INVENTORY OF SUPPLEMENTAL INFORMATION

Supplemental Figures

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SUPPLEMENTAL FIGURES

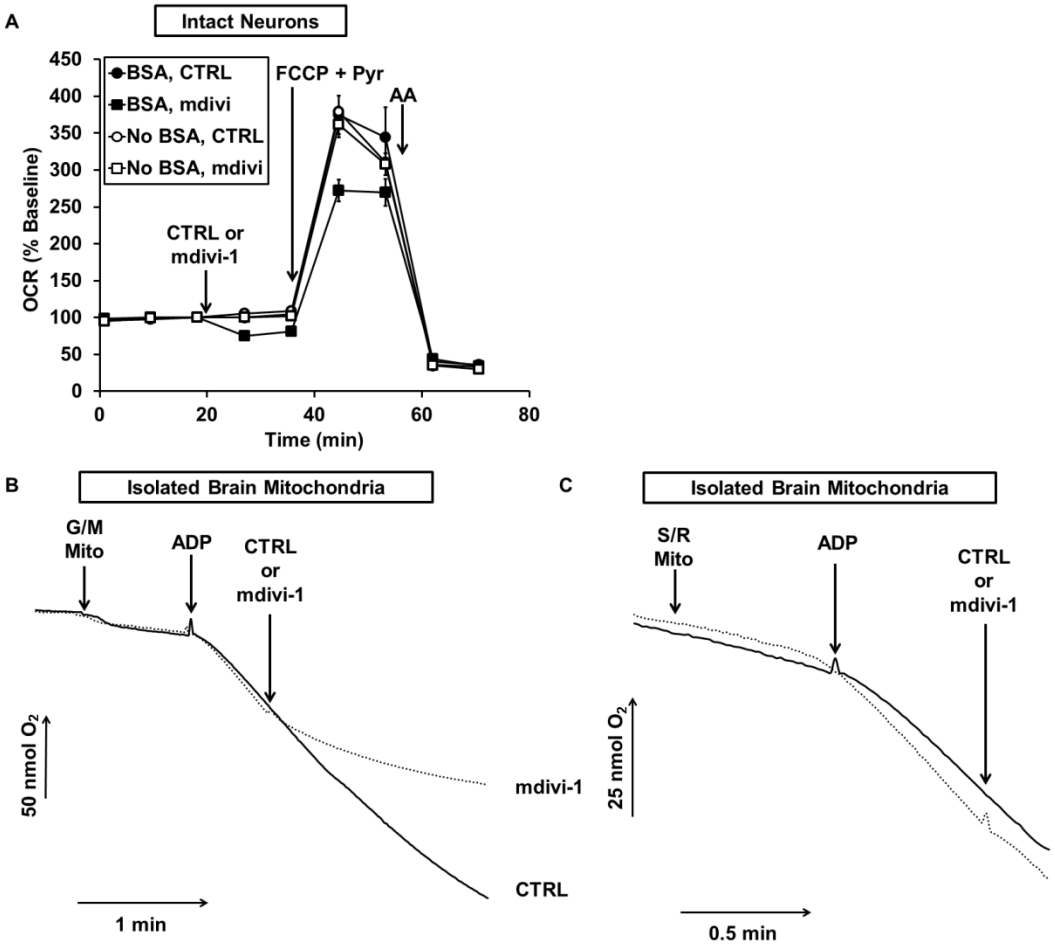

Figure S1. Bovine serum albumin allows mdivi-1 to inhibit neuronal respiration in XF24 assay plates but is not required for respiratory inhibition by mdivi-1, related to Figure 1. (A) Cortical neurons incubated in aCSF with or without 0.4% BSA were treated with DMSO vehicle (CTRL) or mdivi-1 (50 μM), followed by FCCP (3 μM) plus pyruvate (10 mM), and then antimycin A (1 μM). Traces are mean ± SD from three wells. (B) and (C) Isolated mitochondria (0.5 mg/ml) were incubated with Complex I substrates glutamate and malate (G/M, 5 mM each, (B)) or Complex II substrate succinate (S, 5 mM) plus rotenone (R, 2 μM, (C)), and then exposed to mdivi-1 (50 μM) or vehicle (CTRL). Traces are representative of 3 independent experiments. Note that because cell culture media typically used for experiments with mdivi-1 has serum containing albumin, the presence of BSA in our assays is relevant to the literature describing the effects of mdivi-1.

