater, 1991; Deconinck et al., 1997a; Grady et al., 1997a). In *mdx:utrn*$^{-/-}$ double mutants, in contrast, AChRs had a distribution similar to that described above for *adbn*$^{-/-}$ mutants; borders of branches were frayed, and receptors had a patchy distribution within branches (Figure 5c; compare with Figure 2b). This result is consistent with the possibility that a major role of the synaptic DGC is to concentrate α-DB at synaptic sites.

Similar defects were present in older animals, but more difficult to detect. Although synapses remain qualitatively normal in older *utrn*$^{-/-}$ mice, the postsynaptic apparatus in older *mdx* mice breaks up into a series of discrete boutons (Figure 5b), probably as a consequence of myofiber degeneration (Lyons and Slater, 1991). Most synapses in *mdx:utrn*$^{-/-}$ double mutants were also fragmented into discrete boutons, but close examination showed that AChR distribution was often
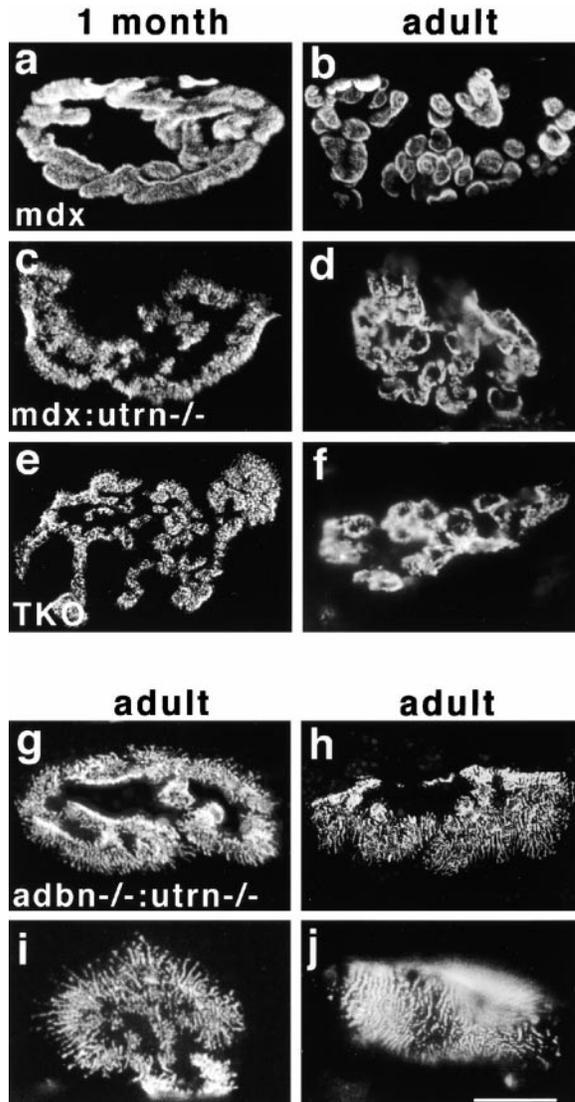
1 month   adult

Figure 5. AChR Distribution at NMJs in Mice Lacking DGC Components

Longitudinal sections of sternomastoid muscle from 1-month-old and adult (>2 months old) mutant mice were stained with rBTX. Synapses from young $utrn^{-/-}$ (data not shown) and mdx (a) mice have essentially normal AChR distribution. In contrast, synapses from young $mdx:utrn^{-/-}$ (c) and $mdx:adbn^{-/-}:utrn^{-/-}$ (TKO) (e) mice have defects similar to those in $adbn^{-/-}$ mice. In older mdx (b), $mdx:utrn^{-/-}$ (d), and $mdx:adbn^{-/-}:utrn$ (f) mice, branches are replaced by multiple boutons as a consequence of muscle regeneration. AChR distribution within the boutons appears relatively normal in mdx mice but is more fragmented in the double and triple mutant. In adult $adbn^{-/-}:utrn^{-/-}$ mice, most synapses resemble those in $adbn^{-/-}$ mice (g), but a minority (20%–30%) are more severely affected (h–j). Scale bar, 15 μm.

granular and patchy within individual boutons (Figure 5d). This defect was perhaps not apparent in previous studies because we (Grady et al., 1997b) and Deconinck et al. (1997b) only examined muscles from older animals (>8 weeks) in which almost all fibers had regenerated. The relative subtlety of the defect might reflect the fact that regenerated fibers are immature.

To test whether α-DB plays DGC-independent roles,

we generated and analyzed $mdx:adbn^{-/-}:utrn^{-/-}$ triple mutants. The AChR distribution at these synapses was similar to that observed in $adbn^{-/-}$ and $mdx:utrn^{-/-}$ mutants, both at 1 month of age, when fibers were intact (Figure 5e), and at 2 months of age, after fibers had degenerated and regenerated (Figure 5f). The similar synaptic phenotypes of $adbn^{-/-}$, $mdx:utrn^{-/-}$, and triple mutants support the idea that α-DB does not play major DGC-independent roles.

In the course of generating $mdx:adbn^{-/-}:utrn^{-/-}$ triple mutants, we recovered $mdx:adbn^{-/-}$ and $adbn^{-/-}:utrn^{-/-}$ double mutants. In $mdx:adbn^{-/-}$ mice examined at 2–4 months of age, synapses were broken into the discrete boutons characteristic of mdx synapses, and AChRs were patchily distributed within boutons, as expected from the $adbn^{-/-}$ phenotype (data not shown). Likewise, most $adbn^{-/-}:utrn^{-/-}$ synapses resembled $adbn^{-/-}$ synapses in showing normal overall geometry but fragmentation of AChRs (Figure 5g). Interestingly, however, a subset of synapses (20%–30%) in $adbn^{-/-}:utrn^{-/-}$ double mutants were more severely disrupted than any $adbn^{-/-}$ synapses (Figures 5h–5j). At these synapses, the normal branching morphology of the AChRs was replaced with coarse striations radiating in a zebra stripe–like pattern. We therefore examined $adbn^{-/-}:utrn^{-/-}$ synapses in the electron microscope to seek additional defects. Nerve terminals and Schwann cells appeared normal at these synapses, but there was an almost complete lack of junctional folds in the postsynaptic membrane. Indeed, the number of folds in these double mutants was lower than that reported above and previously (Grady et al., 1997a, 1997b) for $adbn^{-/-}$ and $utrn^{-/-}$ single mutants and $mdx:utrn^{-/-}$ double mutants (0.09 ± 0.02 folds/μm of primary synaptic cleft in 112 synaptic sites from two adult $adbn^{-/-}:utrn^{-/-}$ mice versus 0.93 ± 0.05, 0.50 ± 0.05, 0.51 ± 0.05, and 0.28 ± 0.04 in wild-type, $utrn^{-/-}$, $adbn^{-/-}$, and $mdx:utrn^{-/-}$ mice, respectively). One reasonable hypothesis, therefore, is that the gross disruption of a minority of $adbn^{-/-}:utrn^{-/-}$ synapses results from the combined loss of α-DB and of junctional folds.

Instability of AChR Clusters in the Absence of α-DB

We used cultured myotubes to investigate the mechanism by which α-DB acts. Myoblasts from $adbn^{-/-}$ and control ($adbn^{+/-}$ or $adbn^{+/+}$) neonates were cultured under conditions that promoted their fusion to form myotubes. No differences in the size, shape, or health of myotubes were noted that correlated with their genotype. Labeling with rBTX revealed a small number of spontaneous AChR clusters on the surface of myotubes (Figures 6a and 6b). The number and size of spontaneous clusters were similar in controls and mutants (Figure 6i, left; data not shown). Thus, in vitro as in vivo, AChRs can cluster in the absence of α-DB.

Next, we asked whether α-DB affects the ability of myotubes to respond to agrin, a nerve-derived organizer of postsynaptic differentiation. Myotubes were treated with a recombinant carboxy-terminal agrin fragment that is capable of activating muscle-specific kinase (MuSK), binding to α-DG and integrins, and inducing AChR clustering (Campanelli et al., 1996; Hopf and Hoch, 1996; Martin and Sanes, 1997). The agrin fragment induced
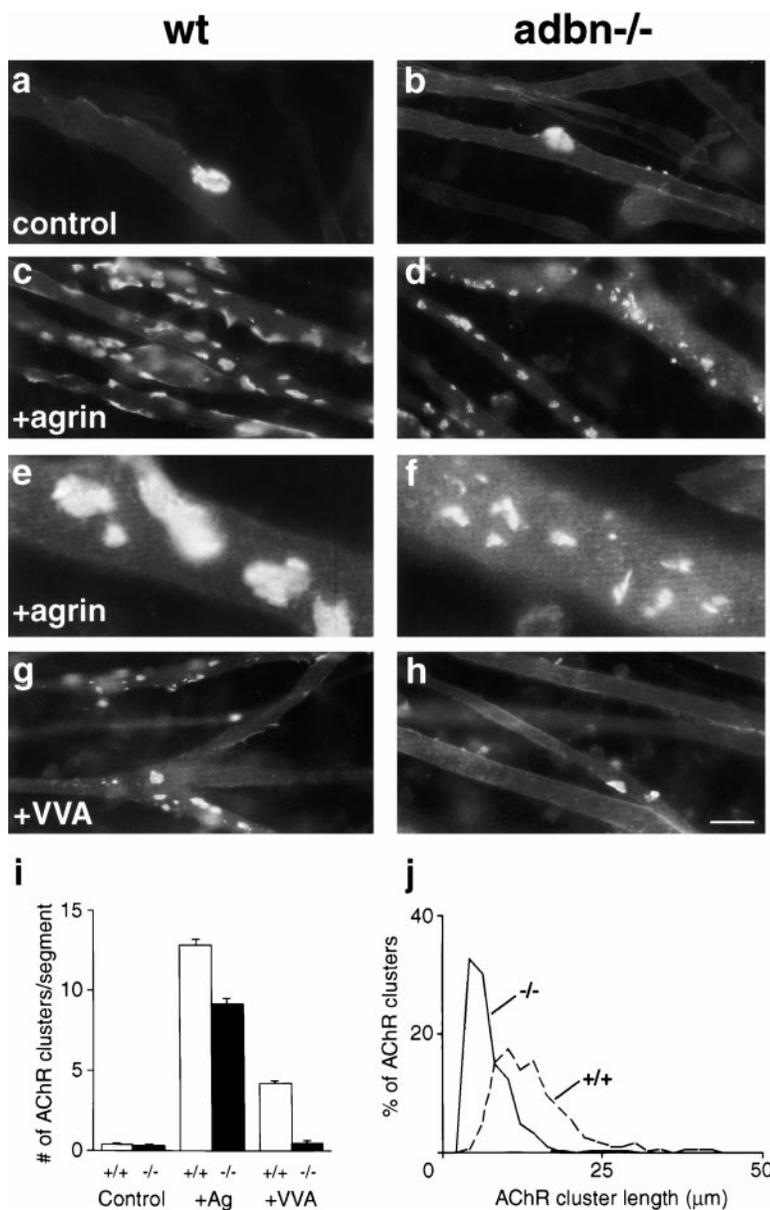
Figure 6. Number and Size of AChR Clusters in Myotubes Cultured from *adbn*$^{-/-}$ Mice

Cultures from wild-type (wt) (a, c, e, and g) or *adbn*$^{-/-}$ (b, d, f, and h) muscles were untreated (control) (a and b), treated for 18 hr with agrin (+agrin) (c–f), or treated for 18 hr with VVA-B$_4$ (+VVA) (g and h), then stained with rBTX. Loss of α-DB has little effect on the number of spontaneous clusters but decreases the number of agrin-induced clusters by ~30% and the number of VVA-B$_4$-induced clusters by ~80% (i). Loss of α-DB leads to ~50% decrease in mean cluster diameter in agrin-treated cultures (j). Clusters >2 μm in diameter were counted or measured in 20 fields from each of three independent experiments in (i). A total of 349 clusters were measured on wild-type myotubes, and a total of 379 clusters were measured on *adbn*$^{-/-}$ myotubes in (j). Scale bar, 10 μm (e and f) and 30 μm (a–d, g, and h).

AChR clustering in both control and *adbn*$^{-/-}$ myotubes (Figures 6c–6f), but the number of clusters per unit length of myotube was reduced by ~30% (Figure 6i, middle), and the average diameter of clusters was reduced by ~50% in *adbn*$^{-/-}$ myotubes (Figure 6j). Thus, α-DB modulates responses to agrin.

α-DB might affect AChR clustering at any of several steps, including activation of the agrin receptor complex (which contains MuSK, plus other unidentified components), signaling downstream of MuSK, growth of clusters, or stabilization of clusters once they have formed. We performed a series of experiments designed to distinguish these alternatives. First, we treated myotubes with a plant lectin, VVA-B$_4$, which binds to N-acetylgalactosamine-terminated glycoconjugates at the synapse. We showed previously that VVA-B$_4$ activates the same pathway as agrin, but at a step downstream of MuSK (Martin and Sanes, 1995; Gautam et al., 1999). The ability of VVA-B$_4$ to induce AChR clusters was nearly abolished

in the absence of α-DB (Figures 6g–6i). Thus, α-DB appears to affect clustering at a step subsequent to binding of agrin and activation of MuSK.

Second, we treated myotubes with agrin for varying periods of time to test whether formation or growth of clusters was slowed in the absence of α-DB. No difference was detectable between control and *adbn*$^{-/-}$ myotubes in the time course of agrin-induced clustering; in both, numbers of clusters were maximal ~4 hr after addition of agrin, then declined over the next 20 hr (data not shown). Thus, α-DB does not markedly affect the initiation of clustering, consistent with our observations in P7 *adbn*$^{-/-}$ mice.

Finally, we treated cultures with agrin for 18 hr, then washed off the agrin and reincubated the cultures in fresh medium to assess the fate of agrin-induced AChR clusters (Figure 7). In control myotubes, clusters dispersed slowly, with a mean half-life of ~10–15 hr following removal of agrin. This degree of stability is similar
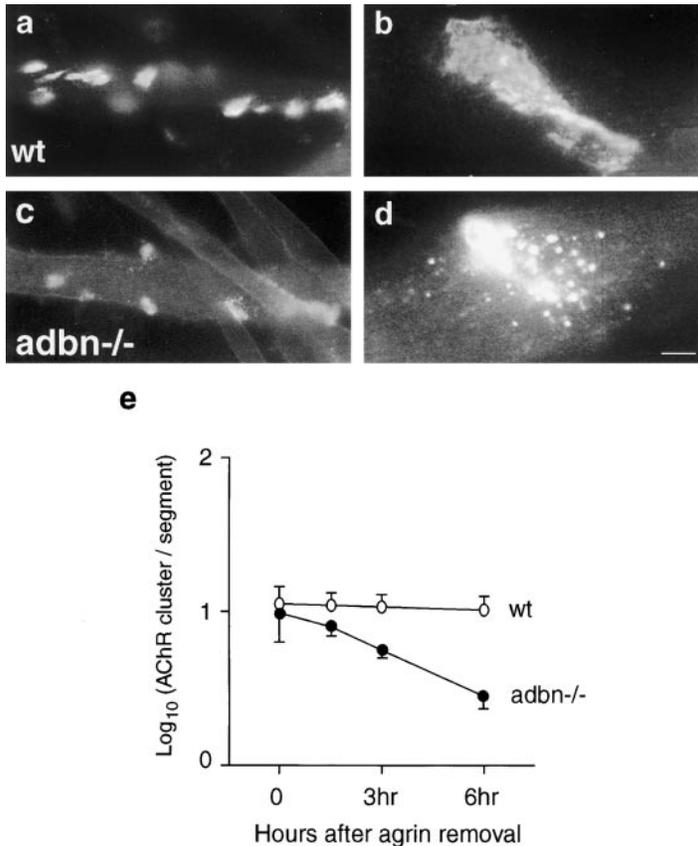
Figure 7. α-DB Stabilizes Agrin-Induced AChR Clusters

