Supporting Information

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SI Materials and Methods

Imaging Analyses. To calculate mito-Case12:mito-RFP ratio in moving and stationary mitochondria, individual mitochondria were outlined and the average fluorescence intensity (normalized to the area of the mitochondria) was determined per time point. Area of each mitochondrion was determined by outlining the mito-RFP signal, and intensity values for mito-RFP and mito-Case12 in the same outlined area were measured in the red and green channel, respectively. Measurements were excluded when mitochondria overlapped each other because accurate determination of intensity was impossible. At least 20 frames (> 100 s) were used to calculate average mito-Case12:mito-RFP ratio. To account for difference in fluorescence intensities between different transfections, the ratio of stationary mitochondria was normalized to the ratio of stationary mitochondria obtained from the first batch of neurons and all ratios were scaled proportionally. The average speed of moving mitochondria was tracked using the Manual Tracking Plugin in ImageJ. At least 20 frames (> 100 s) were used to calculate the average speed of mitochondrial movement. Mitochondrial mobility was determined by counting the percentage of moving mitochondria over the 5-min imaging period. Mitochondrion is labeled as moving if it moved in three consecutive frames (over a 15-s period) over a distance of at least 0.1 μm. To generate kymographs, axons were straightened using the Straighten plugin in ImageJ, resliced, and then *z*-projected (sum intensities). When measuring fold-change in fluorescence intensities before and after drug treatment, fluorescence intensities of all mitochondria in axons or cell bodies were measured (as indicated), and compared with the time point right before drug application. To determine mitochondrial length, the Shape Descriptors plugin in ImageJ was used, and mitochondrial length is defined by the length of the major axis.