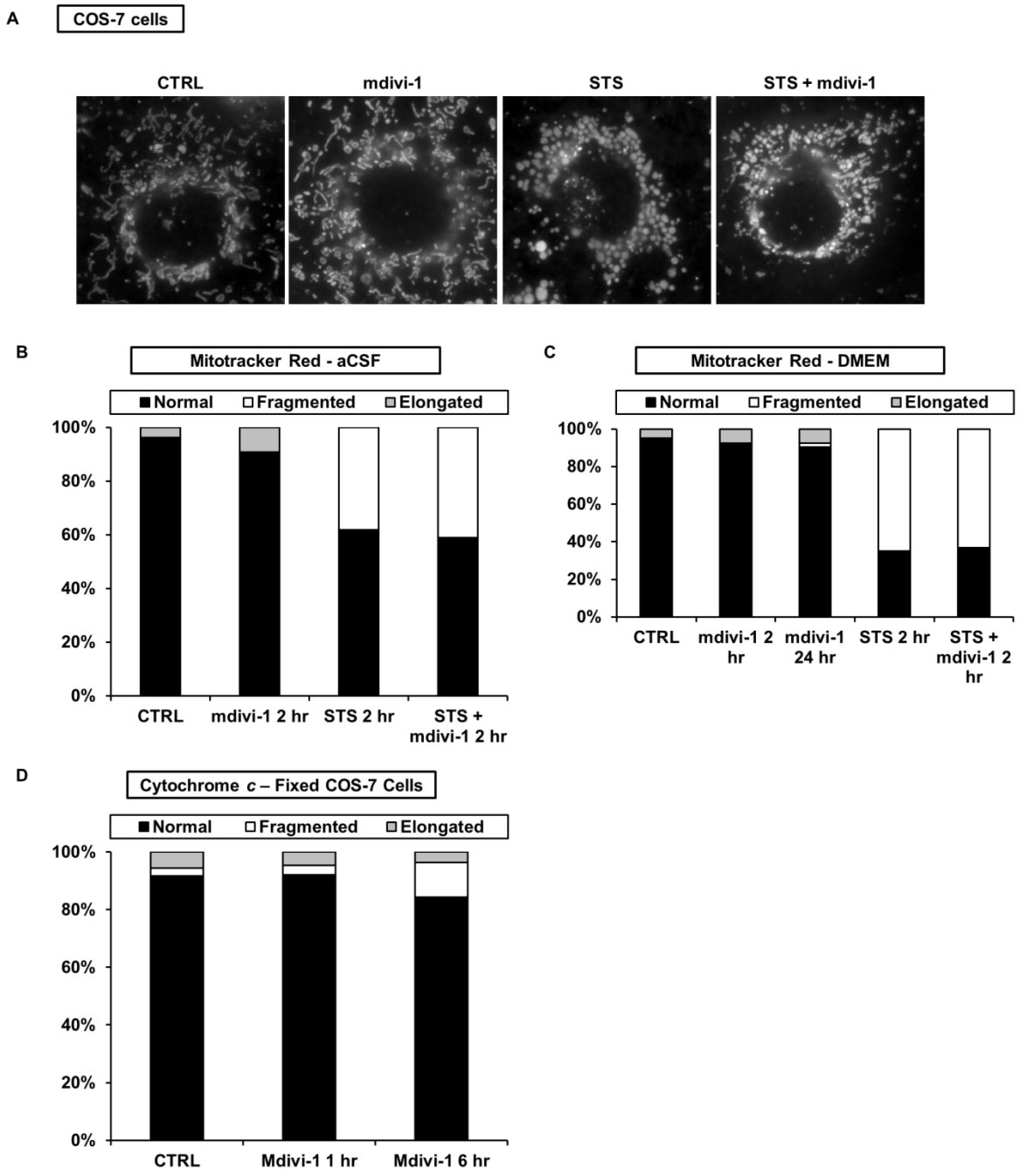

Figure S2. Mdivi-1 fails to influence COS-7 mitochondrial morphology in the absence or presence of staurosporine, related to Figure 2.

