

Dystroglycan Overexpression *In Vivo* Alters Acetylcholine Receptor Aggregation at the Neuromuscular Junction

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Dystroglycan is a member of the transmembrane dystrophin glycoprotein complex in muscle that binds to the synapse-organizing molecule agrin. Dystroglycan binding and AChR aggregation are mediated by two separate domains of agrin. To test whether dystroglycan plays a role in receptor aggregation at the neuromuscular junction, we overexpressed it by injecting rabbit dystroglycan RNA into one- or two-celled *Xenopus* embryos. We measured AChR aggregation in myotomes by labeling them with rhodamine- α -bungarotoxin followed by confocal microscopy and image analysis. Dystroglycan overexpression decreased AChR aggregation at the neuromuscular junction. This result is consistent with dystroglycan competition for agrin without signaling AChR aggregation. It also supports the hypothesis that dystroglycan is not the myotube-associated specificity component (MASC), a putative coreceptor needed for agrin to activate muscle-specific kinase (MuSK) and signal AChR aggregation. Dystroglycan was distributed along the surface of muscle membranes, but was concentrated at the ends of myotomes, where AChRs normally aggregate at synapses. Overexpressed dystroglycan altered AChR aggregation in a rostral–caudal gradient, consistent with the sequential development of neuromuscular synapses along the embryo. Increasing concentrations of dystroglycan RNA did not further decrease AChR aggregation, but decreased embryo survival. Development often stopped during gastrulation, suggesting an essential, nonsynaptic role of dystroglycan during this early period of development. © 2000 Academic Press

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INTRODUCTION

At the neuromuscular junction, acetylcholine receptors (AChRs) aggregate opposite the motor nerve terminal at a much higher concentration than in the rest of the muscle cell membrane. The establishment of this asymmetric distribution is directed by the extracellular matrix molecule agrin. Agrin is a proteoglycan produced by motor neurons that interacts with the muscle-specific kinase (MuSK) receptor on the postsynaptic membrane to stimulate aggregation of AChRs. Before MuSK was identified, several laboratories discovered that agrin binds to

α -dystroglycan (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994). α -Dystroglycan is part of the dystrophin glycoprotein complex in muscle membrane that links the extracellular matrix to the cytoskeleton and whose disruption is associated with several forms of muscular dystrophy (Campbell, 1995). Dystroglycan undergoes posttranslational cleavage to form α - and β -dystroglycan. β -Dystroglycan is a transmembrane protein that binds to dystrophin on the cytoplasmic side of the membrane and to α -dystroglycan on the extracellular side. In addition to agrin, α -dystroglycan binds to laminin and possibly perlecan, all of which contain laminin G (LG) domains (Hemler, 1999). α -Dystroglycan is not the agrin receptor inducing AChR aggregation, since elimination of the dystroglycan-binding domain of agrin does not remove

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its AChR-aggregating activity (Gesemann *et al.*, 1996; Hopf and Hoch, 1996). A current hypothesis is that dystroglycan is involved in the growth and/or maintenance of AChR aggregates by a MuSK-independent mechanism (Montanaro *et al.*, 1998; Jacobson *et al.*, 1998; Grady *et al.*, 2000). To test the role of dystroglycan in regulating AChR aggregation, we overexpressed it in the developing *Xenopus* embryo during synaptogenesis at the neuromuscular junction.

Overexpression of mammalian dystroglycan resulted in a reduction of AChR aggregation during neuromuscular synaptogenesis. This result, like those of knockdown or knock-out experiments (Jacobson *et al.*, 1998; Grady *et al.*, 2000), is not consistent with dystroglycan being a coreceptor for agrin–MuSK signaling. The reduction of AChR aggregation could most easily be explained by agrin binding to the additional dystroglycan instead of MuSK. Since dystroglycan is present throughout the entire muscle membrane during normal development, it could serve as an agrin sink, to reduce AChR aggregation caused by agrin originating from other cell types, including muscle (see Godfrey *et al.*, 1999). Thus the combination of MuSK stimulating AChR aggregation at the site of nerve contact and dystroglycan inhibiting AChR aggregation at other sites could focus synaptic development to the region directly under the nerve terminal.

MATERIALS AND METHODS

RNA Synthesis

Dystroglycan RNA for overexpression in *Xenopus* embryos was synthesized from a rabbit cDNA (Ibraghimov-Beskrovnya *et al.*, 1992). The dystroglycan cDNA was inserted into the pCS2+ plasmid (Rupp *et al.*, 1994; Turner and Weintraub, 1994) and used as a template for RNA synthesis. In a similar manner, the cDNA for a red-shifted form of green fluorescent protein (GFP) was prepared for the synthesis of GFP RNA. Plasmids were linearized and RNA was synthesized with an SP6 mMessage mMachine kit (Ambion, Austin, TX). Agarose gel electrophoresis showed the product of each reaction as a single band whose concentration was determined by comparison with mass standards. RNAs were diluted for embryonic injection (0.5, 1.0, and 2.0 $\mu\text{g}/\mu\text{l}$ for dystroglycan RNA; 0.1 $\mu\text{g}/\mu\text{l}$ for GFP RNA; 2.0 and 3.0 $\mu\text{g}/\mu\text{l}$ for agrin RNA; see Godfrey *et al.*, 1999).

Overexpression

RNA encoding dystroglycan and/or GFP was injected into early embryos of the frog *Xenopus laevis*. Synchronized embryos from a single female were obtained by artificial insemination (Moon and Christian, 1989). Blastomeres were injected with RNAs at the one- or two-cell stage with 4.5 nl from a Nanoject injector (Drummond Scientific, Broomall, PA). Following injection, properly developing embryos were selected at several stages. The first selection was for animals that initiated cleavage. The next day, embryos that successfully completed gastrulation were selected and screened for GFP expression. GFP was generally expressed throughout large contiguous regions (Godfrey *et al.*, 1999), but only embryos with GFP expression in myotomes 5, 6, and 7 were accepted for further

analysis. Following a final selection for normal development to the tail bud stage (1.6 days; st 31), embryos were fixed and prepared for microscopy.

Fixation and Staining

To assay the effect of dystroglycan overexpression on receptor aggregation, AChRs were labeled at the tail bud stage of development (1.6 days, st 31). Embryos were fixed in 4% paraformaldehyde for 1 h at 4°C and washed in Ringers. Skin was removed and AChRs were marked with rhodamine- α -bungarotoxin (1:500; Molecular Probes, Eugene, OR) overnight at 4°C. Stained embryos were mounted on slides for confocal microscopy.