Cultures from wild-type (a and b) or *adbn*^{-/-} (c and d) mice were treated with agrin for 18 hr, washed, reincubated without agrin for 3 hr, and then stained with rBTX. Clusters dissipate rapidly in the absence of α-DB (c); fragmented intermediates are sometimes visible (d). Half-life of AChR clusters following removal of agrin was shorter in *adbn*^{-/-} myotubes than in controls (e). Cultures were all treated with agrin for 18 hr, then washed and reincubated without agrin for the indicated period. Data are the average of two independent experiments. Scale bar, 10 μm (b and d) and 20 μm (a and c).

to that reported by Wallace (1988) for chick myotubes. In the absence of α-DB, in contrast, most AChR clusters dispersed within a few hours of agrin removal; their mean half-life was ~2–3 hr, or ~20% that of controls (Figure 7e). Dispersal appeared to occur by fragmentation of the cluster into microaggregates (Figure 7d), which were reminiscent of those present in the postsynaptic membrane of *adbn*^{-/-} muscle. Thus, α-DB appears to be required for the stabilization of agrin-induced AChR clusters rather than their formation.

Formation of AChR Clusters in the Absence of DG

The observation that cultured *adbn*^{-/-} myotubes show defects in AChR clustering allowed an additional test of the notion that major roles of the synaptic DGC involve α-DB. DG is the major transmembrane component of the DGC; in its absence, the entire DGC fails to assemble (Henry and Campbell, 1999). Although *DG*^{-/-} embryos die before muscles form (Williamson et al., 1997), *DG*^{-/-} embryonic stem (ES) cells have been generated by gene targeting (Henry and Campbell, 1998), and methods are available for generating myotubes from ES cells (Rohwedel et al., 1994, 1998). We found that myotubes generated from *DG*^{-/-} ES cells were similar in size and shape to those formed from control (*DG*^{+/-} or *DG*^{+/+}) ES cells (Figures 8a and 8b). We therefore used these ES cells to compare AChR clustering in the absence of DG with that in the absence of α-DB.

Staining with rBTX revealed that *DG*^{-/-} myotubes formed small numbers of high-density AChR clusters in the absence of exogenous agrin and >5-fold more

clusters following treatment with recombinant agrin (Figures 8c–8f). Heterogeneity among cultures of each genotype (presumably a consequence of the fact that only a subset of ES cells become myoblasts) hindered attempts at quantitative analysis, but the number of clusters on agrin-treated *DG*^{-/-} myotubes was >50% that on corresponding *DG*^{+/-} or *DG*^{+/+} myotubes. Immunohistochemical assays demonstrated that the AChR-associated DGC was disassembled in the absence of DG; utrophin, dystrophin, and α-DB were concentrated at AChR clusters on control myotubes but undetectable at clusters in *DG*^{-/-} myotubes (Figures 8g–8j; data not shown). Thus, cluster formation and agrin signaling both occur not only in the absence of α-DB but also in the apparent absence of an AChR-associated DGC.

On the other hand, examination at high magnification revealed that the structure of AChR clusters was abnormal in the absence of DG; plaques were fragmented into groups of microaggregates (Figures 8o and 8p). This pattern resembles that seen on wild-type myotubes following application of blocking antibodies to DG (Campanelli et al., 1994) and on *adbn*^{-/-} myotubes following removal of agrin (see above). These parallels, along with the known role of DG in basal lamina formation (Henry and Campbell, 1998, 1999), raised the possibility that this defect reflected the lack of cluster-associated "synaptic" basal lamina components. Indeed, the synaptic laminin α5 chain was highly concentrated at AChR clusters on control ES cell–derived myotubes (as is the case in vivo and in some myogenic cell lines; Patton et al., 1997), but no muscle-associated α5 was detectable on mutant myotubes (Figures 8k and 8l). Likewise, antisera

to the common laminin $\gamma 1$ subunit stained control but not $DG^{-/-}$ AChR clusters more intensely than AChR-poor regions of the membrane (Figures 8m and 8n). Thus, DG appears to be required for assembly of an AChR cluster–associated basal lamina, and differences between the structure of $adbn^{-/-}$ and $DG^{-/-}$ AChR clusters might result from the absence of this extracellular scaffold.

