Figure S1

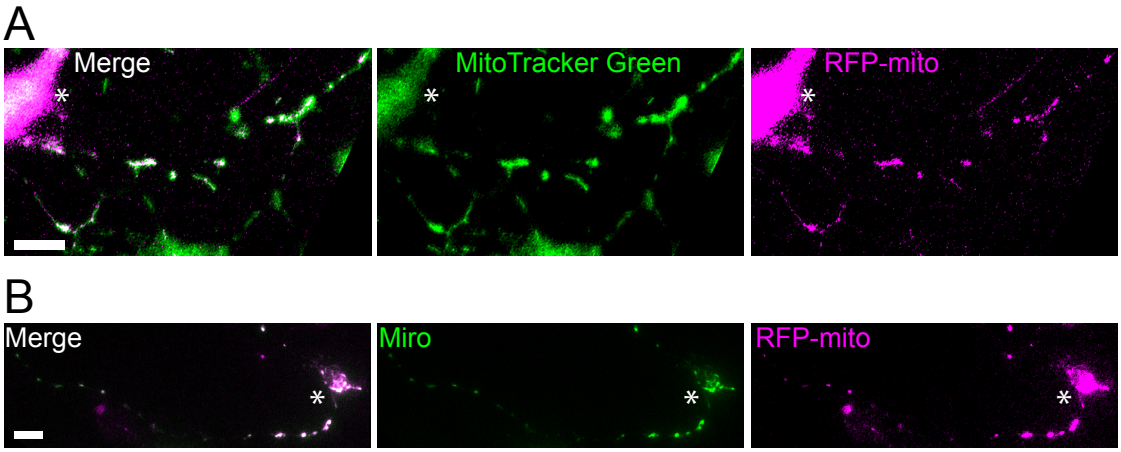


Figure S2

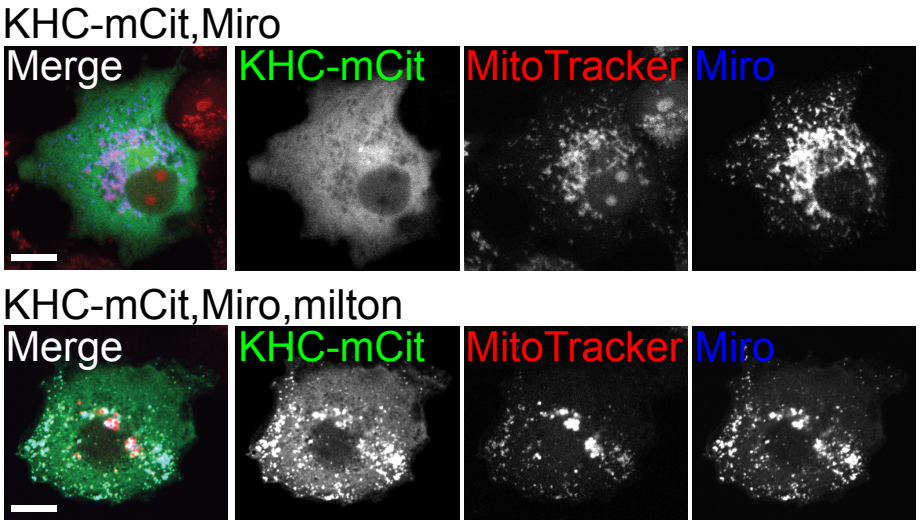

KHC-mCit,Miro
Merge
KHC-mCit
MitoTracker
Miro
KHC-mCit,Miro,milton
Merge
KHC-mCit
MitoTracker
Miro