Immunocytochemistry was used to determine the distribution of dystroglycan in normal embryos and after overexpression. Following fixation, embryos were cryoprotected in sucrose, embedded in TBS tissue freezing medium (Fisher, Pittsburgh, PA), and frozen. Cryostat sections of embryos were placed on Superfrost Plus slides (Fisher) and fixed again (2% paraformaldehyde for 15 min) to secure sections to slides. Sections were incubated in blocking solution (3% BSA in PBS, 1 h), and then primary antibody (1:100, 1 h), a sheep polyclonal to FP-B: a fusion protein corresponding to the cleavage point between α - and β -dystroglycan in rabbit (Ibraghimov-Beskrovnya *et al.*, 1992) that cross-reacts with β -dystroglycan in *Xenopus* (Cohen *et al.*, 1995). After washing, FITC-labeled goat anti-sheep secondary antibody (1:200, 30 min; Vector Laboratories, Burlingame, CA) was used to localize dystroglycan. After final washing, sections were mounted in 90% glycerol with Vectashield (Vector Laboratories) and coverslipped for confocal microscopy. In some cases rhodamine- α -bungarotoxin was added to label AChRs and dystroglycan in the same section.

Confocal Microscopy and Image Analysis

To examine synaptic AChR aggregation and dystroglycan distribution, the ends of myotomes in whole-mount embryos or sections were examined with a confocal microscope (Bio-Rad MRC 600). We obtained stacks of confocal images from synaptic regions and initially chose six representative regions from myotome 5, 6, or 7 in a group of 10 identically treated sibling animals. In later experiments, we analyzed synaptic regions of all three of these myotomes from each of 6 animals.

To quantify data from image stacks, Metamorph (Universal Imaging, West Chester, PA) and Image J (NIH, Bethesda, MD) software was used. The threshold for AChR aggregate staining was set and the percentage of the image area in the entire stack that exceeded threshold was determined. This provides a measure of the size and number of AChR aggregates within identical areas of the embryonic muscle.

Statistics

To compare and analyze data, the Prism (Graphpad, San Diego, CA) graphing and statistical software package was used. Analyses included *t* tests, linear regression, and ANOVA.

RESULTS

Dystroglycan Localization

The distribution of dystroglycan in developing muscle was determined after the formation of functional synapses.

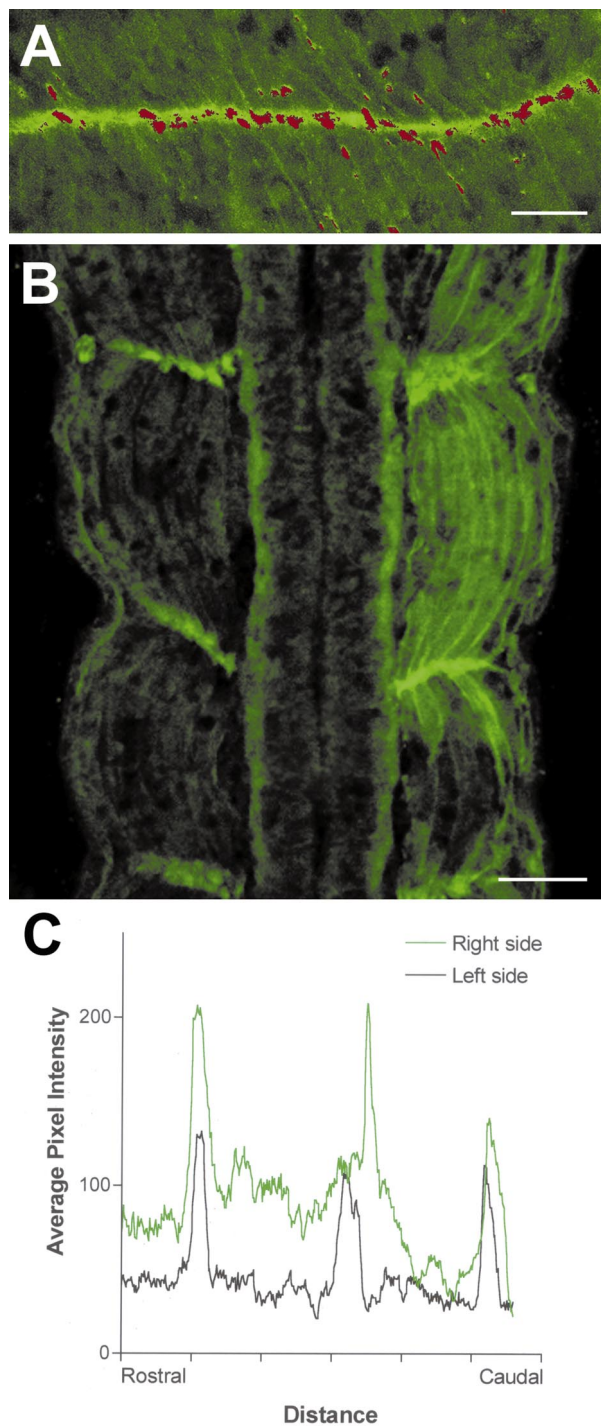


FIG. 1. Dystroglycan distribution. (A) A frozen sagittal section of a normal embryo at 1.6 days (st 31). Dystroglycan is marked with the FP-B antibody (green) and AChRs are marked with rhodamine-labeled bungarotoxin (red). Stacks of confocal images for each color have been projected and red is layered on top of the green. The broad green region is the intermyotomal septum, where synapses normally form. Less intense staining outlines the other surfaces of muscle fibers. AChR aggregates are predominantly located within

The FP-B antibody (Ibraghimov-Beskrovnya *et al.*, 1992; Cohen *et al.*, 1995) was used to stain parasagittal frozen sections of normal *Xenopus* embryos at 1.6 days (st 31; Fig. 1A). A projected stack of confocal images showed a broad region of concentrated dystroglycan immunoreactivity at the intermyotomal septum. Close inspection showed individual muscle cells outlined with fluorescence, indicating that dystroglycan was also expressed in muscle cell membranes away from the intermyotomal septum. This stage is approximately 12 h after the first sign of functional synapses, namely reflexive movements of the embryo (Nieuwkoop and Faber, 1994). The asymmetric distribution of dystroglycan within muscle tissue was readily apparent shortly after the formation of functional synapses.

