Mitochondrial ATP synthase c-subunit leak channel triggers cell death upon loss of its F₁ subcomplex

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Mitochondrial ATP synthase is vital not only for cellular energy production but also for energy dissipation and cell death. ATP synthase c-ring was suggested to house the leak channel of mitochondrial permeability transition (mPT), which activates during excitotoxic ischemic insult. In this study, we purified human c-ring from both eukaryotic and prokaryotic hosts to biophysically characterize its channel activity. We show that purified c-ring forms a large multi-conductance, voltage-gated ion channel that is inhibited by the addition of ATP synthase F₁ subcomplex. In contrast, dissociation of F₁ from F₀ occurs during excitotoxic neuronal death suggesting that the F₁ constitutes the gate of the channel. mPT is known to dissipate the osmotic gradient across the inner membrane during cell death. We show that ATP synthase c-subunit knock down (KD) prevents the osmotic change in response to high calcium and eliminates large conductance, Ca²⁺ and Cs⁺ sensitive channel activity of mPT. These findings elucidate the gating mechanism of the ATP synthase c-subunit leak channel (ACLC) and suggest how ACLC opening is regulated by cell stress in a CypD-dependent manner.

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INTRODUCTION
Mitochondrial F₁F₀ ATP synthase is responsible for ATP synthesis during oxidative phosphorylation and is one of the most abundant proteins in the mitochondrial inner membrane. ATP synthase is a multi-subunit complex consisting of the membrane-embedded F₀ and hydrophilic F₁ subcomplexes. The peripheral and central stalks of ATP synthase link F₀ with F₁ and enhance the catalytic activity and chemo-mechanical coupling of ATP synthase [1].

ATP synthase was shown to contribute to energy dissipation and initiation of cell death through the formation of an uncoupling channel within its c-ring [2–4] or between ATP synthase dimers [5–10]. A model for permeability transition pore opening was suggested recently based on the structural studies of ovine ATP synthase [11]. Cryo-EM maps of ATP synthase exposed to calcium revealed conformational changes within the ATP synthase, including a retracted conformation of subunit e and disassembled c-ring, failing to demonstrate a pore, partially due to the structural determination of protein in detergent [11]. While structural studies can provide valuable information about different conformational snapshots of ACLC on the atomic level, electrophysiological studies are crucial for characterizing the biophysical properties of the channel, defining the gating mechanism of ACLC and its role in mPTP formation. Here we have applied a multidisciplinary approach to characterize the ACLC. We came up with the following set of criteria, in evaluating whether any ATP synthase subunit can form a channel of the mPT: 1) The pore-forming subunit(s) of any ion channel, including the mitochondrial permeability transition pore (mPTP), should form a channel without associated regulatory subunits in its purified form. 2) mPTP is a voltage-gated channel, which means that its channel activity should be modulated in response to changes in transmembrane voltage. 3) Activation and inactivation of ion channels are determined by gating of the channel which regulates the passage of ions. 4) Genetic ablation of the mPTP-forming protein will eliminate its known high (~1.5 nS) conductance activity [12].

In this current report we have addressed these criteria with the following findings: 1) We show that human ATP synthase c-ring forms a large conductance (~1.5 nS) and 2) voltage-gated channel in its purified form without any regulatory subunits. 3) We show that the ATP synthase F₁ subcomplex forms a gate of c-ring channel by inhibiting its activity. In support, we show that F₁ disassembles from F₀ in neurons exposed to glutamate toxicity and that this dissociation is sensitive to CsA, a well-studied inhibitor of CypD-dependent mPT. These data suggest that conformational changes of F₁ towards F₀, or the complete dissociation of these two subcomplexes occurs during permeability

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transition and ACLC opening. In keeping with a central role for ACLC in mPT 4) we demonstrate that significant knockdown (85%) of c-subunit genes eliminates high conductance mPTP-like openings. These findings suggest that the F$_1$ constitutes a gate of ACLC and that the c-ring leak channel comprises a main pore-forming unit of the highly regulated CypD-dependent mPT.

