

# The Dystroglycan Complex Is Necessary for Stabilization of Acetylcholine Receptor Clusters at Neuromuscular Junctions and Formation of the Synaptic Basement Membrane

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**Abstract.** The dystrophin-associated protein (DAP) complex spans the sarcolemmal membrane linking the cytoskeleton to the basement membrane surrounding each myofiber. Defects in the DAP complex have been linked previously to a variety of muscular dystrophies. Other evidence points to a role for the DAP complex in formation of nerve–muscle synapses. We show that myotubes differentiated from dystroglycan<sup>–/–</sup> embryonic stem cells are responsive to agrin, but produce acetylcholine receptor (AChR) clusters which are two to three times larger in area, about half as dense, and significantly less stable than those on dystroglycan<sup>+/+</sup> myotubes. AChRs at neuromuscular junctions are similarly affected in dystroglycan-deficient chimeric mice and there is a coordinate increase in nerve terminal size at these junctions. In culture and in vivo the absence of

dystroglycan disrupts the localization to AChR clusters of laminin, perlecan, and acetylcholinesterase (AChE), but not rapsyn or agrin. Treatment of myotubes in culture with laminin induces AChR clusters on dystroglycan<sup>+/+</sup>, but not <sup>–/–</sup> myotubes. These results suggest that dystroglycan is essential for the assembly of a synaptic basement membrane, most notably by localizing AChE through its binding to perlecan. In addition, they suggest that dystroglycan functions in the organization and stabilization of AChR clusters, which appear to be mediated through its binding of laminin.

**Key words:** synapse formation • acetylcholinesterase • acetylcholine receptors • dystroglycan • dystrophin-associated proteins

## Introduction

The neuromuscular junction (NMJ)<sup>1</sup> encompasses three distinct domains specialized in the efficient transmission of signals from motor neuron to muscle: the nerve terminal, the synaptic basement membrane, and the postsynaptic membrane. During development all three regions differentiate after release of agrin (McMahan, 1990), a heparan sulfate proteoglycan (Denzer et al., 1995), by the nerve. Agrin becomes concentrated in the synaptic basement membrane where it can interact with molecules on the

postsynaptic membrane to initiate the formation of a high density of acetylcholine receptors (AChRs) at sites of nerve–muscle contact (Gautam et al., 1996).

Aggregates of AChRs at mature NMJs are metabolically and diffusionally very stable (Berg and Hall, 1975; Salpeter and Harris, 1983; Akaaboune et al., 1999) and are thought to accumulate in the differentiating postsynaptic membrane by diffusion into a “trap” of membrane-cytoskeletal proteins (Edwards and Frisch, 1976). This accumulation of AChRs in vivo and in culture progresses through two stages. In the first, AChRs assemble into microclusters, which are subsequently condensed and maintained as larger clusters (Anderson and Cohen, 1977; Steinbach, 1981; Wallace, 1988; Cohen et al., 1995; Jacobson et al., 1998). The initial event in the formation of AChR clusters is believed to be the activation of the receptor tyrosine kinase, muscle-specific tyrosine kinase (MuSK), by agrin (Valenzuela et al., 1995; Zhou et al., 1999). The cytoplasmic extensions of AChRs are associated with rapsyn (Burden et al., 1983), a protein which can aggregate to mediate

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<sup>1</sup>Abbreviations used in this paper:  $\alpha$ -Btx,  $\alpha$ -bungarotoxin; AChE, acetylcholinesterase; AChR, acetylcholine receptor; ANOVA, analysis of variance; DAP, dystrophin-associated protein; EHS, Engelbreth-Holm-Swarm; ES, embryonic stem; fas-2, fasciculin 2; HS, horse serum; MASC, myotube-associated specificity component; MuSK, muscle-specific tyrosine kinase; NMJ, neuromuscular junction.

the formation of AChR microclusters (Froehner et al., 1990; Phillips et al., 1991a).

Also contained within microclusters are the dystroglycans, two members of a complex of dystrophin-associated proteins (DAPs) which are found over the entire sarcolemma, but are concentrated at NMJs (Ohlendieck et al., 1991; Matsumura et al., 1992). Encoded by a single gene, the dystroglycan precursor is posttranslationally cleaved to produce the extracellular peripheral membrane protein  $\alpha$ -dystroglycan and the transmembrane protein  $\beta$ -dystroglycan (Ibraghimov-Beskrovnaya et al., 1992; Gorecki et al., 1994; Deyst et al., 1995). These two proteins are bound noncovalently and span the membrane to interact with both the extracellular matrix and cytoskeleton (Ibraghimov-Beskrovnaya et al., 1992; Henry and Campbell, 1999). As such, the dystroglycans form the core of a larger complex of DAPs that function to maintain muscle integrity (Henry and Campbell, 1996) and mediate basement membrane assembly (Williamson et al., 1997) in a variety of tissues, including skeletal muscle (Cohen et al., 1997; Henry and Campbell, 1998; Montanaro et al., 1998; Côté et al., 1999). DAPs have been widely studied since their dysfunction leads to muscular dystrophies (Sunada and Campbell, 1995), but they also have been implicated in the formation of NMJs. This was suggested by evidence that  $\alpha$ -dystroglycan binds agrin (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994) and helps mediate AChR clustering (Campanelli et al., 1994; Gee et al., 1994; Montanaro et al., 1998) during synapse formation in culture (Cohen et al., 1995). More recently, Côté et al. (1999) reported that mouse muscles deficient in dystroglycans have severely disorganized NMJs. In keeping with this, Grady et al. (2000) showed recently that mice null for the DAP dystrobrevin also have disrupted NMJs.

The importance of the dystroglycans in synaptic basement membrane assembly has been highlighted by two related observations. First, laminins are major components of basement membranes (Yurchenco and O'Rear, 1994; Timpl, 1996) and  $\alpha$ -dystroglycan is a laminin receptor (Smalheiser and Schwartz, 1987; Douville et al., 1988; Ibraghimov-Beskrovnaya et al., 1992). Second, laminin and  $\alpha$ -dystroglycan can coassemble on the cell surface (Cohen et al., 1997; Montanaro et al., 1998; Colognato et al., 1999) as well as bind other matrix molecules, including collagen, entactin, and perlecan (Peng et al., 1998; Talts et al., 1999). Acetylcholinesterase (AChE) is another important basement membrane protein that is localized to NMJs via its collagen tail (Massoulié et al., 1993). Indeed, when histological sections of muscle are incubated with the collagen-tailed AChE the enzyme attaches specifically to the sites of nerve-muscle contact on the basement membrane (Rotundo et al., 1997). It was subsequently shown that the AChE molecules can bind to perlecan, which in turn can bind to  $\alpha$ -dystroglycan. These interactions have been proposed to account for synaptic localization of AChE (Peng et al., 1999). In addition, extracellular matrix assembly may also be involved in AChR clustering at sites of nerve-muscle contact since treatment of myotubes in culture with exogenous laminin stimulates AChR aggregation (Vogel et al., 1983) via dystroglycan and independent of MuSK activation (Cohen et al., 1997; Sugiyama et al., 1997; Montanaro et al., 1998).

In this study we further define the role of dystroglycans in AChR clustering and basement membrane assembly using chimeric mice with reduced dystroglycan expression and myotubes generated from dystroglycan-null embryonic stem (ES) cells. Dystroglycan-deficient muscle and dystroglycan-null myotubes were both found to have AChR clusters on their surfaces, though they are comprised of loosely associated microclusters. In culture the absence of dystroglycan did not markedly affect the initiation of AChR clustering by agrin, nor did it affect the colocalization of agrin and rapsyn with AChR clusters, suggesting that dystroglycan functions downstream of agrin and rapsyn by condensing AChR microclusters. However, there was a significant decrease in AChR cluster stability on dystroglycan-null myotubes compared with wild-type controls. This evidence is consistent with a proposed role for dystroglycan in mediating cytoskeletal and possibly basement membrane interactions which trap AChRs (Carbonetto and Lindenbaum, 1995). Furthermore, in muscle myotubes, components of the synaptic basement membrane, including laminin, perlecan, and AChE, were no longer concentrated at AChR clusters, although the distribution of laminin and perlecan over the rest of the myotube appeared unaffected. We conclude that dystroglycans not only are necessary for AChR cluster condensation and stability, but they also coordinate assembly of the synaptic basement membrane.

## Materials and Methods

### Dystroglycan-null ES Cell Generation

ES cell lines rendered null for dystroglycan were described previously (Côté et al., 1999). In brief, targeting vectors were generated by excising a 10.2-kb EcoRI fragment containing exon 2 and portions of the flanking introns from a 17.5-kb clone isolated from a mouse genomic library. Two vectors were generated. The first was constructed by replacing a 0.7-kb StuI-SalI fragment with the hygromycin-resistance gene (*hygro<sup>r</sup>*). This removed the dystroglycan coding sequence corresponding to amino acids 92–117, as well as the splice donor site. In the second vector, the coding sequence corresponding to amino acids 100–117, located between two SacI sites, was replaced with the neomycin-resistance gene (*neo<sup>r</sup>*). The *hygro<sup>r</sup>* and the *neo<sup>r</sup>* vectors were linearized and R1 ES cells (Nagy et al., 1993) were electroporated with the *hygro<sup>r</sup>* vector first. Hygromycin-resistant clones were picked, expanded, and screened for homologous recombination events using a 0.7-kb EcoRI-XhoI fragment to hybridize HindIII-digested DNA. A single clone (2D8) was chosen and electroporated with the *neo<sup>r</sup>* vector. We repeated the selection procedure with G418 sulfate and identified homologous recombinant clones using the same 0.7-kb EcoRI-XhoI fragment as a probe. Two null dystroglycan clones, 3C12 and 3H1, were selected for differentiation in culture.

### Cell Culture and ES Cell Differentiation

Wild-type R1 ES cells and dystroglycan-null 3C12 and 3H1 cells were maintained in a totipotent state by cultivating them as described previously (Abbondanzo et al., 1993). In brief, ES cells were seeded over a layer of neomycin-resistant or neomycin/hygromycin-resistant feeder fibroblasts. The ES cells were fed daily with DME high glucose medium supplemented with 20% ES-cell-qualified FCS (Wisent Canadian Laboratories), L-glutamine (2 mM; Life Technologies), 2-mercaptoethanol ( $10^{-4}$  M; Sigma-Aldrich), and leukemia inhibitory factor (1,000 U/ml; Life Technologies). ES cells were induced to form embryoid bodies as described by Rohwedel et al. (1994), with modifications taken from Dinsmore et al. (1996). ES cells were harvested and fibroblast feeders were removed by preplating twice onto gelatin (0.1%-coated tissue culture dishes. After a 1-h incubation the ES cells were washed off with medium and diluted to a concentration of  $4 \times 10^4$  cells/ml of differentiation medium (DME high glucose supplemented with 15% horse serum; Wisent

Canadian Laboratories), 1% DMSO (Sigma-Aldrich), and 1% penicillin-streptomycin (Life Technologies). Aliquots (20  $\mu$ l) of cell suspension were spotted as drops onto the undersides of lids from 100-mm bacterial petri dishes (Fisher Scientific), which were then inverted over dishes filled with medium to prevent drying. The ES cells were cultivated in this manner for 4 d, then transferred to gelatin (0.1%)-coated tissue culture dishes and cultured for an additional 20–30 d. Differentiating ES cell bodies were maintained in DME high glucose supplemented with 15% horse serum (Wisent Canadian Laboratories) and 1% penicillin-streptomycin.