Colocalization experiments showed the relationship between dystroglycan and AChR aggregates at developing synapses. The projected stack of red images (rhodamine- α -bungarotoxin) was layered on top of the green images and showed the positions of AChR aggregates in the section (Fig. 1A). Most AChR aggregates were also located at the ends of myotomes, the sites of mature synapses; however, the region of high dystroglycan concentration was more extensive than the AChR aggregates. There were also AChR aggregates outside the normal synaptic region, which are shown as red areas surrounded by a thin ring of green dystroglycan staining. The number of these "extrajunctional" AChR aggregates decreased during development, suggesting that the synaptic regions of muscle cell membranes (where dystroglycan is concentrated) can better maintain AChR aggregates. At this stage of development, dystroglycan was colocalized with all AChR aggregates, but aggregates did not form exclusively in regions of concentrated dystroglycan.

To test whether RNA injection resulted in overexpression of dystroglycan during neuromuscular synaptogenesis, we used an antibody to probe for the additional protein.

the broad region of dystroglycan immunoreactivity. (B) A frozen horizontal section of an embryo at 1.8 days (st 33/34) which had one cell injected with dystroglycan RNA at the two-cell stage. A stack of images has been projected. Dystroglycan expression in several segments show much brighter staining on the right side, indicating that dystroglycan protein was successfully overexpressed. Note that on both sides, the outer edge of the spinal cord is stained. (C) Plot profile of equal rectangular areas from each side of the spinal cord of the animal shown in B. The plot is from section 6 of seven in the confocal stack. The peaks correspond to the regions of high intensity at the intermyotomal septum (synaptic regions). The intensities of both the peaks and the background were approximately doubled on the right (injected) side. For all seven sections, the three control peak areas were 54% of the injected side ($P < 0.0001$; paired t test) and the three control baseline areas were 48% of the injected side ($P < 0.0001$; paired t test). Even though the differences were consistent and significant, there was variability in the intensity on the injected side (between the second and the third peaks). Calibration: A, 20 μm ; B, 100 μm .

RNA encoding rabbit dystroglycan (Ibraghimov-Beskrovnaya *et al.*, 1992) was injected into fertilized embryos and dystroglycan protein was localized with the FP-B antibody. Since FP-B recognizes both rabbit and endogenous frog dystroglycan (Cohen *et al.*, 1995), we compared the fluorescence in muscles of injected and uninjected embryos. To minimize interanimal variability in staining, we injected one cell at the two-cell stage with 2.3 ng of dystroglycan RNA. In control experiments with GFP RNA, this produced expression in half the embryo (Godfrey *et al.*, 1999). If injection of dystroglycan RNA results in overexpression, there should be an increase in staining intensity on one side of the animal. An image of a horizontal section through such an animal shows the right side more intensely stained than the left (Fig. 1B), indicating overexpression of rabbit dystroglycan. Muscle fibers on both sides appear normal and are similar in diameter, indicating that dystroglycan has not disrupted muscle development. In this image, the periodic horizontal lines that are more brightly stained than the surrounding tissues correspond to the intermyotomal septa shown at higher magnification in Fig. 1A. The differential staining appeared to be maintained on the injected side. Quantitative analysis of equal areas from each side of the image confirmed that the intermyotomal septa were always more intensely stained on both sides (Fig. 1C). A typical analysis showed that the amount of staining in peak and background areas from each section in this stack was approximately doubled on the injected side (control peaks were 54% of injected peaks, control background was 48% of injected background) and both were significantly different from control ($P < 0.0001$; paired t test). The quantification also showed that overexpression can be variable. The background level between the second and the third peaks on the right side decreased and approached the level on the control side. This suggests that the larger dystroglycan RNA may not diffuse to all of the cells on the injected side as readily as the smaller GFP RNA. Additional staining between the myotome/spinal cord border was greater than background, but not as great as the intermyotomal septum. Thus the boundaries between segments and between some tissue types appear to be sites of dystroglycan accumulation. The embryo in Fig. 1B is from a later stage (1.8 days; st 33/34) than used for testing AChR aggregation (see below). Thus, RNA injection results in the elevation of dystroglycan protein levels, which persist well past the initial stages of neuromuscular synaptogenesis examined in these experiments.

Dystroglycan Reduces AChR Aggregation

Overexpression of dystroglycan did not qualitatively alter AChR aggregation at developing neuromuscular junctions. Following fertilization, animals were injected with a mixture of dystroglycan and GFP RNAs (experimental) or with GFP RNA (control). After translation into protein, the green GFP fluorescence marked animals successfully injected with RNA and synaptic regions appropriate for analysis.

After being labeled with rhodamine- α -bungarotoxin, the intermyotomal synaptic regions were analyzed by confocal microscopy. Projected stacks of images from control (GFP only, Fig. 2A) and experimental (dystroglycan and GFP, Fig. 2B) synaptic regions showed similar localizations of AChR aggregates. The prominent bright line across both images corresponds to the boundary between myotomes where synapses normally form. There were also AChR aggregates outside the normal synaptic regions at this stage in both experimental and control animals. To quantify the differences in AChR aggregation between experimental and control animals, we measured the fluorescent area throughout the entire stack with image analysis software (Metamorph, Universal Imaging). The AChR aggregate area in control (Fig. 2A) and experimental (Fig. 2B) images differed by a factor of 2 (1.24 and 0.68% of the total area in control and experimental images, respectively). The only apparent difference between synaptic regions of experimental and control animals was the quantity of AChR aggregation.

To test whether the reduction in AChR aggregation following overexpression of dystroglycan was significant, we quantitatively analyzed the synaptic regions of myotomes from several groups of animals. Synaptic regions (between myotomes 5/6, 6/7, and 7/8) were imaged, and six representative regions were selected for analysis (Fig. 3A). In the synaptic regions from experiment a, there was some overlap of experimental and control values with the experimental average at 62% of control. In experiment b, there was less overlap with average experimental AChR aggregate volume reduced to less than half (45%) of control (images in Fig. 2 come from experiment a). In 17 of 24 experimental groups, dystroglycan overexpression decreased AChR aggregation below control levels. The largest decrease was to 28.6% of control, with many reductions exceeding 50%. The average change for all 24 experiments (calculated following logarithmic conversion) decreased to 76.1% of control. To test the significance of the decrease, the raw data from all of the synaptic regions receiving each treatment were combined (Fig. 3B). Although this analysis does not take into account the variation between experiments or the possible variation within an experiment due to incomplete diffusion of dystroglycan RNA (see Fig. 1C), it showed that AChR aggregation in experimental synaptic regions decreased significantly ($P = 0.005$; t test) to 77.8% of control. Thus additional dystroglycan decreased AChR aggregation.