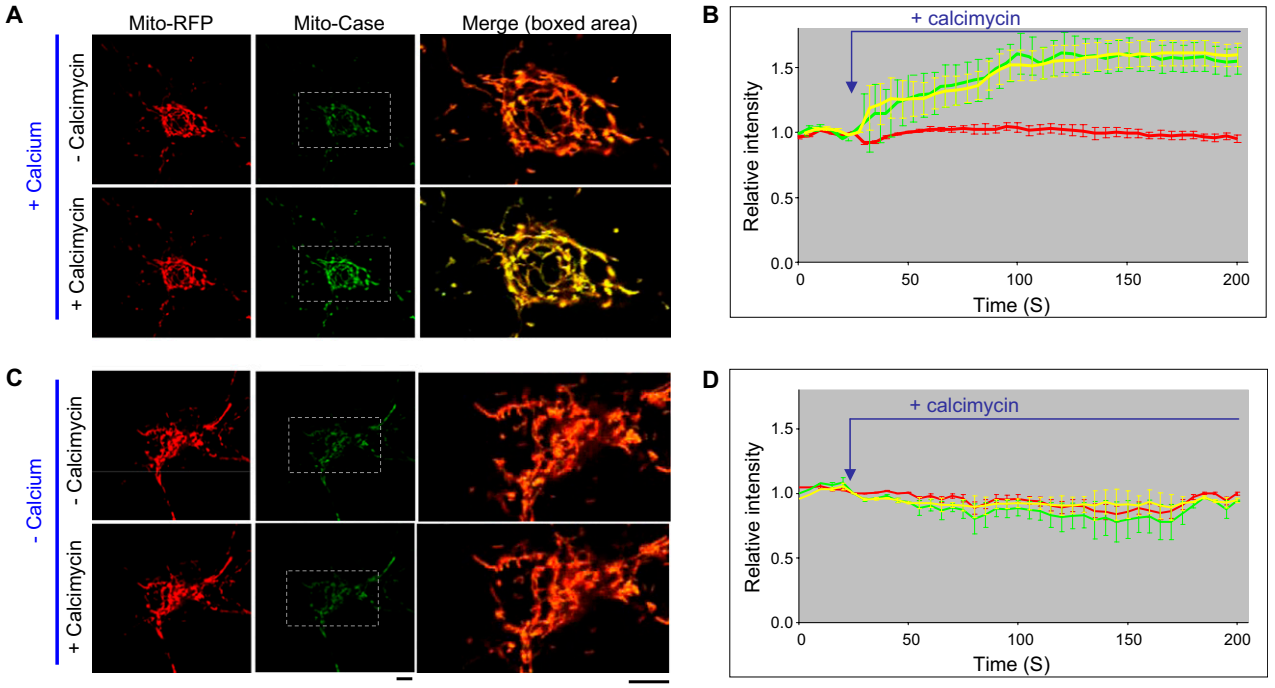


Fig. S1. Mito-Case12 can detect increase in Ca^{2+} following calcimycin treatment. (*A* and *C*) Images of neurons transfected with mito-Case12 (green) and mito-RFP (red) before and after the addition of 2 μg/mL calcimycin, a Ca^{2+} ionophore, in the presence and absence of extracellular Ca^{2+}, respectively. (Scare bars, 10 μm.) Boxed area is magnified in the merged image. (*B*) Fold-change in fluorescence intensities of mito-case12 (green line), mito-RFP (red line), and mito-Case12: mito-RFP ratio (yellow line) before and after addition of calcimycin in normal Ca^{2+} extracellular solution. (*D*) Same as *B*, except done in Ca^{2+}-free extracellular solution. Values represent mean ± SEM, n = 6 neurons (*B*) and 3 neurons (*D*).

