Deletion of mitochondrial calcium uniporter incompletely inhibits calcium uptake and induction of the permeability transition pore in brain mitochondria

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Ca\(^{2+}\) influx into mitochondria is mediated by the mitochondrial calcium uniporter (MCU), whose identity was recently revealed as a 40-kDa protein that along with other proteins forms the mitochondrial Ca\(^{2+}\) uptake machinery. The MCU is a Ca\(^{2+}\)-conducting channel spanning the inner membrane. Here, deletion of the MCU completely inhibited Ca\(^{2+}\) uptake in liver, heart, and skeletal muscle mitochondria. However, in brain nonsynaptic and synaptic mitochondria from neuronal somata/glial cells and nerve terminals, respectively, the MCU deletion slowed, but did not completely block, Ca\(^{2+}\) uptake. Under resting conditions, brain MCU-KO mitochondria remained polarized, and in brain MCU-KO mitochondria, the electrophoretic Ca\(^{2+}\) ionophore ETH129 significantly accelerated Ca\(^{2+}\) uptake. The residual Ca\(^{2+}\) uptake in brain MCU-KO mitochondria was insensitive to inhibitors of mitochondrial Na\(^{+}/\)Ca\(^{2+}\) exchanger and ryanodine receptor (CGP37157 and dantrolene, respectively), but was blocked by the MCU inhibitor Ru360. Respiration of WT and MCU-KO (CGP37157 and dantrolene, respectively), but was blocked by

Mitochondrial Ca\(^{2+}\) uptake and the ability of Ca\(^{2+}\) to regulate mitochondrial functions have been established for many years (1–4). Ca\(^{2+}\) uptake by mitochondria plays an important role for the organelle and for the whole cell. It helps to maintain low cytosolic Ca\(^{2+}\) when Ca\(^{2+}\) influx into the cell is increased (e.g. following stimulation of ionotropic glutamate receptors in neurons) (5–7). The delivery of Ca\(^{2+}\) into the mitochondrial matrix can activate mitochondrial dehydrogenases such as pyruvate, α-ketoglutarate, and isocitrate dehydrogenases and, thereby, can stimulate mitochondrial respiration and ATP production (4). Finally, excessive Ca\(^{2+}\) uptake may lead to mitochondrial damage through induction of the permeability transition pore (PTP) (3, 8). Ca\(^{2+}\) transport into mitochondria is mediated by the Ca\(^{2+}\) uniporter, a Ca\(^{2+}\) channel complex in the inner mitochondrial membrane (9, 10), the molecular identity of which was recently revealed (11, 12).

In recent studies, it was found that mitochondria contain several proteins involved in Ca\(^{2+}\) transport into the organelle: MCU (mitochondrial calcium uniporter) (11, 13), MCUb (14), MICU1 and MICU2 (15–17), EMRE (18), and MCUR (19). Among other components of mitochondrial Ca\(^{2+}\) transport machinery, MCU is believed to be the Ca\(^{2+}\) channel traversing the inner mitochondrial membrane (IMM) (13). In experiments with purified MCU reconstituted into a planar bilayer, ruthenium red, an inhibitor of the Ca\(^{2+}\) uniporter (20, 21), blocked MCU-mediated Ca\(^{2+}\) currents (11). Genetic ablation of MCU completely prevented Ca\(^{2+}\) uptake by skeletal muscle and heart mitochondria and inhibited induction of the PTP but failed to protect cells from cell death and failed to protect cardiac tissue from ischemia–reperfusion injury (22).

The Ca\(^{2+}\)-dependent induction of the PTP is the major mechanism of Ca\(^{2+}\)-induced mitochondrial damage (3, 8, 23, 24). In the brain, PTP induction and consequent mitochondrial damage are the primary mechanisms contributing to glutamate

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2 The abbreviations used are: PTP, permeability transition pore; MCU, mitochondrial calcium uniporter; αD, cyclophilin D; CsA, cyclosporin A; 2,4-DNP, 2,4-dinitrophenol; TPP\(^{+}\), tetraphenylphosphonium; IMM, inner mitochondrial membrane; mNCX, mitochondrial Na\(^{+}/\)Ca\(^{2+}\) exchanger; mNR, mitochondrial ryanodine receptor; NMDA, N-methyl-D-aspartate; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; cyt c, cytchrome c; VDAC1, voltage-dependent anion channel 1; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; KO, knockout.
excitotoxicity (25–27), a major deleterious factor in stroke, secondary traumatic brain injury, and various neurodegenerations. An induction of the PTP requires Ca\(^{2+}\) influx into mitochondria (28). Correspondingly, knockdown of MCU reduced NMDA-induced increases in mitochondrial Ca\(^{2+}\) in neurons, leading to milder mitochondrial depolarization and elevated resistance to excitotoxicity (29). On the other hand, MCU KO failed to protect brain from hypoxic-ischemic damage, despite strongly hindering mitochondrial Ca\(^{2+}\) uptake and PTP induction (30).