Dystroglycan Inhibits Development of AChR Aggregation

Overexpression of dystroglycan could cause AChR aggregation resembling that of synapses at earlier stages of development. Somites form in a rostral-caudal gradient; AChR aggregation on myotomal muscles derived from them should develop in a similar manner. If a gradient in AChR aggregation exists in normal animals, comparing it with animals at the same stage overexpressing dystroglycan

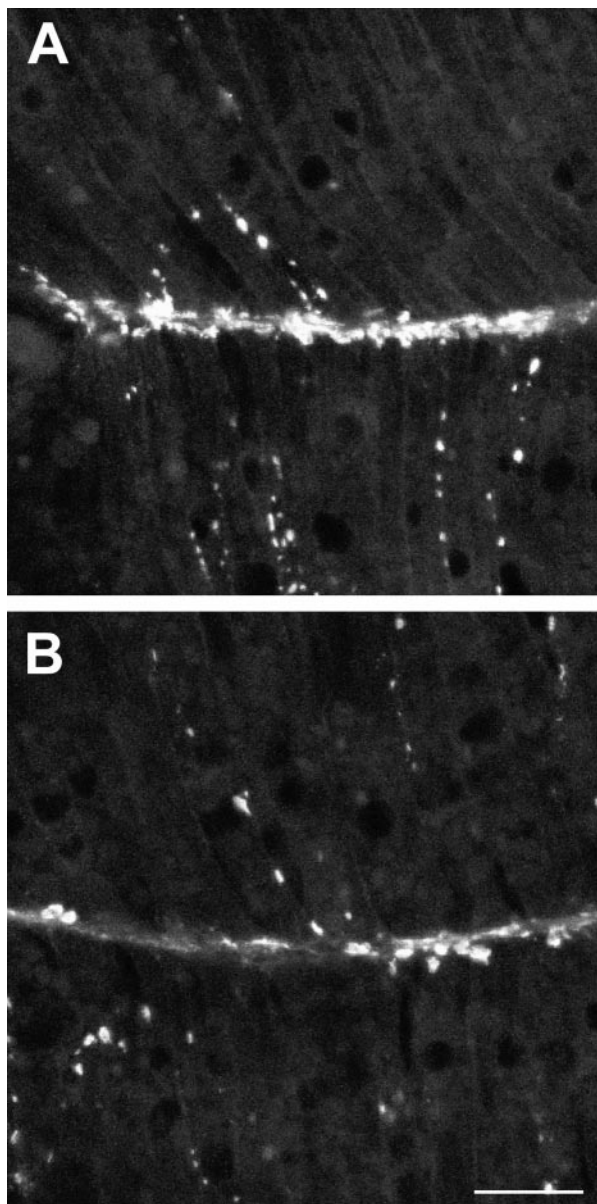


FIG. 2. Synaptic AChR distribution following overexpression. (A) Receptor aggregates in muscles overexpressing GFP. Image from a whole-mount embryo at 1.6 days (st 31) that was injected with GFP RNA at the one-cell stage. Projected stack of confocal images of a synaptic region stained with rhodamine- α -bungarotoxin. The AChR aggregate area is 1.24% of the entire image stack. (B) Dystroglycan overexpression. Image from a sibling injected with GFP and dystroglycan RNAs. The AChR aggregate area is reduced to 0.68%, approximately half that of the GFP control in A. Both images are from myotome 5, have extrasynaptic AChR aggregates, and appear similar except for the AChR aggregate area. Calibration: 20 μ m.

could indicate whether the reduction of AChR aggregation in experimental animals was slowed, stopped, or disrupted. In addition, by examining differences between synaptic

regions in the same animals, more powerful statistical analyses can be used.

To determine if a rostral-caudal gradient of AChR aggregation occurs at neuromuscular synapses in control ani-

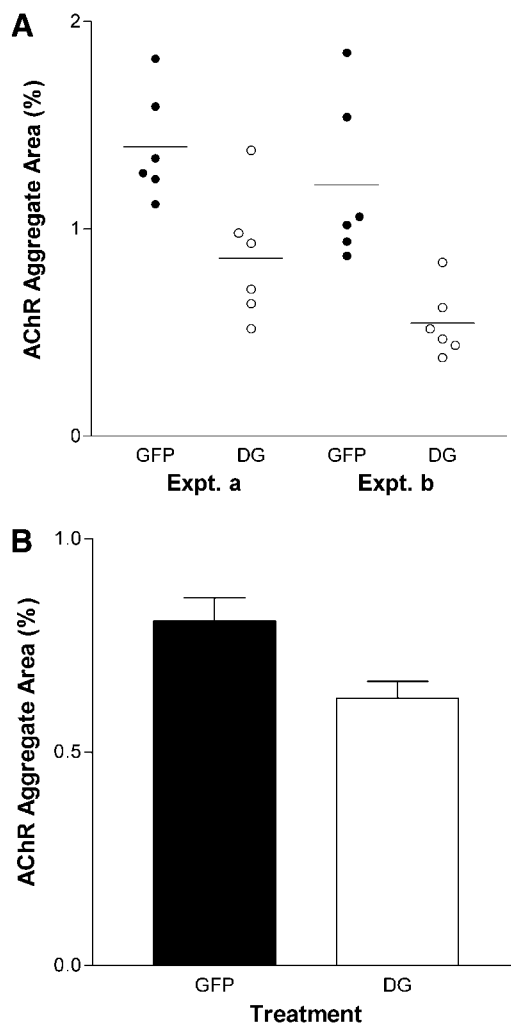


FIG. 3. Dystroglycan overexpression decreased AChR aggregation. (A) Raw AChR aggregation data. Each set of experiments came from siblings: some injected with GFP RNA alone (control, GFP) and others injected with a mixture of GFP and dystroglycan RNAs (experimental, DG). Confocal image stacks from six synaptic regions in each set were analyzed. The AChR aggregate area for each synaptic region sampled in two different sets of experiments is shown, as well as the average for each treatment. Variation occurred within and between experiments. In experiment a, the dystroglycan average was 62% of control ($P = 0.009$; $df = 10$; t test) and in experiment b, it was 45% of control ($P = 0.003$; $df = 10$; t test). Figure 2 images come from experiment a. 17 of 24 experiments showed a decrease in AChR aggregation of DG versus GFP. (B) The AChR aggregate area for all synaptic regions receiving each treatment showed a significant ($P = 0.005$; $df = 250$; t test) dystroglycan-associated decrease to 77.8% of control.