### Antibodies and Toxins

TRITC and Alexa 488-conjugated  $\alpha$ -bungarotoxin ( $\alpha$ -Btx) and TRITC and Oregon green secondary antibodies were obtained from Molecular Probes. Anticollagen IV and antiperlecan (1948) antibodies were purchased from Chemicon International. The antifibronectin antibody was obtained from Telios Pharmaceuticals. The antisynaptophysin antiserum was purchased from Novocastra Laboratories. Fasciculin 2 (fas-2; Sigma-Aldrich) was labeled with TRITC or Oregon green (Molecular Probes) as described previously (Peng et al., 1999). The antisera to laminin  $\alpha$ 2 chain (merosin) and agrin were generously provided by P. Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ) and Z. Hall (University of California at San Francisco, San Francisco, CA), respectively. The anti-laminin and the antidystroglycan antisera were generated in house by inoculating rabbits with Engelbreth-Holm-Swarm (EHS) laminin or a polypeptide corresponding to 15 of the last 16 amino acids of the COOH terminus of  $\beta$ -dystroglycan, respectively. Finally, the antirapsyn monoclonal antibody 1234 was a gift from S. Froehner (University of North Carolina, Chapel Hill, NC).

### Labeling of Muscle Fibers with $\alpha$ -Btx and Fas-2

Intact muscles were dissected, pinned out at resting length, and fixed in 4% paraformaldehyde in 20 mM PBS, pH 7.4. For labeling, bundles of fibers were teased apart and incubated in PBS to remove fixative. The fibers were then washed with PBS containing 10% horse serum (PBS/HS) and stained for 30 min in PBS/HS containing 0.5–1.0  $\mu$ g/ml TRITC- $\alpha$ -Btx and Oregon green-conjugated fas-2 each. The fiber bundles were then washed three times in PBS/HS, twice with PBS alone, and fixed again for 15 min at room temperature with 4% paraformaldehyde in 20 mM PBS. The fibers were then teased apart to give single fibers and mounted in 90% glycerol buffered with 10 mM bicarbonate, pH 9.5, containing 1 mg/ml *p*-phenylenediamine to reduce photobleaching. The specimens were then viewed with a Leica DMR-AX microscope at 40–100 $\times$ . The images were captured using a Princeton Instruments MicroMax CCD camera and analyzed using Metamorph software (Universal Imaging Corp.).

### Immunocytochemistry

Fresh-frozen, superficial gluteal muscles from chimeric mice aged between 3 and 12 wk were sectioned longitudinally (10  $\mu$ m), blocked with 10% goat serum, and stained with TRITC- $\alpha$ -Btx and affinity-purified antidystroglycan antiserum, followed by an Oregon green 488-conjugated secondary antiserum. Myotubes from differentiated ES cells were incubated at 37°C with agrin (500 pM; a gift from M. Ferns, McGill University; Ferns et al., 1993) or EHS laminin (100 nM; Timpl et al., 1982) overnight in ES cell differentiation medium. In all cases AChRs were visualized by staining cultures of live cells. TRITC- or Alexa 488-labeled  $\alpha$ -Btx (2  $\mu$ g/ml) was added directly to the media for 1 h at 37°C. Cultures were then washed twice with PBS and fixed with 2% paraformaldehyde for 15 min at 37°C. This fixation permeabilizes a small fraction of the myotubes and occasionally myotubes will be labeled intracellularly (see Fig. 10 A). After two additional washes with PBS, cells were blocked for 1 h with 10% goat serum in PBS (blocking buffer). When primary antibodies recognizing intracellular epitopes were used, cells were first permeabilized with 0.1% Triton X-100 in blocking buffer for 10 min at room temperature. Primary antibodies were diluted in blocking buffer and incubated at room temperature for 1 h. After several washes with PBS, bound primary antibody was detected with TRITC- or Oregon green 488-conjugated secondary antiserum. Cultures were washed a final time before the addition of Immuno Flore Mounting Medium (ICN Biomedicals) and mounted on coverslips. Fluorescence was viewed with a ZEISS Axioskop fluorescence microscope. To document observed differences in the intensity of antibody labeling, experimental and control samples were photographed and reproduced under identical conditions.

### Quantification of AChR Clusters

ES cell cultures were treated for 16 h with agrin (500 pM) or laminin (100 nM) before incubation with fluorescent- $\alpha$ -Btx for 1 h. Labeled cultures were then washed twice with PBS and fixed with 2% paraformaldehyde for 15 min. The number of spontaneous or induced AChR clusters was quantified from data captured on photographic images taken with a ZEISS Axioskop fluorescence microscope. In each experiment at least 10 nonoverlapping microscope fields from each culture dish and treatment were examined and the average number of AChR clusters per field was quantified. Pains were taken to control the number and caliber of myotubes in each field. In all instances no morphological differences were noted between wild-type R1 myotubes or dystroglycan-null myotubes and individual experiments were repeated at least three times. The area and density of AChR clusters on R1, 3C12, and 3H1 cells were quantified using NIH Image, v1.62, run on a Macintosh G3 (Apple Computer). All cluster images were scanned at 300 dpi and imported into NIH Image software using the scanner interface program FotoLook (v2.07; Agfa) and an Agfa ArcusII scanner. Photographic images were imported into NIH Image in gray scale mode and were “inverted” so that clusters were set to appear black on a white background and the size scale calibration was set to 7.35 pixel/ $\mu$ m.

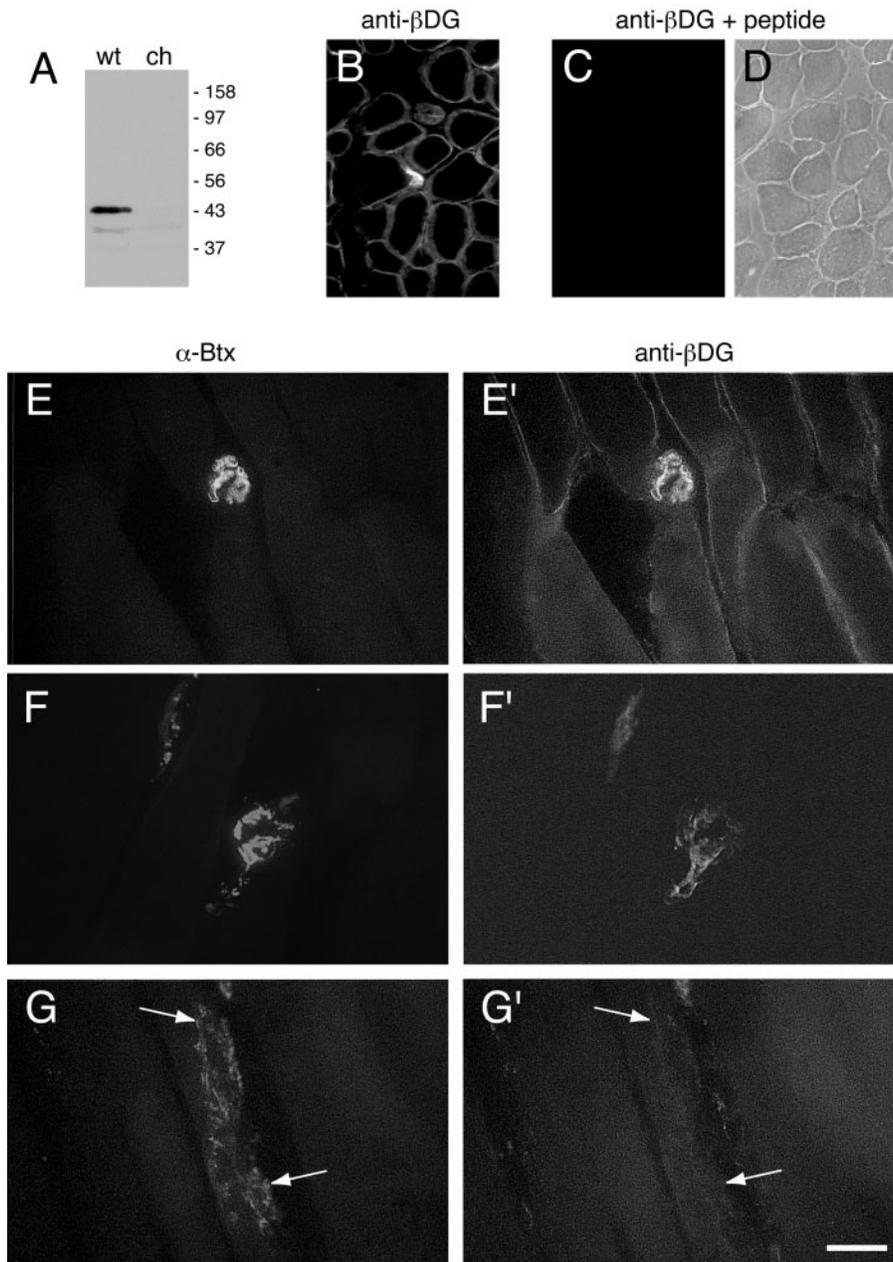
To estimate the density of AChR clusters, the total area occupied by a cluster was determined by circumscribing them using the Free Hand tool function of NIH Image and the Measure command. These clusters were then highlighted using the Density Slice tool and the scale was manipulated so as to accentuate only AChRs within the perimeter of the circumscribed cluster. The selected areas were then compared with the original photographs and the area occupied by AChRs was measured using the Analyze Particles command. The resulting values were summed to give the particle area. The AChR cluster density, given as a percentage, was then derived by dividing the particle area by the total area circumscribed. At least 50 clusters were analyzed for each cell type.

The extent of overlap of extracellular matrix molecules with AChR clusters was quantified using a 40 $\times$  objective on a ZEISS Axioskop fluorescence microscope. The percentage of overlap was determined for each field containing at least one AChR cluster. Plaques of extracellular matrix molecules were considered to overlap with AChRs if the staining for the molecule was completely within the perimeter of an AChR cluster or vice versa. Experiments were repeated at least three times with at least six to seven fields quantified for an extracellular matrix molecule examined in each experiment. The total number of clusters analyzed exceeded 50 for each extracellular matrix molecule. Results were compared using Student's *t* test or analysis of variance (ANOVA), as indicated.