One reason for this controversy could be an incomplete inhibition of Ca\(^{2+}\) uptake in brain mitochondria from MCU-KO mice. However, brain mitochondria from MCU-KO mice are poorly characterized in terms of Ca\(^{2+}\) uptake, membrane potential changes, respiratory activities, and susceptibility to PTP induction. In the present study, we investigated the effect of MCU KO on Ca\(^{2+}\) uptake and membrane potential in isolated brain nonsynaptic and synaptic mitochondria as well as in liver, heart, and skeletal muscle mitochondria. We also compared respiratory activities of brain mitochondria isolated from WT and MCU-KO mice. Finally, we evaluated susceptibility of MCU-KO and WT mitochondria to PTP induction. Our results demonstrate that there is a unique MCU-independent, Ru360-sensitive Ca\(^{2+}\) uptake in brain mitochondria that results in PTP induction in a fraction of vulnerable organelles. Thus, these findings evince a potential mechanism by which deletion of MCU might be not very efficacious in protecting brain mitochondria from PTP induction and, consequently, in protecting the brain from ischemia–reperfusion insults.

Results

The effect of MCU deletion on mitochondrial Ca\(^{2+}\) uptake

All MCU-KO mice used in our experiments were genotyped (Fig. 1A) and all mitochondrial preparations were tested for MCU by immunoblotting (Fig. 1B). The level of MCU expression in brain nonsynaptic mitochondria of MCU-KO mice was below the detection limit of Western blotting. Expression of MCU paralog, MCUb (18), was slightly but statistically significantly lower in brain nonsynaptic mitochondria from MCU-KO mice compared with mitochondria from WT animals. In our study, we used a high-resolution Ca\(^{2+}\)-sensitive electrode that enabled us to evaluate, with high precision, changes in the rate of Ca\(^{2+}\) uptake by mitochondria from MCU-KO and WT mice. To assess the rate and capacity of Ca\(^{2+}\) uptake, multiple pulses of CaCl\(_2\) were applied to mitochondria (Fig. 1, D and E). Mitochondrial Ca\(^{2+}\) uptake was monitored by measuring a decrease in external Ca\(^{2+}\). Brain nonsynaptic mitochondria isolated from WT mice accumulated Ca\(^{2+}\) faster than mitochondria from MCU-KO animals (Fig. 1D). Despite this, Ca\(^{2+}\) uptake capacity, determined as the amount of Ca\(^{2+}\) accumulated by mitochondria before they failed to accumulate additional Ca\(^{2+}\), was not significantly different. BSA significantly increased the Ca\(^{2+}\) uptake capacity (Fig. 1E). The Ca\(^{2+}\) uptake capacity is limited by an induction of the PTP (30). Because BSA binds free fatty acids (31), which are activators of the PTP (32), its inclusion in the incubation medium defers PTP induction and, consequently, increases Ca\(^{2+}\) uptake capacity (33). In the presence of BSA, the Ca\(^{2+}\) uptake capacities of MCU-KO and WT mitochondria remain similar (Fig. 1E).

In parallel experiments, we tested the effect of Ca\(^{2+}\) pulses on mitochondrial membrane potential (Fig. 1, F and G). The decreased rate of Ca\(^{2+}\) uptake by MCU-KO mitochondria (Fig. 1, D and E) could result from these mitochondria being less polarized. However, under resting conditions, mitochondria from MCU-KO and WT mice accumulated comparable amounts of TPP\(^{+}\), suggesting similar polarization of both MCU-KO and WT mitochondria. The repetitive Ca\(^{2+}\) pulses produced fast, transient depolarizations in WT mitochondria (Fig. 1F), whereas in MCU-KO mitochondria, Ca\(^{2+}\) pulses produced slower depolarizations with much smaller amplitude (Fig. 1G). At the end of these experiments, mitochondria were treated with the uncoupler 2,4-dinitrophenol (2,4-DNP) to produce maximal depolarization.
In contrast to brain nonsynaptic mitochondria, mitochondria isolated from liver, heart, and skeletal muscles of MCU-KO mice were not able to take Ca\(^{2+}\) up and depolarize in response to Ca\(^{2+}\) (Fig. 2). The MCU expression in heart mitochondria was the lowest compared with other tissues (Fig. 2). The level of MCU in mitochondria from MCU-KO mice was below the detection limit of Western blotting. The level of MCUb varied among mitochondria from different tissues, with skeletal muscle mitochondria having the least amount of MCUb and heart mitochondria having the greatest amount of MCUb (Fig. 2K).

Expression of MCUb was distinctly lower in skeletal muscle mitochondria from MCU-KOmice compared with MCUb level in skeletal muscle mitochondria from WT animals. ETH129, an electrophoretic Ca\(^{2+}\) ionophore (34), can transport Ca\(^{2+}\) across the membrane independently of the Ca\(^{2+}\) uniporter (35). Ru360, an inhibitor of the Ca\(^{2+}\) uniporter (36), strongly inhibited Ca\(^{2+}\) uptake (Fig. 3B). When the Ca\(^{2+}\) uniporter was blocked by Ru360, ETH129 restored Ca\(^{2+}\) uptake by brain nonsynaptic mitochondria. Similarly, ETH129 significantly increased Ca\(^{2+}\) uptake rate in brain nonsynaptic mitochondria.

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mitochondria from MCU-KO mice (Fig. 3C). This is consistent with unaffected polarization of MCU-KO mitochondria and supports the notion that deletion of MCU was the main cause of inhibition of Ca\textsuperscript{2+} uptake. On the other hand, Ru360 strongly inhibited Ca\textsuperscript{2+} uptake by MCU-KO mitochondria (Fig. 3C). Fig. 3D shows statistical analysis of the data. Fig. 3A shows a calibration of Ca\textsuperscript{2+}-selective electrode with subsequent 100 \mu M CaCl\textsubscript{2} pulses.

