# Supporting Information

## Lin *et al.* 10.1073/pnas.0801683105

### SI Materials and Methods

**Mice.** Transgenic mice expressing c-mag$_{B8}$ were generated as described (1) using the chick N25$_7$C21$_{B8}$_myc construct, which has been described elsewhere (2). The cDNA encoding mouse miniagrin (m-mag$_{z8}$) was obtained by RT-PCR on mRNA isolated from mouse spinal cord. The entire construct was sequenced and inserted into the tetO7-CMV promoter by replacing the cDNA construct described elsewhere (3). MCK-tTA mice (4) were obtained from N. Raben (National Institutes of Health, Bethesda, MD). Transgenic founders were identified by Southern blot analysis and PCR. The following primers were used for PCR genotyping: 5′ ACC CAG CCC CTC AGT ACA TGT and 5′ CTT CTG TTT TGA TGC TCA GC for c-mag$_{B8}$; 5′ CCA ATG TGA CCG CTA GCG AGA AG and 5′ CTG TAG GCC TCC AAG CCA CA for m-mag$_{z8}$. Previously described primers and procedures were used to identify mice that express tTA (3) or YFP (5) or that are deficient for agrin (6).

**Antibodies.** Antibodies directed against chick agrin (7), mouse agrin (8) and MuSK (9) were raised in our laboratory and have been characterized previously. To avoid reactivity with mouse tissue of anti-mouse secondary antibodies, anti-myc antibody 9E10 (10) was biotinylated with biotin-NHS according to the manufacturer's procedure. Other antibodies used were from the following commercial sources: rabbit antibodies against synaptophysin (Dako) or neurofilament (Sigma). Secondary antibodies were coupled to either Cy3 (Jackson ImmunoResearch) or Alexa-488 (Invitrogen).

**Immunoblot and Quantification.** Frozen tissues were pulverized on a metal plate cooled in liquid nitrogen and resuspended in protein extraction buffer [80 mM Tris-HCl pH 6.8, 10 mM EDTA, 2% SDS and 1:50 diluted mix protease inhibitors (Sigma)]. 10 $\mu$g of protein was separated on a 7.5% SDS/PAGE and immunoblotted. Expression levels of the transgenes were quantified by measuring the intensity of the band after subtracting the background in the same blot. For normalization, the intensity of the actin band after Ponceau S staining (Sigma) was used.

**Quantitative Analysis of Synaptic AChR Cluster Bands.** For measuring the width of the synaptic band in the diaphragm, images of whole mounts such as those shown in Figs. S2–S5 were analyzed by AnalySIS software (Soft Imaging System). By using the "measuring Area/Perimeter tool", the periphery of the region comprising AChR clusters contacted by motor nerves was outlined manually and the area of this outline was determined. The average width of the synaptic band was calculated by dividing its length.

**RNA Extraction and PCR Analysis.** Total RNA was extracted from mouse brain, spinal cord and calf muscle as previously described (11). Before reverse transcription, RNA was incubated for 0.5 h with RNase-free DNase (Promega) and purified using RNeasy RNA purification columns (Qiagen). Single-stranded cDNA was prepared from 1.5 $\mu$g of total RNA using SuperScript II Reverse Transcriptase (Invitrogen) according to supplier's instruction. PCR was performed using the following primer sets: GAPDH (sense – 5′ CAT CGT GGA AGG GCT CAT GAC 3′, antisense – 5′ CTT GGC AGC ACC AGT GGA TG 3′), chick miniagrin (sense – 5′ CTT CTG TTT TGA TGC TCA GC 3′, antisense – 5′ ATT GCA TTT GAT GGT AGG 3′). PCR products were analyzed on a 2% agarose gel.