Figure S3

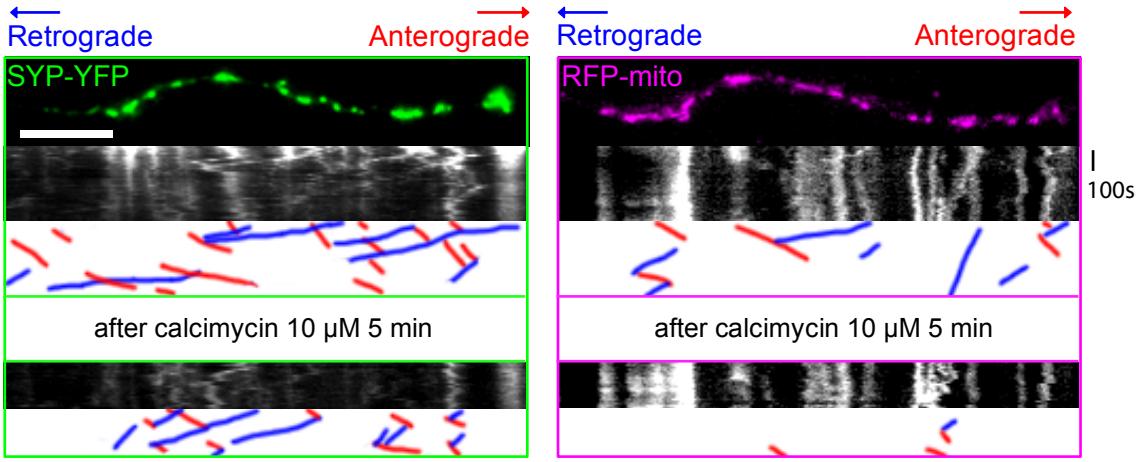
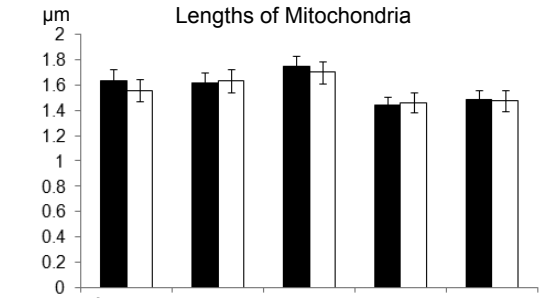

Retrograde
Anterograde
SYP-YFP
after calcimycin 10 μM 5 min
Retrograde
Anterograde
RFP-mito
100s
after calcimycin 10 μM 5 min

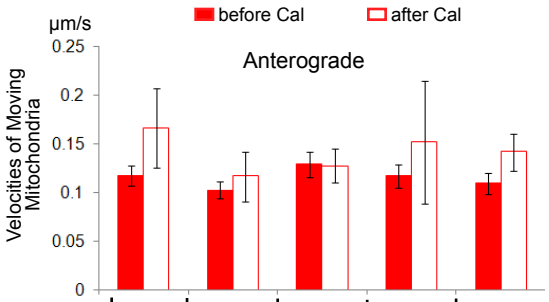
Figure S4

A

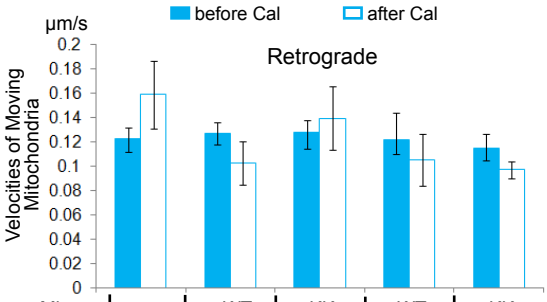


Miro	—	WT	KK	WT	KK
milton	—	—	—	+	+
KHC-mCit	—	—	—	+	+
RFP-mito	+	+	+	+	+
SYP-YFP	+	+	+	—	—

B

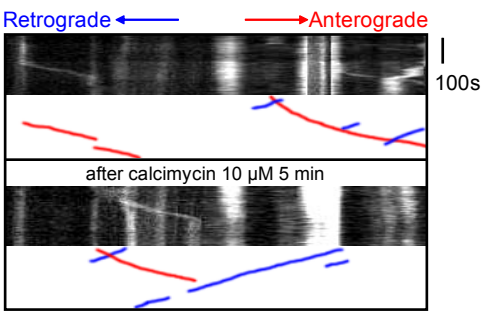


Miro	—	WT	KK	WT	KK
milton	—	—	—	+	+
KHC-mCit	—	—	—	+	+
RFP-mito	+	+	+	+	+
SYP-YFP	+	+	+	—	—



