Supplementary Methods

Constructs

shRNA\textit{ALL}5\textit{β}: To suppress expression of endogenous LL5\textit{β}, two siRNA sequences were selected, modified for expression as shRNA, and transduced into myotubes by lentiviral infection: One was sequence No. 2 from (Kishi et al., 2005), another (No. 4) was designed using the siRNA Selection Server at the Whitehead Institute at the Massachusetts Institute of Technology (http://jura.wi.mit.edu/siRNAext/), see (Yuan et al., 2004). The mouse genome was searched with selected sequences to ensure their specificity. ssDNA oligos were designed based on chosen targeting sequences according to the guidelines of the shRNA expression vector pLKO.1 (Sigma) and purchased from Microsynth (Balgach, Switzerland). After annealing, oligonucleotides were ligated into AgeI/EcoRI-digested pLKO.1. Clones were checked by restriction digest and sequencing. Lentiviral particles were produced in 293T cells by cotransfection of pLKO.1 with pMD2.G and pPAX2, which together encode all necessary lentiviral proteins. Targeting sequences cloned into pLKO.1 were shLL5\textit{β} No. 2: AAGCCTAAGACAGTCGTCAGA; shLL5\textit{β} No. 4: AATGGTAGCTTAGAGGAAGGA; shLL5\textit{β} scrambled: AACGTAATCGCGTACGACGAA; all sequences are denoted in sense orientation. To allow visualization of single shRNA-infected cells, pLKO.1 including a targeting shRNA sequence was modified by replacing the puromycin selection cassette between the BamHI and NsiI sites with cDNA of Histone2B–mRFP (a gift of E. Fuchs, Rockefeller University, New York) amplified by PCR using BglII- and NsiI-flanked primers, analogous to a strategy described elsewhere (Beronja et al., 2010).

\textit{CLASP2}: A C-terminal fragment of human CLASP2 was generated by PCR-amplification of CLASP2 aa 1017-1294, using EcoR1 and BamH1-flanked primers and ligation into pEGFP-C1 (Clontech). The pEGFP-C1 vector alone was used as the GFP control in the indicated experiments.

\textit{AChR-γ}GFP: To visualize AChR dynamics in real-time, GFP-tagged AChR-γ subunit (a gift from Veit Witzemann; described in (Gensler et al., 2001)) was subcloned into the shuttle vector pENTR1A (Invitrogen), using EcoR1, and then recombined into the adenoviral expression vector pAd/CMV/V5-DEST (Invitrogen), using the LR II Gateway
system (Invitrogen) according to the manufacturer’s instructions. AChRs comprising AChR-γ-GFP form ion channels with physiological gating and conduction properties of fetal AChRs; importantly when expressed in adult muscle or in cultured myotubes, such AChRs are incorporated, like wild type AChRs, into the synaptic muscle membrane and agrin-induced AChR clusters, respectively (Gensler et al., 2001).

**Generation and usage of adenoviral and lentiviral particles**

To generate adenoviral particles for protein expression in myotubes, adenoviral vectors were linearized by PacI digest to expose the viral ITR, precipitated, washed, resuspended and transfected into the adenovirus-producing 293Ad cells. The next day medium was exchanged and cells were allowed to accumulate adenoviral particles intracellularly for 10-14 days, until visible regions of cytopathic effect (CPE) were observed. Cells were then scraped off, centrifuged, the pellet was resuspended in PBS and subjected to four consecutive freeze/thaw cycles using methanol at -80°C and cell thawing at 37°C to release adenoviral particles into solution. Cell debris was collected by centrifugation at 3 krpm for 15 minutes at RT. The supernatant containing viral particles was used to again infect 293Ad cells, in order to amplify the viral stock to high titer. After 3-5 days, when visible regions of cytopathic effect were observed, cells were harvested and processed as described above. Finally, viral supernatants were aliquoted and stored at -80°C. In some cases, we were provided with ready-made adenoviral particles, which were either used directly or amplified in 293Ad cells as described above.