The intriguing finding that despite MCU deletion, MCU-KO brain nonsynaptic mitochondria were able to accumulate Ca\textsuperscript{2+} (Fig. 1, D and E), suggested that either MCU is not a Ca\textsuperscript{2+} channel essential for Ca\textsuperscript{2+} uptake and serves a regulatory role or there are alternative pathways for Ca\textsuperscript{2+} influx into mitochondria. Because reconstituted MCU was able to generate Ca\textsuperscript{2+} currents in experiments with planar lipid bilayer (11), the former hypothesis seems unlikely. The alternative pathways for Ca\textsuperscript{2+} uptake by MCU-KO brain mitochondria could include mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, operating in reverse (37, 38), or ryanodine receptor, found in mitochondria (mRR) (39, 40). However, neither CGP37157, an inhibitor of mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (41), nor dantrolene, an inhibitor of ryanodine receptor that inhibited mRR in previous studies (39, 40), suppressed the residual Ca\textsuperscript{2+} uptake by MCU-KO mitochondria (Fig. 4, C–F). Here again, Ru360, strongly inhibited Ca\textsuperscript{2+} uptake by WT and MCU-KO mitochondria (Fig. 4, B and C). Fig. 4A shows calibration of the Ca\textsuperscript{2+}-selective electrode with consecutive 20 \mu M CaCl\textsubscript{2} pulses, whereas Fig. 3F shows statistical analysis of these Ca\textsuperscript{2+} uptake experiments. The difference in the rates of Ca\textsuperscript{2+} uptake shown in Figs. 3 and 4 is most likely due to the difference in the concentration of added Ca\textsuperscript{2+}. In Fig. 3, Ca\textsuperscript{2+} uptake was induced by adding 100 \mu M CaCl\textsubscript{2}, whereas in Fig. 4, 20 \mu M Ca\textsuperscript{2+} was added.

The role of MCU in regulation of mitochondrial respiration

In the mitochondrial matrix, Ca\textsuperscript{2+} activates pyruvate dehydrogenase as well as the Krebs cycle enzymes isocitrate dehydrogenase and \alpha-ketoglutarate dehydrogenase (4), and therefore, inhibition of Ca\textsuperscript{2+} influx into mitochondria might affect mitochondrial respiration. To test this possibility, we performed respirometry experiments using a Clark electrode and isolated brain nonsynaptic mitochondria fueled either with pyruvate plus malate (Complex I substrates) (Fig. 5, A and B) or with succinate (a Complex II substrate) (Fig. 5, C and D). In the latter case, the incubation medium was supplemented with 3 mM glutamate to remove oxaloacetate in transaminase reaction to prevent oxaloacetate accumulation and inhibition of succinate dehydrogenase (42, 43). We analyzed basal respiration...
Ca$^{2+}$ uptake and PTP in MCU-KO brain mitochondria

Figure 5. Respiration of brain nonsynaptic mitochondria isolated from WT and MCU-KO mice. In A and B, respiration of WT and MCU-KO mitochondria, respectively, fueled with a combination of 3 mM pyruvate plus 1 mM malate. Where indicated in parentheses, 200 μM ADP was added to mitochondria. At the end of the experiment, 60 μM 2,4-DNP was added to maximally stimulate respiration. In C and D, respiration of WT and MCU-KO mitochondria, respectively, fueled with a combination of 3 mM succinate plus 3 mM glutamate. Where indicated, 100 μM α-Glu, succinate plus 3 mM glutamate were added to mitochondria. In F, statistical analyses of respiratory rates of WT and MCU-KO mitochondria fueled with 3 mM pyruvate plus 1 mM malate (E) or 3 mM succinate plus 3 mM glutamate (F), determined by proton permeability of the IMM; ADP-stimulated respiration (V$_{ADP}$), determined primarily by activity of the oxidative phosphorylation system; the controlled respiration (V$_{c}$), determined by proton permeability of the IMM and possible contaminations with ATPase activities; and uncoupled respiration (V$_{DNP}$), governed by activity of the mitochondrial electron transport chain. In our experiments, we did not find a significant difference in respiration of mitochondria from MCU-KO and WT mice under any tested conditions. Fig. 5 (E and F) shows statistical analyses of respirometry data. Thus, deletion of MCU does not affect mitochondrial respiration under resting, ADP-stimulated, and uncoupled conditions.

The Ca$^{2+}$ influx into mitochondria may induce the PTP (28), and this may affect mitochondrial respiration (44). Interestingly, this effect depends on the type of oxidative substrates used in these experiments. With WT mitochondria oxidizing pyruvate plus malate, Ca$^{2+}$ produced strong inhibition of respiration that could be rescued by succinate (Fig. 6A). Further improvement was achieved by adding a combination of 3 mM NADH and 30 μg/ml cytochrome c (Cyt c) (Fig. 6B). With MCU-KO mitochondria, Ca$^{2+}$-induced respiratory inhibition was also apparent but was much weaker (Fig. 6C). Similar to experiments with WT mitochondria, succinate and Cyt c plus NADH improved respiration of MCU-KO mitochondria (Fig. 6D), and the effect of succinate was stronger than with WT mitochondria. Glutamate added after succinate failed to further improve mitochondrial respiration (Fig. 6, A–D). Fig. 6E shows statistical analyses of these experiments.

