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Inhibition of synapse assembly in mammalian muscle *in vivo* by RNA interference

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The formation of the vertebrate neuromuscular junction (NMJ) requires the receptor tyrosine kinase MuSK and the adaptor molecule rapsyn. Here, we report that the phenotypes of mice deficient in these two molecules can be reproduced by RNA interference (RNAi) in rat muscle *in vivo*. Specifically, double-stranded RNA (dsRNA) targeting MuSK and rapsyn inhibited the formation of the NMJ in rat muscle fibres *in vivo*, while dsRNA targeting nonessential proteins did not have any effect. Moreover, plasmids that trigger RNAi to MuSK induced the disassembly of existing NMJs. These results thus demonstrate for the first time the functionality of dsRNA in silencing endogenous genes in adult mammalian muscle *in vivo*. Moreover, they show that MuSK is also required for the maintenance of the NMJ, offering a mechanistic explanation for the myasthenia gravis caused by auto-antibodies to MuSK.

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INTRODUCTION

The molecular mechanisms responsible for the formation of synapses are best understood at the neuromuscular junction (NMJ) (reviewed in Sanes & Lichtman, 2001). Several lines of evidence demonstrate that nerve-derived agrin is required and sufficient for the assembly of the entire postsynaptic apparatus (Bezakova & Ruegg, 2003). Moreover, NMJ formation requires the muscle-specific receptor tyrosine kinase MuSK (DeChiara *et al*, 1996) and the cytoplasmic adaptor molecule rapsyn (Gautam *et al*, 1995). All these data were generated by genetically engineering mice that are deficient in the particular protein.

A promising technique that may allow a more straightforward and faster assessment of gene function *in vivo* than current knockout techniques might be RNA interference (RNAi). This technique employs long double-stranded RNA (dsRNA) or 21–23-bp-long short interfering RNA (siRNA) that trigger specific silencing of gene expression (Tijsterman *et al*, 2002). However, long dsRNA has not successfully been applied to mammals because it seems to induce a general shutdown of translation and apoptosis of the cell (Paddison *et al*, 2002). This unspecific reaction is not observed with siRNA (Elbashir *et al*, 2001; Lewis *et al*, 2002; McCaffrey *et al*, 2002). The disadvantage of siRNA is that only some siRNAs are efficient in silencing gene transcription (McManus & Sharp, 2002) and that the effect of siRNA lasts only for a few days. In a further advancement of the technique, siRNA has been replaced by plasmids encoding short hairpin RNA (shRNA), which has enabled prolonged and stable suppression of gene expression *in vivo* (Brummelkamp *et al*, 2002; Yu *et al*, 2002; McCaffrey *et al*, 2003; Rubinson *et al*, 2003). Here we demonstrate that long dsRNA can be used in adult muscle to perturb the function of endogenous genes, and that prolonged exposure of muscle fibres to plasmids encoding hairpin RNA for MuSK induces the disassembly of existing NMJs.

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RESULTS

We investigated whether dsRNA-mediated RNAi could be used to study the function of genes in the formation of postsynaptic structures in muscle fibres *in vivo*. We injected 591–686-bp-long dsRNA, derived from different genes in conjunction with plasmids encoding neural agrin and green fluorescent protein fused to a nuclear localization signal (NLS_GFP), into nonsynaptic regions of rat soleus muscle (Fig 1A). As described previously (Cohen *et al*, 1997; Meier *et al*, 1997), injection of expression plasmids coding for neural agrin was sufficient to induce postsynaptic specializations in nonsynaptic regions (Fig 1A, right). These specializations are characterized by the accumulation of AChRs (red in Fig 1A) on the surface of injected (GFP-positive; green in Fig 1A) and neighbouring noninjected muscle fibres (Fig 1B). To test whether coinjection of dsRNA exerts any unspecific effect on protein synthesis, we injected dsRNA derived from cDNA encoding CD4 (Benoist & Mathis, 1999). As shown in Fig 1C, postsynaptic structures formed in the presence of $dsRNA_{CD4}$ were indistinguishable from controls and AChR clusters were found on injected and neighbouring muscle fibres. To test whether we could observe specific RNAi, we next coinjected dsRNA corresponding to MuSK ($dsRNA_{MuSK}$). MuSK is an essential signalling component for NMJ formation that is activated by neural agrin (Glass *et al*, 1996). Thus, effective $dsRNA_{MuSK}$ should prevent the formation of postsynaptic specializations in response to neural agrin in the injected muscle fibre. Indeed, AChR clusters were only rarely