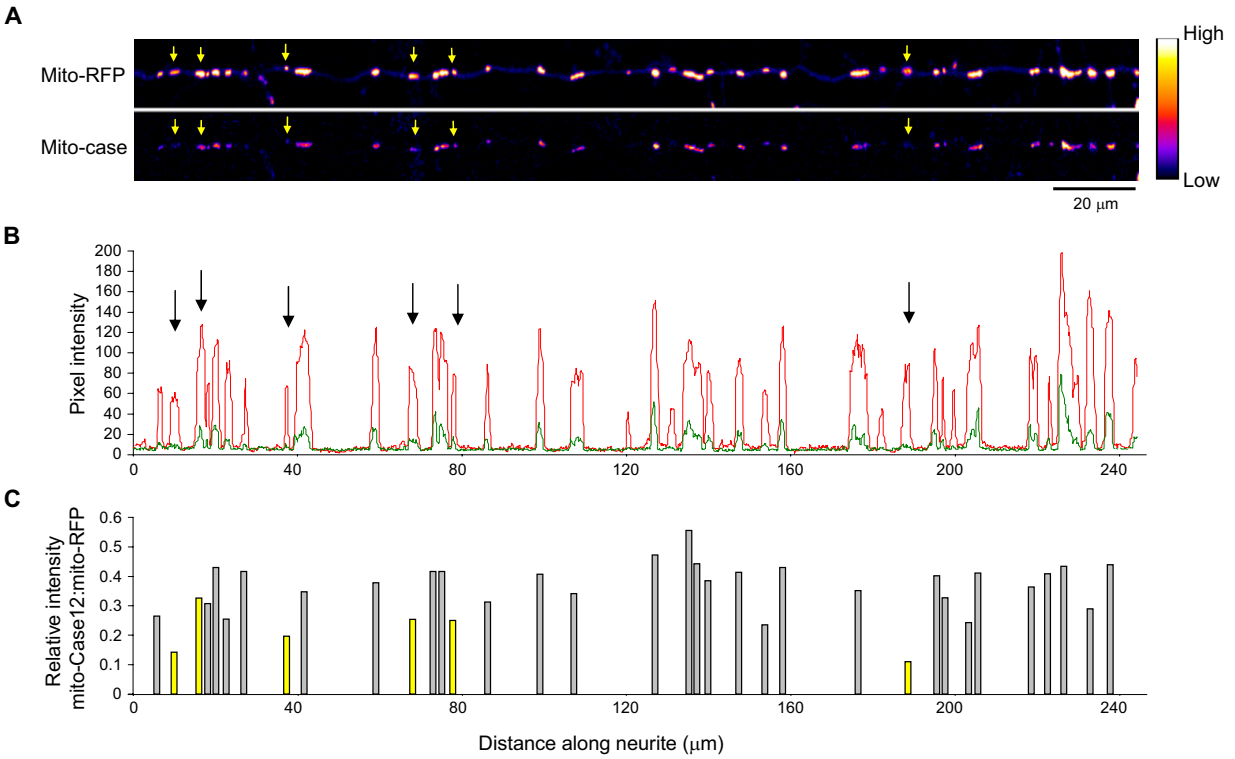


Fig. S2. Moving mitochondria tend to have lower calcium content. (*A*) Pseudocolored images of an axonal segment of a neuron cotransfected with mito-RFP and mito-Case12 as shown in Fig. 1*A*. Arrows point to moving mitochondria. (*B*) Raw intensity profiles of mito-RFP (red) and mito-Case12 (green) as determined using ImageJ. Arrows point to moving mitochondria. (*C*) Ratio of mito-case12 to mito-RFP shown for the axonal segment. Gray bars indicate ratio for stationary mitochondria, whereas yellow bars represent ratio for moving mitochondria.

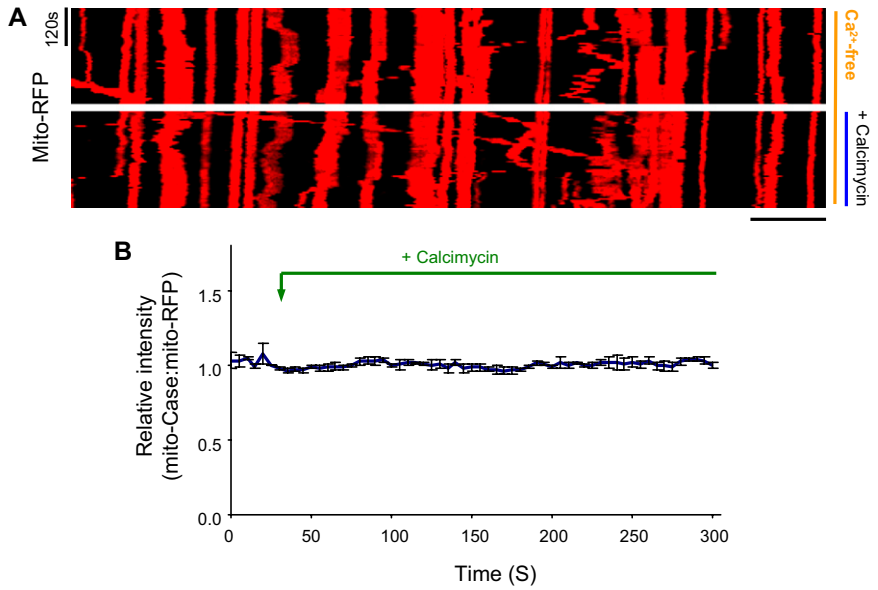


Fig. S3. Calcimycin-induced mitochondrial movement arrest requires Ca^{2+}. (*A*) Kymograph showing mitochondria retain movement upon addition of calcimycin in Ca^{2+}-free extracellular solution. (Scale bar, 10 μm.) (*B*) Calcimycin does not induce changes in mito-Case:mito-RFP ratio in the absence of Ca^{2+}. Values represent mean ± SEM, $n = 4$ axons.