(A) Representative images of COS-7 cells loaded with Mitotracker Red CMXRos (20 nM), and treated with either DMSO vehicle (CTRL), mdivi-1 (50 μM), staurosporine (STS, 1 μM), or STS plus mdivi-1 for 2 hours in aCSF respiration media. (B) and (C) Blinded quantification of mitochondrial morphology in Mitotracker Red-labeled COS-7 cells treated with vehicle (CTRL), mdivi-1 (50 μM), STS (1 μM), or the two drugs combined for the indicated times in aCSF medium (B) or phenol red-free cell culture medium (DMEM) (C). Experiments were performed

in aCSF that contained 0.4% BSA, in addition to in the more standard cell culture medium, because our respiration measurements suggest that mdivi-1 can access mitochondria under those conditions. (D) Blinded quantification of mitochondrial morphology in cytochrome *c*-stained COS-7 cells treated with vehicle (CTRL) or mdivi-1 (50 μM) in DMEM for either 1 or 6 hours. Percentages in (B-D) are based on a total of ≥50 cells per treatment from three independently plated coverslips.

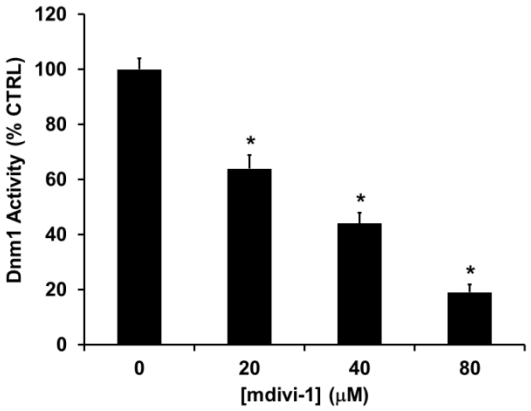

Figure S3. Yeast Dnm1 GTPase activity assay, related to Figure 3.
Purified Dnm1 from yeast was assayed for enzymatic activity in the presence of mdivi-1 or DMSO vehicle using a GTP regenerative assay. Results are mean ± SE, n=3. *$p<0.05$ compared to "0 mdivi-1" control.