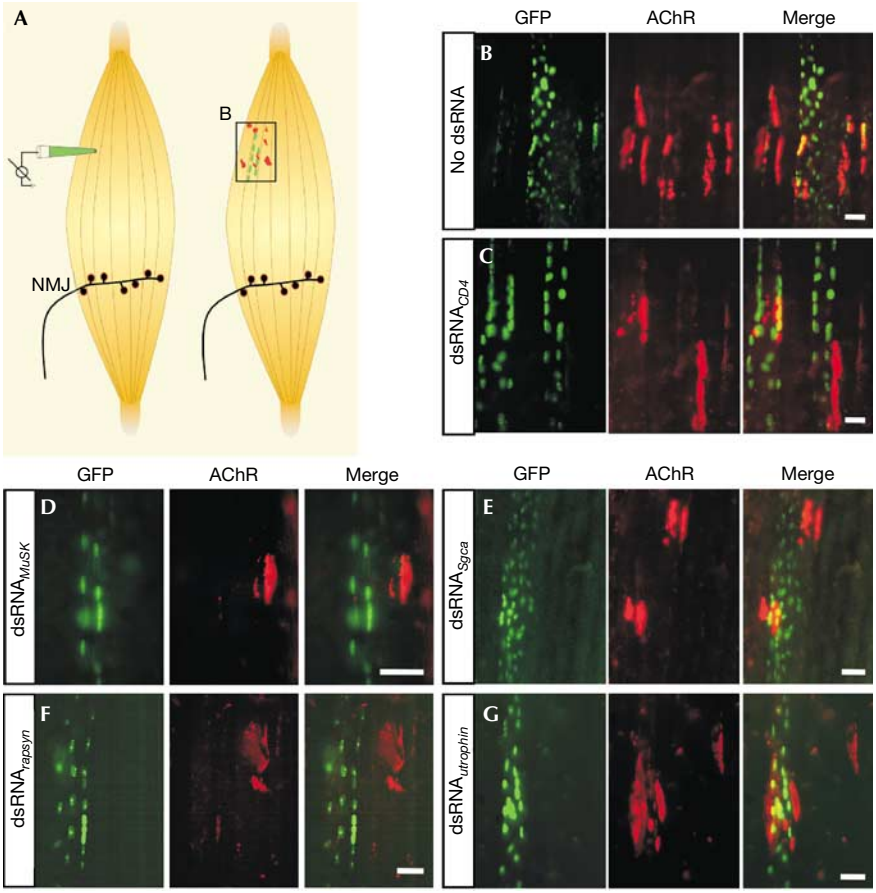

Fig 1 | Inhibition of the formation of postsynaptic structures by dsRNA. **(A)** Schematic representation of the injection of cDNA constructs at nonsynaptic regions of single muscle fibres of rat soleus muscle (left). The injection pipette contains expression plasmids NLS_GFP and neural agrin. dsRNA was added in RNAi experiments. Injected muscle fibres contain GFP-positive myonuclei (green) and aggregates of postsynaptic proteins including AChRs (red). The frame symbolizes the view shown in **(B)**. Postsynaptic structures formed on the surface of the injected and on nearby muscle fibres when no dsRNA **(B)** or dsRNA$_{CD4}$ **(C)** was included. Injection of dsRNA$_{MuSK}$ **(D)** and dsRNA$_{rapsyn}$ **(F)** prevents the formation of postsynaptic structures on injected muscle fibres but not on nearby fibres. Postsynaptic structures formed on muscle fibres injected with dsRNA$_{Sgca}$ **(E)** and dsRNA$_{utrophin}$ **(G)**. Scale bars = 50 μm.