The effect of MCU deletion on PTP opening

The Ca$^{2+}$-induced inhibition of mitochondrial respiration fueled by Complex I substrates was demonstrated previously (44). This could be explained by an induction of the PTP and loss of mitochondrial NADH (45). On the other hand, stimulation of mitochondrial respiration fueled by succinate was also attributed to PTP induction leading to uncoupling of oxidative phosphorylation and the increased respiratory rate (44). Taken together, the effects of Ca$^{2+}$ on mitochondrial respiration suggested a decreased propensity of MCU-KO mitochondria for PTP induction. To test this hypothesis, we monitored simultaneously changes in light scattering of mitochondrial suspension, indicative of mitochondrial swelling, and distribution of lipophilic cation tetrathylphosphonium (TPP$^+$) across the IMM, indicative of changes in mitochondrial membrane potential (Fig. 7). An addition of CaCl$_2$ (100 μM) to WT nonsynaptic mitochondria produced a rapid decrease in light scattering, indicating swelling of the organelles, and an increase of TPP$^+$ concentration in the incubation medium, indicating mitochondrial depolarization (Fig. 7A). To quantify mitochondrial swelling, alamethicin, a pore-forming agent (46), was added to mito-
Ca\(^{2+}\) uptake and PTP in MCU-KO brain mitochondria

Figure 7. PTP induction in brain nonsynaptic mitochondria from WT and MCU-KO mice. A-Western blots demonstrating CyD levels in brain homogenates and nonsynaptic mitochondria isolated from brains of WT and MCU-KO mice. Note that despite the same protein loading for mitochondria and homogenate samples, there is enrichment of CyD and VDAC1 in the isolated mitochondria fraction compared with brain homogenates. MEK1/2 was used as a cytosolic marker to illustrate the purity of the mitochondrial preparation. VDAC1 was used as a loading control. B, statistical analysis of immunoblotting. Data represent a ratio of CyD to VDAC1 band intensities and are shown as mean ± S.D. (error bars) (n = 5). For ADP and ATP measurements (C and D, respectively), nonsynaptic mitochondria were incubated in the standard incubation medium supplemented with 3 mM pyruvate and 1 mM malate for 10 min at 37 °C. Then ADP and ATP levels were measured as described under “Experimental procedures.” Data are shown as mean ± S.D. (error bars), n = 6. *, p < 0.05 comparing ADP content in WT versus MCU-KO mitochondria. a.u., arbitrary units.

The expression of mitochondrial cyclophilin D (CyD) and the levels of ADP and ATP in mitochondria are major factors that regulate PTP induction (47). CyD sensitizes mitochondria to Ca\(^{2+}\)-triggered PTP induction (48, 49). Di- and triphosphate adenine nucleotides antagonize PTP induction by an incompletely understood mechanism (50). We assessed the CyD level in brain nonsynaptic mitochondria isolated from WT and MCU-KO mice and did not find a difference (Fig. 8). In parallel experiments, we assessed the levels of ADP and ATP in WT and MCU-KO brain nonsynaptic mitochondria (Fig. 8). ADP and ATP levels were comparable with those we reported previously (51). The difference in ATP levels was statistically insignificant, whereas ADP was slightly but statistically significantly decreased in mitochondria from MCU-KO mice. The reason for this difference is not clear. These data indicate that suppressed PTP induction in MCU-KO mitochondria was not due to a decrease in CyD expression or elevated ADP and/or ATP levels in these mitochondria.

Figure 8. CyD expression and ADP and ATP content in brain nonsynaptic mitochondria isolated from WT and MCU-KO mice. A Western blots demonstrating CyD levels in brain homogenates and nonsynaptic mitochondria isolated from brains of WT and MCU-KO mice. Note that despite the same protein loading for mitochondria and homogenate samples, there is enrichment of CyD and VDAC1 in the isolated mitochondria fraction compared with brain homogenates. MEK1/2 was used as a cytosolic marker to illustrate the purity of the mitochondrial preparation. VDAC1 was used as a loading control. B, statistical analysis of immunoblotting. Data represent a ratio of CyD to VDAC1 band intensities and are shown as mean ± S.D. (error bars) (n = 5). For ADP and ATP measurements (C and D, respectively), nonsynaptic mitochondria were incubated in the standard incubation medium supplemented with 3 mM pyruvate and 1 mM malate for 10 min at 37 °C. Then ADP and ATP levels were measured as described under “Experimental procedures.” Data are shown as mean ± S.D. (error bars), n = 6. *, p < 0.05 comparing ADP content in WT versus MCU-KO mitochondria. a.u., arbitrary units.
**Ca**

**2+** uptake and PTP in MCU-KO brain mitochondria

Brain nonsynaptic mitochondria used in our study originated from neuronal somata and glial cells, whereas synaptic mitochondria are from nerve terminals and are of purely neuronal origin. We tested synaptic mitochondria from MCU-KO mice and found properties similar to nonsynaptic mitochondria (Fig. 9). The level of MCU in synaptic mitochondria from MCU-KO mice was below the detection limit of Western blotting (Fig. 9A). The level of MCUb in synaptic mitochondria from MCU-KO mice was 2-fold lower than in synaptic mitochondria from WT mice (Fig. 9, A and B). In synaptic mitochondria, deletion of MCU decreased the rate of **Ca**

**2+** uptake but did not completely prevent **Ca**

**2+** influx and membrane depolarization (Fig. 9, C and D). Consistent with this, MCU deletion in synaptic mitochondria inhibited **Ca**

**2+**-dependent PTP induction but failed to completely prevent it (Fig. 9, E–G).

**Discussion**

In the present study, we discovered a unique MCU-independent **Ca**

**2+** uptake mechanism existing in brain mitochondria isolated from MCU-KO mice (Figs. 1 and 9). We found that deletion of MCU significantly slows the rate of **Ca**

**2+** influx into brain mitochondria, but does not completely abolish it. Consistently, the **Ca**

**2+**-triggered induction of PTP in brain mitochondria from MCU-KO mice is hindered, but not completely prevented. Based on these findings, deletion of MCU may have limited ability to protect brain mitochondria from PTP induction and may incompletely protect neurons against glutamate-induced **Ca**

**2+** dysregulation and excitotoxicity, in which an induction of PTP in mitochondria plays a key role (52).

**Ca**

**2+** uptake by mitochondria plays an important role in regulation of cellular and mitochondrial functions. Mitochondria can accumulate large amounts of **Ca**

**2+**, contributing to maintenance of low resting cytosolic **Ca**

**2+** (9). In the mitochondrial matrix, **Ca**

**2+** can activate pyruvate, **α**-ketoglutarate, and isocitrate dehydrogenases, thereby stimulating mitochondrial respiration and oxidative phosphorylation (4). In our study, however, MCU deletion failed to cause significant changes in mitochondrial respiration, suggesting that this kind of regulation might be not essential. This is in line with the notion that rapid **Ca**

**2+** influx into mitochondria mediated by MCU does not regulate resting mitochondrial **Ca**

**2+** levels and, consequently, MCU does not affect the resting mitochondrial respiration. On the other hand, the excessive accumulation of **Ca**

**2+** in mitochondria may lead to the induction of PTP, manifested in mitochondrial depolarization and swelling (8). Both events were evaluated in our study, and both were significantly reduced in MCU-KO mitochondria compared with WT mitochondria.

The **Ca**

**2+**-induced PTP damages mitochondria and, therefore, is ineffectively involved in mitochondrial pathophysiology. The molecular identity of PTP is still a matter of debate (53). However, it is well established that **Ca**

**2+** must enter mitochondria to induce the PTP (28). The **Ca**

**2+** uniporter is the main pathway for **Ca**

**2+** influx into mitochondria (10). Recently, the molecular identity of the **Ca**

**2+** uniporter was revealed (11, 12). It appears that the **Ca**

**2+** uniporter is composed of several subunits, among which MCU serves as the **Ca**

**2+** channel spanning the IMM (9). Consequently, genetic ablation of MCU in MCU-KO mice led to complete inhibition of **Ca**

**2+** uptake in skeletal muscle and heart mitochondria (22). How MCU deletion affects **Ca**

**2+** transport in brain mitochondria was not completely clear. Recently, Nichols et al. (54) reported that **Ca**

**2+** influx in brain mitochondria from MCU-KO mice was inhibited and PTP induction was hindered. However, in this study, mitochondrial depolarization induced by **Ca**

**2+** was not unequivocally attributed to PTP induction by preventing it with PTP inhibitors. In addition, it was not unambiguously proven.
that inhibition of mitochondrial Ca$^{2+}$ uptake is indeed due to MCU deletion. Whether putative inhibition of PTP induction was the result of suppressed Ca$^{2+}$ influx into mitochondria or due to other factors remained unclear.

The lack of difference in CyD expression and comparable or higher levels of ADP and ATP in WT mitochondria are inconsistent with the idea that these factors might contribute to the decreased PTP induction in MCU-KO mitochondria. On the other hand, restoration of PTP induction in MCU-KO mitochondria in the presence of ETH129, an electrophoretic Ca$^{2+}$ ionophore that can deliver Ca$^{2+}$ into mitochondria (34, 35) and accelerate Ca$^{2+}$ influx into MCU-KO mitochondria (Fig. 3C), suggests that inhibition of Ca$^{2+}$ uptake due to MCU deletion is the main cause of PTP inhibition in MCU-KO mitochondria. Nevertheless, the residual Ca$^{2+}$ uptake in MCU-KO mitochondria apparently could induce PTP in the fraction of vulnerable mitochondria. This explains why Ca$^{2+}$ could inhibit respiration of MCU-KO mitochondria fueled by Complex I substrates. Under these conditions, recovery of mitochondrial respiration with succinate is consistent with previously published results (44). Further improvement with Cyt c and NADH suggests the loss of Cyt c and depletion of mitochondrial NADH due to PTP induction (44, 45) as the main mechanisms for respiratory inhibition.

The main unanswered question in our study concerns the mechanism of residual Ca$^{2+}$ uptake in MCU-KO mitochondria. Previously, it was reported that mitochondria may possess an mRR that may contribute to mitochondrial Ca$^{2+}$ uptake (39, 40). In addition, it has been well established that mitochondria possess a Na$^{+}$/Ca$^{2+}$ exchanger (mNCX) responsible for Ca$^{2+}$ efflux from mitochondria (55). It is possible that mNCX, similarly to plasmalemmal NCX (56, 57), may work in reverse under conditions of metabolic inhibition and/or elevated cytosolic Ca$^{2+}$, bringing Ca$^{2+}$ into mitochondria (37, 38). To test these possibilities, we used dantrolene, an inhibitor of ryanodine receptor, previously used in experiments with mitochondria (39, 40), and CGP37157, an inhibitor of mNCX (41). We used these agents in concentrations that previously produced inhibitory effects on the corresponding targets, mRR or mNCX. In our experiments, however, neither of these agents affected the residual Ca$^{2+}$ uptake by MCU-KO mitochondria. On the other hand, Ru360, an inhibitor of the Ca$^{2+}$ uniporter (36), completely blocked Ca$^{2+}$ uptake by MCU-KO mitochondria. The question remains as to which protein(s) interacts with Ru360 in the absence of MCU and how this leads to complete inhibition of residual Ca$^{2+}$ uptake.

