Figure S1. Synapse-specific expression of musk and achr α- and ε-subunit genes does not require neuromuscular NRG/ErbB signaling. In situ hybridization experiments with 35S-labeled riboprobes specific for the respective mRNAs show synaptic localization both in erbb2/4−/− and in wild-type mice. Cross sections processed in the same experiments are shown. Bar, 25 µm.
Figure S2. Double staining of AChR puncta with α-BTX-Alexa 594 and with Mab35 in unpermeabilized muscle resolves changes in AChR migration vs. changes in AChR internalization. Sternomastoid muscle was labeled with α-BTX-Alexa 594 24 h before bathing for 8 h with or without 160 µM dynasore, a drug blocking dynamin-dependent endocytosis (Macia et al., 2006). Surface AChRs were then immunostained without permeabilization using Mab35, and the numbers of puncta stained with α-BTX-Alexa 594, with Mab35, or with both were compared. (a) Labeling protocol for assessing the perisynaptic accumulation of AChRs. (b) High resolution image of an NMJ doubly labeled with α-BTX-Alexa 594 and anti-AChR antibody Mab35, but not treated with dynasore. Few colocalizing fluorescent puncta in the perisynaptic membrane (marked by arrows), and many internalized inside the muscle (asterisks) are seen. (c) High resolution image of a dynasore-treated NMJ (staining as in b) showing increased accumulation of fluorescent puncta in the perisynaptic membrane (marked by arrows) and fewer puncta internalized (marked by asterisks). Thus, the AChR double labeling used resolves changes of AChR internalization from the perisynaptic membrane (here inhibition by dynasore). (d) Graph summarizing quantification of AChR puncta shows an increase in surface puncta and a decrease in internalized puncta in dynasore-treated (31 synapses from 3 animals) compared with control muscle (28 synapses from 2 animals). Bars, 5 µm. (e) Experimental protocol to ascertain effective inhibition of transferrin (Tf) uptake by dynasore in muscle. Sternomastoid muscle was exposed to Tf-Alexa 488 in the presence or absence of 160 µM dynasore. 4 h later, NMJs were stained with α-BTX-Alexa 594 and surface-bound Tf was removed by ice-cold acetic acid/NaCl (0.2 M/0.5 M). Green puncta show Tf bound to internalized TfR. The smaller number of green puncta in dynasore-treated muscle reflects decreased endocytosis of TfR. Bars, 5 µm. (f) Quantitative evaluation of internalized Tf-containing vesicles in the absence or presence of dynasore (21 synapses from 2 nontreated animals compared with 24 synapses from 2 treated animals). (g) Dynasore used before on muscle still inhibits endocytosis of transferrin receptor in COS-1 cells. Cells previously exposed to fluorescent Tf (Alexa 647) on ice were bathed with dynasore that had been used on the sternomastoid muscle, for 15 min (37°C). Surface-bound Tf was removed. Note that dynasore almost completely inhibits endocytosis of Tf-Alexa 647 (red), indicating that the dynasore is still pharmacologically active. Bars, 25 µm. (h) Quantitative assessment of endocytosis of Tf-Alexa 647 in the absence or presence of 160 µM dynasore pre-used on muscle. Quantitative fluorescence analysis was performed as described previously (Hardel et al., 2008).
Figure S3. **Pharmacological inhibition of ErbB2 and ErbB4 receptor kinase activity in wild-type muscle increases AChR puncta containing recycled, but not preexisting AChRs in the perisynaptic membrane.** (a) Labeling method for assessing the accumulation of AChRs in the perisynaptic membrane. Immediately after labeling, recycled receptor muscles were bathed with ErbB blockers AG1478 (ErbB4) and AG879 (ErbB2) for 8 h. Then surface receptors were immunostained with the anti-AChR antibody Mab35 (green) without permeabilization. Superficial synapses were imaged and the number of membranes localized as well as intracellular puncta containing preexisting (blue) and recycled (red) receptors was determined. High resolution image of a portion of synapse from treated compared with untreated muscle shows more colocalized recycled (red) AChR puncta in the muscle surface (green). However, the number of blue puncta containing preexisting AChRs remains roughly unchanged in untreated and treated muscles, indicating that inhibition of ErbB kinase activity promotes the selective migration of recycled AChRs from the synaptic sites. Bars, 5 µm. (b) Graphs summarizing quantifications of fluorescent puncta containing recycled and preexisting AChRs in the perisynaptic membrane (40 control synapses from 3 muscles, and 42 synapses from ErbB-blocked synapses from 4 muscles analyzed). Bars, 5 µm. (c and d) Endplates in wild-type muscle pharmacologically blocked for 8 h and acutely stained with α-BTX–Alexa 594 show an increased number of perisynaptic AChR puncta (40 NMJs from 3 nontreated and 44 NMJs from 4 treated mice) and formation of AChR streaks extending from synaptic into perisynaptic membrane (201 NMJs from 2 nontreated and 151 NMJs from 2 treated mice.). Bars, 5 µm.
Figure S4. **Rapsyn and utrophin co-migrate with AChRs to the perisynaptic membrane.** (a) Sternomastoid muscles from erbb2/−/− mutants 8–10 wk of age were stained acutely with α-BTX–Alexa 594 and after permeabilization with antibodies for rapsyn and utrophin. Note complete colocalization of perisynaptic AChR puncta with rapsyn and utrophin, respectively. Bars, 2.5 µm. (b) Perisynaptic AChR puncta are increased in acutely stained erbb2/−/− mutants (n = 28–32 synapses from 3 animals). (c) Increased number of AChR puncta in erbb mutant synapses colocalize with rapsyn and utrophin, suggesting increased loss of subsynaptic components to the perisynaptic membrane. Graph shows percent colocalization (n = 9–10 synapses).
Figure S5. **Phosphorylation of α-DB1 in C2C12 myotubes is blocked by the ErbB2 blocker AG879 but not by the ErbB4/EGFR blocker AG1478.** All samples were incubated with peroxivanadate and NRG1β either in the absence of blockers or with ErbB2 (AG879) or ErbB4/EGFR (AG1478) blocker and then subjected to immunoprecipitation with anti-syntrophin antibody SYN 1351. Left, 4G10 blot; right, blot with α-DB1 antibody. Unlike in Fig. 7, the samples were subject to centrifugation at 12,000 g for 10 min to reduce background, which, after treatment with AG879, appears to result in the removal of the 89-kD α-DB1 as well as of some other proteins but not of the 75-kD α-DB1 (right) or syntrophin.

References