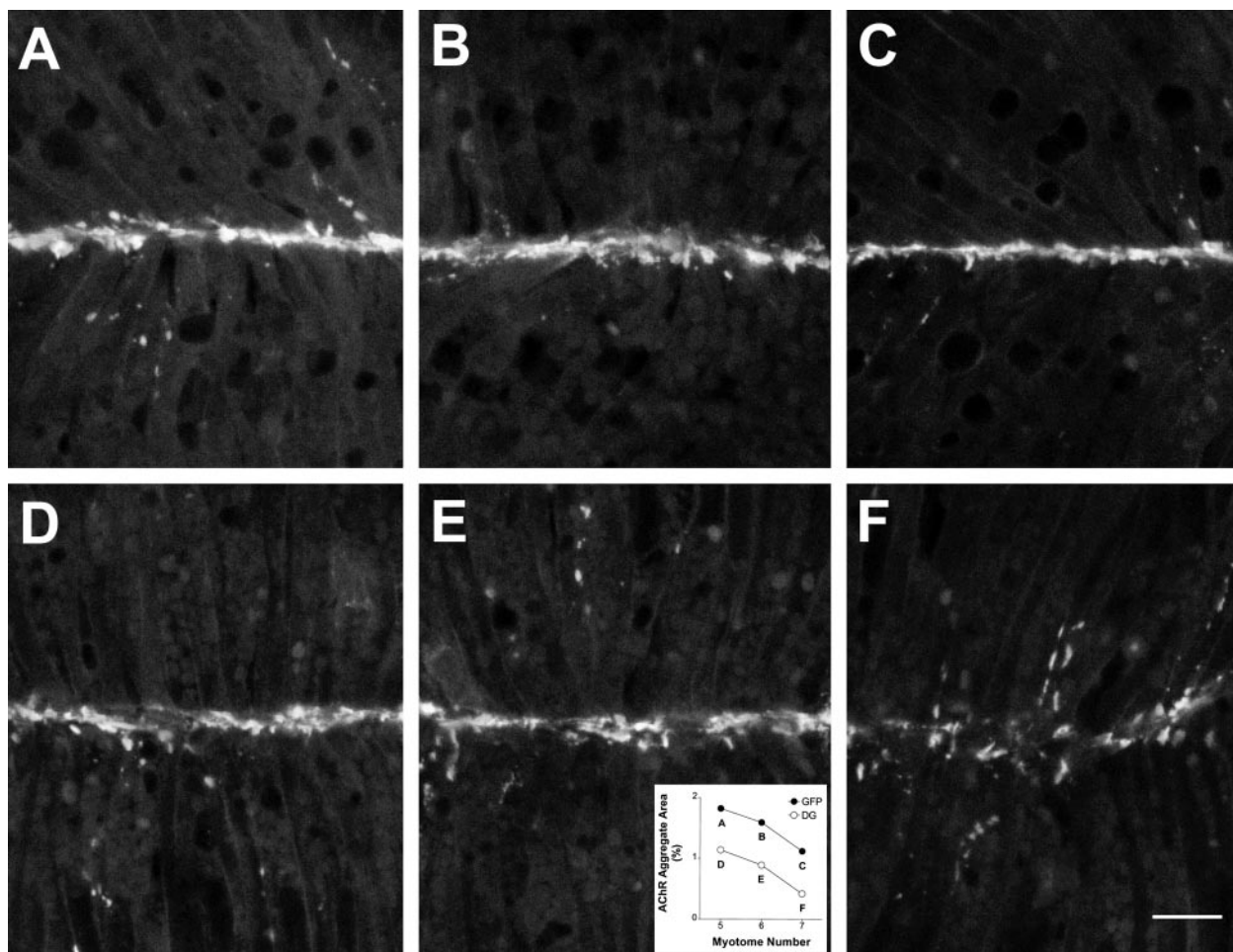


FIG. 4. Rostral-caudal gradient of AChR expression. Top: Images from a whole-mount embryo (1.6 day, st 31) injected with GFP RNA (control). Projected confocal stacks from somites 5 (A), 6 (B), and 7 (C). The greatest AChR aggregation area was at somite 5 (1.82%); it decreased to 1.59% at somite 6 and 1.12% at somite 7. Bottom: Similar series from a sibling embryo injected with GFP and dystroglycan RNAs (experimental). Images are from somites 5 (D), 6 (E), and 7 (F). The respective AChR aggregate areas were 1.14, 0.89, and 0.42%. The inset in E plots the quantitative amounts for each image. Both control and experimental animals showed a rostral-caudal gradient of AChR aggregation. Calibration: 20 μ m.

mals, we obtained images from sequential segmental synaptic regions. Images of synaptic regions between myotomes 5 and 6 (Fig. 4A), 6 and 7 (Fig. 4B), and 7 and 8 (Fig. 4C) were obtained from an animal overexpressing only GFP (control). There was a progressive decrease in the area of rhodamine- α -bungarotoxin-stained AChR aggregates along the rostral-caudal axis in this animal. Measurement of AChR aggregation (Fig. 4E, inset) provides a quantitative confirmation of the result. Although this trend was apparent in many animals, there were exceptions. When data from all control (GFP) animals were combined (Fig. 5), the progressive reduction in AChR aggregation along the rostral-caudal axis was apparent. The synaptic region between myotomes 7 and 8 ("myotome 7") had 70% of the average AChR aggregate volume present in the region

between myotomes 5 and 6 ("myotome 5"). The overall difference across segments was highly significant ($P < 0.001$; repeated-measures ANOVA) for control synaptic regions. Thus there was a distinct gradient in AChR aggregation along the rostral-caudal axis.

To test the patterns of AChR aggregation following overexpression of dystroglycan, we obtained similar images from experimental animals. The three synaptic regions corresponding to the control (Figs. 4A–4C) are shown for a sibling animal (Figs. 4D–4F) receiving experimental treatment (dystroglycan plus GFP RNAs). As in the control, there was a progressive quantitative decrease in AChR aggregation along the rostral-caudal axis of this animal (Fig. 4E, inset). Combining data from all experimental (DG) animals (Fig. 5) shows a progressive reduction in AChR