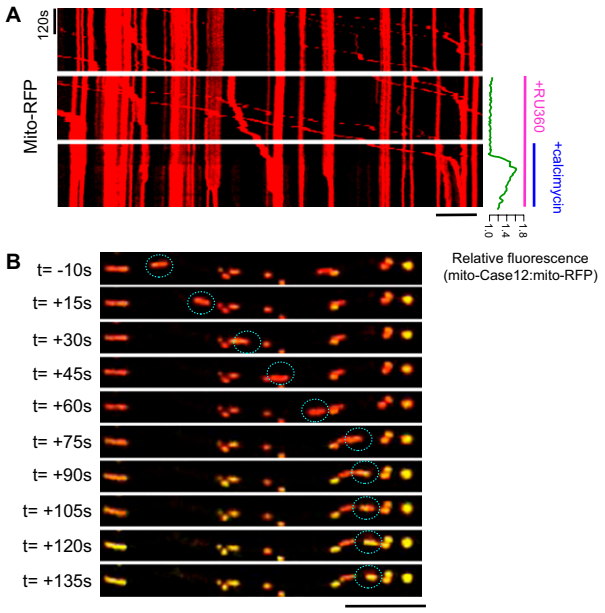


Fig. S4. RU360 delays mitochondrial movement arrest following calcimycin treatment and pause in mitochondrial movement correlates with intra-mitochondrial Ca^{2+} influx. (*A*) Kymograph showing mitochondrial movement for neuron transfected with mito-RFP and mito-Case12. Relative intensity of mito-Case12:mito-RFP signal is shown on the right. Only the last 5 min of RU360 treatment is shown. (*B*) Merged time-lapse images showing mitochondrial movement before (–10 s) and after addition of calcimycin after 15 min of RU360 incubation. Mito-Case12 is in green and mito-RFP is in red, thus increase in mito-Case12 signal is seen as yellow. Blue circle highlights moving mitochondria. Note that RU360 delayed pause in mitochondrial movement and increase in mito-Case12 signal. Increase in mito-Case12 signal indicates increasing mitochondrial Ca^{2+}, which correlated with stop in mitochondrial movement. (Scale bars, 10 μm.)

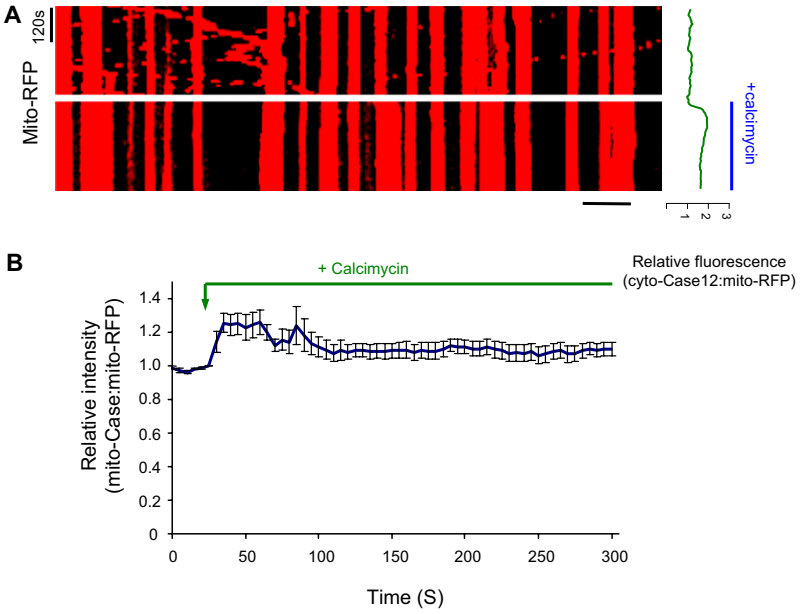


Fig. S5. Calcimycin treatment in extracellular solution containing low Ca^{2+} (0.18 mM Ca^{2+}) is sufficient to pause mitochondrial movement. (*A*) Kymograph of a neuron transfected with mito-RFP and cyto-Case12. Cyto-Case12 profile is normalized to mito-RFP signal to reduce drifts during imaging (shown on the right). (Scale bar, 10 μm.) (*B*) Calcimycin treatment elevates mito-Case:mito-RFP ratio in the axons. Values represent mean ± SEM n = 9 axons.

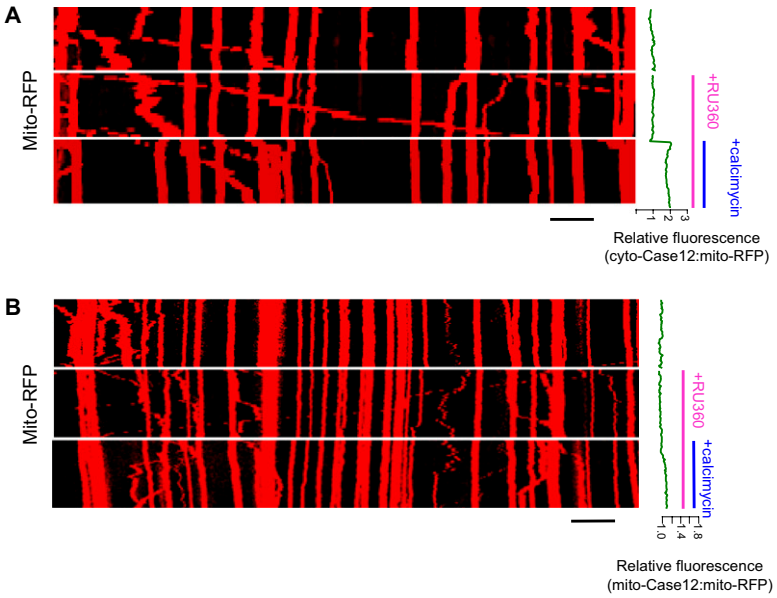


Fig. S6. RU360 treatment prevented calcimycin-induced stop in mitochondrial movement in extracellular solution containing low Ca^{2+} (0.18 mM Ca^{2+}). (*A*) Kymograph of a neuron transfected with mito-RFP and cyto-Case12. Cyto-Case12:mitoRFP profile in the absence and presence of RU360 and calcimycin is shown on the right. RU360 treatment maintained mitochondrial mobility despite high cytoplasmic Ca^{2+} elevation. (*B*) Kymograph of a neuron transfected with mito-RFP and mito-Case12 before and after RU360 and calcimycin treatment. Mito-Case12:mito-RFP profile is shown on the right. (Scale bars, 10 μm.)

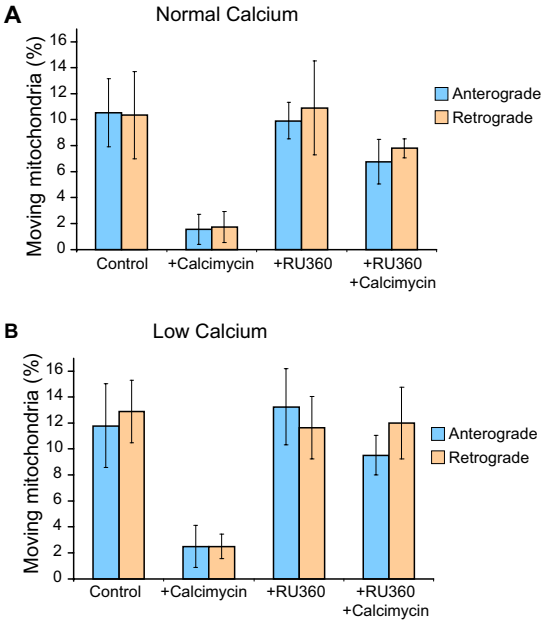


Fig. S7. Calcimycin and RU360 treatment did not change directionality of mitochondrial transport. Percentage of mitochondria moving in anterograde or retrograde direction as indicated for each treatment. (*A*) Summary of results in normal extracellular solution (1.8 mM Ca^{2+}) and (*B*) in extracellular solution containing low Ca^{2+} (0.18 mM Ca^{2+}). Note that the proportion of mitochondria moving in anterograde and retrograde direction remained the same. $n > 100$ mitochondria from at least five axons in the different conditions. Values represent mean ± SEM.

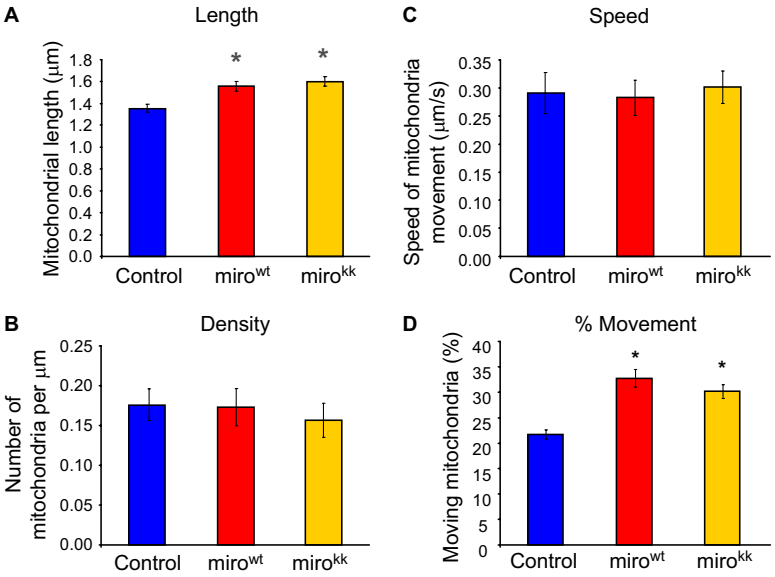


Fig. S8. Neurons expressing miro^{wt} and miro^{kk} altered mitochondrial length and percentage of moving mitochondria, but not mitochondrial density nor the average speed of mitochondrial movement in axons. (*A*) Average length of mitochondria. (*B*) Density, or the number of mitochondria per micrometer, in axons. (*C*) Average speed of movement determined from mobile mitochondria. (*D*) Percentage of moving mitochondria. Values represent mean ± SEM and $n > 180$ mitochondria examined per condition from at least four axons in *A*, *B*, and *D*. In *C*, $n = 40–50$ mitochondria per condition. *$P < 0.05$.

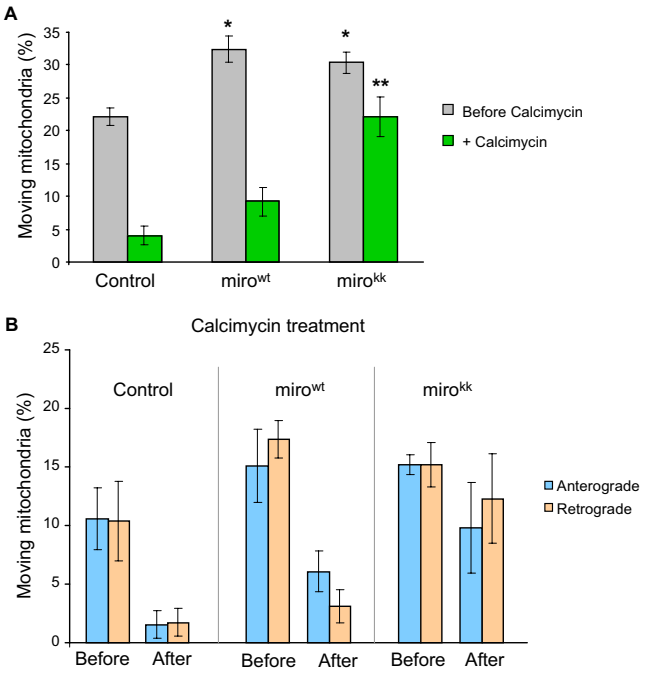


Fig. S9. Calcimycin treatment did not arrest mitochondrial movement in miro^{kk}-transfected neurons. (*A*) Percentage of moving mitochondria before and after calcimycin treatment as indicated. *$P < 0.05$ compared with control before the addition of calcimycin. **$P < 0.05$ compared with control after calcimycin treatment. (*B*) Percentage of mitochondria moving in anterograde or retrograde direction before and after calcimycin treatment. All values represent mean ± SEM, $n > 110$ mitochondria from at least four axons per condition.

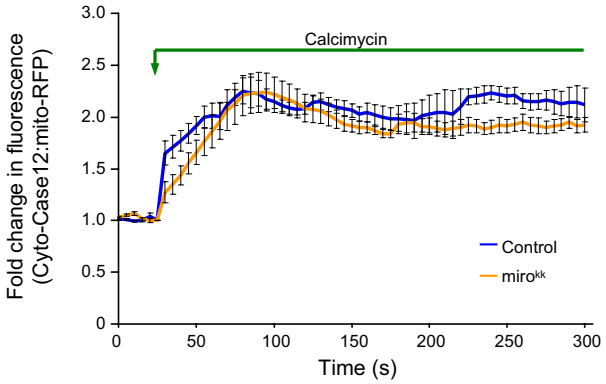


Fig. S10. Miro^{kk} does not alter Ca^{2+} influx into the cytoplasm. Fold-change in cytoplasmic Case 12 signal is normalized to that of mito-RFP to reduce variation due to drifts in *z* axis during imaging. Values represent mean ± SEM, *n* = 4 axons for each.

