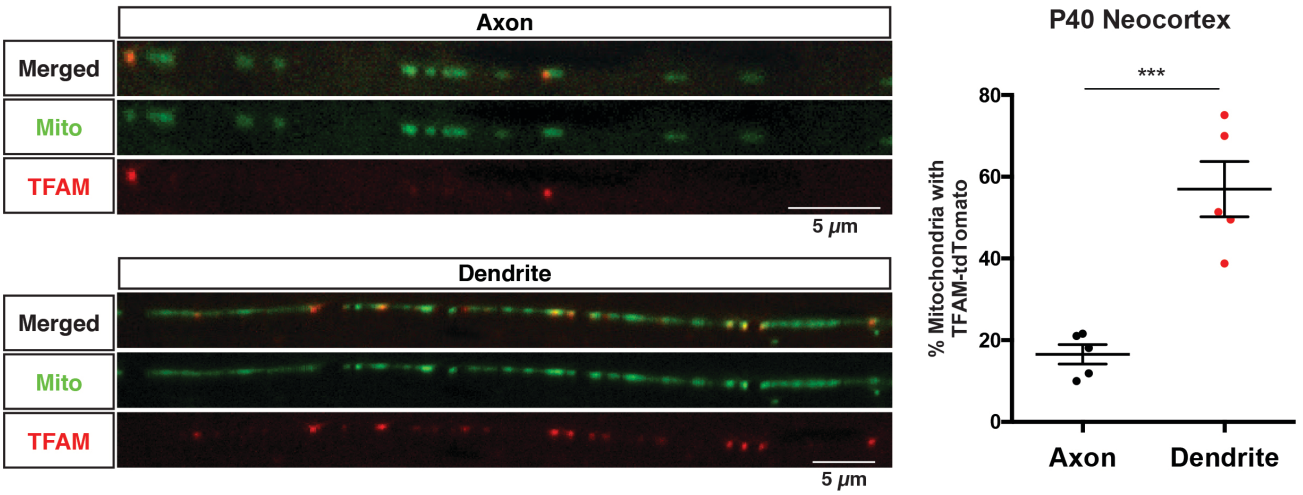
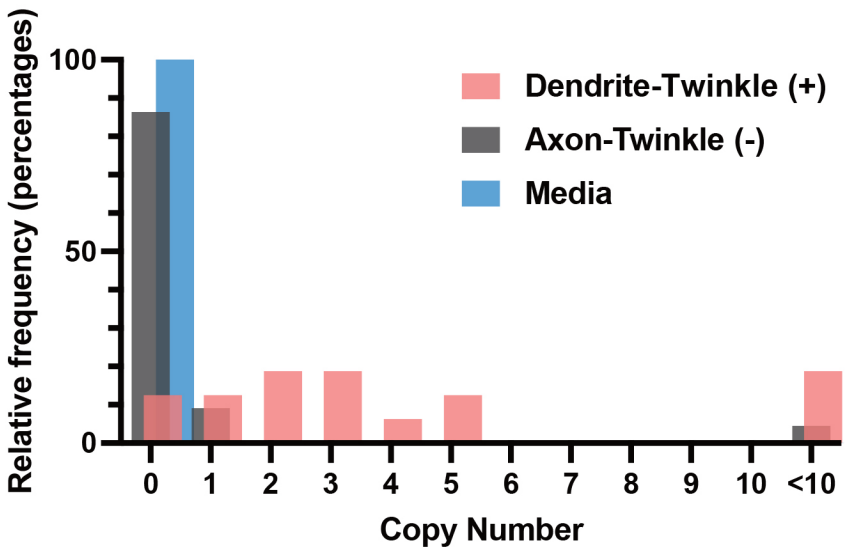


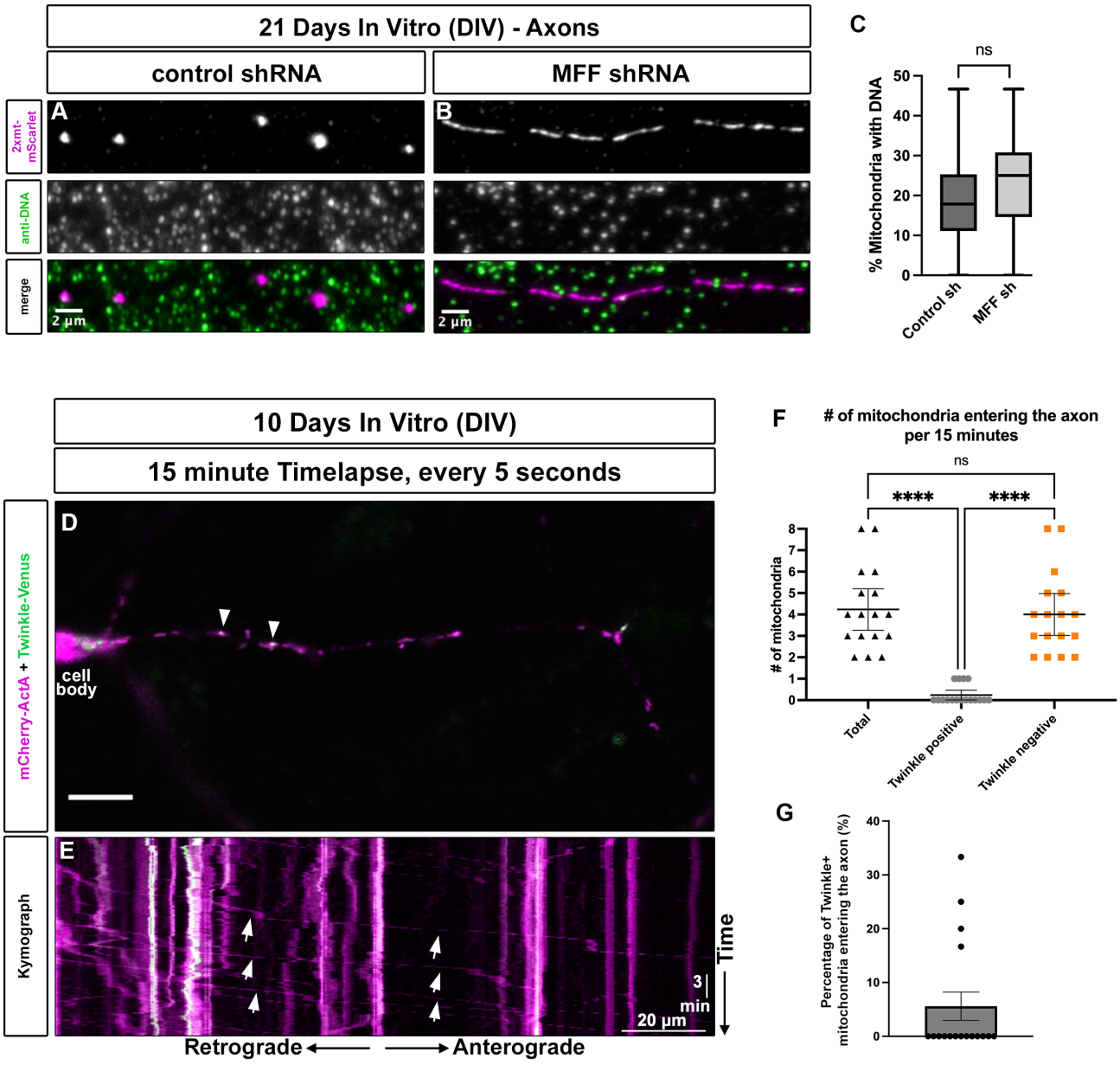
Supplemental Figure 1: A low fraction of axonal mitochondria contain mtDNA-associated protein TFAM compared to dendritic mitochondria *in vivo*. Related to Figure 1.

(**A-B**) Representative images of the axon (A) and a dendrite (B) of a layer 2/3 CPNs *in vivo* expressing TFAM-tdTomato and IMM-targeted mt-YFP at P40. Scale bar: 5μm. (**C**) Percentage of mitochondria positive for TFAM in the axon or dendrites. Numbers of mitochondria counted are shown in each column. 5 neurons from 2 mice at P40 and P69 were used for quantifications. *** p=0.0004 by paired t-test.