Discussion

Agrin is a critical nerve-derived organizer of postsynaptic differentiation at the NMJ (McMahan, 1990; Gautam et al., 1996; Burgess et al., 1999). The discovery that agrin binds tightly and specifically to α-DG (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994) suggested that the DGC plays crucial roles in synaptogenesis. Subsequently, however, it was found that fragments of agrin unable to bind DG are capable of promoting AChR clustering (Gesemann et al., 1996; Hopf and Hoch, 1996). Nonetheless, other lines of evidence have implicated the DGC in postsynaptic differentiation (references in Introduction), so several groups suggested that its roles might instead involve maturation or maintenance of the NMJ (Deconinck et al., 1997a; Grady et al., 1997a; Balasubramanian et al., 1998; Jacobson et al., 1998; Nawrotzki et al., 1998). Here, using mice lacking one, two, or three components of the DGC (α-DB, utrophin, and dystrophin), as well as cultured myotubes lacking α-DB or DG, we have tested this hypothesis and provided direct evidence in its favor.

α-DB and the NMJ
NMJs appear to form normally in mice lacking α-DB, but their postnatal maturation is compromised. Most striking is a rearrangement of AChRs and their associated cytoplasmic proteins, rapsyn and utrophin. In addition, junctional folds, which form postnatally, are reduced in number, and the ultrastructural distinction between the crests and depths of the folds is diminished. Thus, α-DB is dispensable for formation of the NMJ but essential for its maturation.

At least two isoforms of α-DB, α-DB1, and α-DB2, are present at the NMJ (Peters et al., 1998). Both are lost in the $adbn^{-/-}$ mutant, but several observations suggest that α-DB1 may be more critical than α-DB2 is for synaptic organization. First, α-DB1 is confined to the postsynaptic membrane in muscle fibers, whereas α-DB2 is present both synaptically and extrasynaptically (Balasubramanian et al., 1998; Peters et al., 1998). Second, α-DB1 binds directly to utrophin, which is selectively associated with the synapse, whereas α-DB2 binds to dystrophin but not utrophin (Peters et al., 1998). Third, α-DB1, but not α-DB2, is concentrated at AChR clusters in a myogenic cell line (Nawrotzki et al., 1998). Fourth, α-DB1, but not α-DB2, is subject to tyrosine phosphorylation and therefore potentially involved in signaling functions (Wagner et al., 1993; Balasubramanian et al., 1998). Finally, no α-DB1 is detectable, whereas at least some α-DB2 is retained, at $mdx:utrn^{-/-}$ synapses (Peters et al., 1998), yet the synaptic phenotypes of $mdx:utrn^{-/-}$ and $adbn^{-/-}$ mice are similar. Together, these results suggest that α-DB2 is primarily responsible for maintaining muscle stability, while α-DB1 promotes synaptic

maturation, perhaps through a mechanism involving tyrosine phosphorylation.

Although the molecular links between α-DB and synaptic structure remain to be determined, our studies in vitro provided some insight into the cellular mechanism of the defect: the stability of AChR clusters following removal of exogenous agrin is decreased severalfold in the absence of α-DB. This defect suggests that α-DB is required for the maturation and/or stabilization of the postsynaptic apparatus once it has been induced by agrin. Although α-DB might also affect early steps in cluster formation, the reductions in cluster number and size observed in vitro may reflect an alteration in the normal balance between ongoing cluster growth and dissolution. It is also important to note that the defects observed in cultured myotubes provide strong evidence that α-DB acts cell autonomously in the postsynaptic apparatus rather than indirectly, for example, through effects on the nerve or Schwann cell.

α-DB and the DGC
Because α-DB is a component of the DGC, we considered whether or how DGC dysfunction contributed to the synaptic phenotype observed in $adbn^{-/-}$ mice. First, we asked whether loss of α-DB led to disassembly of the synaptic DGC. Key cytoskeletal (dystrophin and utrophin) and transmembrane (β-DG and β-sarcoglycan) DGC components remain concentrated at $adbn^{-/-}$ synaptic sites, but levels of $\alpha 1$, $\beta 1$, and $\beta 2$ syntrophins, and of the DGC-associated enzyme nNOS, were decreased at $adbn^{-/-}$ synapses. Thus, α-DB's effect upon the NMJ may be mediated in part through nNOS and the syntrophins. This seems unlikely to account for the entire phenotype, however, in that nNOS-deficient mice and mdx mice (which have dramatically reduced levels of syntrophins and nNOS) do not display the abnormalities observed at $adbn^{-/-}$ synapses (Lyons and Slater, 1991; R. G. M. and J. R. S., unpublished data).

Second, we compared the structures of synapses in $adbn^{-/-}$ mutant mice with those in $mdx:utrn^{-/-}$ double mutants, in which levels of many DGC components are dramatically reduced. We reasoned that synaptic phenotypes of $mdx:utrn^{-/-}$ and $adbn^{-/-}$ mice would be similar if the principal functions of the synaptic DGC are mediated by α-DB; on the other hand, $mdx:utrn^{-/-}$ mice would be more severely impaired than $adbn^{-/-}$ mice if the DGC plays α-DB-independent synaptic roles. Once influences of age and muscular dystrophy had been taken into account, the synaptic defects in $mdx:utrn^{-/-}$ mice appeared to be very similar to those in $adbn^{-/-}$ mice. This result suggests that a main function of the synaptic DGC may be to localize or concentrate α-DB in the postsynaptic membrane.