Miro	—	WT	KK	WT	KK
milton	—	—	—	+	+
KHC-mCit	—	—	—	+	+
RFP-mito	+	+	+	+	+
SYP-YFP	+	+	+	—	—

C



D

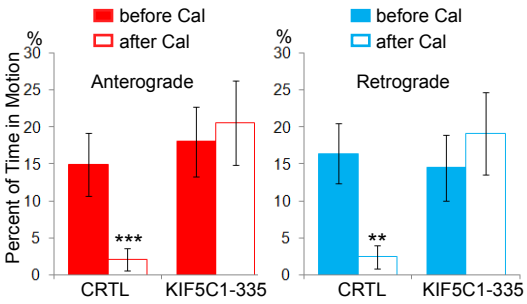
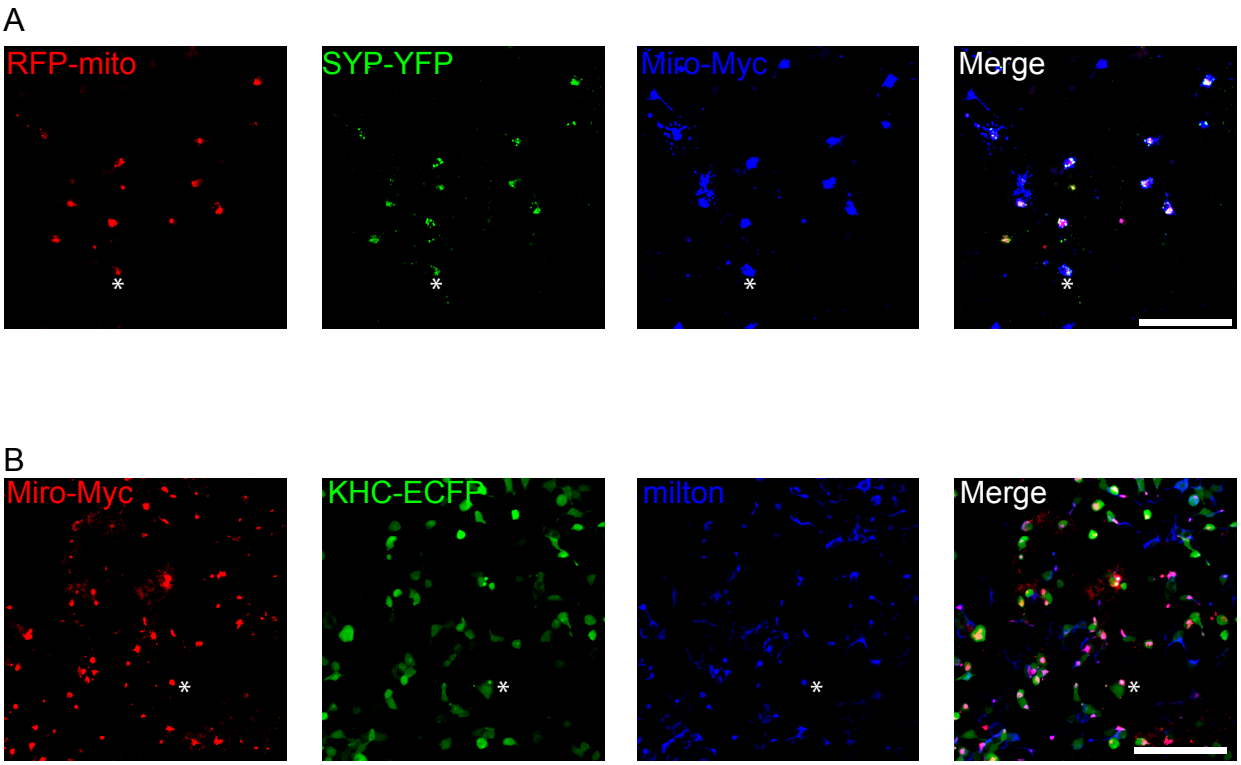


Figure S5


A
RFP-mito
SYP-YFP
Miro-Myc
Merge
B
Miro-Myc
KHC-ECFP
milton
Merge

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. RFP-mito Localizes to Mitochondria in Rat Hippocampal Neurons.