## Results

### Dystroglycan Depletion in Skeletal Muscle Results in Aberrant NMJs

In a previous report we described the dystrophic phenotype of chimeric mice deficient in dystroglycan and noted that NMJs, especially in older mice, were disorganized (Côté et al., 1999). At that time we were unable to rigorously evaluate the amount of dystroglycan expressed at individual endplates because high levels of endogenous mouse immunoglobulins were present at the periphery of wild-type muscle fibers and within degenerating fibers of chimeric muscle (Côté et al., 1999). This resulted in a very high level of labeling with the anti-mouse secondary antisera used against the available monoclonal antibody to  $\beta$ -dystroglycan. Thus, in chimeric skeletal muscle, where each myofiber has hundreds to thousands of nuclei, muscles which appeared to be completely derived from dystroglycan-null stem cells, when assayed by the extremely sensitive method of glucose phosphate isomerase electrophoresis (De Lorenzo and Ruddle, 1969), may have had some residual dystroglycan. To evaluate this, we have generated and characterized a rabbit antiserum against  $\beta$ -dystroglycan. The specificity of this antibody is shown in an



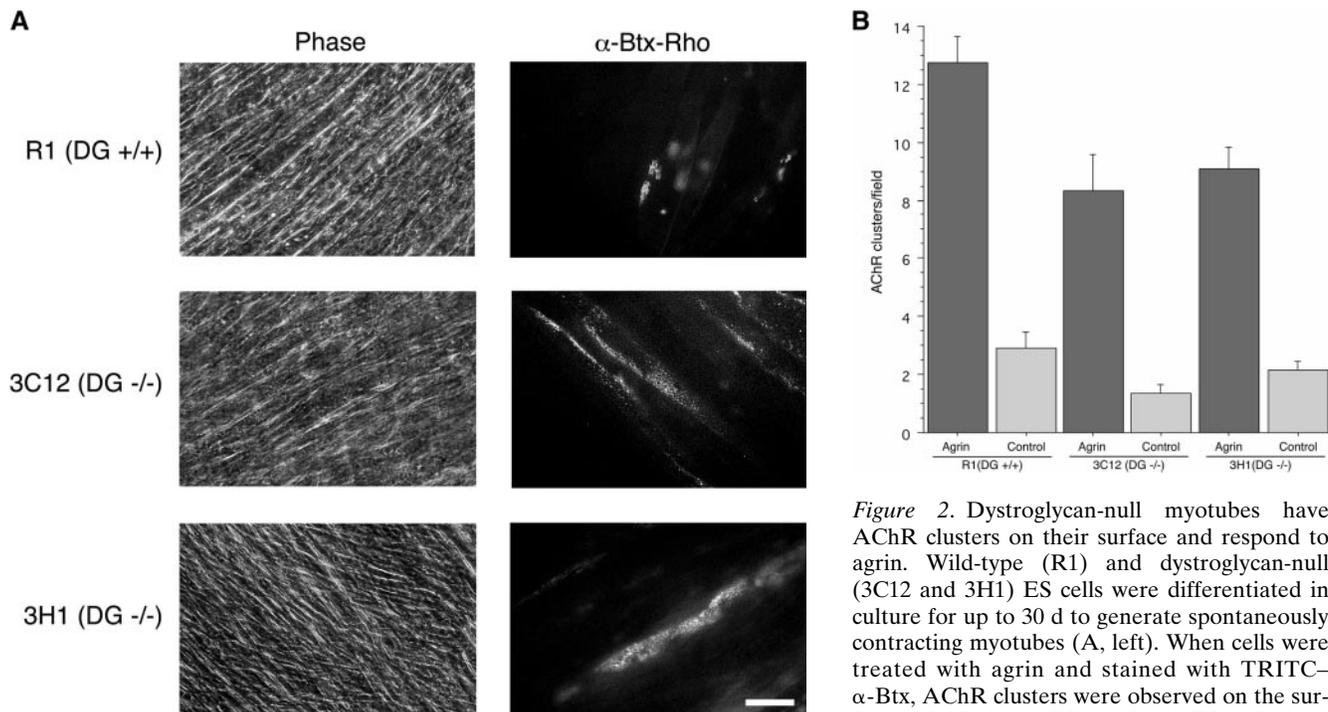
**Figure 1.** Adult NMJs are disorganized in dystroglycan-deficient muscles. (A) The specificity of the polyclonal antiserum used in this study against  $\beta$ -dystroglycan was tested by immunoblot of total muscle extracts of wild-type (wt) and dystroglycan-depleted chimeric muscle (ch). Additionally, wild-type muscle sections were probed immunocytochemically using either antiserum against  $\beta$ -dystroglycan (B) or the antiserum preincubated with 100-fold excess of the peptide used for immunization (C and D; phase corresponding to C). Longitudinal sections of the superficial gluteal muscle were stained with TRITC- $\alpha$ -Btx and antisera to  $\beta$ -dystroglycan (anti- $\beta$ DG). In muscle fibers with near normal levels of dystroglycan expression (E') there is a concentration of dystroglycan at endplates in a pattern corresponding to AChR staining (E) and equivalent to that seen in wild-type mice (not shown). In muscle fibers with no detectable extrajunctional dystroglycan there may be some dystroglycan at end-plates (F') and significantly disorganized AChRs (F). In instances where there is no dystroglycan whatsoever (G'), AChRs are grossly disorganized and broken up into microclusters spread over the fiber surface (G). Bar: (B, C, and D) 100  $\mu$ m; (E, F, and G) 30  $\mu$ m.

immunoblot (Fig. 1 A), where the antiserum identifies a single major band at 43 kD, the size of  $\beta$ -dystroglycan, in wild-type muscle (Fig. 1 A; lane wt). This band is undetectable in muscle extracts from dystroglycan-deficient chimeras (Fig. 1 A; lane ch). Also, immunostaining of wild-type muscles with this antiserum is restricted to the surfaces of these myofibers (Fig. 1 B) and is blocked by addition of the peptide used to generate the antiserum (Fig. 1, C and D). Dystroglycan expression was then assayed by immunohistochemistry on similar muscle sections in conjunction with fluorescently labeled  $\alpha$ -Btx to identify NMJs. When viewed en face, we observed endplates on muscle fibers with variable levels of dystroglycan expression. In many cases, in younger mice these junctions appeared like those in muscles from wild-type littermates (Fig. 1, E and E'). In other cases, there was a large reduction of dystroglycan extrasynaptically and the residual dystroglycan had been re-

cruited to partially disorganized endplates (Fig. 1, F and F'); in still other cases, there were fibers devoid of any detectable dystroglycan with very diffuse clusters of AChRs (Fig. 1, G and G'; arrows).

#### ***Myotubes Generated from Dystroglycan-null ES Cells Do Not Form Condensed Clusters of AChRs either Spontaneously or in Response to Agrin***

To assess the roles of dystroglycan in the organization of the sarcolemma and the organization of AChRs, ES cells were grown (see Materials and Methods) and allowed to differentiate for up to 30 d. Under these conditions dystroglycan-null ES cells differentiated into several cell types, including neurons, blood cells, and myoblasts (Côté, P.D., and S. Carbonetto, unpublished results). By 20 d in culture myoblasts had fused into myotubes with multiple aligned nuclei which expressed myogenin when assayed immuno-



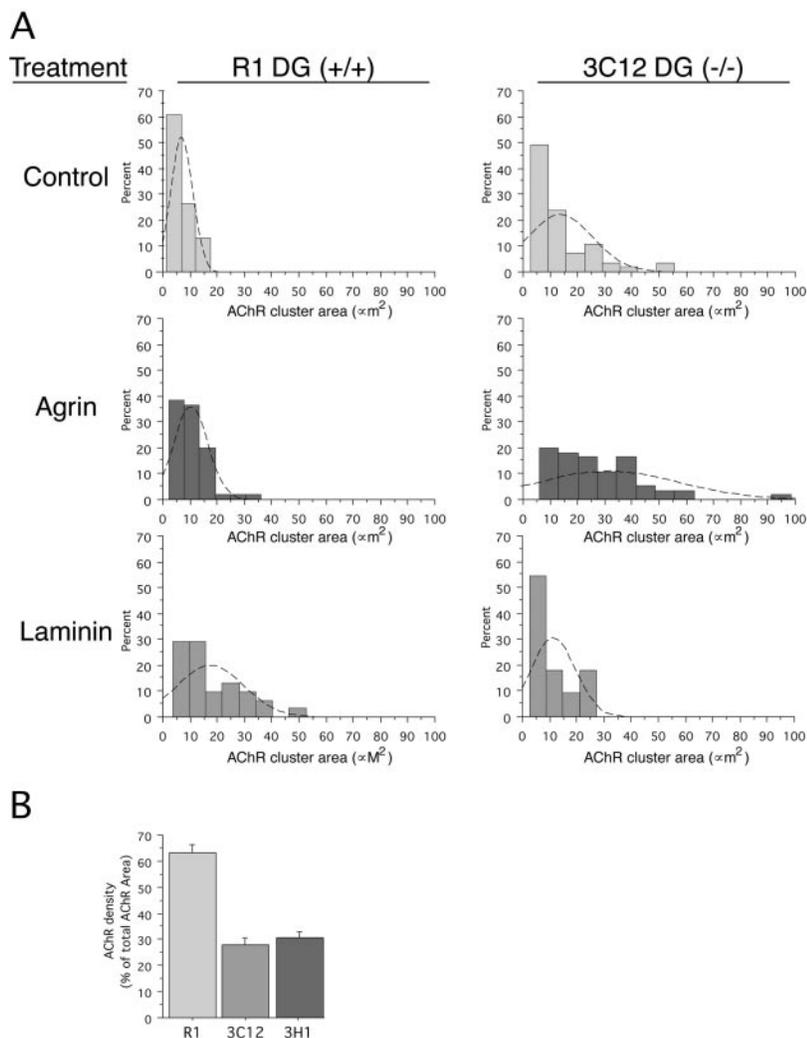
**Figure 2.** Dystroglycan-null myotubes have AChR clusters on their surface and respond to agrin. Wild-type (R1) and dystroglycan-null (3C12 and 3H1) ES cells were differentiated in culture for up to 30 d to generate spontaneously contracting myotubes (A, left). When cells were treated with agrin and stained with TRITC- $\alpha$ -Btx, AChR clusters were observed on the surfaces of all three differentiated ES cell lines. Dystroglycan-null myotubes (3C12 and 3H1) have AChR clusters comprised of diffuse arrays of microclusters. This contrasts with the AChR clusters on R1 cells, which are in smaller and more uniformly fluorescent plaques (A, right). (B) Overnight treatments with 500 pM agrin induced an approximately fourfold increase in the number of clusters for all three cell lines compared with untreated cultures. The data presented represent at least three separate experiments with 10–20 microscope fields of myotubes quantified per experiment. Significance was tested using Student's *t* test. Bar, 10  $\mu$ m.

cytochemically (data not shown). Myotubes in cultures of wild-type (R1) and dystroglycan-null ES cell lines (3C12 and 3H1) were indistinguishable under phase optics (Fig. 2 A, left) and contracted spontaneously. After incubation with TRITC-labeled  $\alpha$ -Btx, wild-type and dystroglycan-null myotubes were both found to have AChR clusters, though there was a clear difference in their morphology. AChRs from wild-type (R1) myotubes were in tight, uniformly fluorescent clusters, whereas clusters of AChRs on dystroglycan-null myotubes (3C12 and 3H1) were punctate and covered a larger area than those on wild-type myotubes (Fig. 2 A). Similar results were obtained whether clusters had formed spontaneously or were induced by agrin (Fig. 2 A). The small clusters of AChRs appeared similar to the microclusters that first form on the surface of muscle after innervation and which are known to contain dystroglycan (Cohen et al., 1995). Based on these observations, we hypothesized that dystroglycan either was necessary for the condensation of AChR microclusters into larger clusters, or was required for stabilization of preexisting clusters which had formed normally but then dispersed in the absence of dystroglycan.