1. Moll J, *et al.* (2001) An agrin minigene rescues dystrophic symptoms in a mouse model for congenital muscular dystrophy. *Nature* 413:302–307.
2. Meier T, *et al.* (1998) A minigene of neural agrin encoding the laminin-binding and acetylcholine receptor-aggregating domains is sufficient to induce postsynaptic differentiation in muscle fibres. *Eur J Neurosci* 10:3141–3152.
3. Meinen S, Barzaghi P, Lin S, Lochmuller H, Ruegg MA (2007) Linker molecules between laminins and dystroglycan ameliorate laminin-alpha2-deficient muscular dystrophy at all disease stages. *J Cell Biol* 176:979–993.
4. Ghersa P, *et al.* (1998) Highly controlled gene expression using combinations of a tissue-specific promoter, recombinant adenovirus and a tetracycline- regulatable transcription factor. *Gene Ther* 5:1213–1220.
5. Feng G, *et al.* (2000) Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 28:41–51.
6. Lin W, *et al.* (2001) Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* 410:1057–1064.
7. Gesemann M, Denzer AJ, Ruegg MA (1995) Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. *J Cell Biol* 128:625–636.
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9. Scotton P, *et al.* (2006) Activation of muscle-specific receptor tyrosine kinase and binding to dystroglycan are regulated by alternative mRNA splicing of agrin. *J Biol Chem* 281:36835–36845.
10. Evan GI, Lewis GK, Ramsay G, Bishop JM (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* 5:3610–3616.
11. Ksiazek I, *et al.* (2007) Synapse loss in cortex of agrin-deficient mice after genetic rescue of perinatal death. *J Neurosci* 27:7183–7195.