In a hippocampal neuron transfected with RFP-mito (magenta), the RFP colocalizes with the mitochondrial dye MitoTracker Green (A) and with immunoreactivity for the mitochondrial protein Miro (B). Cell bodies of transfected neurons are indicated with asterisks. Scale bars, 10 µm.

Figure S2. Miro Requires Milton to KHC to Mitochondria.

COS cells were transfected with KHC-mCit (green) and Miro-Myc (blue), with (top) or without (bottom) milton cotransfection. Mitochondria were labeled with MitoTracker. KHC-mCit is diffusely cytoplasmic when only Miro has been expressed but colocalizes in puncta with MitoTracker and Miro when milton is also expressed. Scale bars, 10 µm.

Figure S3. Calcimycin Does Not Affect Synaptic Vesicle Movement.

Motility of axonal mitochondria (labeled by transfection with RFP-mito) and vesicles containing synaptophysin (SYP-YFP). The first frame and kymograph of SYP-YFP or RFP-mito are paired with hand-drawn traces that extract those puncta moving anterograde (red) or retrograde (blue). Kymographs were made before (upper) and after (lower) 5 min in the presence of calcimycin. Calcimycin arrested mitochondrial movement, but SYP-YFP puncta continued to move. Quantifications of movement parameters of SYP-YFP are in Table S2. Scale bar, 10 µm.

Figure S4. Movement Parameters of Mitochondria before and after Calcimycin Addition.

(A) Lengths of mitochondria, in neurons transfected as indicated, were quantified before and

after a 5-minute incubation with calcimycin (Cal). One-Way ANOVA for all 10 groups (5 classes of transfection, before and after calcimycin) is 0.098, n=111-122. (B) The average instantaneous velocities of moving mitochondria, transfected as in A. One-Way ANOVA for all 20 groups (5 classes of transfection, in anterograde and retrograde directions, before and after calcimycin) is 0.312; n=4-29 moving mitochondria. (C, D) Overxpression of the motor domain of KIF5C interferes with Ca^{++} arrest of mitochondrial movement. (C) Mitochondrial motility before and after calcimycin addition, represented by kymographs of an axon transfected with KIF5C1-335-YFP and RFP-mito. (D) From kymographs like those in C, the percent of time mitochondria were in motion was calculated. Prior to calcimycin, motor-domain expressing and control (CTRL) neurons were similar, but no significant inhibition of movement was observed upon addition of calcimycin (Cal) to the former. Control axons were transfected with RFP-mito and SYP-YFP. n=48-57 mitochondria from 6 axons and 3 separate transfections.

Figure S5. Retrospective Staining of Multiply Transfected Hippocampal Neurons.

When multiple plasmids were transfected into hippocampal cultures, neurons that expressed one construct almost always expressed all. See Supplementary Methods for details of quantification. (A) A representative 25×magnification view of cultured rat hippocampal neurons transfected with RFP-mito, SYP-YFP and Miro-Myc. Mitochondria within the neuronal cell body appear as red RFP-mito spots (e.g. one marked by white asterisks) that are also labeled in green (SYP-YFP) and blue (Miro-Myc). For each field, the total number of cells labeled with all three colors (green, red, blue) was counted and divided by the total number of cells that were both red and green to determine the percent of neurons of the type whose axons were imaged that were also expressing the Miro-Myc transgene. 98.56±0.99% of the cells with SYP-YFP and RFP-mito were also