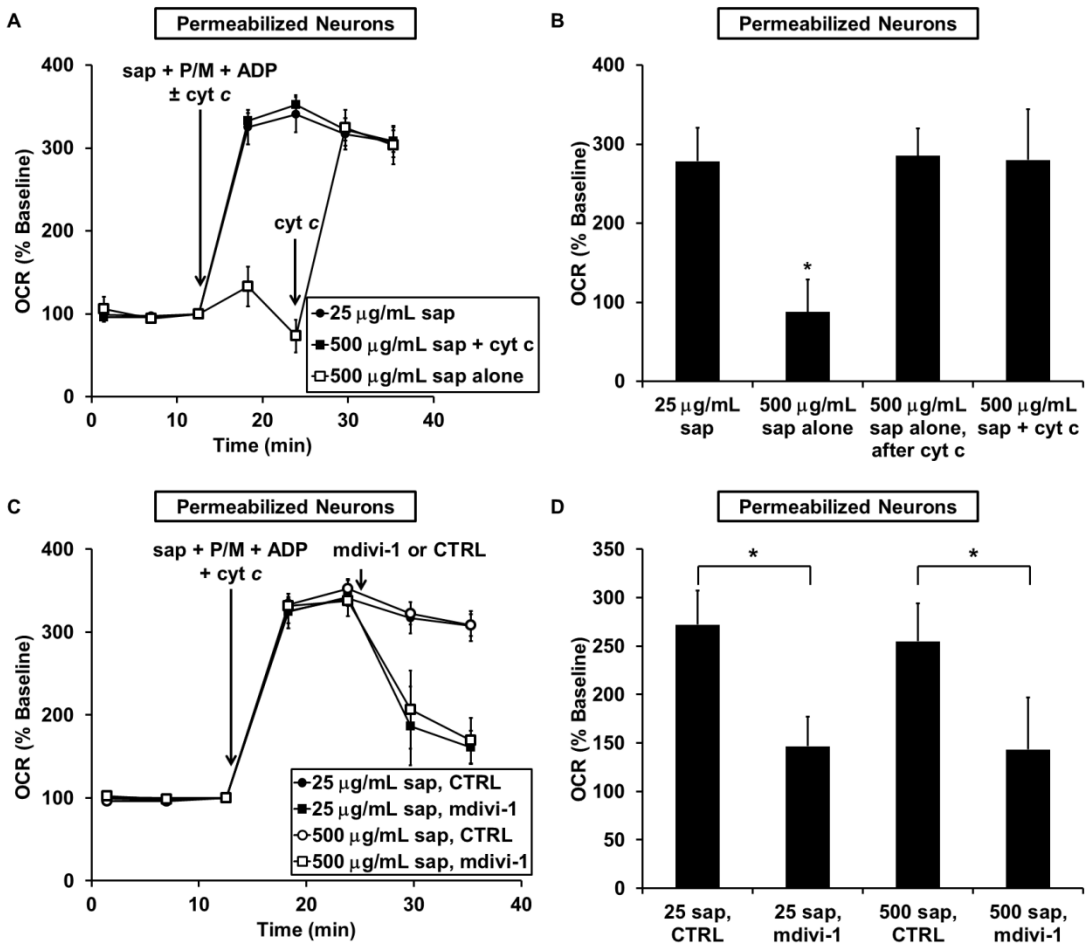

Figure S4. A mitochondrial outer membrane target is not required for mdivi-1 to inhibit respiration, related to Figure 4.

(A) Saponin (25 or 500 μg/ml) was added to neurons together with the Complex I substrates pyruvate and malate (P/M, 5 mM each) and ADP (1 mM) to stimulate respiration. Permeabilizing the mitochondrial outer membrane releases cytochrome *c* from the electron transport chain (Clerc and Polster, 2012). To demonstrate outer membrane permeabilization by 500 μg/ml saponin, purified cytochrome *c* (100 μM) was added either in conjunction with saponin or after two additional OCR measurements, as indicated. (B) Bar graph of the data depicted in (A). (C) Saponin (25 or 500 μg/ml) was added to neurons together with P/M and ADP as in (A). Cytochrome *c* (100 μM) was also present when 500 μg/ml saponin was added to allow respiration following mitochondrial outer membrane compromise. Mdivi-1 (50 μM) or vehicle control (CTRL) were then injected after two OCR measurements. (D) Bar graph of the data depicted in (C). Traces are mean ± SD from three wells and are representative of three independent experiments. Bar graphs are mean ± SD, n=3. * $p<0.05$ compared to control. There was no significant difference in the extent of respiratory suppression by mdivi-1when the mitochondrial outer membrane was intact (25 μg/ml saponin, filled symbols in (C)) or permeabilized (500 μg/ml saponin, open symbols in (C)).

