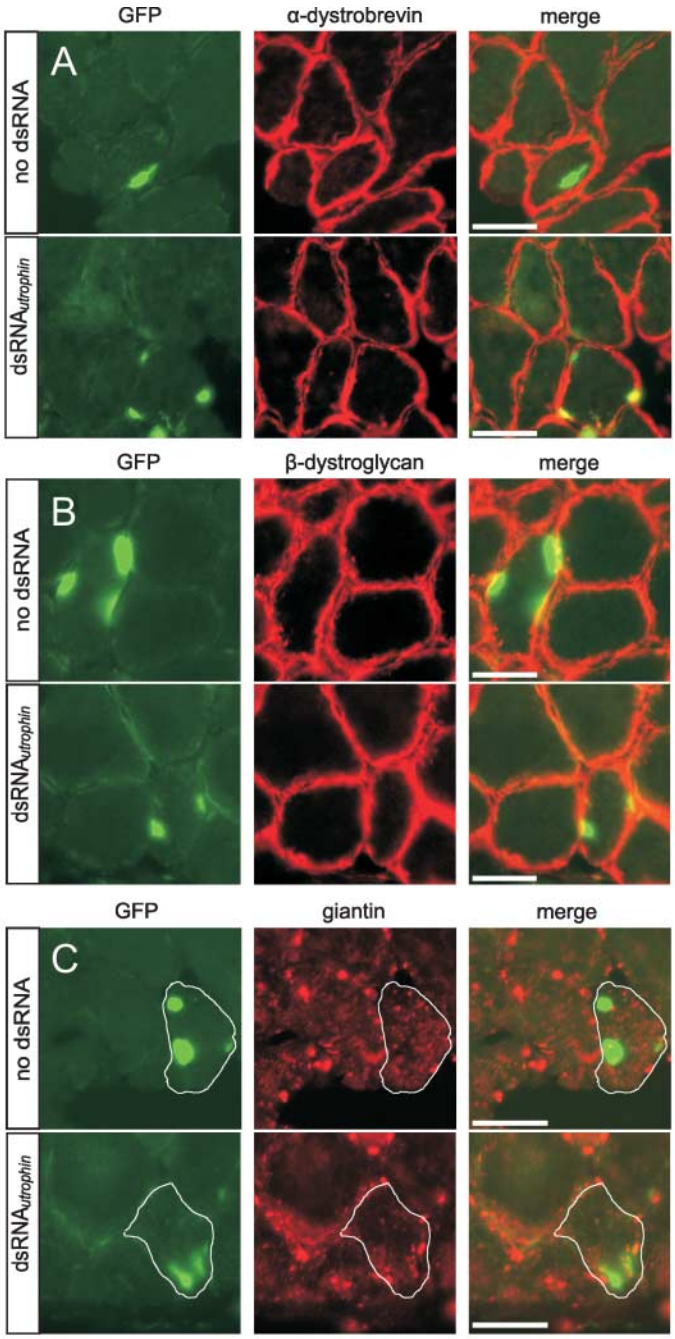
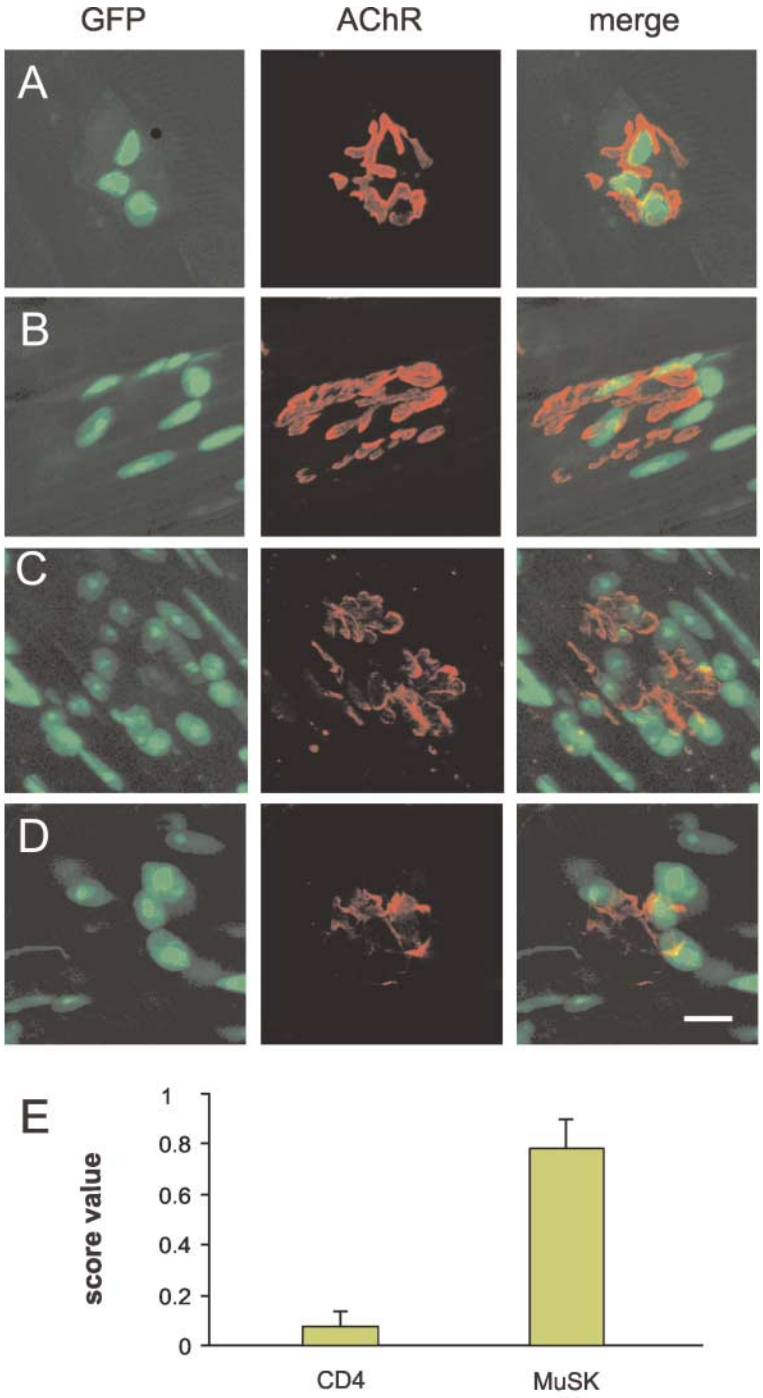
Supplementary Information