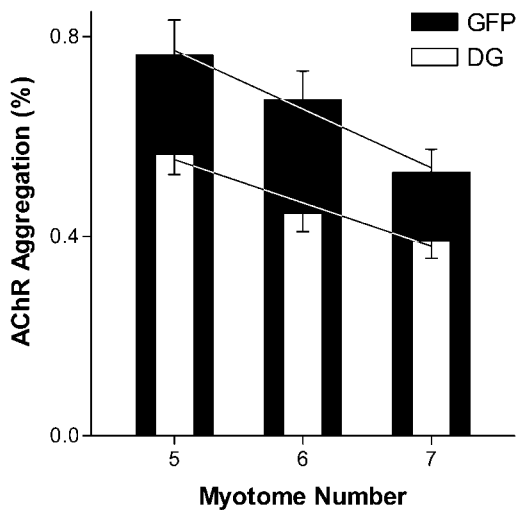


FIG. 5. Dystroglycan inhibits AChR aggregation along a rostral-caudal gradient. Average AChR aggregation volume (with SEM) for all synaptic regions at each myotome border is shown. All three myotomes were analyzed in control (GFP; $n = 54$) and experimental (DG; $n = 86$) animals. A gradient is evident with the greatest and least AChR aggregation in the most rostral (5) and caudal (7) myotomes, respectively. Repeated-measures ANOVA showed significant overall differences between AChR aggregation areas across the three myotomes in both GFP and DG animals ($P < 0.001$ for each). Of all combinations within each group, only the synaptic regions between myotomes 5 and 6 in GFP-treated animals were not significantly different ($P = 0.08$; compared using an unpaired t test). Regression analysis of the data for each experimental group showed similar slopes (-0.12 for GFP and -0.09 for DG). The roughly parallel lines suggest that the experimental treatment inhibited AChR aggregation by a constant amount at each segment. The experimental (DG) value at myotome 5 is similar to the control (GFP) value at myotome 7, indicating that development in experimental animals lagged two segments behind controls.

aggregation along the rostral-caudal axis. The magnitude of the decrease was 70% between myotomes 5 and 7. Repeated-measures ANOVA showed a highly significant overall difference ($P < 0.001$; t test) between AChR aggregation in these synaptic regions. As with controls, there was a gradient along the rostral-caudal axis.

Similar rostral-caudal gradients in control and experimental animals would suggest that dystroglycan inhibits or slows development. Since the morphological structures characteristic of developmental stage were the same and muscle fiber sizes were similar (diameters are approximately $10 \mu\text{m}$) in experimental and control animals, excess dystroglycan had a differential effect on AChR aggregation. The rostral-caudal data for experimental and control groups showed similar trends, but experimental values for each somite decreased to approximately 70% of control (Fig. 5). These differences were reduced from experiments that did not distinguish between different axial levels (Fig. 3C) and were significant at each somite ($P < 0.05$).

Regression analysis showed a linear trend in AChR aggregate volume among the segments at the three synaptic regions (Fig. 5). Both control (GFP) and experimental (DG) groups have similar slopes (-0.12 for GFP and -0.09 for DG), confirming their parallel appearance. In animals overexpressing dystroglycan, the synaptic region of myotome 5 had roughly the same amount of AChR aggregation as synaptic regions at myotome 7 in controls. The similar slopes of AChR aggregation along the rostral-caudal axis (Fig. 5) suggest that experimental synaptic regions are two somites behind controls in their development. Thus dystroglycan apparently retarded the aggregation of AChRs and presumably the function of these developing synapses.

Developmental Effects of Dystroglycan Overexpression

Dystroglycan appeared to reduce AChR aggregation in a constant and linear fashion; it also appeared to disrupt development at earlier stages. Fertility and successful development varied for each batch of eggs. To determine whether dystroglycan affected embryogenesis, we measured the survival of over 15,000 animals from 16 groups that received three different treatments: (1) injection with dystroglycan and GFP, (2) injection with GFP, and (3) no injection. The percentage of uninjected controls that started cleaving and successfully reached the tail bud stage (st 31) was twice that of either injected group of siblings (Fig. 6A). Injection of both dystroglycan and GFP RNA resulted in significantly lower survival than in uninjected controls ($P < 0.001$; t test), but they did not differ from each other, suggesting that RNA injection affected embryo survival. However, embryos injected with dystroglycan RNA frequently showed developmental defects that were associated with gastrulation (Fig. 7). Multiple sites of gastrulation, incomplete gastrulation, and exogastrulation were among the defects found in embryos that did not survive.

To determine whether dystroglycan affected gastrulation, we injected different amounts of dystroglycan RNA and measured embryo survival. To eliminate potential variability between RNA preparations, we serially diluted the RNA from a single preparation and injected 2.3 ([DG]), 4.6 (2[DG]), and 9.2 (4[DG]) ng into each embryo. Only the largest amount of dystroglycan RNA (4[DG]) decreased survival below that of control (GFP) embryos (Fig. 6B). It is unlikely that the reduction in survival was due to the greater quantity of exogenous RNA, since equal and greater amounts of the similar-sized RNA encoding the carboxyl-terminal portion of agrin 0,0 (Godfrey *et al.*, 1999) RNA (4[AG] or 9.2 ng and 6[AG] or 13.8 ng) did not have this effect (Fig. 6B). Surprisingly, we did not detect a dose-dependent decrease in AChR aggregation in response to higher amounts of dystroglycan RNA (data not shown).

We measured viability at three embryonic stages to determine if gastrulation was specifically affected. First, we measured the percentage of embryos that successfully started cleaving (Fig. 6C). Since embryos injected with GFP