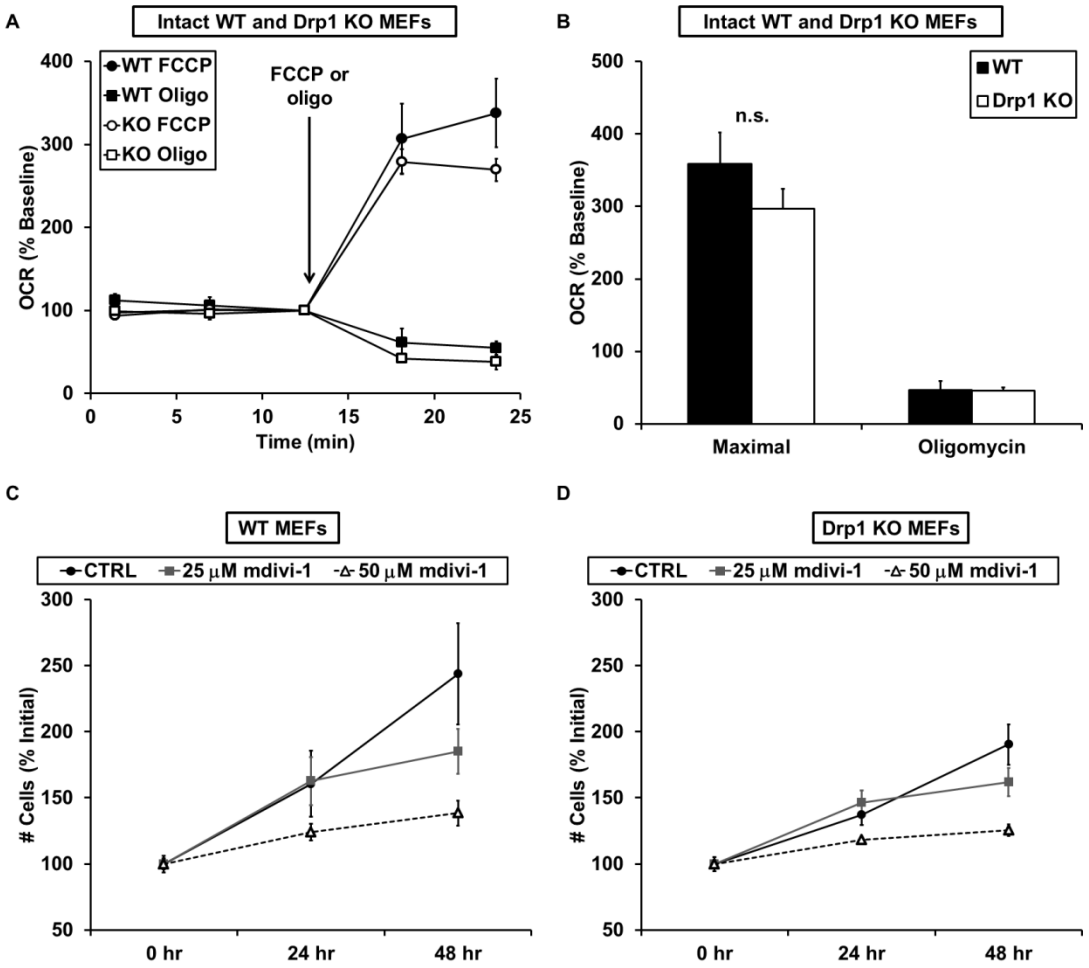

Figure S5. Drp1 KO does not alter FCCP-stimulated respiration or the effect of mdivi-1 on cell proliferation, related to Figure 4.

(A) WT or Drp1 KO MEFs were treated with FCCP (3 μM) plus pyruvate (10 mM) or oligomycin (0.5 μg/ml) while OCR was measured. OCRs are normalized to the final measurement prior to FCCP addition. (B) FCCP response in WT and Drp1 KO cells, expressed as a percentage of the baseline OCR prior to drug addition (mean ± SD, n=4, n.s., not significant). WT (C) or Drp1 KO (D) MEFs grown in DMEM cell culture medium without pyruvate were treated with vehicle (CTRL), 25 μM mdivi-1, or 50 μM mdivi-1 for 0, 24, or 48 hr. Cells were counted at each time point and the cell number is expressed as a percentage of the initial cell number.