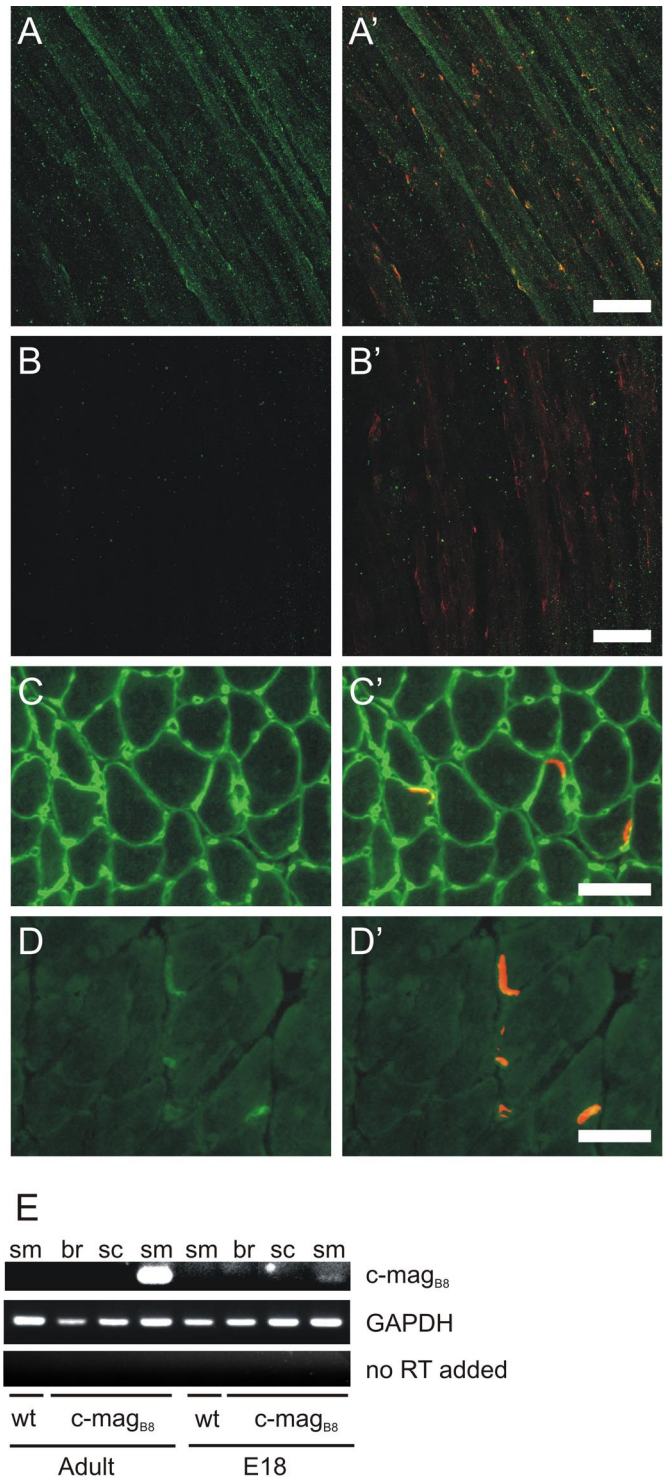



**Fig. S1.** Expression of c-mag$_{B8}$ in embryonic and adult tissue. (*A, B*) Whole mounts of diaphragm muscles from E13.5 c-mag$_{B8}$ transgenic (*A, A'*) or wild-type (*B, B'*) mice. Muscles were stained with an antibody raised against chick agrin (Gesemann M, Denzer AJ, Ruegg MA (1995). *J Cell Biol* 128:625–636) followed by an Alexa-488 conjugated secondary antibody (green; *A, B*). AChRs were visualized with Alexa-555-$\alpha$-bungarotoxin (red; *A', B'*). The transgenic protein is detected already at E13.5 (compare A to B). (*C, D*) Cross-sections of soleus muscle from 6 week-old c-mag$_{B8}$ transgenic (*C, C'*) or wild-type (*D, D'*) mice. Staining procedure was the same as described above. The transgenic protein is detected in the muscle basal lamina without a particular enrichment at AChR clusters. Note: immunoreactivity in wild-type mice originates from the cross-reactivity of the antiserum with mouse agrin. (*E*) RT-PCR of RNA from brain (br), spinal cord (sc) and skeletal muscle (sm) of wt and c-mag$_{B8}$ transgenic mice. Miniagrin mRNA can be detected in adult and embryonic (E18) skeletal muscle but not in brain or spinal cord of c-mag$_{B8}$ transgenic mice. Scale bars: 50 $\mu$m (*A, B*) and 25 $\mu$m (*C, D*).