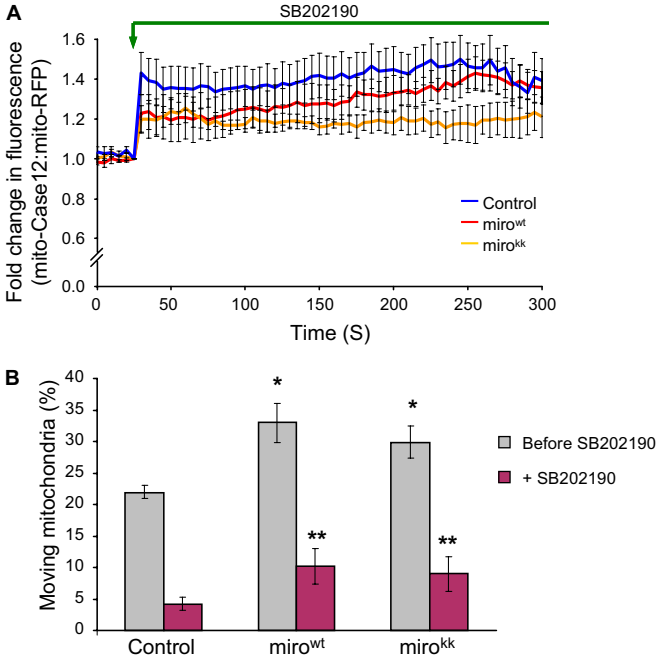


Fig. S11. SB202190 treatment paused mitochondrial transport in both miro^{wt}- and MIRO^{KK}-transfected neurons. (*A*) Mito-Case12 to mito-RFP ratio before and after SB202190 treatment in axons. (*B*) Percentage of moving mitochondria before and after SB202190 treatment as indicated. *$P < 0.05$ compared with control before the addition of SB202190. **$P < 0.05$ compared with control after SB202190 treatment. Values represent mean ± SEM, $n > 160$ mitochondria from four to eight axons analyzed per condition.

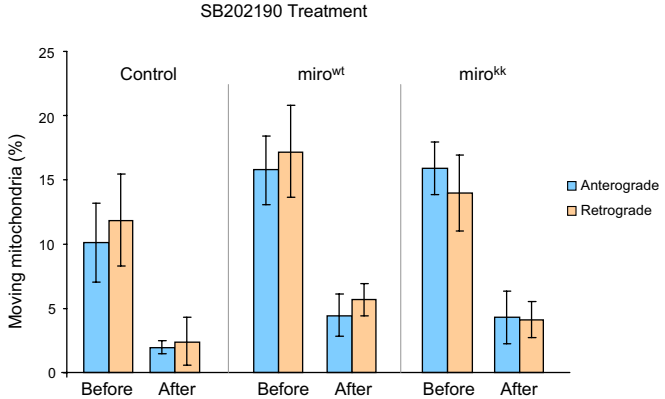
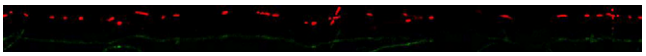
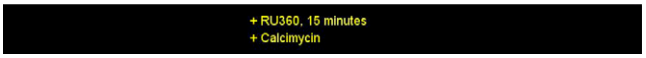


Fig. S12. SB202190 treatment did not change the directionality of mitochondrial transport. Percentage of anterogradely and retrogradely moving mitochondria as indicated for each condition. Values represent mean ± SEM, $n > 160$ mitochondria from four axons per condition.



Movie S1. Calcimycin treatment increases mitochondrial calcium and stops mitochondrial movement. Axonal segment of neuron cotransfected with mito-RFP (*Upper*) and cyto-Case12 (*Lower*) imaged for 5 min before and after calcimycin treatment. Images were acquired at 5-s intervals, compressed to 15 frames per second in the movie.

Movie S1



Movie S2. Inhibition of mitochondrial calcium uniporter allows continued mitochondrial movement in the presence of high intracellular calcium. Neuron cotransfected with mito-RFP (*Upper*) and cyto-Case12 (*Lower*) was incubated with RU360 for 15 min, and calcimycin was added at the beginning of this movie. Images were acquired at 5-s intervals, compressed 15 frames per second in the movie.

Movie S2