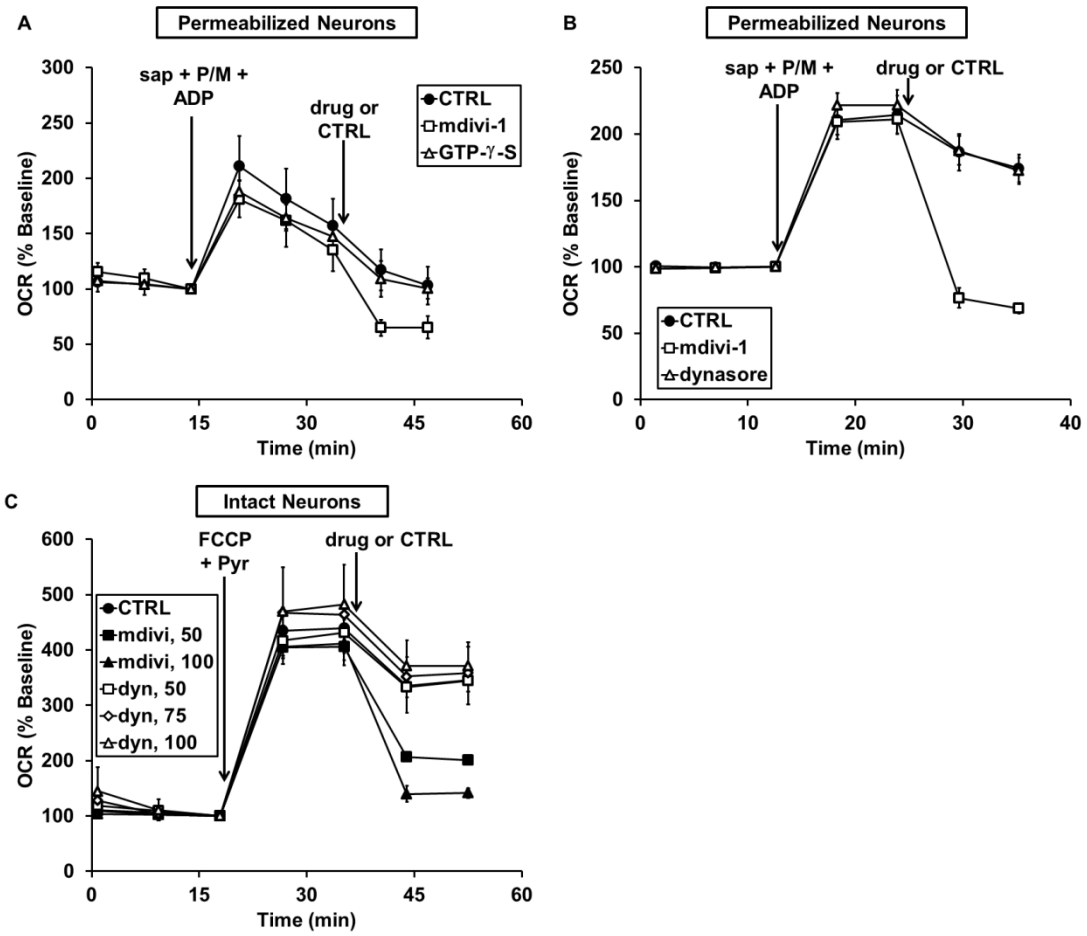

Figure S6. General GTPase or dynamin inhibition does not impair Complex I-dependent neuronal respiration, related to Figure 4. Saponin (sap), pyruvate plus malate (P/M), and ADP were injected as in Figure 1D while neuronal respiration was monitored. At the second arrow, neurons were treated with GTP-γ-S (0.5 mM, A) or dynasore (50 μM, B), and the response was compared to that of mdivi-1 (50 μM) or DMSO vehicle (CTRL) injection. (C) Respiration of intact neurons was measured, followed by injection of FCCP (3 μM) plus pyruvate (10 mM) to stimulate maximal respiration (first arrow), and then CTRL, mdivi-1, or dynasore (second arrow). Numbers in figure legend are mdivi-1 (mdivi) or dynasore (dyn) concentration in μM. Traces are mean ± SD from three wells and are representative of three independent experiments.