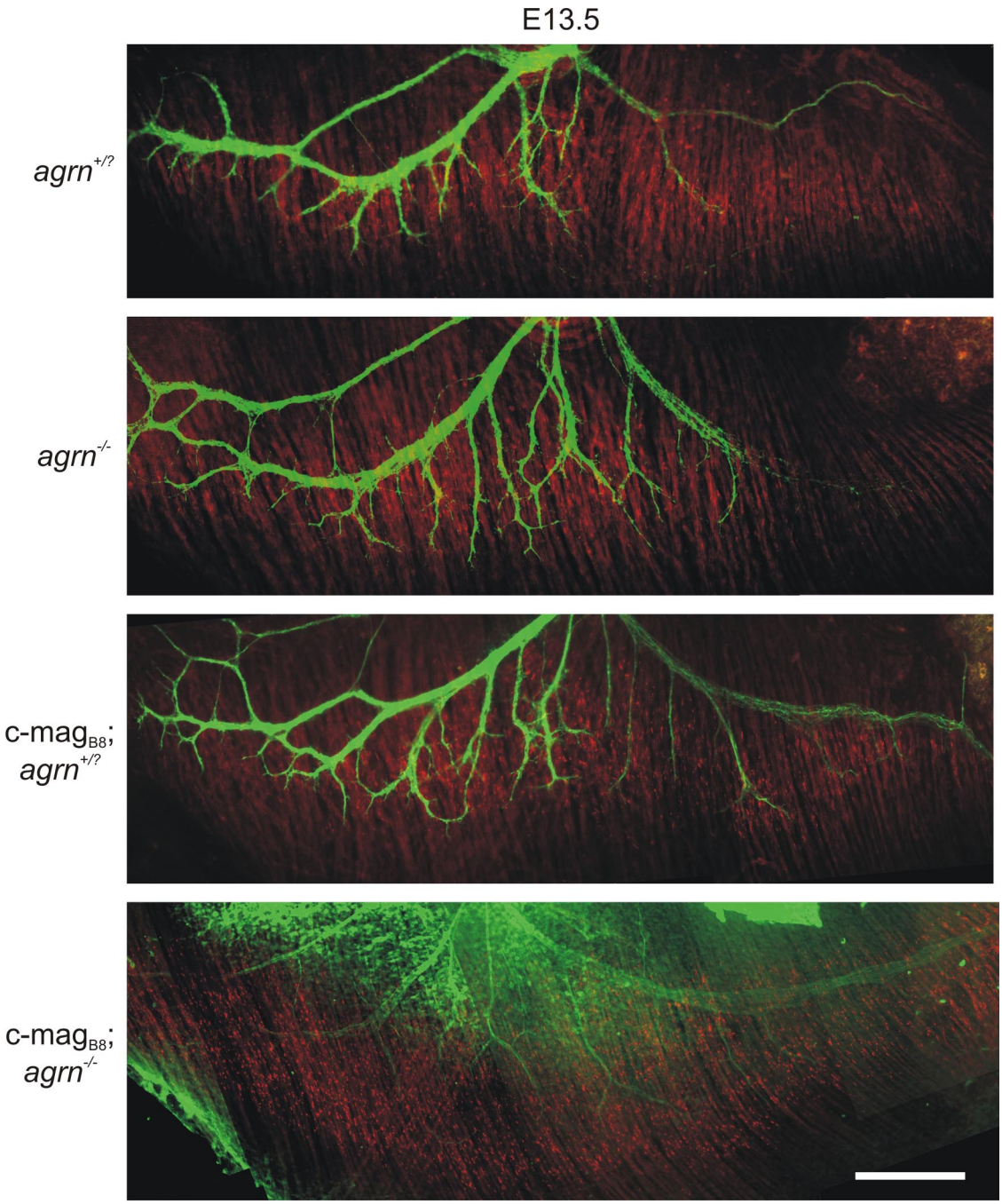



**Fig. S2.** Whole mounts of diaphragm muscles from E13.5. AChR clusters were visualized by Alexa-555-$\alpha$-bungarotoxin. Motor neurons are visualized either by YFP expression or by an antibody mixture against neurofilament and synaptophysin. (Scale bars: 250 $\mu$m.)

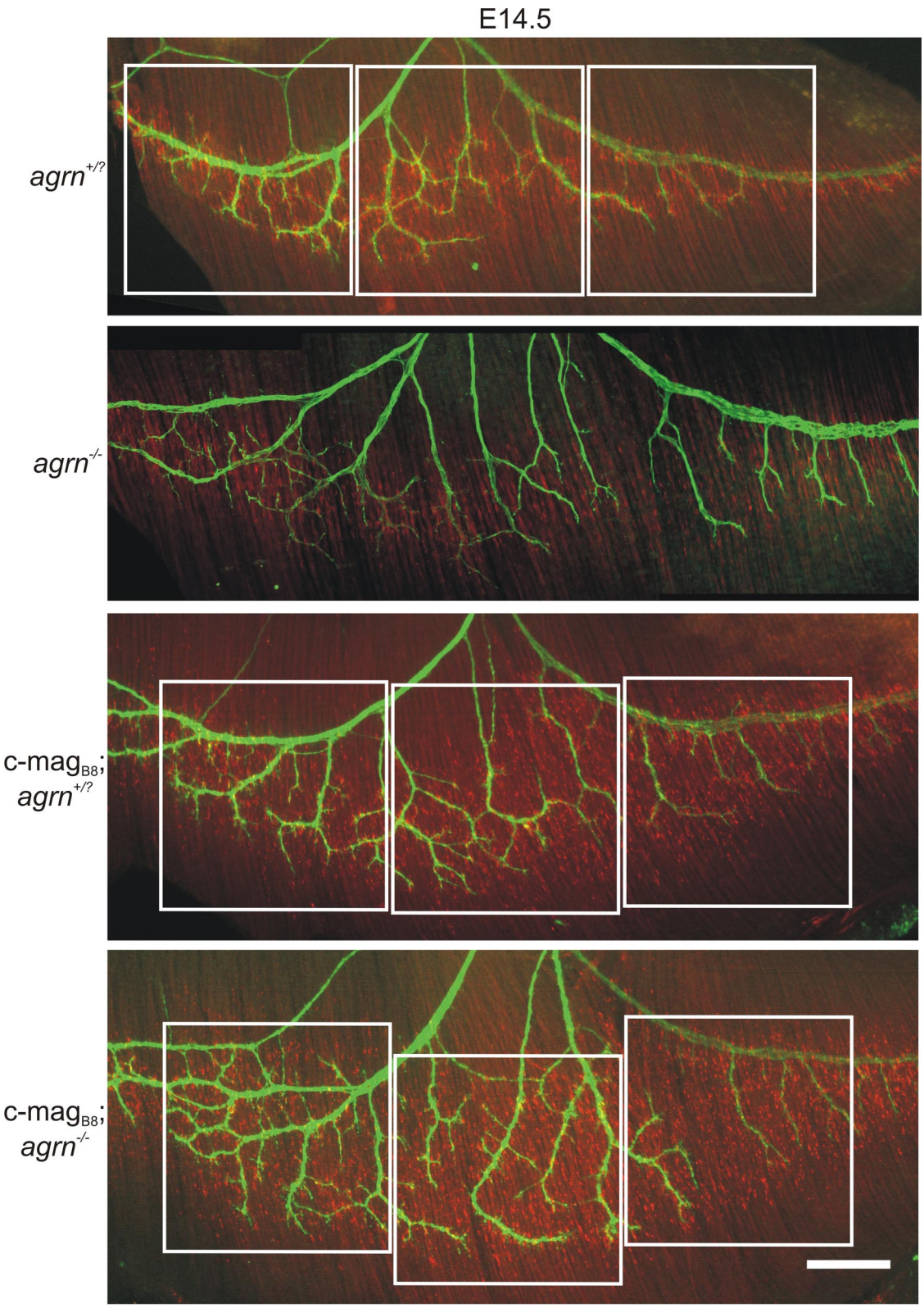



**Fig. S3.** Whole mounts of diaphragm muscles from E14.5. AChR clusters were visualized by Alexa-555-$\alpha$-bungarotoxin. Motor neurons are visualized either by YFP expression or by an antibody mixture against neurofilament and synaptophysin. White frames indicate the regions used for quantification shown in Fig. 3 and Fig. S6. (Scale bars: 250 $\mu$m.)