RESULTS
ATP synthase c-ring purified from different hosts forms a large multi-conductance voltage-gated channel sensitive to ATP

To study the biophysical characteristics of the c-ring as a pore-forming component of mPTP, we purified human c-ring from HEK 293 cells (Fig. 1A). We have optimized the c-subunit overexpression and purification procedure reported previously [3] to increase the purity of the preparation. Shown in Fig. 1A is a silver-stained gel of this purified protein which illustrates that it is free from contamination by other proteins. The purified c-subunit is also not contaminated with ATP synthase F$_1$ (Supplementary Fig. S1A). We confirmed the preservation of the native ring structure of purified c-subunit with negative stain EM studies (Fig. 1B) and clear native PAGE (Supplementary Fig. S1B).

To further rule out the possibility of purified c-ring contamination with other mitochondrial proteins, we heterologously overexpressed and purified the human c-ring from E. coli. Human ATP synthase c-subunit is encoded by three different genes ATP5G1, ATP5G2, and ATP5G3. Each of the three genes has a distinct mitochondrial targeting sequence but encodes the identical 75-amino acid-long mature protein. Here in this study, the sequence of human ATP synthase c-subunit corresponding to the mature protein was cloned into the bacterial pEX-1 expression vector. DNA codon optimization strategy was used to increase the expression level of protein in E. coli cells (Supplementary Fig. S1C). The protein was tagged with six histidine residues at its C-terminus to facilitate purification (Ni-NTA). Interestingly, overexpressed mature c-subunit traffics into E. coli membranes (Supplementary Fig. S1D), therefore the N-terminal sequence of c-subunit seems to be dispensable for membrane targeting in E. coli. We found that our preparation of c-subunit purified from E. coli is not contaminated with ATP synthase F$_1$ (Supplementary Fig. S1E). The purified c-subunit demonstrated the same oligomeric composition as the c-subunit purified from HEK 293 cells as assessed by clear native page (Supplementary Fig. S1B). In both cases in the absence of detergent with SDS, human c-subunit was detected at ~250 kDa, suggesting the presence of tetramers of octameric rings formed by purified c-subunits, as has been observed previously [3, 13]. This is also in agreement with the recent report of the cryo-EM structure of porcine ATP synthase tetramers [14]. Partial to complete disassembly of higher-order oligomeric states of c-ring was observed in the presence of SDS as demonstrated by the presence of bands at ~66 and ~8 kDa positions (Supplementary Fig. S1B). There is remarkable variability in c-ring oligomeric composition in different species with c-rings composed of between 8–17 subunits [15–17] and the determining factor for the ring stoichiometries is likely to be the amino acid sequence of the protein [18], as we observed here in the case of human c-subunit overexpression in E. coli.

Next, we set out to investigate the single-channel activity of purified c-rings reconstituted in liposomes (Fig. 1C, D). Shown in Fig. 1D is a patch-clamp recording of purified c-ring (HEK 293) reconstituted in liposomes. The current recordings demonstrate large multi-conductance channel activity with an average peak conductance of 1.1 nS (Fig. 1D) similar to previously published data [3, 19]. We concatenated eleven consecutive traces (10-second long) of channel activity for amplitude histogram analysis. The amplitude histogram demonstrates subconcentrations of 70–100 pS, 300 pS, 600 pS, 700 pS and a presumed fully open state of 1100 pS (Fig. 1D). Data shown here are consistent with a continuously expanding pore such as reported previously for the mitochondrial megachannel [19]. Similar low and high conductance states were reported for mPTP during electrophysiological recordings of isolated mitochondria [12, 20].