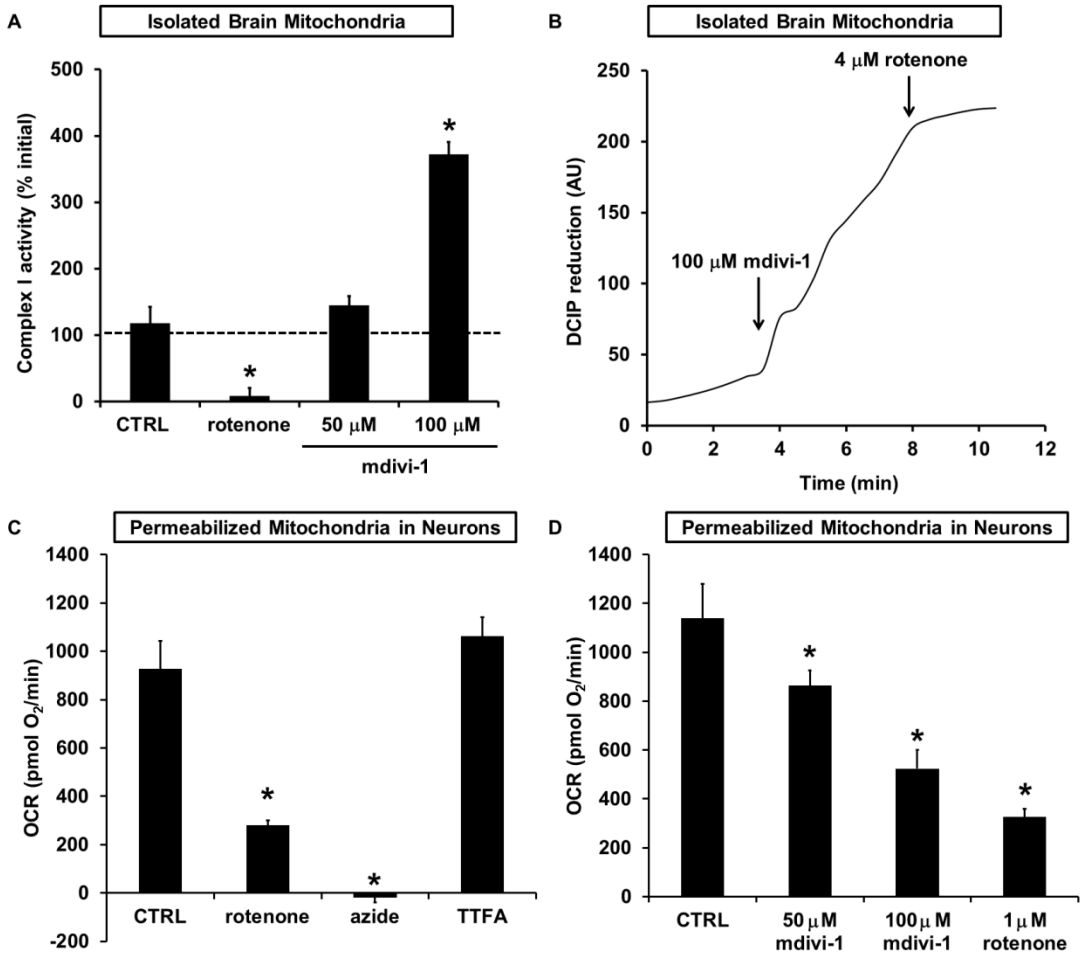

Figure S7. Mdivi-1 inhibits Complex I-III-IV linked activity, but not the ability of Complex I to reduce an artificial electron acceptor, related to Figure 5.

(A) Complex I activity was quantified by DCIP reduction in brain mitochondria exposed to mdivi-1 (50 or 100μM), rotenone (4μM), or vehicle. (B) Representative trace of data quantified in A. (C) Neuronal OCR was measured in the presence of saponin (25 μg/ml), alamethicin (40 μg/ml), NADH (0.5 mM), and cytochrome *c* (100 μM). Rotenone (1 μM), sodium azide (5 mM) or TTFA (10 μM) were present when indicated. Note that negative OCR following azide treatment is due to inaccuracy at the detection limit of the instrument. (D) OCR was measured as in (C) and mdivi-1 or rotenone was present when indicated. Data in (A), (C), and (D) are mean ± SE, n=3. * $p<0.05$ compared to control.

SUPPLEMENTAL MOVIES

Movie S1. Mitochondrial structure over time in vehicle-treated COS-7 cells, related to Figure 2.

Mito-GFP-transfected COS-7 cells incubated in phenol red-free DMEM culture medium were imaged for 65 minutes (5 minutes per frame) following DMSO addition.

Movie S2. Mitochondrial structure over time in mdivi-1-treated COS-7 cells, related to Figure 2.

Mito-GFP-transfected COS-7 cells incubated in phenol red-free DMEM culture medium were imaged for 65 minutes (5 minutes per frame) following mdivi-1 (50 μM) addition.