To generate lentiviral particles for RNAi-mediated gene silencing in myotubes and myofibers, the lentiviral vector pLKO.1 (Invitrogen) containing the respective targeting sequences was co-transfected with pPAX2 and pMD2.G, which together encode all necessary lentiviral proteins. Vectors were used in molar ratio 3:2:1. The next day medium was changed and 48 hours later, viral supernatant was collected. For usage on cells, lentiviral supernatant was concentrated 100x by using Lenti-X concentrator (Clontech) and viral suspensions in PBS were aliquoted and stored at -80°C.

**Antibodies and chemicals**
LL5β antibody was a gift from Joshua Sanes, Harvard University, and is described elsewhere (Kishi et al., 2005). Polyclonal rabbit anti-human EB3 has been described (Schmidt et al., 2012). All other antibodies were commercial products: anti-GFP (from chicken) was from Invitrogen, anti-β-Tubulin was from BD Bioscience, anti-phospho-GSK3β (Ser9), anti-total GSK3β, anti-phospho-AKT (Ser 473), anti-total AKT were from Cell Signaling. Secondary antibodies were Alexa-conjugated goat anti–rabbit, goat anti–mouse, or goat anti–rat antibodies (Invitrogen) as well as donkey anti–chicken (Jackson ImmunoResearch). HRP-conjugated secondary antibodies were from Santa Cruz. AChRs were labeled with α–BTX–Alexa488, -594, or -647 (Invitrogen). Ringer’s solution was obtained from Braun. Phosphatase inhibitors PIC1 and 2 were purchased from Sigma-Aldrich, Complete protease inhibitors from Roche and transfection reagent Fugene HD from Promega. Antibiotic/antimycotic solution was purchased from Gibco, basic FGF was purchased from Invitrogen. ZSTK474 (LC Laboratories) was used at 5 µM on cultured myotubes. All other reagents were applied in the following concentrations: Nocodazole (Sigma) 10µM, Cytochalasin D (Sigma) at 2 µm.

Immunoblotting.

Myotubes were lysed in 3x SDS-lysis buffer (150 mM Tris, pH 6.8, 300 mM DTT, 6% SDS, 0.2% bromophenol blue, and 30% glycerol) supplemented with phosphatase inhibitors (1:100), protease inhibitors (1/10 tablet per ml lysis buffer), and 100 mM DTT. Lysates were denatured at 95°C for 5 min and separated by SDS-PAGE. Gels were electro-transferred onto PVDF membranes and developed with ECL after incubation with primary and secondary antibodies.
**Supplemental Figure Legends**

**Supplemental Figure S1: Knockdown of LL5β abolishes MT capturing at AChR clusters**

Primary myotubes cultured on agrin patches were infected with scrambled and shLL5β lentivirus, stained for AChRs (blue), anti-EB3(red), and anti- LL5β (red), and imaged under identical acquisition settings. Note the depletion of LL5β and the loss of EB3 enrichment at clusters (enlarged box 1) vs. outside clusters (enlarged box 2) upon knockdown. Scale bars = 10 μm, inset scale bar = 2.5 μm

**Supplemental Figure S2: CLASP2-C expression reduces AChR cluster size in myotubes**

Primary myotubes cultured on agrin patches were transfected with the indicated constructs, and AChR cluster size and density was quantified in GFP-expressing cells. A) Representative images from myotubes expressing GFP or GFP-CLASP2-C and stained with α-BTX-594. Clusters are outlined in white. Scale bar = 20μm

B) Quantification of AChR cluster size. Bars depict means ± s.e.m.; N = 50 clusters for GFP, 42 clusters for GFP-CLASP2-C; **=p<0.01.