Miro-Myc positive. When Miro^{KK}-Myc was used instead of Miro-Myc, the overlap was 97.92±0.95%. In similar experiments on cells transfected with four constructs (KHC-mCit, RFP-mito, Miro-Myc and milton), almost all the cells with mCit (green) and RFP (red) were also immunoreactive for Miro-Myc (98.75±0.68%) or milton (98.78±0.66%). In cultures transfected with KHC-mCit (green), RFP-mito (red), Miro^{KK}-Myc and milton, 98.50±1.03% of mCit and RFP positive cells also expressed Miro^{KK}-Myc and 97.54±1.32% expressed milton. The neurons were at the same stage as the neurons for live-imaging, but the laser power and magnification were lower than that used for live imaging. (B) Coexpression of multiple transgenes in HEK cells. A representative 25×magnification view of HEK cells transfected with Miro-Myc (red), KHC-ECFP (green) and milton (blue). A representative triple-labeled cell is marked (asterisk). The Miro-Myc signal does not fill the cell, but instead is concentrated on mitochondria, often clustered at the edge of the cell. For each view, the total number of cells of three colors (green, red, blue) was counted and divided by the total number of cells of one single color. Therefore, for cells transfected with Miro-Myc (red), KHC-ECFP (green) and milton (blue), the percentage of the number of cells with all three constructs out of the number of cells with KHC-ECFP, or Miro-Myc, or milton, was 99.01±0.31%, 98.99±0.30%, 98.67±0.68% respectively; and 99.45±0.31%, 99.29±0.38%, 97.47±0.76% respectively when Miro^{KK}-Myc was used instead of Miro-Myc. Scale bars, 100 μm.

SUPPLEMENTARY METHODS

Cell Culture

Rat hippocampal cells were dissected and dissociated from day 18 embryos and plated at 50-100,000/well on poly-ornithine and laminin-coated 24-well plates in Neurobasal supplemented with B27 (Invitrogen, Carlsbad, CA) for 8-11 days prior to transfection. Cultured hippocampal neurons were transfected with Lipofectamine 2000 (Invitrogen) and imaged 2-4 days later. COS and HEK293T cells were cultured in DME supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin and transfected with calcium phosphate.

Quantification of Retrospective Staining of Multiply Transfected Hippocampal Cultures and HEK cells

The extent of coexpression of multiple constructs transfected into neurons was determined by retrospective immunostaining: each coverslip was fixed as previously described (Glater et al., 2006; Stowers et al., 2002). Those cultures transfected with three constructs were stained with mouse anti-Myc (9E10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:300. Those coverslips that were transfected with four constructs were, broken in half and stained and one half was labeled with mouse anti-milton (2A108, Glater et al., 2006) at 1:50 and the other with mouse anti-Myc at 1:300. Cy5-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:500 was used as a secondary antibody. For HEK cells, each coverslip was fixed and stained with mouse anti-Myc (9E10) at 1:300 and rabbit anti-milton (P1-152, Stowers et al., 2002) at 1:100, and Cy3-conjugated anti-mouse and Cy5-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at 1:500. Cells were imaged at room temperature (20°C) with a 25×/NA0.75 Plan-Apochromat objective on a laser scanning confocal microscope (LSM 510 META/NLO; Carl Zeiss MicroImaging, Inc., Thornwood, NY) with LSM software 3.2 (Carl Zeiss MicroImaging, Inc.). Examples of retrospective staining are shown in

Figure S5. For quantification, 5 views from each coverslip (or half coverslip) were randomly selected under 25×, and the percent of RFP, YFP (mCit) and Cy5 positive cells out of total RFP and YFP (mCit) positive cells, or out of total single color positive cells, was averaged across the 5 views from 3 coverslips of three independent transfections.

Live Imaging of Mitochondrial Movements

Live cells were maintained in CO_2 independent medium (Hibernate E, Brainbits, IL) with 1.8 mM Ca^{++} at 37 °C for less than an hour. Calcimycin (Sigma, St Louis, MO) was applied at 10 µM. Glutamate (Sigma) was applied at 30 µM with 1 µM glycine. MitoTracker Green (Invitrogen) was applied at 100 nM for 1 minute. Images were collected on a Zeiss LSM 510 confocal microscope using a 63×/N.A.0.90 water IR-Achroplan objective with excitation at 488/543nm. Lasers were used at 10-60% to minimize damage, and pinholes were opened maximally to allow the entire thickness of the axon to be imaged.