To distinguish between these two possibilities, we treated cultures with agrin and monitored the onset of AChR clustering. From the earliest time points at which they were visible (8 h), clusters induced by agrin (500 pM) on 3C12 and 3H1 cells were more diffuse than those on wild-type R1 cells. On 3C12 cells there was no evidence of normal-looking AChR clusters which would subsequently disperse into microclusters. In addition to the difference in distribution of AChR clusters, there was also a decrease in

their number on dystroglycan-null myotubes treated with agrin. There were 12.1 clusters per field on R1 cells and only 8.0 and 9.0 clusters per field on 3C12 and 3H1 myotubes, respectively (Fig. 2 B). The reduction in the number of AChR clusters on the two dystroglycan-null lines 3C12 and 3H1 was statistically significant when individually compared with R1 cells ( $P < 0.01$ ;  $n \geq 3$ ). However, in all three cell lines there was an approximately fourfold increase in the number of clusters on cells treated with agrin compared with untreated controls.

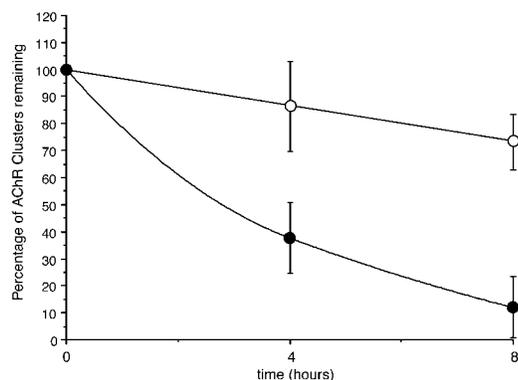
Detailed morphometric analysis allowed quantification of the differences in size and density of AChR clusters on R1 and 3C12 myotubes. Cluster area was determined for untreated and agrin-treated R1 and 3C12 myotubes, and the resulting values were used to calculate the average cluster size as well as to generate a frequency distribution of cluster areas (Fig. 3 A). Agrin treatment of wild-type (R1) and null (3C12) myotubes resulted in a much larger increase in AChR cluster area in 3C12 cells (13.69 to 31.35  $\mu$ m<sup>2</sup>) than in R1 cells (6.93 to 10.3  $\mu$ m<sup>2</sup>; Fig. 3 A, broken line). The increase in area with agrin treatment within a cell line and the difference in areas between the two different lines were statistically significant ( $P < 0.0001$ ). The difference in AChR density was quantified by determining the ratio of surface area covered with  $\alpha$ -Btx fluorescence to nonfluorescent area within the perimeter occupied by individual R1 and 3C12 AChR clusters. AChRs occupied ~63% of the area within the perimeter of a wild-type AChR cluster (Fig. 3 B), which was approximately double the 28–30% of the area that receptors occupied in each dystroglycan-null myotube cluster ( $P < 0.0001$ ;  $n > 50$ ). The



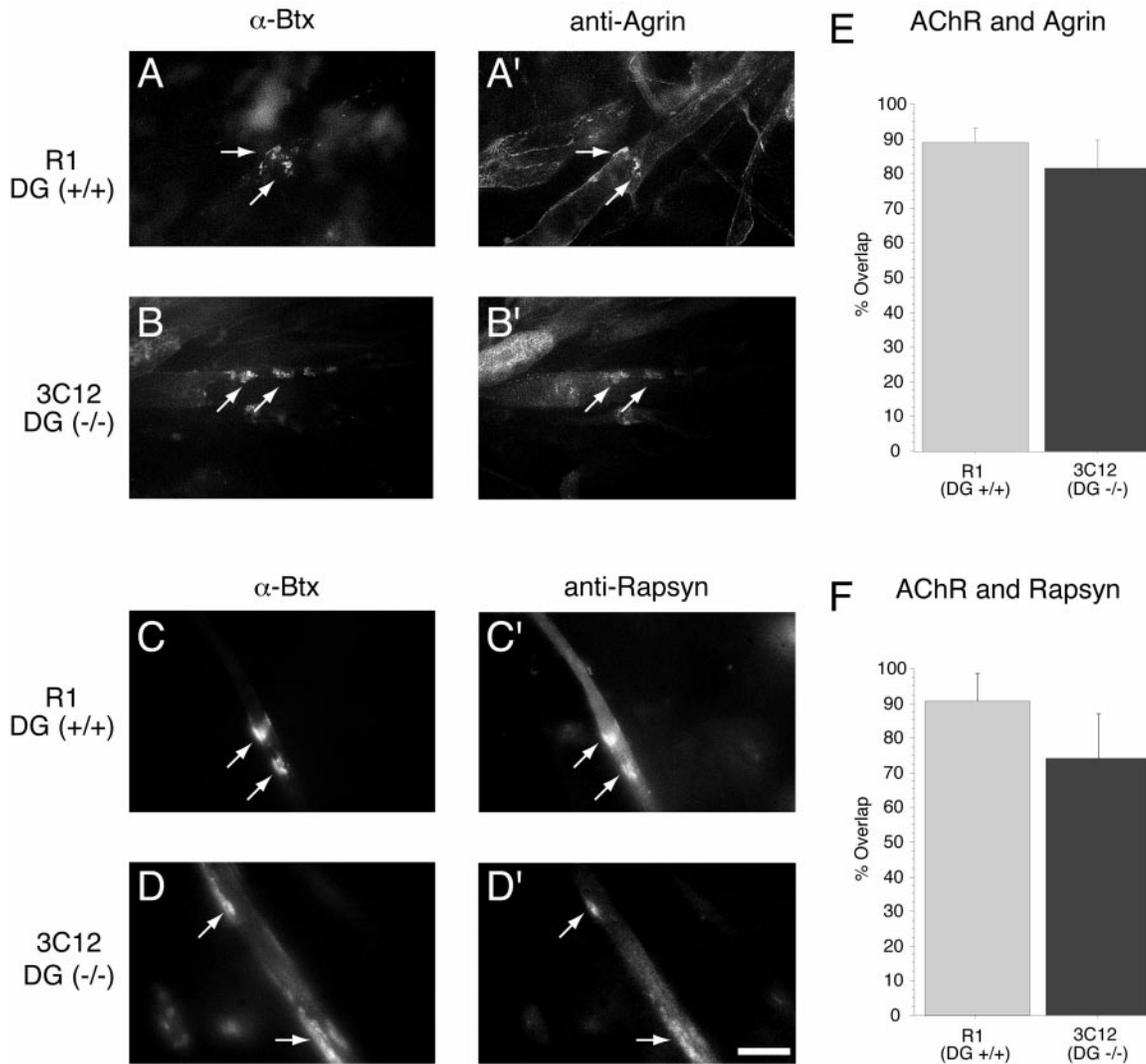
**Figure 3.** AChR cluster area and density is altered in dystroglycan-null myotubes. (A) The histograms show size distributions for the wild-type (R1) and dystroglycan-null (3C12) cell lines in untreated cultures (Control) or in cultures treated with 500 pM agrin or 100 nM laminin. The average cluster area is represented by the peak of the dashed line on each graph. Agrin and laminin treatment of R1 cells resulted in an increase in cluster area from 6.9 to 10.3 and 17.6  $\mu\text{m}^2$ , respectively. A larger increase in AChR cluster size was noted for agrin-treated 3C12 cells, 31.4  $\mu\text{m}^2$ , up from 13.7  $\mu\text{m}^2$ . Laminin increased the size of clusters on R1 cells but was ineffective on 3C12 cells. At least 10 microscope fields containing AChR clusters were quantified in five separate experiments for each cell line and treatment. (B) The density of AChR clusters was estimated (see Materials and Methods) for the three cell lines after agrin treatment. AChR clusters on 3C12 and 3H1 cells were twice as diffuse as those on R1 cells. AChRs covered 28 and 30% of the area within clusters on dystroglycan-null myotubes versus 63% of the area on wild-type clusters. At least 50 clusters were quantified per cell line over five experiments to determine AChR density and the significance tested using a Student's *t* test ( $P < 0.0001$ ).

relative level of AChR expression on each ES cell line was estimated by multiplying the number of clusters within each microscope field (Fig. 2) by the average cluster area for each cell type (Fig. 3 A) and the density of AChRs (see above). By this measure the area covered by AChRs on R1, 3C12, and 3H1 myotubes was 78.71, 70.22, and 89.73  $\mu\text{m}^2$ , respectively. When expressed relative to values on R1 cells, the values on null myotubes were approximately com-

parable, being 89% (3C12) or 114% (3H1) of the levels on R1 cells. The lack of dystroglycan thus resulted in AChR clusters which were three times larger and twice as diffuse as those found on wild-type cells, though the myotubes appeared to contain similar numbers of AChRs. Taken together, these data suggest that dystroglycan-null cells were able to respond to agrin initially to form microclusters but unable to condense them into larger, synaptic-like clusters.



**Figure 4.** AChR cluster stability is reduced in dystroglycan-null myotubes. Differentiated ES cells were incubated overnight with or without 500 pM agrin, washed, then maintained in fresh medium. Cultures were labeled with TRITC- $\alpha$ -Btx at 0, 4, and 8 h after washing, fixed, and the number of AChR clusters per field was determined. The number of spontaneous clusters in untreated cultures was subtracted from each of the agrin treated time points and the resulting value was expressed as a percentage of the value at time zero. AChR clusters on 3C12 cells dispersed at approximately three times the rate of clusters on wild-type cells. By 8 h there was an 88% drop in the number of AChR aggregates on 3C12 cells (filled circles), compared with a 27% drop on R1 cells (open circles). There was no significant difference between the number of clusters remaining on 3C12 cells at 8 h and on untreated cultures. The data represent the average of three trials. Significance was tested by ANOVA ( $P < 0.0001$ ).



**Figure 5.** Agrin and rapsyn colocalize with AChR clusters on dystroglycan-null myotubes. Myotubes from wild-type (R1) and dystroglycan-null ES cells (3C12) were treated overnight with 500 pM agrin and stained with TRITC- $\alpha$ -Btx and either antiagrins or antirapsyn antibodies. In both cell types there is colocalization of AChR clusters with agrin (arrows in A, A', B, and B') and with rapsyn (arrows in C, C', D, and D'). The percentage overlap (E and F) indicates the percentage of AChR clusters with corresponding concentrations of agrin and rapsyn. In both 3C12 and R1 lines we found no significant difference in the level of colocalization of AChR aggregates with agrin (E) or rapsyn (F). The histograms represent the average of three separate experiments in which 10–20 microscopic fields containing at least one AChR cluster per field. Bar, 10  $\mu$ m.