Third, we compared synapses in $mdx:utrn^{-/-}$ double mutants with those in $mdx:adbn^{-/-}:utrn^{-/-}$ triple mutants. We reasoned that if α-DB played DGC-independent roles, the defects might be additive, in which case the synaptic phenotype would be more severe in the triple mutant than in the double mutant. In fact, synaptic structure did not differ significantly between double and triple mutants, consistent with the idea that α-DB function requires an intact DGC. Such a requirement could be limited to the DGC's role in concentrating α-DB at the synapse or could involve molecular cooperation between α-DB and other DGC components.
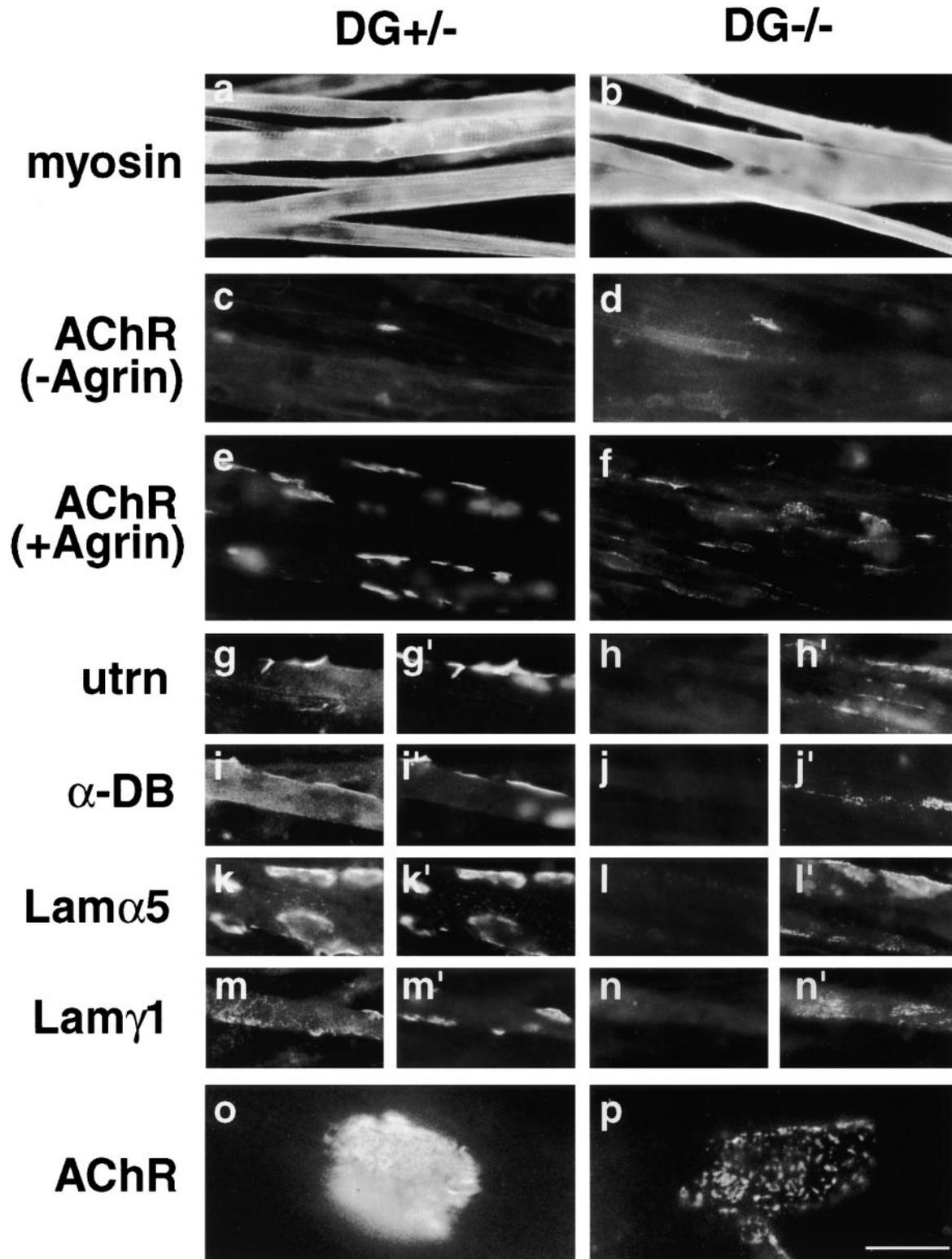
Figure 8. AChR Clustering in the Absence of DG

Myotubes generated from control ($DG^{+/-}$) or $DG^{-/-}$ ES cells were stained with anti-myosin (a and b) or rBTX (c–f, o, and p), or doubly stained with anti-utrophin (g and h), anti-α-DB (i and j), anti–laminin α5 (k and l), or anti–laminin γ1 (m and n) plus rBTX (g'–n'). Cultures in (e) through (p) were treated with agrin overnight before staining. Mutant myotubes are agrin sensitive and form AChR clusters despite lacking a detectable AChR-associated DGC or basal lamina. However, clusters are broken into microaggregates in the absence of DG. Scale bar, 40 μm (a–n') and 10 μm (o and p).

DG, the DGC, and the NMJ

As a final test of the relationship between α-DB and the synaptic DGC, we assessed AChR clustering in myotubes lacking DG. DG is a single-pass membrane protein that is cleaved to form a transmembrane protein (β-DG) that binds dystrophin/utrophin and an attached peripheral membrane protein (α-DG) that binds laminins and agrin. In the absence of DG, no DGC forms (Henry and Campbell, 1998, 1999). Although $DG^{-/-}$ mice die soon after implantation (Williamson et al., 1997), we were able to generate myotubes from $DG^{-/-}$ ES cells.

Myotubes formed normally in the complete absence of DG and retained the ability to form numerous AChR clusters following treatment with agrin. Recently, Carbonetto and colleagues reported normal myotube formation but markedly reduced AChR clustering following 70%–85% attenuation of DG levels in a myogenic cell line by expression of antisense sequences (Jacobson et al., 1998; Montanaro et al., 1999). In their studies, the possibilities remained that myogenesis required the 15%–30% residual DG and/or that complete removal of DG would completely abolish AChR clustering. Our results show that this is not the case and thereby provide conclusive evidence that DG is dispensable for myogenesis and agrin signaling. Moreover, no sarcolemma-associated DGC components were detectable in $DG^{-/-}$ myotubes, either at AChR clusters (Figure 8) or elsewhere in the myotube (K. P. C., unpublished data). We therefore conclude that the entire DGC may be dispensable for agrin signaling.

It is important to note that AChR clustering is not disrupted much more in the absence of the entire DGC than in the absence of α-DB. These results support the conclusion discussed above, that major roles of the synaptic DGC in vivo are likely to be mediated by α-DB. Equally important, however, is a difference between $adbn^{-/-}$ and $DG^{-/-}$ myotubes: only in the absence of DG do AChR clusters form pools of microaggregates that fail to coalesce into large clusters. Three lines of evidence suggest that this difference results from an extracellular role of DG. First, similar microaggregates are seen in the presence of antibodies to α-DG, which block ligand binding but do not disrupt the DGC (Campanelli et al., 1994). Second, AChR clusters on myotubes from $mdx:utrn^{-/-}$ double mutants, in which cytoplasmic portions of the DGC are disassembled, do not contain microaggregates but instead resemble those on $adbn^{-/-}$ myotubes (H. Z. and R. M. G., unpublished data). Third, the specialized basal lamina that is associated with AChR clusters in control myotubes does not form in $DG^{-/-}$ myotubes. Together, these results suggest that the DGC may play two roles in maturation of the postsynaptic apparatus, one that involves its intracellular components, especially α-DB, and a second that involves the DG-dependent assembly of a synaptic basal lamina.

Synaptic and Extrasynaptic Roles of the DGC

Taken together with our previous analysis of muscular dystrophy in $adbn^{-/-}$ mice (Grady et al., 1999), the present results reveal both similarities and differences between synaptic and extrasynaptic roles of α-DB. Synaptic and extrasynaptic DGC are largely intact in $adbn^{-/-}$ mice, suggesting that α-DB is more likely to play signaling than structural roles in both locations. Likewise, synaptic (myasthenic) and extrasynaptic (dystrophic) defects are similar in $mdx:utrn^{-/-}$ and $mdx:adbn^{-/-}:utrn^{-/-}$ mice, suggesting that critical roles of α-DB involve the DGC in both locations. On the other hand, the muscular dystrophy observed in $mdx:utrn^{-/-}$ double mutants is dramatically worse than that in $adbn^{-/-}$ mice, but synaptic defects of the single and double mutants are similar. The relationship between α-DB and DGC thus appears to differ markedly between synaptic and extrasynaptic regions of the muscle fiber. With respect to muscle stability, the DGC appears to play both structural and signaling roles, with only the signaling functions requiring α-DB, thereby accounting for the more severe dystrophic phenotype of $mdx:utrn^{-/-}$ compared with $adbn^{-/-}$ mutants. At the synapse, in contrast, the main roles of the DGC seem to involve its interaction with α-DB, as shown by the similar synaptic phenotype of $mdx:utrn^{-/-}$ and $adbn^{-/-}$ mutants.

Distinct Mechanisms for Synapse Formation and Synapse Maintenance

Perhaps the most interesting feature of the synaptic $adbn^{-/-}$ phenotype is its "late onset" both in vivo and in vitro. In vivo, synapses appear to form properly but fail to mature normally. In vitro, α-DB is dispensable for agrin-induced AChR clustering but required for maintenance of clusters following withdrawal of agrin. Together, these results indicate that separate mechanisms regulate the formation of the postsynaptic membrane and its subsequent maturation and/or maintenance and implicate α-DB and the DGC in the latter processes.