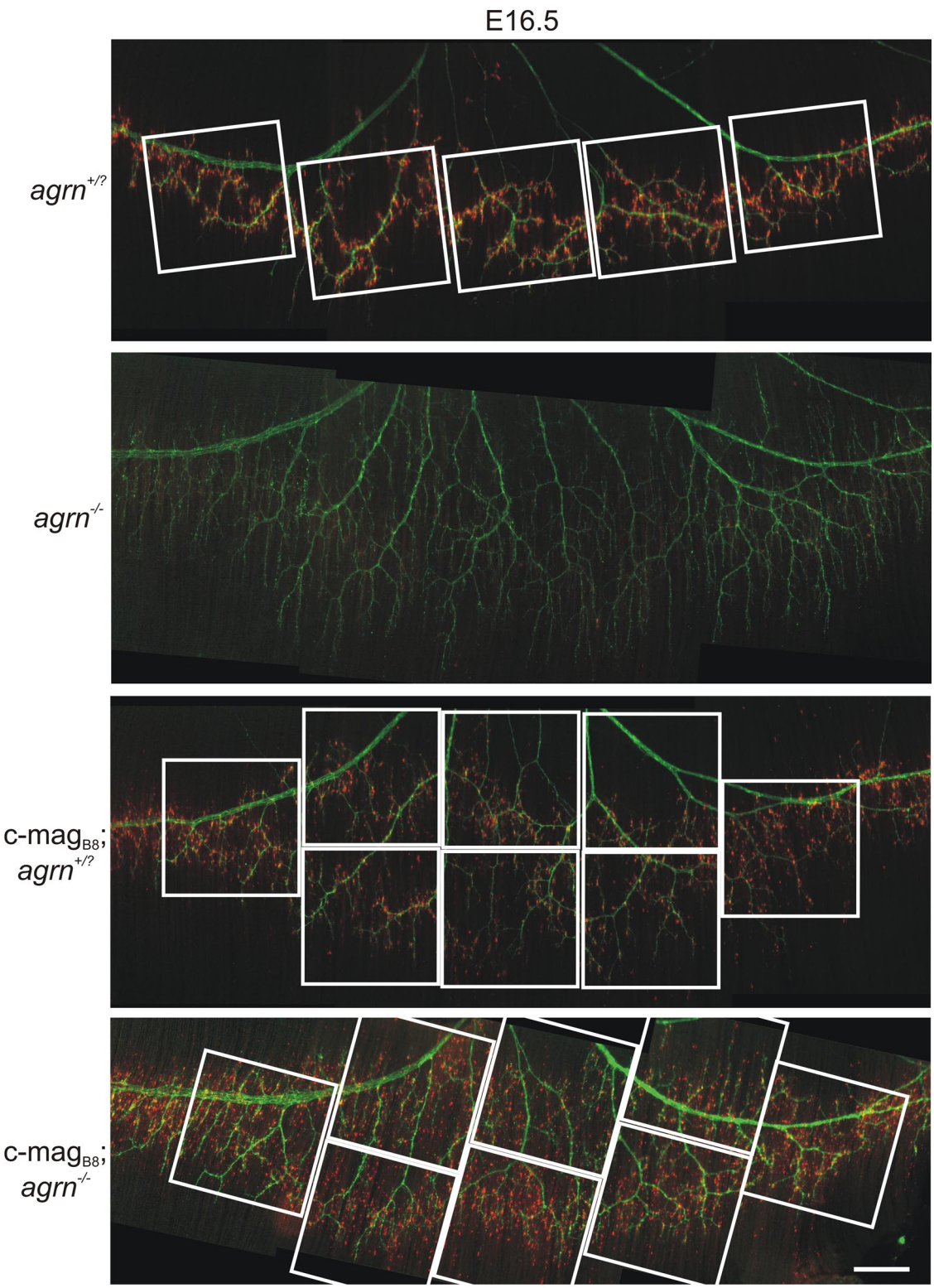



**Fig. S4.** Whole mounts of diaphragm muscles from E16.5. AChR clusters were visualized by Alexa-555-$\alpha$-bungarotoxin. Motor neurons are visualized either by YFP expression or by an antibody mixture against neurofilament and synaptophysin. White frames indicate the regions used for quantification shown in Fig. 3 and Fig. S6. (Scale bars: 250 $\mu$m.)