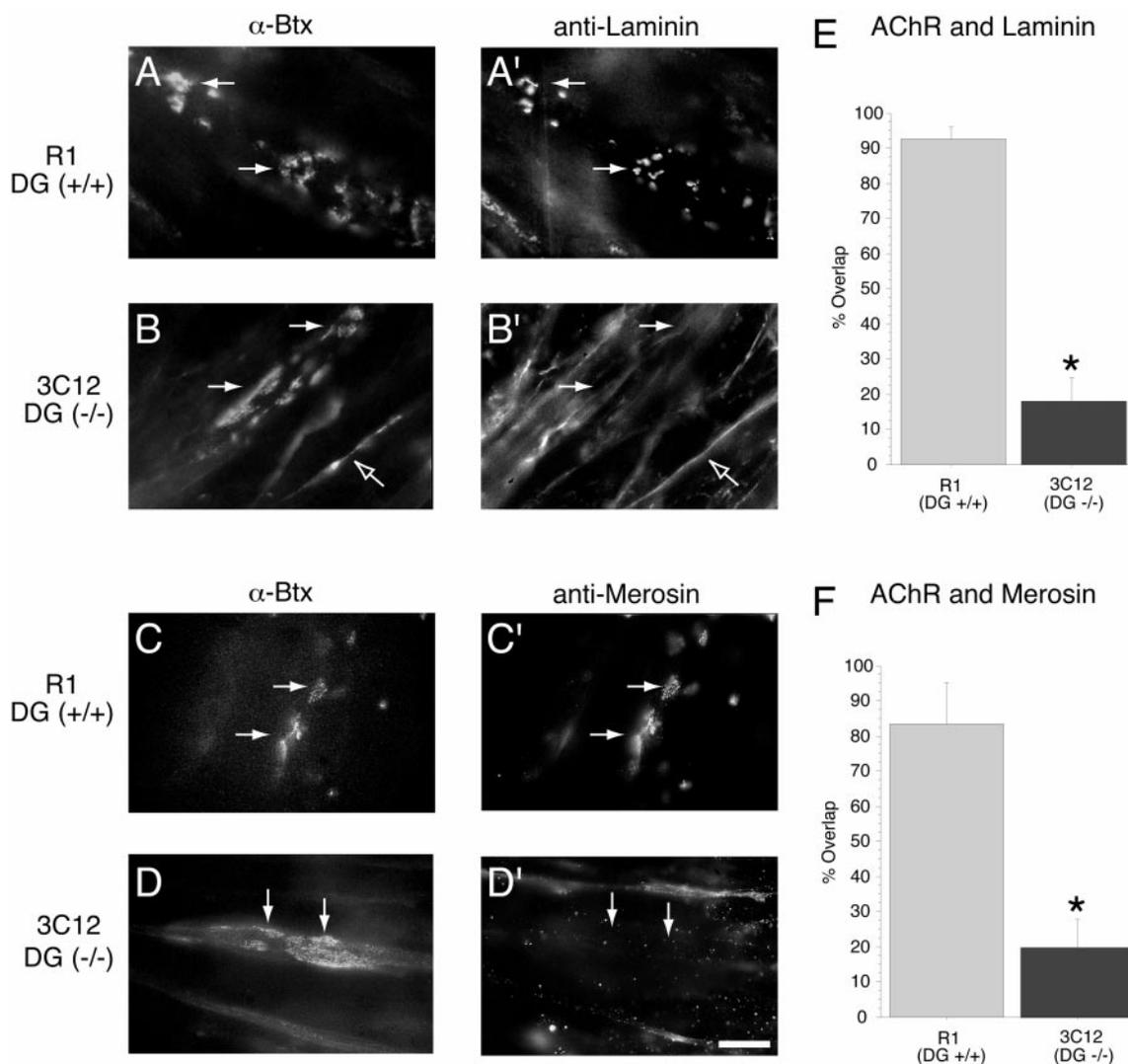
### *Dystroglycans Increase the Stability of AChR Clusters*

In view of its position as a transmembrane complex and its role in condensing AChRs, it is reasonable to propose that  $\alpha$ - and  $\beta$ -dystroglycan contribute to the stabilization of AChR clusters once formed. Cluster stability was determined by quantifying the number of AChR clusters on wild-type and dystroglycan-null myotubes after treatment overnight with 500 pM agrin, then replacing the agrin-containing medium with fresh medium and assaying the number of AChR clusters after 0, 4, or 8 h. Within 4 h there was a significant difference in the number of AChR clusters on R1- and 3C12-derived myotubes (Fig. 4). Clusters on R1 cells had decreased by 11%, but on 3C12 cells by 62%. This downward trend continued, with R1 myotubes declining a further 16% and 3C12 myotubes 26% by 8 h (Fig. 4). Indeed, by 8 h, the vast majority of agrin-induced

AChR clusters remained on R1 cells, whereas they had essentially dispersed on 3C12 cells. These data suggest that condensation of AChR clusters mediated by dystroglycan enhanced their stability.

### *Agrin and Rapsyn Colocalize with AChR Clusters Independently of Dystroglycan*

Agrin binds directly to  $\alpha$ -dystroglycan (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994) and is found in microcluster of AChRs within 1 h of synapse formation (Cohen et al., 1995). In addition,  $\beta$ -dystroglycan has been reported to aggregate with rapsyn and AChRs when coexpressed in heterologous cells and to bind to rapsyn in biochemical assays (Apel et al., 1995; Cartaud et al., 1998). We examined whether the microclusters on dystroglycan-null cells were similar in composition



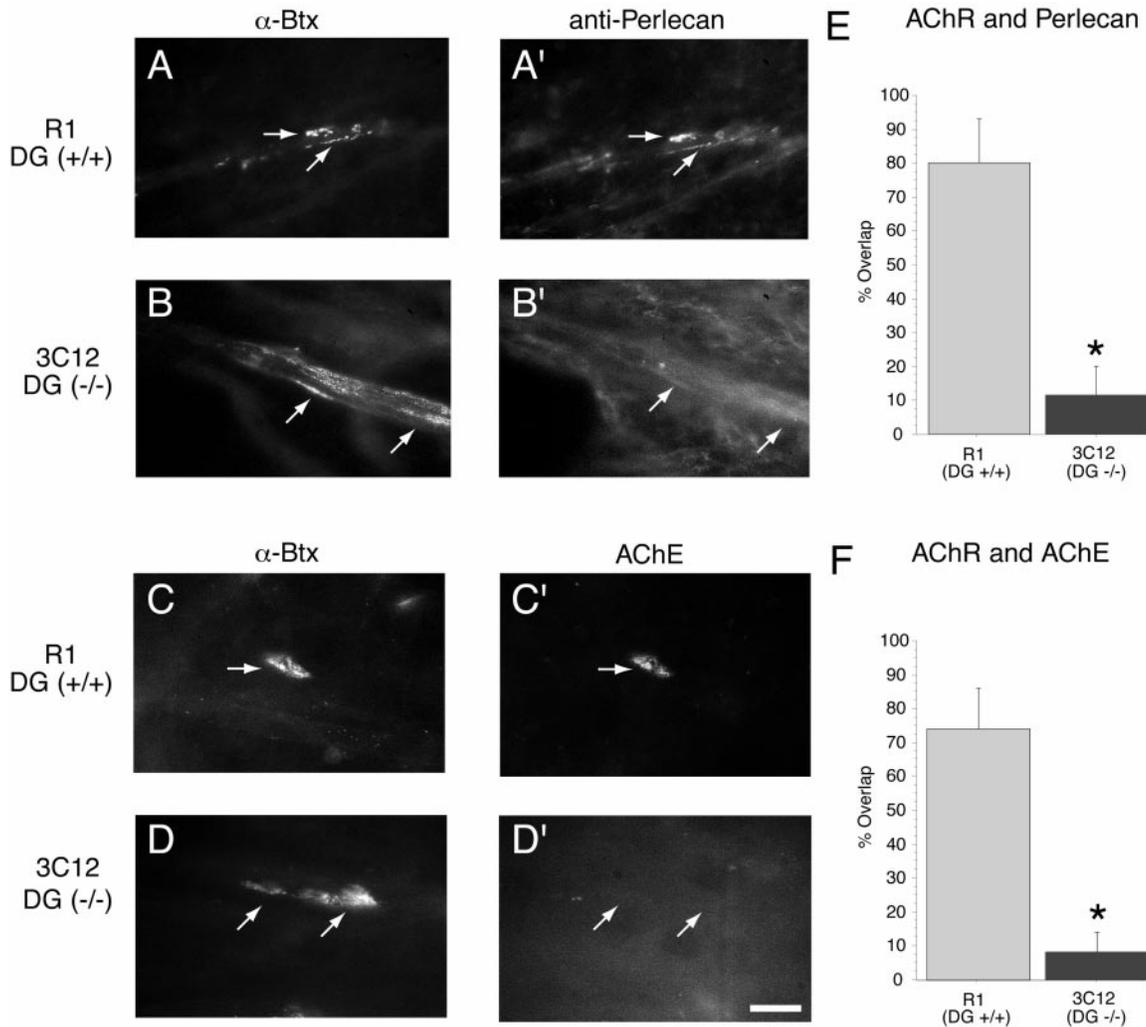
**Figure 6.** Laminin colocalization with AChR aggregates is disrupted on dystroglycan-null myotubes. Wild-type (R1) and dystroglycan-null myotubes (3C12) were treated overnight with 500 pM agrin and subsequently fixed and stained for AChRs in conjunction with either anti-EHS laminin (A, A', B, and B') or antilaminin  $\alpha 2$  chain (merosin) antibodies (C, C', D, and D'). The filled arrows in all panels indicate the location of AChR clusters (A–D), laminin (A' and B'), or merosin (C' and D'). AChR clusters on the tops of wild-type myotubes contained both laminin and merosin whereas those on dystroglycan-null cells have little or no laminin and merosin. We did note some ribbons of AChRs along the sides of myotubes which contain laminin (open arrows). The amount of overlap with AChR clusters for each laminin isoform is shown in E and F. Histograms represent the quantification of three separate experiments with 10–20 microscope fields containing at least one AChR cluster per field. Significance was determined using Student's *t* test (\**P* < 0.0001). Bar, 10  $\mu$ m.

to those at nascent synapses and whether they contained agrin. Fixed ES cell myotubes were stained with an anti-agrin polyclonal antiserum in conjunction with live staining with TRITC- $\alpha$ -Btx to determine whether agrin codistributed with AChR clusters. We found that agrin was localized to AChR clusters on wild-type (R1) myotubes (Fig. 5, A and A') as well as dystroglycan-null (3C12) myotubes (Fig. 5, B and B'). R1 and 3C12 cells had  $88.9 \pm 4.0\%$  and  $81.7 \pm 8.0\%$  overlap of agrin labeling with AChR clusters, respectively (Fig. 5 E). Antirapsyn antibodies labeled  $91.7 \pm 8.3\%$  of R1 AChR clusters (Fig. 5, C, C', D, and D') and  $75 \pm 13.1\%$  of 3C12 clusters (Fig. 5 F). However, despite similar levels of colocalization there were differences in the intensity and distribution of rapsyn staining in 3C12 myotubes. In wild-type myotubes rapsyn was evenly

distributed throughout each AChR cluster, but in dystroglycan-null myotubes rapsyn was restricted to the largest and brightest regions of all clusters. The presence of agrin and rapsyn in microclusters of AChRs in dystroglycan-null cells is consistent with the notion that these are microclusters similar to those at developing NMJs and suggest that they are localized there independent of dystroglycan.