detected on injected muscle fibres while they were readily detected on neighbouring, noninjected muscle fibres (Fig 1D). Thus, the effect of dsRNA remains restricted to the injected muscle fibres, indicating that dsRNA does not cross cell boundaries in mammalian muscle, a phenomenon that has been reported in *Caenorhabditis elegans* (Winston *et al*, 2002). As a further test for the specificity of the inhibitory activity of dsRNA$_{MuSK}$, we also coinjected dsRNA$_{Sgca}$ derived from α-sarcoglycan, a protein that is highly expressed in muscle fibres but is not necessary for the formation of NMJs (Duclos *et al*, 1998). In this case, AChR clusters were formed both on injected and neighbouring muscle fibres (Fig 1E). To test the universality of the method, we also examined the effect of dsRNA$_{rapsyn}$, which was derived from rapsyn, an adaptor molecule essential for the clustering of AChRs (Gautam *et al*, 1995). No AChR clusters were detected in dsRNA$_{rapsyn}$-containing muscle fibres, whereas such clusters were found on neighbouring fibres (Fig 1F). Finally, we injected dsRNA$_{utrophin}$ because utrophin is highly concentrated at the postsynaptic site of NMJs and at ectopic postsynaptic structures (Meier *et al*, 1997), but its inactivation in mice does not impinge on the initial formation of the NMJ (Deconinck *et al*, 1997; Grady *et al*, 1997). As expected, AChR clusters were still formed on injected muscle fibres (Fig 1G).

For quantification, we counted the number of AChR clusters on injected and on neighbouring, noninjected muscle fibres as schematically shown in Fig 2A. From Fig 2B, this quantification demonstrates that injection of dsRNA$_{MuSK}$ or dsRNA$_{rapsyn}$ resulted in a highly significant reduction of AChR clusters in the injected muscle fibres, whereas 'no dsRNA', dsRNA$_{CD4}$, dsRNA$_{utrophin}$ and dsRNA$_{Sgca}$ did not inhibit AChR cluster formation. In summary, these results demonstrate that dsRNA, when injected into single, adult muscle fibres *in vivo*, knocks the expression of the targeted protein down to the extent that it mimics the phenotype of the corresponding knockout mouse.

To measure the effect of dsRNA-induced RNAi on a particular gene directly, we next quantified the amount of utrophin and dystrophin found at ectopic postsynapses. As shown earlier, agrin-induced formation of postsynaptic specializations at ectopic sites requires local transcription of synaptic proteins in the myonuclei underlying these sites (Briguet & Ruegg, 2000; Moore *et al*, 2001). Moreover, agrin-induced ectopic postsynaptic structures form

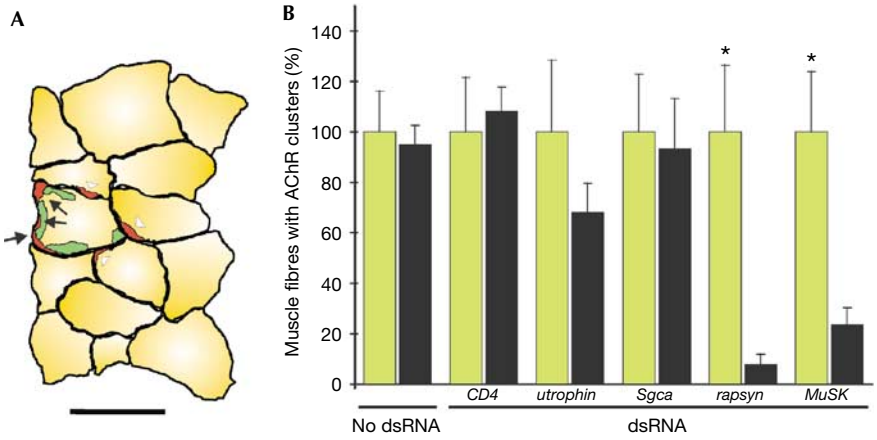

Fig 2 | Quantification of the number of AChR clusters formed on injected and neighbouring muscle fibres. **(A)** Camera lucida drawing of a cross-section through an injection site, 2 weeks after injection. In this particular case, the injection cocktail contained expression constructs for neural agrin and NLS_GFP, and dsRNA$_{utrophin}$. In the cross-section shown, AChR clusters (red) were found along the circumference of the injected (GFP-positive nuclei; green) and the neighbouring, noninjected muscle fibres. AChR clusters on injected fibres are marked with solid arrows, and AChR clusters on neighbouring fibres are marked with open arrowheads. Bar = 50 μm. **(B)** Quantification of three independent experiments for each experimental condition. For each muscle, the number of AChR clusters in neighbouring, noninjected muscle fibres (green) was normalized to 100%. Significant differences ($P < 0.01$; Wilcoxon test) between the number of AChR clusters on noninjected, neighbouring (green) and injected (black) muscle fibres are indicated by asterisks. For experimental details, see supplementary information online.

