Supplementary Fig. 1

% residual surface $^{125}\text{i-}\alpha\text{-BT}$

Time (hours)

- `src-/-;fyn-/-`
- `src-/-;fyn-/- + Cholesterol`
- `wild-type`
Supplementary Data

Legend to Supplementary Figure 1:

**Turnover of AChRs is not affected by the absence of SFKs or by cholesterol treatment.** The degradation rate of surface AChRs was analyzed in wild-type and src-/;fyn-/ myotubes using $^{125}$I-$\alpha$-BT according to an established protocol (Wang et al., 1999). Cells were treated with $^{125}$I-$\alpha$-BT for 90 min to label AChRs at the surface, washed, and incubated at 37°C. The radioactivity released into the medium (originating from AChR degradation) was measured at the times indicated, and the data were used to calculate the turnover rates. Cholesterol was added overnight before start of the experiment, and was present throughout the experiment. src-/;fyn-/ myotubes did not tolerate prolonged cholesterol treatment (i.e. beyond the 20 h time point). Data reflect mean ± SEM from n=5 experiments. The data show, in agreement with Wang et al. (1999), that the half-life time of AChRs in wild-type myotubes is about 35 h. This turnover is not affected in src-/;fyn-/ myotubes. Furthermore, cholesterol treatment has no effect on the turnover of AChRs in the mutant cells.

Legend to Supplementary Figure 2:

**The overall cellular distribution of AChRs is not affected by the absence of SFKs, or treatments with cholesterol or MβCD that influence cluster stability.** C2C12 (C2), src-/;fyn-/ and wild-type myotubes were incubated with 50 μM cholesterol overnight or with 5 mM MβCD for 1.5 h as indicated. AChRs at the surface and in intracellular compartments were quantitated using $^{125}$I-$\alpha$-BT. For surface AChRs, para-formaldehyde-fixed cells were incubated with $^{125}$I-$\alpha$-BT; for labeling of intracellular AChRs, cells were first incubated with excess cold toxin to saturate surface binding sites, then washed, fixed with para-formaldehyde, permeabilized with Triton X-100 and incubated with $^{125}$I-$\alpha$-BT. Surface AChRs were expressed as % of all (= surface + intracellular) receptors (mean ± SEM from n=3 experiments). The data show, in
agreement with Moransard et al. (2003), that about 80% of all AChRs are at the surface in C2C12 and wild-type myotubes. This is not affected by the treatments with MβCD or cholesterol, or by the absence of SFKs in src⁻/⁻:fyn⁻/⁻ myotubes.

Methods:

**AChR turnover.** Metabolic turnover of AChRs was assayed as described earlier by Wang et al., 1999. Cells were incubated with 5 nM ¹²⁵I-α-BT (150 Ci/mmol, Amersham Biosciences, Freiburg, Germany) for 90 min to label AChRs at the surface, and unbound toxin was removed by washing twice with PBS. Growth medium was added and myotubes were returned to the incubator. At the indicated time points thereafter, duplicate aliquots of medium were taken and replaced with fresh medium. After removal of the last aliquots (29 h), cells were solubilized using 1% NP-40 in PBS. Radioactivity in both the aliquots and cells was measured using a gamma counter (1282 CompuGamma, LKB Wallac). The total radioactivity on the cell surface after labeling at the beginning of the experiment was calculated by addition of that found in the medium and solubilized cells and set to 100%. Cell-associated radioactivity was calculated accordingly for each time point and plotted, revealing appropriate degradation kinetics. Nonspecific binding of ¹²⁵I-α-BT, determined by pre-incubation with excess (10 μM) cold toxin, was low and subtracted. In experiments with cholesterol, cholesterol (50 μM) was added overnight before start of the experiment, and was present throughout the experiment.

**Distribution of AChRs between surface and intracellular compartments.** To label AChRs at the myotube surface, cells were fixed with 4% para-formaldehyde and incubated with 2.5 nM ¹²⁵I-α-BT for 30 min. For labeling of intracellular AChRs, cells were first incubated with 10 μM cold α-BT to saturate surface binding sites, then washed with PBS, fixed with 4% para-formaldehyde, permeabilized with 0.1% Triton X-100, and incubated with 2.5 nM ¹²⁵I-α-BT for 30 min. After radioactive labeling, all cells were washed with PBS to remove unbound toxin, scraped into eppendorf tubes and counted in a gamma counter (1282 CompuGamma, LKB Wallac). Non-specific binding of ¹²⁵I-α-
BT, determined by pre-incubation of cells with 10 μM cold toxin, was low in all cases (less than 4%) and subtracted. Surface AChRs were calculated as percentage of total AChRs (= surface + intracellular). We obtained the same results when surface AChRs were assayed on unfixed cells.

References:
