Supplementary Methods

Golgi-Cox staining

We used a modified Golgi staining method adapted from the protocols used by Bayram-Weston et al. (1). *Ank3* cKO mice were sacrificed and then perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brain was removed, incubated for 24 h at 4°C in 4% paraformaldehyde, and dehydrated for 48 h at 4°C in PBS containing 30% sucrose. Subsequent procedures were carried out according to the manufacturer's instructions for FD Rapid GolgiStain Kit (FD Technologies, Columbia, MD, USA). Mounted sections of 120 μm thickness were imaged on a microscope (LSM 510; Carl Zeiss) using a 63x oil lens with AxioCam software from Zeiss (Oberkochen, Germany). For apical dendritic spine quantification, only the first branch of each apical dendrite over 30 μm away from cell body was included for the analysis of spine linear density (# of spines/10 μm dendritic length). For dendrite analysis, images were acquired on a Nikon (Amsterdam, Netherlands) Ti2 wide field microscope using a 20x lens over the full slice. Traces of dendrites were drawn and analyzed with Sholl analysis in ImageJ.

Plasmids and antibodies

GFP-Ankyrin-G-190, RFP-Ankyrin-G-270, and EB3-dtTomato were purchased from Addgene (Watertown, MA, USA; #31059, #42566, #50708). RNAi constructs were purchased from Origene (Rockville, MD, USA) in the pGFP-V-RS vector with a turboGFP element to enable identification of transfected cells. The target sequence used was GGCAGAACGAGACGCCAAGTGGAAGCCTA. RNAi-resistant GFP-Ankyrin-G-190, RFP Ankyrin-G-270 and C70A mutant (2) constructs were generated using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA) by inserting three non-coding mutations into the RNAi target sequence. All constructs were sequenced for verification. pEGFP-N2 and pmCherry-C1 (Clontech, Mountain View, CA, USA; now Takara Bio USA, Inc.) were overexpressed in cultured neurons as a cell field. The following primary monoclonal and polyclonal antibodies (mAb and pAb, respectively) were used: AnkG mAb clone N106/20 (western blot) and clone N106/36 (immunocytochemistry) from Neuromab (Davis, CA, USA), PSD-95 (Neuromab), turboGFP mAb (to identify RNAi expression, Origene,), DsRed pAb (to identify mCherry expression, Clontech, Mountain View, CA, USA), and GFP chicken pAb (to identify GFP expression, Abcam, Cambridge, MA, USA).

Neuronal cell culture and transfection

Dissociated cultures of primary cortical neurons were prepared from E18 Sprague-Dawley rat embryos. Brains were dissected in ice-cold Hank's buffered salt solution, and cortical tissue isolated, digested with papain (Sigma; diluted in Neurobasal with EDTA (0.5 mM) and DNaseI (2 units/mL), activated with L-cysteine (1 mM) at 37°C), and mechanically dissociated in neuronal feeding media (Neurobasal + B27 supplement (Invitrogen, Carlsbad, CA, USA) + 0.5 mM glutamine + penicillin/streptomycin). Dissociated neurons were plated at high density (300 000 cells/cm^2) on 1.5 mm thickness glass coverslips of 18 mm diameter (Warner instrument, Hamden, CT, USA)) coated polylysine. One h after plating, media was replaced. Neuronal cultures were maintained at 37°C in 5% CO_2. Neuronal feeding medium was supplemented with 200 μM D,L-amino-phosphonovalerate (D,L-APV, Ascent Scientific, Bristol, UK) beginning on day 4 in vitro (DIV4). Neurons were transfected at DIV21 with Lipofectamine 2000 (Invitrogen), providing a transfection efficiency of 0.5-1%. Plasmids (1-10 μg total DNA) and Lipofectamine 2000 were diluted in Dulbecco's Modified Eagle Medium + HEPES (10 mM), mixed thoroughly together, and incubated for 20-30 min at 37°C before addition to cultured cells. Following transfection, neurons were supplanted in antibiotic-containing feeding media containing half conditioned and half fresh media, and allowed to express constructs for 3 days or as indicated.