de novo, which makes the protein levels at the ectopic sites grossly independent of protein turnover. In controls, all postsynaptic structures induced by neural agrin contained a high concentration of utrophin (Fig 3A), whereas the staining was less intense at AChR clusters of muscle fibres injected with dsRNA$_{utrophin}$ (Fig 3B). When we stained for dystrophin, which may compensate for utrophin (Deconinck *et al*, 1997; Grady *et al*, 1997), we found that staining for dystrophin was very similar in injected and neighbouring muscle fibres (Fig 3C,D). Quantification of the fluorescence intensity for utrophin and dystrophin in the different experimental paradigms is shown in Fig 3E. No difference between the staining intensity for utrophin at AChR clusters formed on injected and neighbouring muscle fibres was observed in controls (no dsRNA). The levels of utrophin at AChR clusters of dsRNA$_{utrophin}$-injected muscle were significantly lower (<40%) than at AChR clusters in neighbouring fibres. The low level of utrophin that is still detected at the ectopic postsynaptic sites of dsRNA$_{utrophin}$-containing muscle fibres might be due to either some utrophin expressed in nonsynaptic regions and/or the aggregation of utrophin diffused from the NMJ. We also could not detect any unspecific suppression of gene products that are not concentrated at AChR clusters (supplementary Fig 1 online). Note that the level of dystrophin is slightly increased in the muscle fibres injected with dsRNA$_{utrophin}$ compared to neighbouring fibres. This lends support to the idea that dystrophin compensates for the lack of utrophin in the knockout mice.

In a further step, we asked whether RNAi could also be used to study the requirement of genes for the stability of the nerve–muscle synapse. To address this question, we chose to target MuSK because (i) it is required for the formation of postsynapses and (ii) auto-antibodies to MuSK cause myasthenia gravis (Hoch *et al*, 2001). This disease is characterized by muscle weakness and loss of AChRs, suggesting that NMJs might disassemble. As NMJs represent only 0.1% of the total surface of a muscle fibre, dsRNA injection into single muscle fibres did not allow a global and sustained perturbation of gene expression at the NMJs. We therefore used plasmids that express functional long dsRNA or siRNA (Brummelkamp *et al*, 2002; Yu *et al*, 2002; Yi *et al*, 2003). Three different MuSK–shRNA plasmids were designed while only one long hairpin RNA was used. As a control, plasmids derived from CD4 were transfected. On examining the electroporated muscles after 2 weeks, we could not detect a clear effect on the structure of their NMJs (data not shown), and we therefore concentrated our examination to 6 weeks. In CD4-targeted muscle fibres, NMJs overlying GFP-positive myonuclei were indistinguishable from NMJs on muscle fibres that were not transfected (Fig 4A). The alterations of the postsynaptic structures after applying MuSK shRNA plasmids ranged from fragmentation (Fig 4B) to severe disassembly of postsynaptic AChR clusters (Fig 4C). In response to the abrogation of postsynapse integrity, presynaptic nerve terminals began to sprout (arrowheads in Fig 4C,D). In some severe cases (Fig 4D), the entire postsynaptic structure was lost and only the remaining motor nerve terminal indicated that an NMJ had been present before. In all GFP-negative muscle fibres, NMJs were not different from nontreated animals (data not shown). Quantification revealed that NMJs were not altered by electroporation of shRNA plasmids directed to CD4 and to one sequence of MuSK (M1). Two other shRNA plasmids to MuSK (M2 and M3) showed a clear effect on NMJ structure (Fig 4E). Similarly, plasmids encoding functional long dsRNA to MuSK led to the disassembly of postsynaptic AChR clusters (supplementary Fig 2 online).