#### *The Synaptic Extracellular Matrix Is Disorganized in Dystroglycan-null Myotubes*

In view of reports (Montanaro et al., 1998; Peng et al., 1998, 1999) that  $\alpha$ -dystroglycan is a receptor for laminin, perlecan, and indirectly AChE, all of which are important components of the synaptic basement membrane, we examined the distribution of these molecules in differenti-

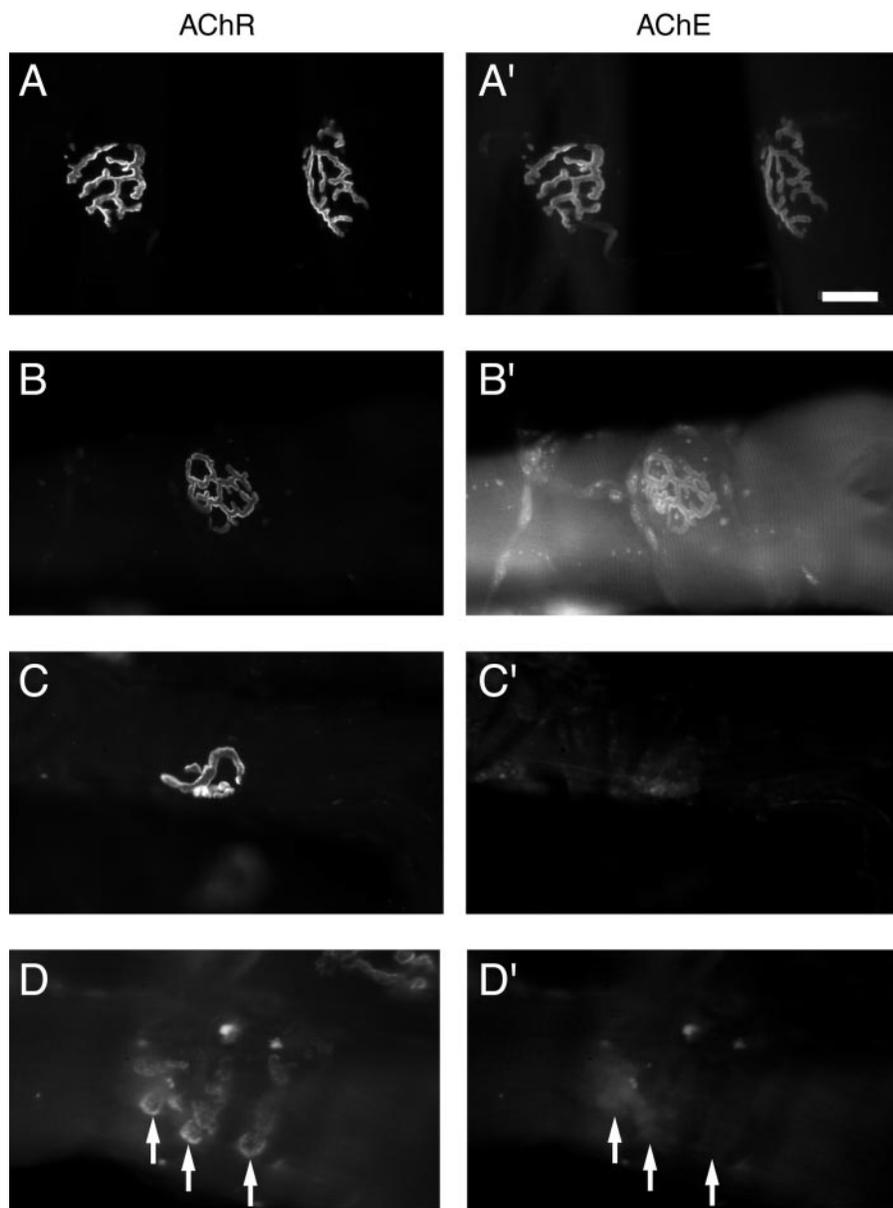


**Figure 7.** Perlecan and AChE do not colocalize with AChR clusters in dystroglycan-null myotubes. Agrin-induced (500 pM overnight) wild-type (R1) and dystroglycan-null (3C12) myotubes were stained with TRITC- $\alpha$ -Btx in conjunction with antiperlecan antibodies or fas-2, a fluorescently labeled AChE-specific toxin. The arrows indicate AChR clusters in A–D, perlecan staining in A' and B', and AChE staining in C' and D'. Perlecan and AChE do not colocalize with AChR clusters in dystroglycan-null (3C12) myotubes. The level of colocalization is reflected in the histograms at the right (E and F). The data represented in the histograms were collected as above with the significance determined using Student *t* test (\**P* < 0.0001). Bar, 10  $\mu$ m.

ated ES cell cultures null for dystroglycan. We initially stained myotubes with two laminin-specific antibodies. With an antiserum to EHS laminin, staining was found to be highly concentrated at AChR clusters in wild-type myotubes (Fig. 6, A and A'), but not in dystroglycan-null myotubes (Fig. 6, B and B'). In many cases the size and shape of these laminin clusters mirrored that of the underlying AChRs. Laminin colocalized with AChRs on wild-type ( $92.1 \pm 2.6\%$ ) and dystroglycan-null ( $16.3 \pm 6.0\%$ ) myotubes (Fig. 6 E). Similar results were obtained with antiserum specific for the laminin  $\alpha$ 2 chain (Fig. 6, C, C', D, and D'): the  $\alpha$  chain is part of laminins 2 and 4, which are the predominant isoforms on the muscle surface (Ehrig et al., 1990). When quantified, laminin  $\alpha$ 2 staining overlapped with  $90 \pm 4.3\%$  of AChR clusters on R1 myotubes and  $20 \pm 10.7\%$  on the surface of 3C12 myotubes (Fig. 6 F).

Perlecan is another extracellular matrix molecule concentrated at the NMJ early during differentiation (Bayne

et al., 1984; Anderson, 1986). In ES cell myotubes perlecan colocalization with AChRs was dependent on dystroglycan expression. Perlecan was found to be highly colocalized with AChRs in R1 myotubes (Fig. 7, A and A') and the absence of dystroglycan resulted in the loss of perlecan at AChR clusters (Fig. 7, B and B'). Quantitatively, perlecan colocalized with AChRs in  $72.9 \pm 12\%$  of clusters in wild-type cells and only  $10.7 \pm 7.7\%$  in null myotubes (Fig. 7 E). The loss of perlecan might be expected to affect the distribution of AChE, which can bind to perlecan and is localized to NMJs and AChR clusters (Peng et al., 1999). Wild-type and dystroglycan-null myotubes stained with TRITC- $\alpha$ -Btx and Oregon green fas-2 to label AChE revealed AChE at  $73.3 \pm 11.7\%$  of R1 AChR clusters (Fig. 7, C, C', and F), but only  $8.3 \pm 5.6\%$  of 3C12 AChR clusters (Fig. 7, D, D', and F). We also examined the distribution of AChRs and AChE in teased muscle fibers of dystroglycan-deficient chimeric mice. As shown in Fig. 1, and for reasons explained earlier, we observed a gradation in



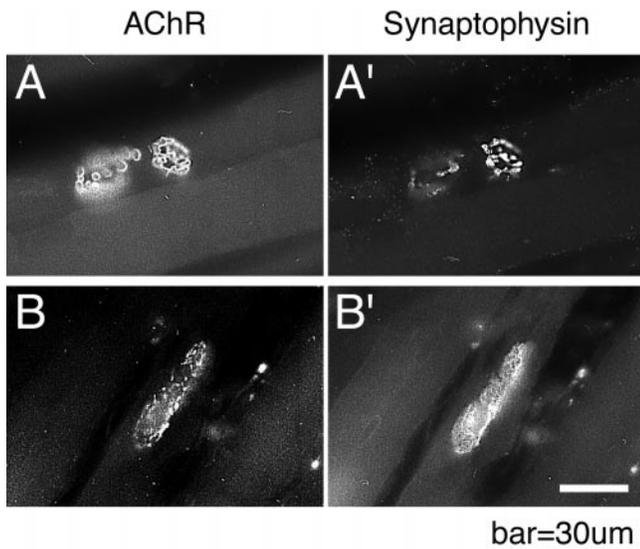
**Figure 8.** AChE distribution is altered at the endplates of dystroglycan-deficient chimeric mouse muscle. Teased muscle fibers from wild-type (A) and chimeric mice (B–D) were isolated and stained with TRITC- $\alpha$ -Btx and fas-2. AChR labeling at endplates varies from nearly normal (B) to severely depleted (C and D). AChE staining is similarly graded and is virtually lost from the most severely disorganized endplates (C' and D'). The arrows in D and D' point to punctate aggregates of AChR and the sites to which AChE should be localized within the same field respectively. Bar, 10  $\mu$ m.

the disruption of NMJs after labeling with TRITC- $\alpha$ -Btx, reflecting the level of dystroglycan expression at the junction. These defects ranged from very mild (Fig. 8 B), where synaptic gutters or folds in the postsynaptic membrane were abundant and similar in intensity, to wild-type staining at the edges (Fig. 8 A), to very severe (Fig. 8 D) with no distinguishable gutters and punctate AChR labeling. The intensity of fas-2 staining was correlated with the level of disruption of the AChR distribution. When AChR staining was strong, fas-2 staining was intense and its distribution matched that of  $\alpha$ -Btx along the synaptic gutters (Fig. 8 B'), as in wild-type muscle (Fig. 8 A'). However, when the AChR distribution was abnormal, fas-2 staining was either weak (Fig. 8 C') or completely absent (Fig. 8 D'), indicating that AChE localization was dependent on dystroglycan expression at NMJs as at AChR clusters in culture.

In dystroglycan-deficient muscle the size of the nerve terminal, as shown by immunohistochemistry for the syn-

aptic vesicle protein synaptophysin, was also affected (Fig. 9). When compared with wild-type AChR clusters and nerve terminals (Fig. 9, A and A', respectively), endplates from chimeric animals ( $n = 3$ ) could be found that were larger with obviously disorganized AChR clusters (Fig. 9 B) and a similarly large area of synaptophysin staining (Fig. 9 B'). These results suggest that dystroglycan not only was necessary for the localization of several basement membrane components to the synapse, but also might directly or indirectly regulate alignment between the pre- and postsynaptic specializations.

In contrast to the distribution of AChR clusters in culture, the distribution of laminin, perlecan, agrin, collagen IV, and fibronectin over the remaining surface of myotubes was grossly the same on all lines of cultured ES cells (Fig. 10). This is consistent with their distribution in the extrajunctional muscle of dystroglycan-deficient mice (Côté et al., 1999). However, on close examination there was evidence of a difference in the pattern of laminin



**Figure 9.** Nerve terminal size correlates with AChR distribution in dystroglycan-deficient chimeric mice. Longitudinal sections from wild-type (A and A') and chimeric mice (B and B') were stained with TRITC- $\alpha$ -Btx and antisynaptophysin antisera to label nerve terminals. Synaptophysin staining and AChR aggregates were found to be in precise alignment at synapses in both wild-type and dystroglycan-deficient muscle, whereas nerve terminals on dystroglycan-deficient myofibers appeared enlarged (compare A and A' with B and B'). Bar, 10  $\mu$ m.

staining on the surface of cells. Wild-type myotubes appeared to have large plaques of laminin on the surfaces (Fig. 10, arrow), whereas these plaques were absent on dystroglycan-null cells and the laminin is more fibrillar in appearance (Fig. 10, A and A'). Perlecan staining was the same on myotubes derived from wild-type or null ES cells, and was diffuse over the cell surface (Fig. 10, B and B') with very little perlecan bound to myotubes outside of AChR clusters. Collagen IV and fibronectin staining was also unaffected by the lack of dystroglycan and was uniformly filamentous over wild-type and null myotubes (Fig. 10, C, C', D, and D'). Finally, agrin staining was diffusely distributed over most of the myotubes, with some concentrations found at the edges of myotubes in both cell types (Fig. 10, E and E'). Together, these results suggest that the expression of extracellular matrix proteins is essentially normal over most of the myotube surface in the absence of dystroglycan (Côté et al., 1999) despite clear defects within AChR clusters.

#### **AChR Clusters Induced by Laminin Are Defective on Dystroglycan-null Myotubes**

The absence of laminin, but not agrin (compare Fig. 5, top, to Fig. 6), from AChR clusters suggested that laminin, rather than agrin, bound to dystroglycan participates in the condensation of microclusters of AChRs into larger clusters. To test this possibility, we examined the effect of exogenously added laminin on AChR clustering on R1 and 3C12 myotubes. Laminin induced the formation of AChR clusters on the surface of myotubes derived from wild-type ES cells, but not on dystroglycan-null myotubes (Fig. 11). Laminin-treated R1 cells had 7.2 vs. 3.2 clusters

per field in untreated myotubes, whereas dystroglycan-null 3C12 myotubes treated with laminin had 1.5 vs. 2.3 clusters per field in untreated cultures ( $P < 0.001$ ,  $n = 3$ ). Laminin-induced AChR clusters on R1 myotubes (Fig. 3, bottom left) were  $\sim 1.7$  times larger ( $17.6 \mu\text{m}^2$ ) than those induced by agrin ( $10.3 \mu\text{m}^2$ ;  $P < 0.0001$ ,  $n = 50$ ). In comparison, AChR clusters on laminin-treated 3C12 cells ( $11.4 \mu\text{m}^2$ ) were similar in appearance (compare Fig. 3 A, top right with bottom right) and size to spontaneous clusters ( $13.7 \mu\text{m}^2$ ) on untreated cells. The inability of laminin to induce AChR clusters on dystroglycan-null myotubes, in combination with the change in appearance of spontaneous and agrin-induced clusters, suggested that laminin-dystroglycan interactions were important in the formation of dense AChR clusters and that disruption of these interactions is responsible for the diffuse, unstable clusters in culture and in vivo.

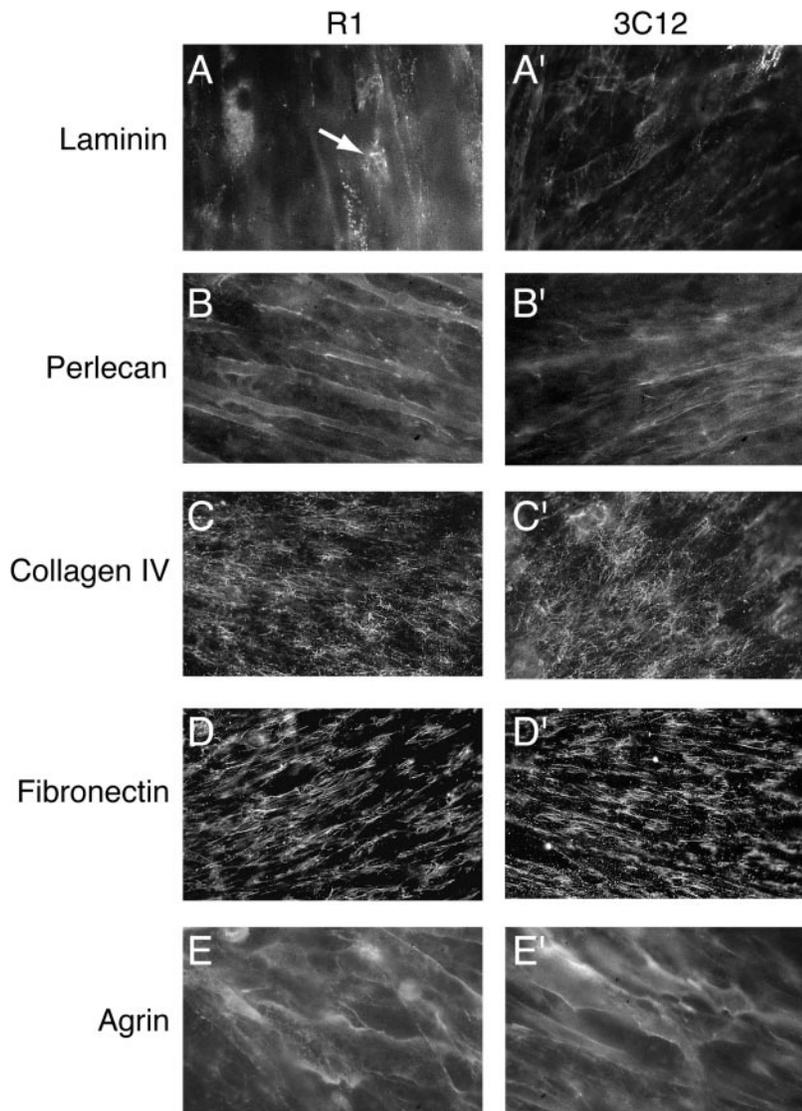
#### **Discussion**

ES cells with targeted disruptions in both dystroglycan alleles were used to generate chimeric mice deficient in dystroglycan and to form myotubes devoid of dystroglycan. Using these two systems, we have found that dystroglycans are necessary for the condensation of AChR microclusters and stabilization of AChRs within the plasma membrane, as well as for the assembly of normal synaptic components, including AChE. These results highlight the importance of DAPs in multiple aspects of synapse formation.

#### **DAPs Are Necessary for the Formation of AChR Clusters in Culture and NMJs**

In previous studies, we and others have found that DAPs are involved in agrin-induced AChR clustering (Campa-nelli et al., 1994; Gee et al., 1994; Jacobson et al., 1998; Montanaro et al., 1998; Grady et al., 2000) and we recently reported initial observations that dystrophic mice deficient in dystroglycan had disorganized NMJs (Côté et al., 1999). Here we have focused on the NMJs and analyzed muscle from young chimeric animals (3–12-wk-old) whose muscles showed minimal signs of muscular dystrophy. The animals did not show any obvious muscle weakness as they walked, or the waddling gait described previously (Côté et al., 1999) and histological examination revealed few degenerating fibers, evident by their small diameter with disrupted myofibrillar apparatus, and few regenerating fibers with central nuclei. In these animals there was a graded disruption of synapses, reflecting the level of dystroglycan expressed (Fig. 1). Similarities in the synaptic defects at NMJs in chimeric mice and in myotubes in culture, which lack any dystroglycan, argue that deficiencies in dystroglycan in the muscle fiber per se and not in the innervating neuron or surrounding Schwann cells (Yamada et al., 1996a,b) are responsible for defective NMJs.

AChR clusters on dystroglycan-null myotubes were three times larger (Fig. 3 A), but had approximately half the AChR density compared with clusters on wild-type myotubes (Fig. 3 B). In previous studies with C2C12 muscle cell lines that had reduced levels of dystroglycan as a result of the introduction of an antisense cDNA, we reported that there was a corresponding decrease in the number of AChR clusters after agrin treatment (Jacobson



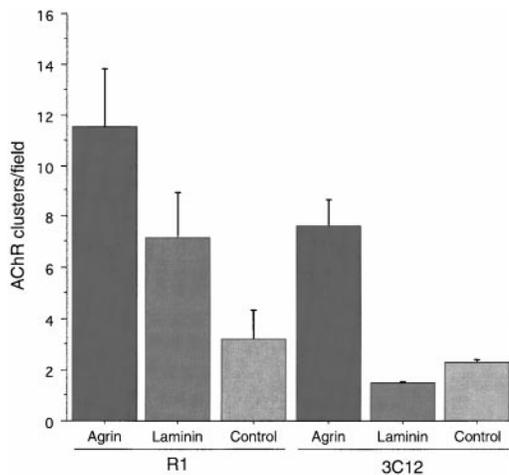
*Figure 10.* The distribution of various basement membrane molecules on myotubes derived from wild-type and dystroglycan-null ES cells. Wild-type (R1) and dystroglycan-null myotubes (3C12) were fixed gently and stained with antibodies for the basement membrane molecules laminin, perlecan, collagen IV, fibronectin, and agrin. Occasionally this fixation permeabilizes myotubes, which results in some intracellular labeling that highlights nuclei (A, left corner), though the vast majority of the cells in this and other panels show surface labeling and have no visible intracellular labeling. Photomicrographs were taken of fields of myotubes lacking AChR clusters. Over most of the sarcolemma there appeared to be few morphological differences in basement membranes of wild-type and dystroglycan-null myotubes, with the exception of laminin, which was found consistently to form large plaques on wild-type myotubes (arrow in A), but not on dystroglycan-null myotubes (A').

et al., 1998). It is important to note that in this earlier study clusters  $<2.5 \mu\text{m}$  in diameter were not counted, which would have excluded the microclusters that predominate on dystroglycan-null myotubes (Jacobson et al., 1998) and this likely accounts for the apparent reduction in clusters on myotubes immediately after agrin treatment not seen in the present study. The AChR microclusters on dystroglycan-null myotubes are similar in appearance to those observed by Campanelli et al. (1994) using a monoclonal antibody to inhibit  $\alpha$ -dystroglycan function (Gee et al., 1994), as well as to the microclusters seen at early stages of synaptogenesis (Anderson and Cohen, 1977; Steinbach, 1981) or induced by rapsyn in heterologous cells (Froehner et al., 1990; Phillips et al., 1991b). These rapsyn-containing microclusters appear to form a subunit of the mature postsynaptic density of AChRs which are knit together by DAPs.

The 25–33% decrease in the number of AChR clusters on dystroglycan-null versus wild-type myotubes probably results from their diffuse nature and increased size rather than a difference in AChR levels and response to agrin. With no clear boundaries, counts of clusters on dystroglycan-null myotubes may have been skewed toward lower

absolute numbers. This is supported by previous studies in dystroglycan-deficient C2C12 cells, where we found similar levels of agrin induction (Fig. 2 B) and little difference in MuSK and AChR  $\beta$ -subunit phosphorylation (Jacobson et al., 1998), implying that dystroglycan depletion has little or no effect on agrin signaling. The diffuse appearance of AChR clusters on dystroglycan-null myotubes could then reflect either a failure of large dense clusters to form or their rapid dispersal once formed. In dystroglycan-null cells, we found no evidence for condensed clusters. Moreover, the AChR clusters on dystroglycan-null myotubes were also less stable than wild-type AChR clusters and, on removal of agrin, dispersed at approximately twice the rate as those in wild-type cultures. In principle, this instability may result from increased turnover of receptors in microclusters or diffusion of receptors away from them. Xu and Salpeter (1997) reported that the degradation of AChRs in denervated muscle devoid of dystrophin is increased and this may obtain in dystroglycan-null myotubes.

In related work, Grady et al. (2000) reported that the DAP dystrobrevin is also necessary for NMJ formation.