It is possible that mitochondria possess MCU-independent pathways for Ca$^{2+}$ uptake (58, 59). One of the possible candidates for the MCU-independent Ca$^{2+}$ uptake pathways is MCUb (14), which was detected in isolated brain mitochondria (Figs. 1B and 9A). Although Raffaello et al. (14) proposed that MCUb forms a nonconducting channel and this correlated with the lack of Glu-257 in MCUb, the structure study by Oxenoid et al. (60) argued against that conclusion. They found that the Glu-257 is dispensable for Ca$^{2+}$ uptake, thus implying that MCUb could also conduct Ca$^{2+}$. In addition, it is known that Ser-259 of MCU is responsible for inhibition of the MCU-mediated Ca$^{2+}$ uptake by Ru360 (12, 60). This Ser-259 is conserved both in MCU and MCUb. These previous studies therefore raised the possibility that MCUb could be responsible for the MCU KO–resistant and Ru360-sensitive mitochondrial Ca$^{2+}$ uptake found in the present study. Alternatively, our data obtained with brain mitochondria do not rule out the possibility that MCU might not be a Ca$^{2+}$ channel, but instead could be an accessory, regulatory subunit of the mitochondrial Ca$^{2+}$ transport complex. However, our experiments with liver, heart, and skeletal muscle mitochondria as well as experiments with MCU reconstituted in the planar lipid bilayer, in which MCU ion channel activity has been detected (11), argue against these hypotheses.

Interestingly, a low expression of MCU and high expression of MCUb in WT heart mitochondria correlated with high Ca$^{2+}$ uptake capacity of heart mitochondria compared with liver and skeletal muscle mitochondria. The amount of Ca$^{2+}$ that can be accumulated by mitochondria is limited due to PTP induction (30). Consequently, our findings suggest that the levels of expression of MCU and MCUb may determine the propensity to PTP induction in mitochondria.

The failure to completely block Ca$^{2+}$ uptake by genetic ablation of MCU in brain mitochondria and, subsequently, incomplete prevention of PTP induction suggests that targeting MCU alone might not be very efficacious in protecting neurons from glutamate-induced Ca$^{2+}$ dysregulation and glutamate excitotoxicity. It is conceivable that combined deletion of MCU and CyD might be much more effective in neuroprotection. CyD deletion increases mitochondrial Ca$^{2+}$ uptake capacity, inhibits PTP induction, and augments resistance of neurons to deleterious effect of glutamate (52). However, this neuroprotection is limited. An exposure to higher glutamate concentrations that cause larger Ca$^{2+}$ influx in the cell and, subsequently, into mitochondria, may overcome neuroprotection achieved by CyD deletion (52). It would be very interesting to test in future studies whether a combination of CyD and MCU deletions would result in stronger neuroprotection and whether simultaneous pharmacological inhibition of CyD and MCU could be a valid approach in protecting brain cells from insults associated with elevation of Ca$^{2+}$ influx into neurons.

**Experimental procedures**

**Materials**

Pyruvate, malate, succinate, glutamate, EGTA, ADP, oligomycin, 2,4-dinitrophenol, CGP37157, ETH129, dantrolene, and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), rat Cyt c, and NADH were purchased from Sigma. Tetraphenylphosphonium chloride was from Fluka (Buchs, Switzerland). Percoll was from GE Healthcare. Ru360 was from Calbiochem. BSA, free from free fatty acids, was from MP Biomedicals (Irvine, CA). Alamethicin was from Enzo (Farmingdale, NY).

**Animals**

All procedures with animals were performed in accordance with the institutional animal care and use committee–approved protocol. The breeders of MCU-KO mice were obtained from Dr. Toren Finkel (Center for Molecular Medicine, NHLBI, National Institutes of Health), and breeding col-
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onies were established in the Laboratory Animal Resource Center at Indiana University School of Medicine (Indianapolis, IN). The mice were maintained on a CD1 (Charles River Laboratories, Wilmington, MA) background, and experimental homozygous mice were obtained by heterozygous crosses (22). Age- and sex-matched WT littermates were used as controls. The mice were housed under standard conditions with free access to water and food. In our experiments, we used animals of both sexes. However, in every individual experiment, we only used animals of the same sex. We did not find any significant difference in the results obtained with either sex, and therefore, the data were pooled together for statistical analysis.

**Genotyping**

All offspring were genotyped using a PCR assay on tail DNA with the forward primer: GT F2 (5′-GGAGTAAAGTCAT-GAGCCTGCTAT-3′) and reverse primers GT R2 (5′-CTG-GCTTAGTTGGCAGATTC-3′) and V76R (5′-CCAATAGAACCTCTTGTGCAGTG-3′) (22) and the Platinum PCR Super Mix (Invitrogen) for amplification. The PCR conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s) and then 5 min at 72 °C. Reaction products were analyzed on 1.2% agarose gel run at 100 V for 60 min with Tris acetate–EDTA running buffer containing 1 × GelRed™ nucleic acid gel stain (Biotium, Fremont, CA).