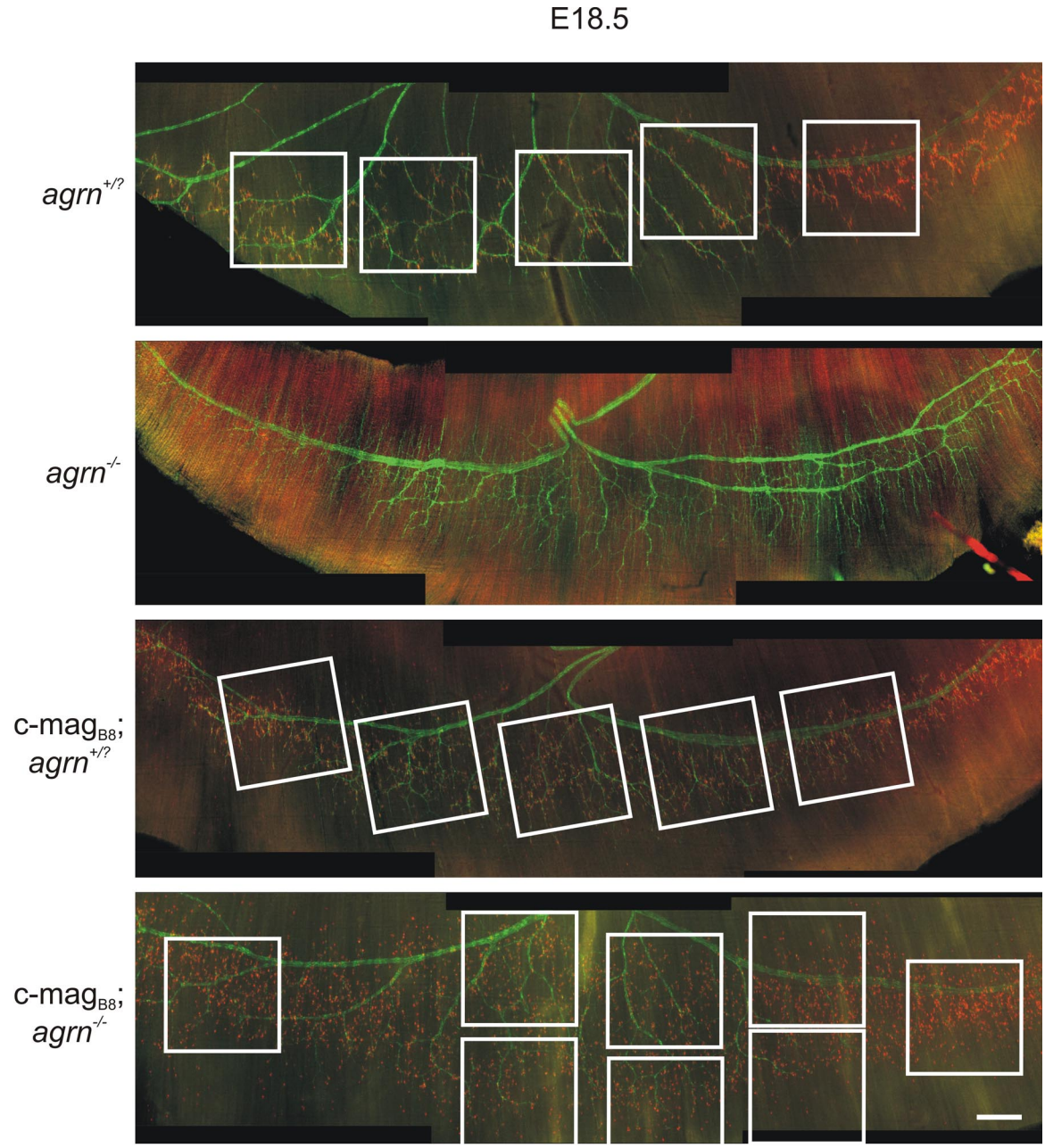



**Fig. S5.** Whole mounts of diaphragm muscles from AChR clusters were visualized by Alexa-555-$\alpha$-bungarotoxin. Motor neurons are visualized either by YFP expression or by an antibody mixture against neurofilament and synaptophysin. White frames indicate the regions used for quantification shown in Fig. 3 and Fig. S6. (Scale bars: 250 $\mu$m.)