DISCUSSION

Although dsRNA can mediate RNAi in cultured mammalian cells (Elbashir *et al*, 2001), its specific effect is overwritten by the activation of the dsRNA-dependent interferon response, which triggers general inhibition of protein translation and induces

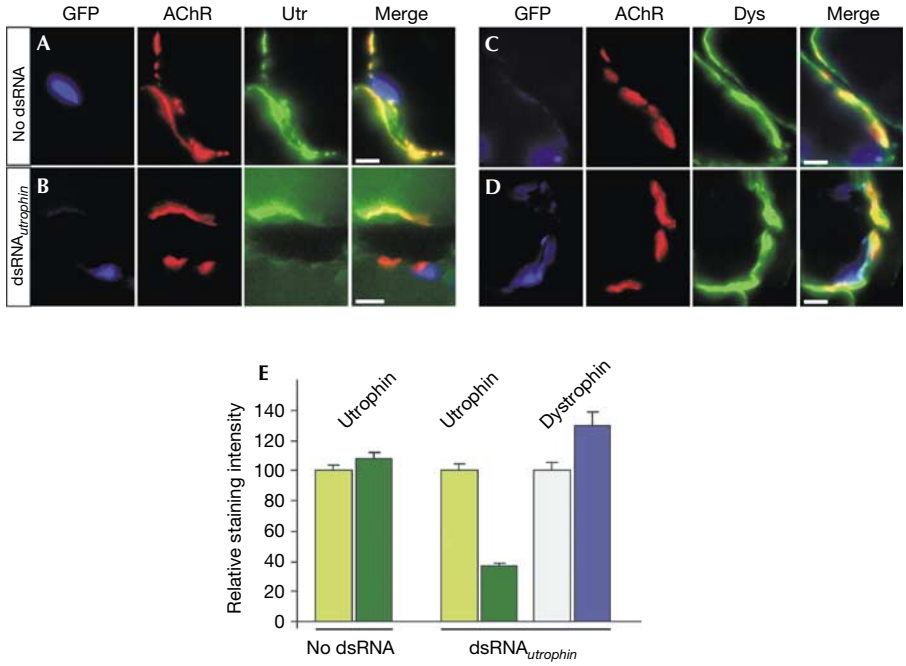

Fig 3 | Staining of ectopic postsynaptic structures for utrophin and dystrophin. **(A)** No difference in the staining intensity for utrophin was seen when dsRNA was omitted. **(B)** Although AChR clusters were formed on the muscle that contained dsRNA$_{utrophin}$ (GFP-positive), these clusters were often devoid of any utrophin. **(C)** Dystrophin was more uniformly distributed along the entire plasmalemmal membrane, with only some enrichment at AChR clusters. **(D)** In dsRNA$_{utrophin}$-expressing muscle fibres, dystrophin was found at sites of AChR accumulation. **(E)** Quantification of the amount of utrophin (green bars) and dystrophin (blue bars) in neighbouring (light colours) and injected muscle fibres (dark colours). Bars in **(A–D)** = 5 μm.

apoptosis of cells (Paddison *et al*, 2002). Here, we used dsRNA and show for the first time that dsRNA-induced RNAi is highly reproducible and sequence specific. For example, dsRNA directed to utrophin clearly reduced the amount of utrophin at the ectopic postsynapses but did not affect the expression levels of its homologue dystrophin. Second, the use of dsRNA did not cause inhibition of protein translation in general, indicated by the fact that the number of AChR clusters formed on muscle fibres injected with dsRNA directed to CD4, α-sarcoglycan or utrophin was not different from muscle fibres that were not injected with any dsRNA. Moreover, we could not detect any off-target effect (Jackson *et al*, 2003, but see also Chi *et al*, 2003; Semizarov *et al*, 2003) because the amount of several proteins was not affected by the expression of dsRNA (supplementary Fig 1 online). Third, the effect of dsRNA was confined to the injected muscle fibre and did not spread across cell boundaries. This allowed comparison of perturbed and nonaffected muscle fibres in the same muscle and easy control of the method.