For quantification of the movements, as described in the Experimental Methods, we excluded bias caused by the drift in the system: fixed axons were imaged and the movement parameters were calculated as a control. The average velocities of the fixed mitochondria were, anterograde 0.0033±0.0002µm/s, retrograde 0.0034±0.0004µm/s (n=140). The maximum velocity was 0.0125µm/s. A threshold velocity was therefore defined as 0.05µm/s which was equivalent to a displacement of 1 pixel over a time lapse of 1 pixel and exceeded the bias caused by recording drift and mouse-positioning. Velocities less than 0.05µm/s were considered zero.

Immunoreagents

For probing immunoprecipitates the following reagents were used: mouse anti-Myc (9E10) at 1:500, rabbit anti-GFP (Invitrogen) at 1:5000, mouse anti-milton (2A128, Glater et al., 2006) at 1:50, mouse anti-T7 (Novagen, Madison, WI) at 1:1000, mouse anti-KHC (H2, gift of Dr. K. Verhey) at 1:400, rabbit anti-hMiro1(Fransson et al., 2003) at 1:1000, or rabbit anti-OIP106 at 1:500 (SAI1, gift of Dr. G. Hart), and goat anti-mouse or rabbit HRP at 1:5000 (Jackson ImmunoResearch Laboratories, Inc.).

Microtubule cosedimentation blots were probed with the following reagents: rabbit anti-GFP (Invitrogen) at 1:5000, anti-milton monoclonal 2A128 (Glater et al., 2006) at 1:50, anti-Myc (9E10) at 1:500, or anti-tubulin (DM1A) at 1:5000, and goat anti-rabbit or mouse HRP at 1:5000 (Jackson ImmunoResearch Laboratories, Inc.).

Preparation of Mitochondrial Fractions

Cultured rat hippocampal neurons (4×10^7) were mechanically broken and further homogenized by auto and needle homogenization in isolation buffer: 200 mM sucrose, 10mM TRIS/MOPS (pH 7.4), and 1 mM EGTA/TRIS or 2 mM $CaCl_2$. Nuclear waste was pelleted by 200g for 5 min. The supernatant (containing mitochondria) was centrifuged again at 10,000g for 10 min to pellet the mitochondria fraction. The supernatants were collected as the non-mitochondrial fraction.

SUPPLEMENTARY MOVIES

For all movies, anterograde movement is to the right, retrograde movement to the left. 1 second = 50 seconds real time. Movies are integrated with a kymograph underneath based on the movie. “Calcimycin” indicates 5-minute incubation with 10 µM calcimycin prior to the next episode of the movie. Scale bars, 10 µm.

Movie S1 A control axon without transfected Miro. RFP-mito is shown in red. Right arrows in the first frame indicate a mitochondrion that will move anterograde and its corresponding line in the kymograph. Asterisks in the first frame indicate a mitochondrion that will move both anterograde and retrograde and its corresponding line in kymograph.

Movie S2 An axon overexpressing Miro. RFP-mito is shown in red. Arrows in the first frame indicate mitochondria that will move anterograde (right arrow) and retrograde (left arrow) and their corresponding lines in the kymograph. Asterisks in the first frame indicate a mitochondrion that will move in both directions and its corresponding line in the kymograph.

Movie S3 A $Miro^{KK}$-overexpressing axon. RFP-mito is shown in red.

Movie S4 A Miro/milton/KHC-mCit-overexpressing axon. RFP-mito is in magenta, and KHC-mCit in green.

Movie S5 A $Miro^{KK}$/milton/KHC-mCit-overexpressing axon. RFP-mito is in magenta, and KHC-mCit in green. Right and left arrows in the first frame indicate mitochondria that will move anterograde and retrograde and their corresponding lines in the kymograph.