Supplementary Figure 1: Staining of cross-sections from rat soleus muscle. Muscle fibers were injected with expression plasmids encoding neural agrin and NLS_GFP (no dsRNA) or co-injected with dsRNA derived from utrophin ($dsRNA_{utrophin}$). Protein levels for α-dystrobrevin (**A**), β-dystroglycan (**B**) and giantin (**C**) were the same in injected (GFP-positive) and the neighboring non-injected muscle fibers. The same results were obtained for α-actinin, myosin, parvalbumin, β-catenin (data not shown). Outline in **C** indicates the circumference of injected muscle fibers. Scale bars = 50 mm.



Supplementary Figure 2: Postsynaptic structures, six weeks after *in vivo* electroporation of mouse soleus muscle with long hairpin RNA-plasmids. Electroporated muscle fibers are marked by NLS_GFP (GFP, green), AChRs were stained to visualize postsynapses (AChR, red). (**A**) Postsynapses are not altered by long hairpin RNA to CD4. (**B–D**) Disassembly of postsynapses by long hairpin RNA to MuSK. While some postsynaptic structures were not altered (**B**), in some cases, postsynaptic AChR aggregates were fragmented to various degrees (**C** & **D**). Scale bars = 10 mm. (**E**) The extent of postsynaptic disassembly on each transfected muscle fiber was quantified using a scale from zero (no fragmentation), one (fragmentation) to two (severe fragmentation) as shown in **B**, **C** and **D**, respectively. For each condition, muscles from three independent experiments were analyzed. The graph shows the mean value for each condition. CD4: long hairpin RNA targeting CD4; MuSK: long hairpin RNA targeting MuSK.