We did not investigate why we found no evidence for a general silencing of translation in dsRNA-injected muscle fibres. It could well be that muscle fibres do not respond to dsRNA in this unspecific way. Indeed, vector-mediated delivery of dsRNA has also been shown to induce sequence-specific RNAi in cultured C2C12 cells, a cell line that forms myotubes. As in our case, the silencing of endogenous genes was not accompanied by a global effect on translation (Yi *et al*, 2003). To restrict expression of long dsRNA to skeletal muscle, we used the muscle creatine kinase promoter (Moll *et al*, 2001) instead of the U6 promoter used for shRNA plasmids, to drive expression of long hairpin RNA. Our work also introduces two alternative methods for applying RNA interference. While electroporation is the method of choice to reach many muscle fibres and allows assessment of gene function in the entire muscle fibre including the NMJ, microinjection of single muscle fibres allows one to fine-tune the experimental settings. In particular, the amount of dsRNA and expression plasmids can be well controlled. Moreover, the distance of the injection site from the endogenous NMJ can be chosen. All these parameters influence the outcome of the experiment, and thus microinjection is well suited to study the molecular details of how postsynaptic structures are induced *in vivo*. In summary, both methods will facilitate the functional characterization of unknown genes in muscle *in vivo*, which is a significant advantage over the current methods using conventional gene-targeting techniques in mice. Moreover, this method may also be beneficial for studies aimed at the treatment of particular diseases.

Another aspect of our work is that we provide direct evidence that MuSK expression is necessary to warrant the integrity of the NMJ. The recent discovery that auto-antibodies to MuSK cause myasthenia gravis (Hoch *et al*, 2001) is suggestive of a role of MuSK in warranting the integrity of postsynaptic structures. However, no direct evidence has yet been provided. We describe here that MuSK perturbation causes pronounced disassembly of the entire NMJ. MuSK perturbation also resulted in the sprouting of the presynaptic nerve terminal, indicating that a compact postsynaptic structure is also required to maintain presynaptic integrity. Denervation of NMJs may be a secondary consequence in patients who suffer from myasthenia gravis caused by MuSK auto-antibodies.