**Figure 11.** Laminin induces AChR clusters on myotubes derived from wild-type (R1) but not dystroglycan-null (3C12) ES cells. Cultures were treated overnight with either 500 pM of agrin or 100 nM EHS laminin, or left untreated (control). The cultures were then stained with  $\alpha$ -Btx and the number of AChR aggregates quantified. Agrin and laminin induced a significant increase in the number of AChR clusters on R1 cells but only agrin was effective in inducing clusters on 3C12 myotubes (ANOVA,  $P < 0.001$ ,  $n = 3$ ). The values represent the mean  $\pm$  SD of three individual experiments in which at least 10 microscopic fields per treatment were quantified.

The distribution of AChRs in dystrobrevin null mice appears similar to that of AChRs in dystroglycan-null myotubes, as shown here and as reported previously by Grady et al. (2000), as well as to the distribution in vivo (Côté et al., 1999). Our data on dystroglycan-null myotubes in culture extend those of Grady et al. (2000) by quantifying the changes in AChR clusters, synaptic basement membrane proteins, while also measuring the effects of agrin treatment and the stability of induced AChR clusters. Interestingly, AChRs on dystrobrevin null myotubes are unstable, similar to those in dystroglycan-null myotubes (Fig. 4) and dystroglycan is present in AChRs lacking dystrobrevin (Grady et al., 2000). This suggests that dystroglycan is upstream of dystrobrevin in the pathway responsible for AChR aggregation/stabilization.

Rapsyn is a 43-kD intracellular protein that is associated with AChRs (Sobel et al., 1977; Froehner et al., 1981; Burden et al., 1983). This association, in conjunction with rapsyn aggregation, contributes to the formation of microclusters of AChRs when both are expressed in heterologous cells (Froehner et al., 1990; Phillips et al., 1991b). Moreover, mice with a targeted mutation in the rapsyn gene have NMJs with diffusely organized AChRs (Gautam et al., 1995). Rapsyn can bind directly to  $\beta$ -dystroglycan (Cartaud et al., 1998) and when coexpressed with dystroglycan in heterologous cells has been reported to recruit dystroglycan into AChR-rich microclusters (Apel and Merlie, 1995). Thus,  $\beta$ -dystroglycan has been hypothesized to mediate interactions with rapsyn important to AChR clustering. In our studies rapsyn was localized to AChR clusters in both the R1 and 3C12 cell lines, although in null myotubes rapsyn staining was reduced or absent outside of intense areas of AChR localization (Fig.

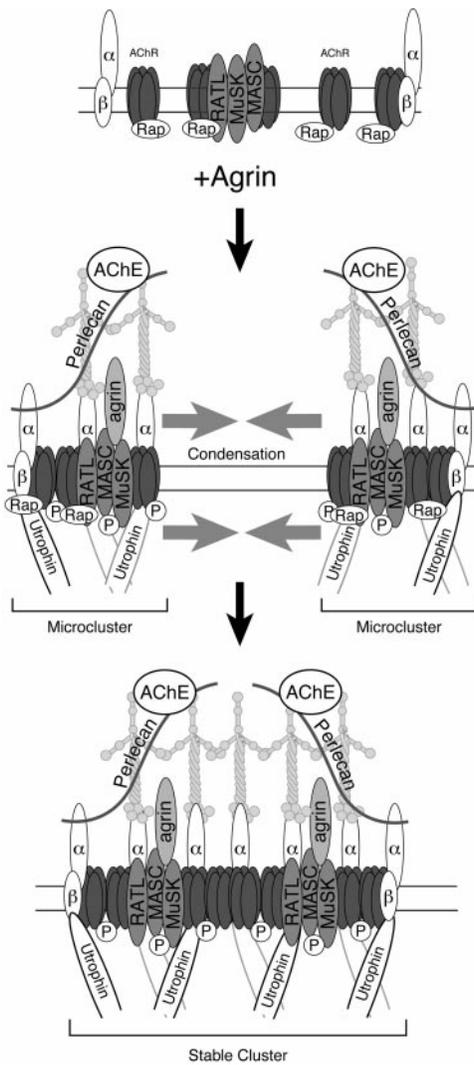
5, C' and D'). These results suggest that some fraction of rapsyn may interact with  $\beta$ -dystroglycan, but that this interaction is not necessary for localization of rapsyn within AChR clusters.

### **Dystroglycan Organizes Rapsyn, AChE, Perlecan, and Laminin at the NMJ**

Asymmetric isoforms of AChE are tightly bound within the basement membrane through interactions of their collagen-like tail with heparan sulfate proteoglycans (Rossi and Rotundo, 1996), in particular perlecan (Peng et al., 1999). Perlecan is concentrated at NMJs in vitro where it is thought to bind to dystroglycan via its laminin-like G domains (Peng et al., 1998). We have found that both perlecan and AChE are depleted at AChR clusters in vitro (Fig. 7) and that AChE was depleted in vivo (Fig. 8), which strongly suggests that this interaction occurs in situ. However, perlecan is expressed at high levels throughout the entire basement membrane and in prior studies there was no obvious reduction in its levels in dystroglycan-deficient muscle in vivo (Côté et al., 1999). This may occur because perlecan is concentrated early during synapse formation (Bayne et al., 1984; Anderson, 1986) and this concentration is later occluded by deposition of perlecan in the extrajunctional basement membrane. For example, the concentration of  $\alpha$ -dystroglycan at mouse NMJs is sometimes difficult to resolve, but is obvious in the muscle of mdx mice where the extrajunctional dystroglycan is very low (Leschziner et al., 2000).

Dystroglycan was originally isolated as a component of the DAP complex (Yoshida and Ozawa, 1990; Ervasti et al., 1991) and individually as a high affinity laminin binding protein (Smalheiser and Schwartz, 1987; Douville et al., 1988; Ibraghimov-Beskrovnyaya et al., 1992; Gee et al., 1993). Subsequently, several lines of evidence suggested that  $\alpha$ -dystroglycan mediates AChR clustering, including binding of agrin to  $\alpha$ -dystroglycan (Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), colocalization of  $\alpha$ -dystroglycan, agrin, and AChRs at developing and mature synapses (Cohen et al., 1995), and reduced AChR clustering in dystroglycan antisense lines (Jacobson et al., 1998). Hence, it is somewhat surprising that even in the absence of dystroglycan, agrin was still associated with upwards of 82% of AChR clusters (Fig. 5, top). Either dystroglycan does not function as an agrin receptor in AChR clustering, or several other molecules compensate for it. Both the MuSK/myotube-associated specificity component (MASC) receptor complex (Glass et al., 1996) and the  $\alpha_v\beta_1$  integrin (Martin and Sanes, 1997) bind to agrin and may localize it to the synapse in the absence of dystroglycan.

In contrast to the distribution of agrin in culture, we found that laminin, specifically laminins 2 and 4, which contain the  $\alpha 2$  chain, were no longer concentrated at AChR clusters on the surface of dystroglycan-null myotubes (Fig. 6). We were also unable to induce the aggregation AChRs by application of exogenous laminin on these same myotubes (Fig. 11). Dystroglycan thus appears to be the primary receptor for laminin at the synapse. This result confirms results generated by Montanaro et al. (1998) in C2C12 cells expressing reduced levels of dystroglycan, but without the complication presented by residual dystrogly-



**Figure 12.** A model illustrating the role of dystroglycan in the condensation of AChR microclusters and the localization of specific synaptic basement membrane molecules. On uninnervated myotubes components of the postsynaptic apparatus are diffusely distributed throughout the sarcolemma (top). The subsequent binding of agrin to the MuSK receptor complex initiates a cascade of events (middle) that induces the formation AChR microclusters and the clustering of cytoskeletal and basement membrane molecules such as laminin, perlecan, and AChE. These microclusters continue to condense into larger clusters through the recruitment of dystroglycan and associated basement membrane and cytoskeleton components resulting in a more stable cluster of AChRs (bottom).

can expression in C2C12 cells. In vivo, the absence of laminins has been correlated with minor defects in nerve terminal structure and function (Carbonetto, 1977; Noakes et al., 1995). In fact, the nerve terminals on dystroglycan-null muscle in vivo can be larger, corresponding to the larger size of the endplate (Fig. 9). For future work, we are producing reagents that will allow colocalization of synaptophysin, dystroglycan, and AChRs in the same histological sections to correlate differences in synaptic morphology with the expression of dystroglycan in the muscle. It is well-known that developmentally the size of nerve terminals is matched to that of the muscle fiber, presumably to

ensure sufficient release of neurotransmitter to activate the muscle (Kuno et al., 1971). Dystroglycan may contribute to this regulation either directly or indirectly through laminin.

We also analyzed the staining of extracellular matrix molecules over extrajunctional regions of myotubes to determine whether dystroglycan gene ablation had a significant effect on the basement membrane. Previously, several groups have shown that the depletion of the dystrophin–glycoprotein complex appears to have little effect on the gross formation of the basement membrane in vivo (Dickson et al., 1992; Matsumura et al., 1993; Côté et al., 1999). Outside of AChR clusters the expression of laminin, perlecan, collagen IV, fibronectin, and agrin on myotubes was relatively normal, with the exception (Fig. 10) that laminin was no longer found as plaques on the surface of null myotubes, but rather in fibrils (compare Fig. 10 A with 10 A'). Cohen et al. (1997) and Montanaro et al. (1999) found that dystroglycan was associated with similar plaques in *Xenopus* and C2C12 cultures. These plaques have been proposed to reflect sites of basement membrane assembly wherein laminin is concentrated at the membrane by binding to  $\alpha$ -dystroglycan and enhances the polymerization of extracellular matrix proteins (Cohen et al., 1997; Montanaro et al., 1998; Colognato et al., 1999).

### A Role for Dystroglycan in Synapse Formation

Several groups have shown that it is possible to bypass agrin signaling and to stimulate the formation of large AChR clusters by the addition of laminin (Vogel et al., 1983; Sugiyama et al., 1997; Montanaro et al., 1998) and that this clustering is mediated by dystroglycan (Montanaro et al., 1998; Fig. 10). Since laminin is deficient from AChR clusters on dystroglycan-null myotubes, these data suggest that  $\alpha$ -dystroglycan functions as a laminin receptor in AChR clustering. These results also raise questions concerning the mechanism of condensation and stabilization of AChRs. Laminin can self-assemble in solution via its short arms (Yurchenco et al., 1985) and this also occurs on the muscle cell surface (Cohen et al., 1997). Interestingly, laminin treatment of myotubes, together with an antilaminin antiserum, results in much smaller clusters of AChRs, similar to microclusters (Montanaro et al., 1998), possibly because of inhibition of homophilic binding of the laminin short arms. Thus, an essential aspect of the synapse formation appears to involve binding of laminin to dystroglycan and its assembly with other proteins within the basement membrane. Our studies suggest that this is the case for AChE, which binds dystroglycan indirectly through perlecan (Figs. 7 and 8). One could imagine that assembly of a basement membrane through dystroglycans contributes to a diffusion trap for synaptic proteins by mediating coordinate assembly of a cytoskeletal matrix. As such, dystroglycans are uniquely suited to bring DAPs into the trap. We propose a model (Fig. 12) wherein dystroglycans are preferentially clustered to synaptic sites via activation of MuSK and the resulting density of dystroglycan facilitates interactions with laminin and perlecan, as well as between perlecan and AChE, to assemble a synaptic basement membrane at the same time that  $\beta$ -dystroglycan contributes to the formation of a cytoskeletal network responsible for condensation and stabilization of AChRs.

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