Immunocytochemistry

Cells were fixed for 10 min in 4% formaldehyde/4% sucrose in PBS and then in methanol pre-chilled to -20°C for 10 min. Fixed neurons were permeabilized and blocked simultaneously in PBS containing 2% normal goat serum and 0.2% Triton-X-100 for 1 h at room temperature. Primary antibodies were added in PBS containing 2% normal goat serum overnight at 4°C, followed by 3 x 10 min washes in PBS. Secondary antibodies were incubated for 1 h at room temp, also in 2% normal goat serum in PBS. Three further washes (15 min each) were performed before coverslips were mounted using ProLong antifade reagent (Life Technologies).

Confocal microscopy

Confocal images of immunostained neurons were obtained with a Nikon (Amsterdam, Netherlands) C2 confocal microscope. Images of neurons were taken using the 63x oil-immersion objective (numerical aperture (NA) = 1.4) as z-series of 7-12 images, averaged 2 times, taken at 0.4 μm intervals, with 1024x1024 pixel resolution. Detector gain and offset were adjusted in the channel of each cell fill (GFP or mCherry) to include all spines and enhance edge detection. Intensity plot profiles for dendrite/spine localization were performed in ImageJ. A 4 μm line was

drawn across 3-5 spines per neuron and averaged across neurons to produce average intensity plot profiles ± SEM. Spine density, width, and length were analyzed with ImageJ. Epifluorescence images were obtained with a 10x objective, and traces of dendrites were drawn and analyzed with Sholl analysis in ImageJ. In culture, pyramidal neurons conserve their polarity and develop a long dendrite, which we refer to as the apical dendrite, and shorter dendrites near the soma, which we refer to as basal dendrites for Sholl analysis.

Structured illumination microscopy imaging and analysis

Imaging and reconstruction parameters were empirically determined with the assistance of the expertise in the Nikon Imaging Center at Northwestern. Acquisition was set to 10 MHz, 14 bits with EM gain and no binning. Auto-exposure was kept between 100-300 ms, and the EM gain multiplier restrained below 300. Conversion gain was held at 1x unless necessary to increase signal with 2.4x. Laser power was adjusted to keep the look-up table (LUT) within the first quarter of the scale (<4000). Three reconstruction parameters (illumination modulation contrast, high resolution noise suppression and out of focus blur suppression) were extensively tested to generate consistent images across experiments without abnormal features or artifacts and produce the best Fourier transforms. Reconstruction parameters (0.96, 1.19, and 0.17) were kept consistent across experiments and imaging sessions. 3D dendritic spine reconstruction was done on a NIS instrument (Nikon). Intensity plot profiling for dendrite analysis was performed with ImageJ (National Institutes of Health, *http://rsbweb.nih.gov/ij*). A punctate distribution at the membrane corresponds to a larger fluctuation in fluorescence intensity, which will result in a larger standard deviation (Puncta index).

Acyl biotinyl exchange assay

For acyl biotinyl exchange (ABE) experiments, rat cortical neurons cultured as described above were lysed directly in buffer (50 mM HEPES pH 7.0, 2% SDS, 1 mM EDTA plus protease inhibitor mixture (PIC, Roche) and 20 mM MMTS (to block free thiols). Following lysis, excess MMTS was removed by acetone precipitation. Pellets were dissolved in buffer containing 4% (wt/vol) SDS. Samples were diluted and incubated for 1 h in either 0.7 M hydroxylamine (NH2OH) pH 7.4 (to cleave thioester bonds) or 50 mM Tris pH 7.4, both containing sulfhydryl-reactive (HPDP-) biotin, and incubated for 1 h at room temperature. Acetone precipitation was performed to remove unreacted HPDP-biotin and hydroxylamine and pellets were resuspended in lysis buffer without MMTS. SDS was diluted to 0.1% (wt/vol) and biotinylated proteins in the samples were affinity-purified using neutravidin-conjugated beads. Beta-mercaptoethanol [1% (vol/vol)] was used to cleave HPDP-biotin and release purified proteins from the beads. The released proteins in the

supernatant were denatured in SDS sample buffer and processed for SDS-PAGE. Adult rat forebrain was dissected, rapidly cooled in ice-cold recording buffer (3) and homogenized in 10 volumes of 4 mM HEPES, 320 mM sucrose, pH 7.4, containing fresh PIC and 20 mM MMTS. Samples were centrifuged to remove debris, brought to room temperature, and SDS was added to 1% (v/v) final concentration. Samples were then centrifuged at 27,000 x *g* for 30 min at 4°C and supernatants subjected to acetone precipitation and ABE as described above.

Statistical analysis

All statistical tests were performed with GraphPad Prism. Data were tested for normality with D'Agostino and Pearson methods to determine use of non-parametric (Mann-Whitney, Kruskal-Wallis, Spearman correlations) or parametric (unpaired t-test, ANOVA, Pearson correlations) tests. Post-hoc tests were included in analyses with multiple comparisons. Bar graphs the mean ± SEM, unless otherwise noted. Differences were considered significant if $p \leq 0.05$. N values refer to number of cells per condition, unless otherwise stated.

Supplementary References

1. Bayram-Weston Z, Olsen E, Harrison DJ, Dunnett SB, Brooks SP. Optimising Golgi-Cox staining for use with perfusion-fixed brain tissue validated in the zQ175 mouse model of Huntington's disease. J Neurosci Methods. 2016;265:81-8.

2. He M, Jenkins P, Bennett V. Cysteine 70 of ankyrin-G is S-palmitoylated and is required for function of ankyrin-G in membrane domain assembly. J Biol Chem. 2012;287(52):43995-4005.

3. Thomas GM, Hayashi T, Chiu SL, Chen CM, Huganir RL. Palmitoylation by DHHC5/8 targets GRIP1 to dendritic endosomes to regulate AMPA-R trafficking. Neuron. 2012;73(3):482-96.

Figure Supp. 1

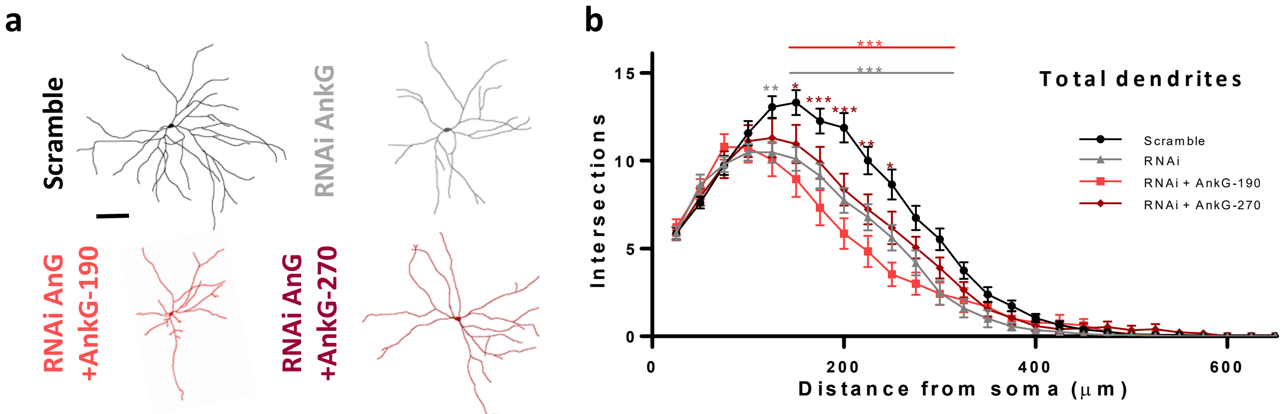

Figure S1. (**a**) Representative traces of cultured rat neurons expressing scramble or AnkG RNAi with GFP, GFP-AnkG-190, or GFP-AnkG-270 for three days, scale=100 µm. (**b**) Sholl analysis graph for total dendrites showing a decrease in dendrite complexity with AnkG-190 (17 to 23 neurons, 2-way ANOVA with Dunnett's post-test, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, ±SEM).

Figure Supp. 2

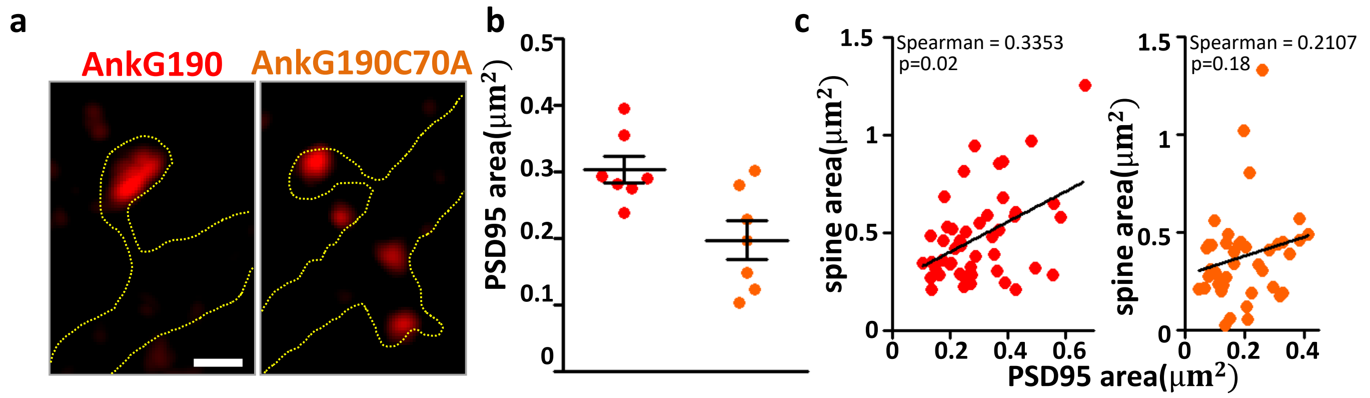

Figure S2. (**a**) SIM images of PSD-95 from neurons expressing GFP-AnkG-190 and GFP-AnkG-190-C70A, scale=0.5 μm. (**b**) Scatter plot showing a decrease of PSD-95 area in spines with C70A mutation compared with WT AnkG-190 (n=7 neurons, Mann Whitney, *p≤0.05). (**c**) Correlation plot of spine area vs. PSD-95 area (40 to 50 spines) with AnkG-190 or AnkG-190-C70A overexpression.

Figure Supp. 3

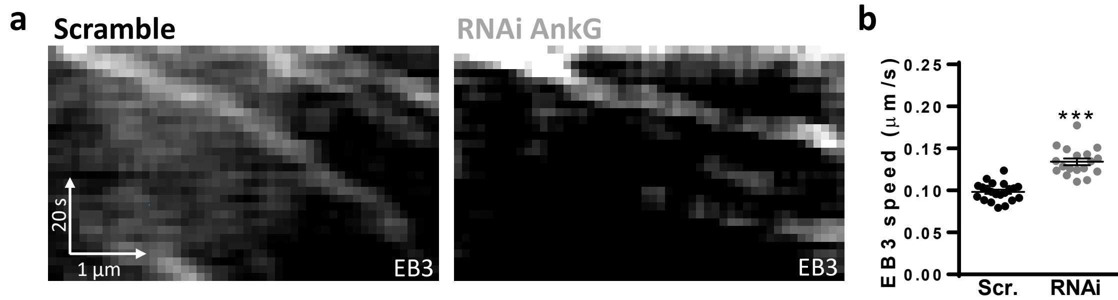

Figure S3. (**a**) Representative kymographs of axons from cultured rat neurons expressing for three days EB3-tdTomato plus either scramble or AnkG RNAi. (**b**) Scatter plot showing an increase in EB3-GFP speed in AnkG knockdown axons (n=18-21 neurons, t-test, ***p≤0.001).