**Supplemental Figure S3: LL5β at the NMJ postsynaptic membrane increases AChR density**

A) LL5β is recruited via its PH domain to the synaptic membrane. Soleus muscles were electroporated with expression constructs for WT-LL5β-GFP (wild type) or a LL5β (Mut-LL5β-GFP). Two weeks later, muscles were stained for AChRs (red), and NMJs were identified in cryosections by their α-BTX staining. GFP fluorescence at synaptic AChR clusters relative to that in nonsynaptic membrane was taken as a read-out for LL5β localization. Scale bars: 25 μm, inset: 5 μm. Graph shows ratio of relative GFP fluorescence at NMJs in fibers electroporated with the respective constructs. N = 8 each; *=p<0.05.
B-D) The density of synaptic AChRs is reduced at NMJs upon knockdown of LL5β. Histone2B-RFP expressing pLKO.1-shLL5β or pLKO.1-shScrambled (control) were electroporated into sternomastoid muscles, and muscles were stained to saturation with α-BTX-Alexa 488 and analyzed as outlined in scheme B).

C) Examples of synapses imaged (in pseudocolour) and nuclei marked by RFP in their subsynaptic nuclei used for identification of successfully electroporated fibers. Scale bar = 10 µm.

D) Graphs summarizing the effect of LL5β knockdown on synaptic AChR density. Black numbers in columns give numbers of synapses analyzed, bars give average AChR density relative to that at t = 0. Bars ± s.e. are given. *** = p < 0.0001, two sided t-test.

Supplemental Figure S4: CytoD treatment does not interfere with signaling cascade linking agrin to CLASP2-dependent MT capture.

A) Western blots showing that agrin-induced levels in phospho-AKT and in phospho-GSK3β are not affected by pretreating myotubes with CytoD. This indicates that PIP3 levels required for LL5β recruitment to cluster membrane and phospho GSK3β (inactive GSK3β) levels required for CLASP2-dependent MT capture are unchanged.

B) CytoD treatment does not abolish of LL5β recruitment to agrin-induced AChR clusters. Primary myotubes stained with α-BTX-594 (red) and anti-LL5β, 1 hour after CytoD treatment. Scale bars = 7.5 µm

Supplemental Figure S5: Intracellular AChRs are transported along dynamic MTs at AChR clusters

Volume rendering of an image stack of 6 slices taken at 0.11 µm step-size on a Structured Illumination microscope. Intracellular AChRs (red) in primary myotubes cultured on agrin substrate were visualized using α-BTX-647. Dynamic MTs were visualized by immunostaining with Tyrosinated tubulin (green). Arrowheads indicate colocalization of internal AChRs with dynamic MTs. Scale bars = 5 µm
**Supplementary Movie Legend 1:**

**Dynamics of AChR vesicle fusion at agrin-induced clusters in primary myotubes.**

Time-lapse video of control myotube (see Figure 6) expressing AChR-γGFP and scrambled shRNA imaged with TIRF microscopy after photobleaching (movie is accelerated to 7 frames per second). Vesicle fusion events (numbered blue circles) were identified by automated tracking software and visualized with the MtrackJ plugin for ImageJ (see Materials and Methods). Note that vesicle fusion appears to occur mostly as discrete events, although some show lateral movement within the bleach region as well. Frames were acquired every 300 ms for 30 s, using a 100X APO Tirf objective from Nikon (NA=1.49) on a Nikon Ti Eclipse microscope. Bleaching was performed for 585 milliseconds. Images are inverted for better visualization of fusion events.

**Supplementary Movie Legend 2:**

**AChR vesicle fusion is reduced upon LL5β knockdown.**

Video of myotube (see Figure 6) expressing AChR-γGFP and LL5β shRNA imaged with TIRF microscopy after photobleaching. Note the decrease in number of fusion events upon LL5β depletion. Imaging, acquisition, and analysis parameters were the same as in movie 1.
Supplementary References

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FIGURE S1
FIGURE S2
FIGURE S3
A

| agrin 30 min + CytoD 30min | agrin 60 min | agrin 30min | ctrl |
|-----------------------------|--------------|-------------|------|
| phospho-GSK3 β              | pan GSK3 β   | phospho-AKT | pan AKT |

B

ctrl | AChR | LL5β

cytoD

FIGURE S4