**Isolation of brain, liver, heart, and skeletal muscle mitochondria**

Percoll gradient–purified brain nonsynaptic and synaptic mitochondria from homozygous MCU-KO mice and WT littermates were isolated as we described previously (61). Briefly, brains from three mice of each strain were harvested and processed simultaneously. Following brain tissue homogenization in a 15-ml glass Dounce homogenizer (10 strokes with pestle A, 30 strokes with pestle B) on ice, homogenates were diluted with 30 ml of Isolation Buffer 1 and centrifuged for 10 min at 2,400 rpm in the Beckman centrifuge Avanti J-26XP, rotor JA-25.50 (700 × g). This and all other procedures and centrifugations were performed at 0–2 °C. Then supernatant was centrifuged for 10 min at 12,500 rpm (18,900 × g) in the Beckman centrifuge Avanti J-26XP, rotor JA-25.50. Supernatant was discarded, and the pellet was resuspended in 35 ml of Isolation Buffer 2 and centrifuged for 10 min at 12,500 rpm (18,850 × g) in the Beckman centrifuge Avanti J-26XP, rotor JA-25.50. Next, the pellet was resuspended in 5 ml of Isolation Buffer 3. The suspension was layered onto the top of the Percoll gradient (26%/40%) in Beckman Ultra-Clear centrifuge tubes and centrifuged for 28 min at 15,500 rpm (41,100 × g) in the Beckman ultracentrifuge Optima L100K, bucket rotor SW41Ti. Nonsynaptic mitochondria were resuspended in Isolation Buffer 3 and centrifuged for 20 min at 15,500 rpm (41,100 × g) in the Beckman ultracentrifuge Optima L100K, bucket rotor SW41Ti. The pellet was resuspended in Isolation Buffer 3 and centrifuged again for 20 min at 15,500 rpm (41,100 × g) in the Beckman ultracentrifuge Optima L100K, bucket rotor SW41Ti. The pellet of nonsynaptic mitochondria was collected, resuspended in 0.15 ml of Isolation Buffer 3, and stored on ice. Isolation Buffer 1 contained 225 mM mannitol, 75 mM sucrose, 10 mM Hepes, pH 7.4, adjusted with KOH, 0.1% BSA, free from fatty acids, and 1 mM EGTA. Isolation Buffer 2 contained 225 mM mannitol, 75 mM sucrose, 10 mM Hepes, pH 7.4, adjusted with KOH, 0.1 mM EGTA. Isolation Buffer 3 contained 395 mM sucrose, 0.1 mM EGTA, 10 mM Hepes, pH 7.4. 26% Percoll in Percoll Buffer was prepared with 5.2 ml of Percoll (Sigma) and 14.8 ml of Percoll Buffer; 40% Percoll in Percoll Buffer was prepared with 8 ml of Percoll and 12 ml of Percoll Buffer. Percoll Buffer contained 320 mM sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.4.

Synaptic mitochondria were isolated from synaptosomes by the nitrogen cavitation method using a nitrogen cell disruption bomb, model 4639 (Parr Instrument Co., Moline, IL), cooled on ice (42) with some modifications. Briefly, the synaptosomes obtained during preparation of nonsynaptic mitochondria were transferred to a cooled beaker and placed into the nitrogen bomb on ice under 1,100 p.s.i. for 13 min. Then synaptosomes were layered on top of the discontinuous Percoll gradient (24%/40%) and centrifuged at 15,500 rpm (41,100 × g) for 28 min in a ultracentrifuge Optima L100K, bucket rotor SW41Ti. The mitochondrial fraction in the interface between Percoll layers was transferred into a fresh tube; diluted 1:5 with medium containing 395 mM sucrose, 0.1 mM EGTA, 10 mM Hepes, pH 7.4; and centrifuged for 20 min at 15,500 rpm (41,100 × g) in the Beckman ultracentrifuge Optima L100K, bucket rotor SW41Ti. The pellet was resuspended in 0.15 ml of the latter medium and kept on ice.

Liver, heart, and skeletal muscle mitochondria were isolated and purified in the same way as nonsynaptic brain mitochondria, but without Percoll gradient purification. Liver tissue from one mouse per strain was homogenized using 30 ml of Potter–Elvehjem homogenizers, and then mitochondria were isolated using differential centrifugation as described for nonsynaptic mitochondria before Percoll gradient purification. Hearts from three mice per strain as well as skeletal muscle (quadriceps of one hind limb) from one mouse per strain were disintegrated using Tissue Master 125 grinder (OMNI International, Kennesaw, GA). Then tissues were homogenized using 30-ml Potter–Elvehjem homogenizers, and mitochondria were isolated using differential centrifugation as described for nonsynaptic mitochondria without Percoll gradient purification. Mitochondrial protein concentration was measured by the Bradford method (44) with BSA as a standard.