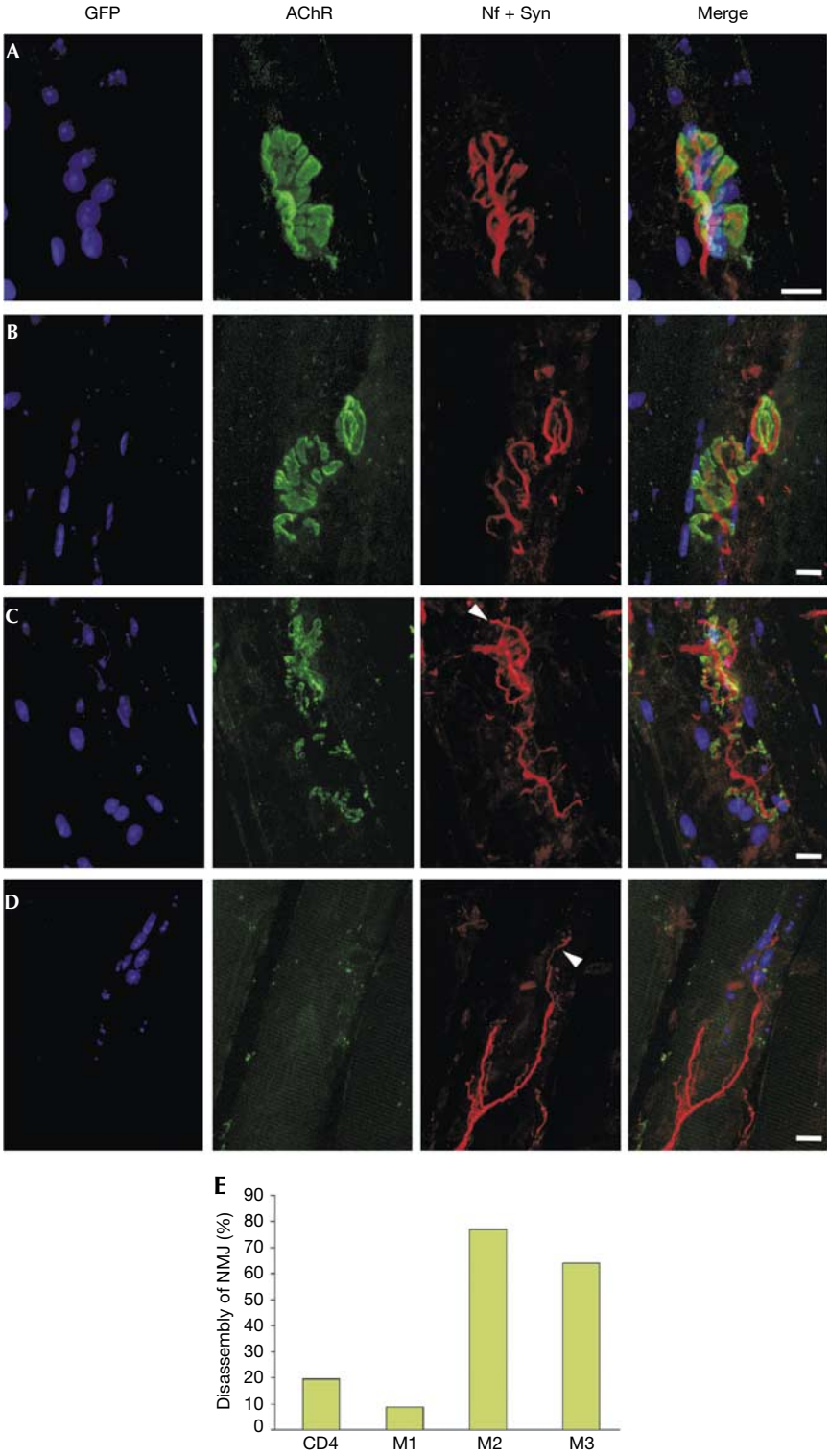

Fig 4 | Whole mounts of NMJs, 6 weeks after *in vivo* electroporation of mouse soleus muscle with siRNA plasmids. Electroporated muscle fibres are marked by NLS_GFP (GFP). AChRs were stained to visualize postsynapses, and a mixture of antibodies to neurofilament (Nf) and synaptophysin (Syn) was used to label the presynaptic motor neurons. **(A)** NMJs are not altered by an siRNA plasmid to CD4. **(B–D)** Disassembly of NMJs by siRNA plasmids to MuSK. See text for details. Scale bars = 15 μm. **(E)** Quantification of the effect of plasmid-mediated siRNA (see supplementary information online). CD4, siRNA plasmid targeting CD4; M1, M2 and M3, three different siRNA plasmids targeting MuSK. Note that M1 does not show any effect, which is consistent with observations by others that most but not all siRNA constructs are functional (McManus & Sharp, 2002).

METHODS

shRNA plasmids and injections. Full-length chick agrin cDNA and the NLS_GFP construct have been described previously (Denzer *et al*, 1995; Jones *et al*, 1999). Vectors encoding shRNAs were constructed according to Yu *et al* (2002) using the loop sequence TTCAAGAGA (Brummelkamp *et al*, 2002). The murine 21 nt target sequences correspond to nucleotides 125–145 (M1), 352–372 (M2) and 525–545 (M3) of MuSK (NCBI accession: NM_010944) and 494–514 of CD4 (M36850). Injection into rat muscle fibres was performed as described (Meier *et al*, 1997).

dsRNA preparation. PCR-generated transcription templates contained T7 or T3 promoter sequences on the 5′ end of the sense or antisense template. RNAs were synthesized using the Megascripts kit (Ambion) and annealed as described (Wianny & Zernicka-Goetz, 2000). The target sequences in rats correspond to nucleotides 5–608 of MuSK (U34985), 44–687 of rapsyn according to murine homologue gene (NM_009023), 114–704 of CD4 (M15768), 55–740 of α-sarcoglycan according to murine homologue gene (NM_009161) and 2132–2747 of utrophin (AJ002967). PCR products of rapsyn, α-sarcoglycan and utrophin were sequenced.

Electroporation of cDNA into muscle fibres. A 5–10 μl mix of cDNAs (2 μg/μl of each construct) was injected into the soleus muscle of C57BL/6 mice (>6 months). Electroporation was performed as described previously (Gehl & Mir, 1999) using an ECM 830 electroporation system (BTX). Eight pulses were applied for 20 ms and at a frequency of 1 Hz. The voltage was set to 200 V/cm. After 2–6 weeks, the electroporated muscle was analysed.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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