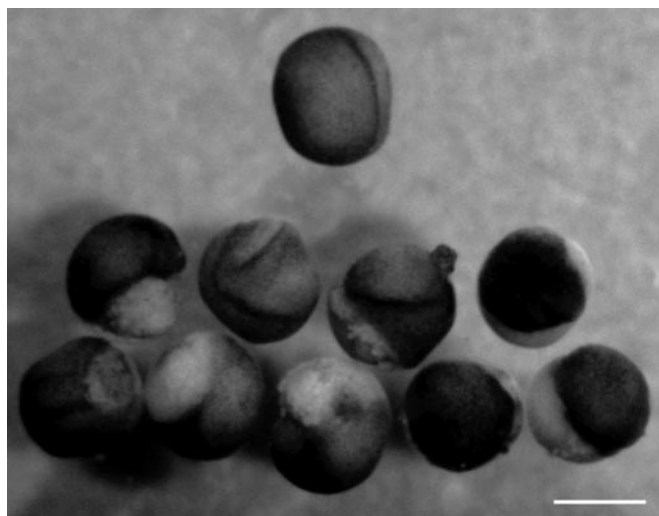
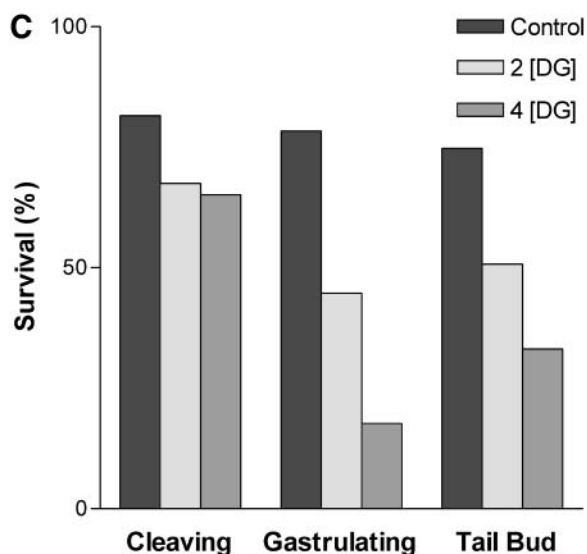
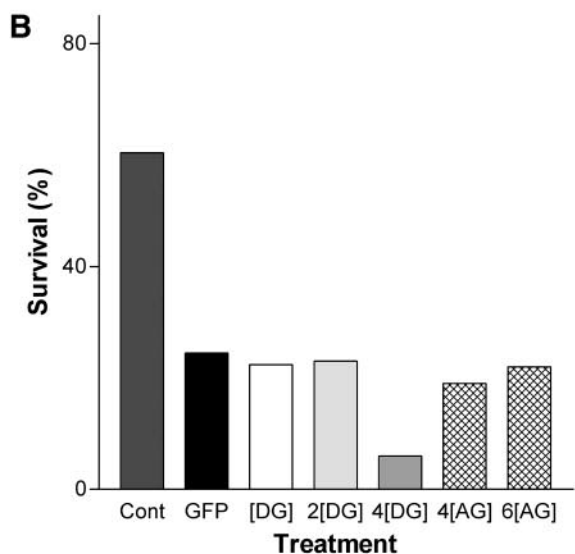
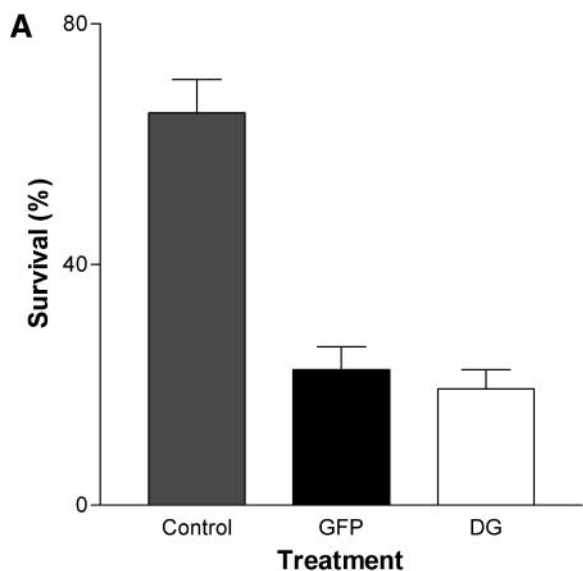


FIG. 7. Developmental defects following overexpression of dystroglycan. Embryos injected with dystroglycan showed decreased survival between cleavage and neurulation. Many of the animals showed defects in gastrulation, including multiple sites of gastrulation, incomplete gastrulation, and exogastrulation. A number of defective embryos injected with dystroglycan RNA are shown next to a single normal sibling (top) that gastrulated and underwent successful neurulation. Calibration: 1 mm.

or the lowest concentration of dystroglycan had a survival frequency similar to that of those injected with the intermediate concentration of dystroglycan RNA, only the 2[DG] is shown. Survival was less than that of controls, but neither concentration of dystroglycan prevented embryos from starting to cleave. Those surviving to the next day

FIG. 6. Dystroglycan overexpression affects embryonic survival. (A) RNA injection decreased the survival of cleaving embryos to tail bud stage. Data are from more than 15,000 embryos produced by 16 females. Repeated-measures ANOVA shows that survival was significantly different across the three groups ($P < 0.001$). The Newman-Keuls posttest showed that the survival of animals injected with GFP RNA or a mixture of dystroglycan and GFP RNAs was significantly decreased from that of uninjected controls ($P < 0.001$) but not from each other. (B) Survival from cleavage to tail bud was examined for animals injected with different concentrations of dystroglycan ($n = 492$ (Control), 661 (GFP; 0.5 ng), 690 ([DG]; 2.3 ng), 596 (2[DG]; 4.6 ng), 666 (4[DG]; 9.2 ng), 473 (4[AG]; 9.2 ng), 157 (6[AG]; 13.8 ng). Animals receiving the greatest amount of dystroglycan RNA (4[DG] or 9.2 ng) showed decreased survival but this was not evident in animals injected with equal or greater amounts of agrin RNA (4[AG] or 9.2 ng and 6[AG] or 13.8 ng) in other experiments. (C) To determine when additional dystroglycan disrupts embryogenesis, survival was analyzed at several stages. Since the effects of GFP and [DG] were similar to those of 2[DG] RNA, only the latter is shown. The largest quantities of DG RNA decreased survival the greatest during gastrulation.

were in the middle of neurulation, but most injected with the highest concentration of dystroglycan RNA did not survive (Fig. 6C). These embryos had multiple sites of gastrulation, incomplete gastrulation, and exogastrulation (Fig. 7) and were unable to continue normal development. Those that successfully completed gastrulation had a better chance of surviving to the tail bud stage (Fig. 6C). Thus, overexpression of high concentrations of dystroglycan decreased survival during gastrulation.

DISCUSSION

The Developmental Role of Dystroglycan

Dystroglycan is part of the dystrophin glycoprotein complex in muscle cells. Two dystroglycan proteins are produced from a polypeptide that is posttranslationally modified. Dystroglycan apparently plays a critical role in early development, since knockout mice are unable to form basement membranes and stop developing early in embryogenesis (Williamson *et al.*, 1997; Henry and Campbell, 1998). In Duchenne muscular dystrophy, dystrophin is missing and dystroglycan is dramatically reduced (Ervasti *et al.*, 1990), thereby eliminating the link between the cytoskeleton and the extracellular matrix. Presumably, the proper localization of dystroglycan is important for muscle integrity (Durbeej *et al.*, 1998a).

The role of dystroglycan during early development may be related to its function in forming basement membranes, a component of extracellular matrix. Mice lacking dystroglycan are unable to make Reichert's membrane and stop developing quite early (Williamson *et al.*, 1997). Electron microscopy showed that embryonic stem cells missing the dystroglycan gene make structures that lack basement membranes, but transfection with functional dystroglycan cDNA restores their ability to do so (Henry and Campbell, 1998). Our experiments increased dystroglycan expression and often resulted in defective gastrulation. Thus, too little or too much dystroglycan can disrupt early development. Although we did not examine basement membranes, gastrulation marks the start of extensive cell movements in the amphibian embryo, which are dependent upon cell attachment to extracellular matrix molecules. An inappropriate linkage between the cytoskeleton and the extracellular matrix could disrupt cell migration during gastrulation. Dystroglycan links the cytoskeleton to the extracellular matrix in differentiated muscle cells and may have a similar function early in development.