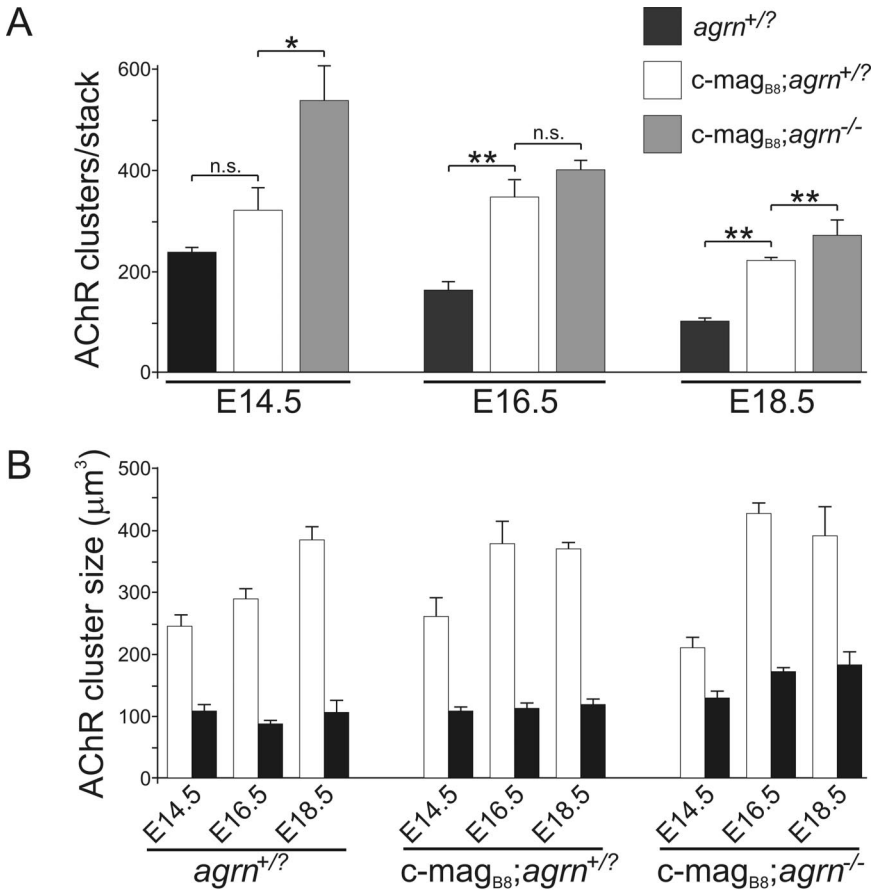



**Fig. S6.** (*A*) Total number of AChR clusters per stack and (*B*) the size of synaptic (open bars) and non-synaptic (filled bars) AChR clusters. Parameters were set the same as those used for Fig. 3. *P* values (two tailed Student's *t* test): **: $P \leq 0.01$; *: $P \leq 0.05$; n.s.: $P > 0.05$.

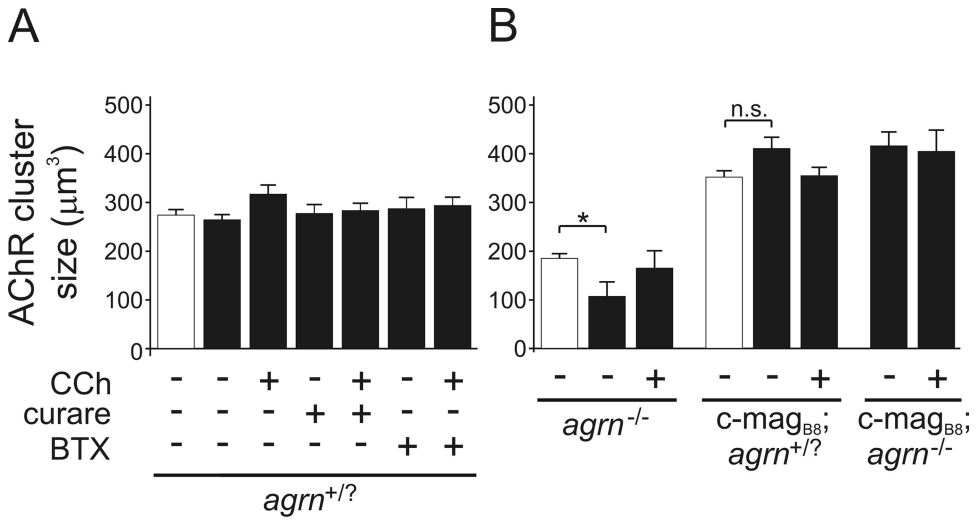
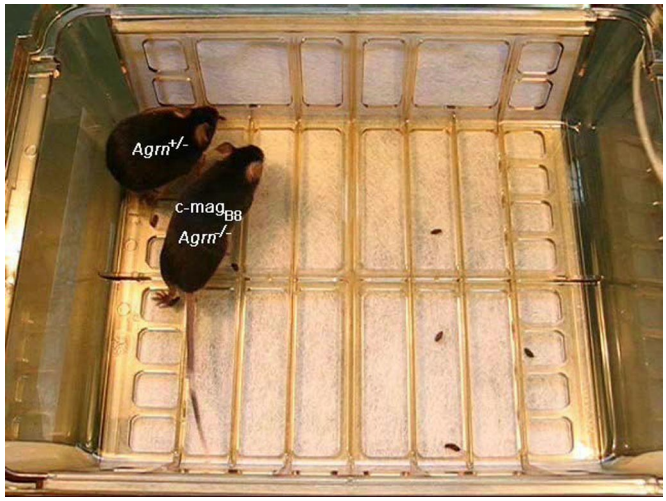



**Fig. S7.** The size of AChR clusters was quantified before (open bars) and after cultivation (filled bars). Parameters were set the same as those used for Fig. 4. *P* values (two tailed Student's *t* test): *: $P \leq 0.05$; n.s.: $P > 0.05$.




**Movie S1.** Transgenic expression of $mag_{B/z8}$ in muscle prevents perinatal death of agrin mutant mice. A video recording of two 8 week-old littermates: the rescued mouse (c-$mag_{B8}$; *agrn*$^{-/-}$) behaves the same as the control (*agrn*$^{+/-}$).

Movie S1 (AVI)