Supplemental Figure 2: mtDNA copy number distribution. Related to Figure 3

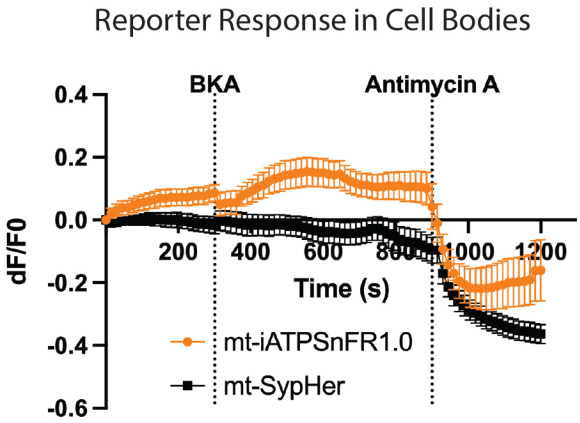
Relative frequency histograms of the copy number of mtDNA determine by qPCR for each isolated mitochondrion with Twinkle from dendrites (black), the isolated mitochondria without Twinkle from axons (red), and the culture media (blue; negative control).



Supplemental Figure 3: Fraction of mtDNA+ mitochondria in axons is independent of fission and determined as they enter the axon. Related to Figure 3.

(**A-B**) Axonal mitochondria in CPNs electroporated with control shRNA are punctate and mostly lack DNA labeling (A). Blocking of mitochondrial fission using shRNA-mediated knockdown of Mff (B), elongates axonal mitochondria but does not impact DNA positivity, arguing that the coupling of mitochondrial fission to mtDNA replication is not a major mechanism for regulating mtDNA levels in the axon. (**C**) Quantification of the percentage of axonal mitochondria containing DNA following knockdown with the indicated constructs. $N_{control\ shRNA}$ = 335 mitochondria from 18 axon segments from 3 independent

cultures. $N_{Mff\ shRNA}$ = 314 mitochondria from 18 axon segments from 3 independent cultures. Graphs are minimum to maximum box plots with 25th, 50th, and 75th percentiles marked. ns = not significant by Brown-Forsythe and Welch ANOVA. (**D**) Representative image of a cell body and emerging axon of a CPN in culture (DIV10) labeled with mCherry-ActA (mitochondria) and a mtDNA-associated protein (Twinkle-Venus). (**E**) Kymograph of the axon shown in A, imaged every five seconds for fifteen minutes. (**F**) Quantification of the number of mitochondria entering the axon (left: total mitochondria), (middle: mitochondria from total with Twinkle labeling), (right: mitochondria from total without Twinkle labeling). (**G**) Quantification of the percentage of mitochondria entering the axon with Twinkle-Venus labeling, demonstrating that the majority of mitochondria entering the axon already lack markers of a nucleoid. n = 71 mitochondria from 17 axons from 3 independent cultures, ns = not significant, ****$p \leq 0.0001$ by Kruskal-Wallis test.



Supplemental Figure 4: BKA treatment does not significantly alter mitochondrial pH. Related to Figure 4.

The soma of cortical pyramidal neurons expressing either mt-iATPSnFR1.0 (orange) or mt-SypHer (black) were timelapse imaged for 20 minutes. At 5 minutes, bath application of bongkrekic acid (BKA, an inhibitor of the ATP transporter- adenine nucleotide translocase (ANT)) at 50µM was added and fluorescence intensity of the fluorescent reporter was monitored until 15 minutes, when Antimycin A at 1.25µM was added. As expected, in the cell body (just like in dendritic mitochondria- see Figure 4), BKA addition caused an increase in mt-iATPSnFR1.0 fluorescence over the next five minutes, followed by a dramatic decrease in mt-iATPSnFR1.0 fluorescence following Antimycin A. No observable difference in mt-SypHer fluorescence was observed for the 10 minutes following BKA addition, while a decrease was observed following Antimycin A addition. This argues that the increase observed in iATPSnFR1.0 is a result of ATP increase in the matrix and not a change in pH, while most of the effect observed following Antimycin A is a result of matrix acidification. $N_{mt\text{-}iATPSnFR1.0}$ = 10 neurons from 3 independent cultures. $N_{mt\text{-}SypHer}$ = 10 neurons from 3 independent cultures.

Supplemental Movie 1

Isolation of a single axonal mitochondrion using a nanopipette from the culture for 7DIV. Scale bar: 5 µm.