There is an asymmetric distribution of dystroglycan during normal development. Many epithelial cell types express dystroglycan on their basal side during differentiation (Durbeej *et al.*, 1995) and as mature cells (Durbeej *et al.*, 1998b). The embryonic brain and spinal cord express dystroglycan RNA (Schofield *et al.*, 1995), which could account for the immunoreactivity on the basal surface of the neural tube. In skeletal muscle, dystroglycan is present in the sarcolemmal membrane and is concentrated at the

neuromuscular and myotendinous junctions (see Durbeej *et al.*, 1998b). In embryonic *Xenopus* myotomes, neuromuscular junctions are located at the intermyotomal septum, which is homologous to the myotendinous junction in adults (Cary and Klymkowsky, 1995). The enrichment of dystroglycan immunoreactivity at the ends of myotomes is likely due to the coincidence of neuromuscular and myotendinous junctions in *Xenopus* embryos. This asymmetry was not evident in cultured myotomal muscle cells (Cohen *et al.*, 1995), perhaps because myotendinous junctions do not form in culture (Cary and Klymkowsky, 1995) and neuromuscular junctions differentiate over several days following nerve-muscle contact (Kullberg *et al.*, 1977). The enrichment of dystroglycan at normal neuromuscular junctions may contribute to the extensive basal lamina associated with junctional folds at the synapse.

A Potential Role for Dystroglycan at the Developing Synapse

The binding of α -dystroglycan to agrin has stimulated interest in a potential role for dystroglycan at the developing synapse. The agrin hypothesis (McMahan, 1990) states that postsynaptic differentiation of the neuromuscular junction is induced by agrin, a protein secreted by the motor nerve terminal. The discovery that dystroglycan bound agrin (Bowe *et al.*, 1994; Campanelli *et al.*, 1994; Gee *et al.*, 1994; Sugiyama *et al.*, 1994) suggested that dystroglycan might be the agrin receptor. However, agrin fragments which no longer bound dystroglycan caused AChR aggregation (Gesemann *et al.*, 1996; Hopf and Hoch, 1996). Agrin signaling is likely mediated by a complex of MuSK (DeChiara *et al.*, 1996) and a myotube-associated specificity component (MASC; Glass *et al.*, 1996). Dystroglycan could mediate AChR aggregation via a MuSK-independent pathway (Montanaro *et al.*, 1998) that involves interactions with laminin (Sugiyama *et al.*, 1997). Cells with reduced levels or no α -dystroglycan showed reduced AChR aggregation in response to laminin and agrin, suggesting that α -dystroglycan could contribute to the growth and/or stabilization of AChR aggregates (Montanaro *et al.*, 1998; Jacobson *et al.*, 1998; Grady *et al.*, 2000). A reasonable hypothesis based on this work is that dystroglycan overexpression should increase AChR aggregation by the MuSK-independent pathway, yet our experiments show a decrease in AChR aggregation. One possible explanation is that excess dystroglycan *in vivo* overcame stimulation of the MuSK-independent pathway and inhibited synaptic AChR aggregation. Inhibition of AChR aggregation by dystroglycan supports the conclusion of Jacobson *et al.* (1998) that dystroglycan is not the MASC component of MuSK signaling.

How could dystroglycan decrease AChR aggregation? Since α -dystroglycan binds to agrin strongly, it could act as a competitive inhibitor of agrin by preventing interaction with the MuSK/MASC complex. This would increase the agrin threshold. Another possibility is that excess

β -dystroglycan could bind to utrophin and inhibit cytoskeletal binding of AChRs. Dystroglycan associates with rapsyn (Apel *et al.*, 1995; Cartaud *et al.*, 1998) and utrophin, a dystrophin-like protein concentrated at the synapse (James *et al.*, 1996). β -Dystroglycan is part of a complex formed by AChR with utrophin, rapsyn, and various synaptic kinases, but it is not needed to maintain the complex (Fuhrer *et al.*, 1999). Thus we favor the idea that dystroglycan inhibits agrin through α -dystroglycan rather than the AChR receptor complex through β -dystroglycan. This provides a simple explanation for the parallel reduction of AChR aggregation at neighboring myotomes in experimental and control animals. Agrin binds to excess dystroglycan, so it cannot bind to MuSK and induce AChR aggregation. This would occur in both experimental and control animals, but additional dystroglycan would retard AChR aggregation.

What is a potential role of dystroglycan in muscle cell development? It is unlikely that excess dystroglycan is produced during normal development, but its ability to decrease AChR aggregation in our experiments could reflect a biologically relevant phenomenon. A muscle isoform of agrin increases AChR aggregation when overexpressed *in vivo* (Godfrey *et al.*, 1999). Dystroglycan could act as a buffer to inhibit ectopic aggregation caused by muscle agrin. At the site of nerve contact, focal release of agrin would eventually overcome the inhibition of dystroglycan. The higher concentration of dystroglycan at the synaptic region would ensure that only the membrane directly opposite an active nerve terminal would contain AChR aggregates. Recent work suggests a potentially complementary function of dystroglycan in the formation of basement membranes by interaction with laminin to assist in depositing other basement membrane proteins (Williamson *et al.*, 1997; Henry and Campbell, 1998) such as perlecan and acetylcholinesterase (Peng *et al.*, 1999). Muscle cells are surrounded by basement membranes, including the specialization at the neuromuscular junction. Muscle isoforms of agrin, like laminin, could interact with dystroglycan around the entire muscle cell to promote formation of its basement membrane. Dystroglycan could interact with proteins containing LG domains (laminin, agrin isoforms, perlecan; Peng *et al.*, 1998) to form basement membranes around muscle cells. At the neuromuscular junction, the additional agrin from nerve terminals would saturate dystroglycan before recruiting the receptors that effect AChR aggregation.

During development of the myotomal neuromuscular system of *Xenopus*, there is a distinct rostral-caudal sequence in AChR aggregation. Somites in *Xenopus* form in a rostral-caudal sequence (Nieuwkoop and Faber, 1994). Thus, caudal myotomes could express fewer AChRs and/or caudal motor neurons could express less agrin to direct AChR aggregation. In either case, myotomal cells could receive synapses on opposite ends, which would recruit AChRs produced by the single muscle nucleus. In higher vertebrates, muscle cells receive and maintain a genetic tag characteristic of their rostral-caudal position in the embryo

(Donoghue *et al.*, 1991). Although the differences in rostral-caudal timing of development are not as obvious in lower vertebrates, they result in significant differences in AChR aggregation at synapses of neighboring segments. These differences could have functional consequences by affecting embryonic movements prior to hatching.

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