Supplementary Method

Long hairpin constructs. The long hairpin constructs were generated by a two step overlapping PCR using the following primers:

5' primer of MuSK (M) or CD4 (C):

M: CCCATCGATGAGAGCTTGTCAACATTCCA

C: CCCATCGATAGCAGAACTGCCCTGCGAGA

Middle primer:

M: GGCACAGCTTACTCCAAACTTCTCTTGAAAGTTTGGAGTAAGCTGTGCC

C: AACTTTGCAGAGGAAAACGGTCTCTTGAACCGTTTTCCTCTGCAAAGTT

3' primer:

M: CCGCTCGAGGAGAGCTTGTCAACATTCCA

C: CCGCTCGAGAGCAGAACTGCCCTGCGAGA

The middle primers contained the loop sequence TTCAAGAGA (Brummelkamp *et al.*, 2002). Cloning sites Cla1 and Xho1 were introduced at the 5' and 3' ends. In the first step, forward and reverse megaprimers were produced in two separate PCRs using either the 5' or the 3' primer in combination with the middle primer. Purified megaprimers were mixed, annealed at 56 °C and elongated at 72 °C for 5 cycles. The resulting product was amplified by 35 additional cycles after addition of 5' and 3' primers. The final PCR products were purified by gel electrophoresis and subsequently inserted between the muscle creatine kinase promoter (Larochelle et al., 1997; Moll et al., 2001) and a polyA transcription stop sequence in pBluescript II KS+ plasmids. Insertions were verified by restriction enzyme digestions. The murine ~600 nt target sequences correspond to nucleotides 5-608 of MuSK (NCBI accession number: NM_010944) and 150-746 of CD4 (M36850).

Immunohistochemistry and antibodies. AChRs on rat muscles were visualized using rhodamine a-bungarotoxin (Molecular Probes). To stain for AChRs, synaptophysin and neurofilament, mouse muscle was fixed by 2% paraformaldehyde and stained with biotin-xx conjugated a-bungarotoxin, followed by streptavidin-APC (Molecular Probes). After permeabilization (1% Triton X-100), the anti-synaptophysin (DAKO) and anti-neurofilament polyclonal antibodies (Sigma) were incubated for 2 days at 4°C in PBS, 1% BSA. For detection, Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) was used. For staining of a-actinin, b-catenin, myosin, (antibodies for all three proteins: Sigma), a-dystrobrevin (Blake et al., 1998), giantin (Linstedt and Hauri, 1993.), b-dystroglycan, dystrophin, utrophin (antibodies for all three proteins: Novocastra) and parvalbumin (SWant), 12µm-thick cross-sections were fixed for 5 min with 1% paraformaldehyde, washed briefly with PBS, and preincubated for 15 min in PBS supplemented with 5% horse serum, 1% BSA and 0.01% Triton X-100. After overnight incubation with the primary antibodies were detected with Alexa 350-/Alexa 488-conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes).

Quantification. Quantification of AChR clustering was done as described elsewhere (Briguet and Ruegg, 2000) with slight modifications. Briefly, cross-sections through the whole GFP-positive area of an injected muscle fiber were prepared. For each injection site, the number of AChR clusters was counted on GFP-positive (injected) and on neighboring (non-injected) muscle fibers. Number of AChR clusters was calculated for each injection site separately. After normalization to the number of

cross-sections with GFP-positive sites, the mean number of AChR clusters on injected and on neighboring fibers was calculated. For each experimental condition, at least 20 injected muscle fibers (of at least three independent experiments) were counted. The average of all normalized values of AChR cluster-positive neighboring sites on each fiber was taken as 100%.

Quantification of utrophin and dystrophin expression is presented as the average of staining intensity per area, subtracted by the background intensity and normalized to the intensity of the rhodamine α-bungarotoxin staining of the same area. The area for quantification is defined by the size of the AChR cluster on each consecutive cross-section made across the entire injection site. A muscle fiber was defined cluster-positive when a clear and sharp rhodamine α-bungarotoxin staining was detected on its membrane. Quantification was done on unprocessed images (taken by a Leica DC 300F camera) using the program Adobe Photoshop 5.0.

To quantify the effect of plasmid-mediated siRNA on NMJ structure, the degree of fragmentation of postsynaptic AChRs and of the apposition with the presynaptic nerve terminal specializations was scored. To this end, bundles of muscle fibers were stained with fluorescently labeled a-bungarotoxin and a mixture of antibodies against neurofilament and synaptophysin (see Fig. 4). By examining the samples in a double blind manner, four colleagues determined the extent of disassembly of each muscle on a scale of zero (low) to two (high). Scores for each sample and each observer were added and normalized to the theoretically maximal score. For each condition, four muscles of two independent experiments were examined.

Supplementary References

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