**Mitochondrial Ca²⁺ uptake measurements**

Mitochondrial Ca²⁺ uptake was measured with a miniature Ca²⁺-selective electrode in a 0.3-ml chamber at 37 °C under continuous stirring. A decrease in Ca²⁺ concentration in the incubation medium indicated mitochondrial Ca²⁺ uptake. Here and in other experiments with isolated mitochondria, the standard incubation medium contained 125 mM KCl, 0.5 mM MgCl₂, 3 mM KH₂PO₄, 10 mM Hepes, pH 7.4, 10 μM EGTA and was supplemented with 3 mM pyruvate plus 1 mM malate. In addition, in some experiments, the incubation medium was supplemented with 0.1 mM ADP and 1 μM oligomycin as described previously (30) and with 0.1% BSA (free from fatty acids, MP Biochemicals, Santa Ana, CA) as indicated. Ca²⁺ was delivered to mitochondria as 10, 20, or 100 μM CaCl₂ pulses.
Ca\(^{2+}\) uptake rates were quantified as nmol of Ca\(^{2+}\)/min/mg of mitochondrial protein.

**Mitochondrial respiration**

Mitochondrial respiration was assessed in a 0.4-ml continuously stirred chamber containing the standard incubation medium, which was supplemented either with 3 mM pyruvate plus 1 mM malate or 3 mM succinate plus 3 mM glutamate. In experiments with succinate, glutamate was used to prevent inhibition of succinate dehydrogenase by oxaloacetate due to elimination of oxaloacetate in the reaction catalyzed by aspartate aminotransferase (43, 62). The chamber was maintained at 37°C and was equipped with a Clark-type oxygen electrode and a tightly sealed lid. The slope of the oxygen consumption trace corresponded to the respiratory rate.

**Mitochondrial swelling and membrane potential**

Mitochondrial swelling was evaluated in a 0.3-ml continuously stirred chamber at 37°C by following changes in light scattering of mitochondrial suspension at 525 nm with an incident light beam at 180° relative to the photodetector. The incubation medium for light scattering measurements contained 215 mM mannitol, 70 mM sucrose, 0.5 mM MgCl\(_2\), 3 mM KH\(_2\)PO\(_4\), 10 mM Hepes, pH 7.4, 10 mM EGTA, 3 mM pyruvate, 1 mM malate. A decrease in light scattering of mitochondrial suspension indicated mitochondrial swelling. Maximal mitochondrial swelling was induced by alamethicin (30 ng/ml), which was considered as 100% swelling. Ca\(^{2+}\)-induced swelling was calculated as a percentage of maximal, alamethicin-induced swelling (63). Mitochondrial membrane potential was assessed simultaneously with mitochondrial swelling with a TPP\(^+\) electrode by monitoring TPP\(^+\) distribution between the incubation medium and mitochondria (64). A decrease in external TPP\(^+\) concentration corresponds to mitochondrial polarization, whereas an increase of TPP\(^+\) concentration in the incubation medium corresponds to depolarization.

**Immunoblotting**

Brain isolated mitochondria pretreated with Protease Inhibitor Mixture (Roche Diagnostics) were solubilized by incubation in NuPAGE LDS sample buffer (Invitrogen) supplemented with a reducing agent at 70°C for 15 min. BisTris Mops gels (12%; Invitrogen) were used for electrophoresis (10 g of protein/lane). After electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Blots were incubated for 1 h at room temperature in blocking solution of 5% BSA, PBS, pH 7.2, and 0.15% Triton X-100. Then blots were incubated with one of the following primary antibodies: mouse monoclonal anti-Complex II 70-kDa subunit (Invitrogen; 1:1000), mouse monoclonal anti-cyclophilin D antibody (Calbiochem; 1:500), and rabbit polyclonal anti-VDAC1 (Calbiochem; 1:1000), rabbit monoclonal anti-MEK1/2 (Pierce; 1:1000). For detection of MCU, we used goat polyclonal antibody (Santa Cruz Biotechnology, Inc. (Dallas, TX), catalog no. sc-163985; 1:250). Blots were incubated with goat anti-mouse or goat anti-rabbit IgG (1:20,000) coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed with Supersignal West Pico chemiluminescent reagents (Pierce). Molecular mass markers See Blue Plus 2 Standards (5 ml) and HiMark prestained high-molecular weight protein standards (10 ml) (Invitrogen) were used to determine molecular masses of the bands. NIH ImageJ version 1.48 (National Institutes of Health) was used to quantify band densities.

**Mitochondrial ADP and ATP content**

ADP and ATP levels were determined using a luciferin/luciferase-based ATP bioluminescent somatic cell assay kit (Sigma) and a GloMax 20/20 luminometer (Promega, Madison, WI). Mitochondria were incubated for 10 min at 37°C in the standard incubation medium supplemented with 3 mM pyruvate plus 1 mM malate, and then ADP and ATP were measured. Measurements were made in 4% perchloric acid extracts neutralized by KOH following the kit manufacturer’s suggestions. ADP was converted to ATP using pyruvate kinase in the presence of phosphoenolpyruvate (67).

**Statistics**

Data are shown as mean ± S.D. of the indicated number of separate experiments. Statistical analysis of the experimental results consisted of unpaired t test or one-way analysis of variance followed by Bonferroni’s post hoc test if applicable (GraphPad Prism® version 4.0, GraphPad Software Inc., La Jolla, CA). Every experiment was performed using several different preparations of isolated mitochondria.

**Author contributions**—J. H. conducted most of the experiments, analyzed the results, and contributed to writing the paper. T. B. maintained the MCU-KO breeding colony, conducted genotyping and immunoblotting experiments, and analyzed the results. J. E. R. and Z. L. maintained the MCU-KO colony and conducted genotyping. Y. M. U. analyzed the results and contributed to writing the paper. N. B. conceived the idea for the project, analyzed the results, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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