Recent genetic studies have placed several synapse-associated proteins in a pathway that induces synaptic differentiation: agrin is a nerve-derived organizer of postsynaptic differentiation, MuSK is a critical component of the agrin receptor complex, and the cytoplasmic protein rapsyn is essential for AChR clustering per se (Gautam et al., 1995, 1996; DeChiara et al., 1996; Burgess et al., 1999). The postsynaptic apparatus fails to form in mutant mice lacking any of these proteins. These studies have not, however, addressed the fate of synapses once they have formed. Indeed, NMJs undergo dramatic changes in structure and function postnatally and retain into adulthood an ability to remodel in response to trauma or altered activity (reviewed by Sanes and Lichtman, 1999). Comparatively little is known about the molecules and mechanisms that promote maturation or that enforce stability while permitting rearrangement. Further analyses of the DGC may be useful in understanding these crucial processes at the NMJ. Moreover, components of the DGC are present at interneuronal synapses (Lidov et al., 1990; Blank et al., 1999) and might be involved in regulating the synaptic rearrangements in the CNS.

Experimental Procedures

Animals

mdx mice with a naturally occurring mutation in the $dystrophin$ gene were obtained from Jackson Laboratories. Generation of mice bearing targeted mutations in the utrophin ($utrn^{-/-}$) and α-DB ($adbn^{-/-}$) genes, as well as double ($adbn^{-/-}:utrn^{-/-}$, $mdx:utrn^{-/-}$, and

mdx:adbn$^{-/-}$) and triple mutants (*mdx:adbn$^{-/-}$:utrn$^{-/-}$*), has been described previously (Grady et al., 1997a, 1997b, 1999). Mutants were maintained on a 129SVJ-C57/BL6 hybrid background. Genotypes were determined by PCR, as described previously (Grady et al., 1997a, 1997b, 1999).

Histological Analysis

To assess synaptic geometry, sternomastoid muscles were dissected from mutants and control littermates and fixed for 20 min in 1% paraformaldehyde in phosphate-buffered saline (PBS). The muscles were then equilibrated with sucrose, frozen in liquid nitrogen–cooled isopentane, and sectioned longitudinally at 40 μm in a cryostat. Sections were incubated with rBTX (Molecular Probes) or with a mixture of rBTX and primary antibodies. Following extensive washing, the muscles were reincubated with fluorescein-conjugated secondary antibodies, washed again, and mounted in 90% glycerol/10% PBS containing para-phenylenediamine to retard fading.

For routine immunohistochemical studies, muscles were frozen without fixation and cross-sectioned at 8 μm. Sections were stained with primary antibodies in PBS containing 1% bovine serum albumin, washed in PBS, reincubated with a mixture of second antibodies and rBTX, and mounted as above. Primary antibodies to the following antigen were used: SV2 (K. Buckley, Harvard University); neurofilament, VVA-B$_4$, and nNOS (Sigma); acetylcholinesterase (T. Rosenberry, Mayo Clinic); agrin (Regeneron Pharmaceuticals); α1, β1, and β2 syntrophin and dystrobrevin (DB2 and DB638; S. Froehner, University of North Carolina); utrophin, dystrophin, β-DG, and β-sarcoglycan (Novocastra Laboratories); rapsyn and dystrobrevin (DB692; Grady et al., 1999); laminin γ1 (Chemicon); and laminin α5 (Patton et al., 1997). Secondary antibodies used were FITC-conjugated goat anti-rabbit immunoglobulin G (IgG; Cappel) and FITC-conjugated goat anti-mouse IgG1 and IgG2a (Boehringer Mannheim).

For electron microscopy, muscles were fixed in 4% paraformaldehyde plus 4% glutaraldehyde in 0.1 M cacodylate buffer, washed, refixed in 1% OsO$_4$, dehydrated, and embedded in resin. Thin sections were stained with lead citrate plus uranyl acetate. The density of junctional folds was measured on electron micrographs as described previously (Grady et al., 1997a). For ultrastructural localization of AChRs, muscles were incubated successively with biotin-BTX (Molecular Probes) and avidin-HRP (Sigma) before fixation, then processed as described in Missias et al. (1997).

Cultures

Mononucleated cells were dissociated from limb muscles of neonatal control and mutant mice, plated on gelatin-coated glass coverslips, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum, 5% newborn calf serum, penicillin, and streptomycin. After 2–3 days in culture, cells were switched to DMEM containing 2% horse serum to promote fusion of myoblasts and formation of myotubes; 3–4 days later, a recombinant carboxy-terminal fragment of z-agrin (x = 12, y = 4, z = 8 form; Ferns et al., 1993; generated from transfected cells generously provided by Richard Scheller, Stanford University) or *Vicia villosa* agglutinin B$_4$ (VVA-B$_4$, 50 μg/ml, Sigma; used as described by Martin and Sanes [1995]) was added to some cultures. Following overnight incubation, these cultures were then incubated with rBTX for 1 hr at 37°C, rinsed with PBS, fixed with 2% paraformaldehyde, and mounted for fluorescence microscopy. AChR clusters were counted in 20 randomly chosen fields from each culture. Length of rhodamine-labeled AChR clusters was measured using images captured with a charge-coupled device camera and analyzed with IPLab software (Signal Analytics, Vienna, VA).

DG$^{+/-}$ and *DG$^{-/-}$* ES cells were generated as described previously (Henry and Campbell, 1998). The ES cells were cultured as described by Rohwedel et al. (1994, 1998) to generate myoblasts, which then fused to form myotubes. Once myotubes appeared mature (20–25 days after plating), the cells were treated with agrin and then stained as described above.

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