We also characterized the single-channel activity of the human c-ring purified from E. coli by performing planar lipid bilayer recordings (Fig. 1E, Supplementary Fig. S1G–I). Shown in Fig. 1E are lipid bilayer recordings at different voltages from different experiments. The group data of single-channel recordings demonstrate that c-ring purified from either HEK 293 or E. coli forms voltage-gated channel activity with a similar peak conductance and probability of opening (NPF; see methods) (Supplementary Fig. S1H, I). Control recordings were performed in the presence of membrane scaffold protein, MSP (Supplementary Fig. S1J) to show that a known non-channel forming protein fails to produce ion channel activity. Shown in Supplementary Fig. S1F is a silver-stained gel of the purified MSP that was used for recordings. Control recordings were also performed with empty bilayers (Supplementary Fig. S1K) to rule out the possibility of membrane leak formation due to the presence of residual detergent or physical rupture of the membrane during recordings.

To determine possible contamination of purified c-subunit with other channel-forming mitochondrial proteins, such as the adenine nucleotide carrier (ANT), we have carried out channel recordings of human c-ring (purified from HEK 293 cells) in the presence of bongkrekic acid (BA), a specific inhibitor of ANT [21]. Figure 2A shows that c-ring channel activity is not affected by the addition of BA during the channel recordings, confirming a lack of contamination with ANT.

One of the biophysical characteristics of the mPTP is its sensitivity to adenine nucleotides [20, 22]. We have reported previously that purified ATP synthase from porcine heart mitochondria and c-subunit (purified from HEK 293 cells) both demonstrate ATP-sensitive channel activities with different binding affinities [3, 4]. Here, we show that ATP addition inhibits channel activity during a continuous bilayer recording of the human c-ring (purified from E. coli) (Fig. 2B, F). In a previous report, we showed that channel activity was equally attenuated by ATP, ADP and AMP, suggesting that binding of these adenine nucleotides rather than ATP hydrolysis is required for channel inhibition [3]. We have previously shown that another well-known inhibitor of mPTP, CsA, fails to inhibit the c-ring channel activity [3] that lacks F$_1$ and stator components, since the binding site for its interacting partner, CypD, is located in the OSCP subunit [10, 23].

ACLC is gated by purified F$_1$

Mitochondrial ATP synthase consists of two structural domains, hydrophilic F$_1$, located in the matrix and membrane-embedded F$_0$, which are linked together by the central and peripheral stalks. According to high-resolution structures of ATP synthase, the central stalk subunits of F$_1$, γ, δ, and ε interact with the c-ring [14]. We have shown earlier that separation of F$_1$ from F$_0$ occurs in isolated mitochondria during exposure to high Ca$^{2+}$ that induces the mPTP opening [3] suggesting that F$_1$ could constitute a channel gate and that conformational changes in ATP synthase are needed to open the channel. Here we set out to study if the application of purified F$_1$ during channel recordings will inhibit the voltage-dependent c-ring channel activity. Supplementary Fig. S2A, B demonstrate the SDS-PAGE and immunoblot analysis of purified F$_1$, respectively. Figure 2C demonstrates a continuous patch-clamp recording of c-ring reconstituted proteoliposomes. The addition of purified F$_1$ to the bath caused the inactivation of the channel (Fig. 2C, G). Ramp voltage recordings before and after the addition of F$_1$ show that channel conductance is inhibited by F$_1$ at all voltages between −100 mV and −100 mV (Fig. 2H). To confirm these findings, we studied if F$_1$ application would inhibit
the c-ring channel activity during planar lipid bilayer experiments. Indeed, we observed channel inactivation upon F₁ application (Fig. 2D, Supplementary Fig. S2C). In contrast to the inhibition found after F₁ addition, the addition of ATP synthase αβ₃ complex, lacking the central stalk subunits gamma, delta and epsilon (Supplementary Fig. S2D) did not inhibit the channel activity (Fig. 2E, G) suggesting that specific interactions between the central stalk subunits gamma, delta and epsilon with the c-ring are necessary for channel inhibition with F₁. We also show that the addition of boiled (denatured) F₁ during the recordings did not inhibit the c-ring channel activity (Fig. 2G). These findings suggest that F₁ forms a gate of the ACLC.

**Dissociation of ATP synthase F₁ from F₀ occurs during excitotoxic neuronal death**

Our findings thus far show that ATP synthase c-ring demonstrates voltage dependent single-channel activity in the absence of F₁ and that the addition of F₁ inhibits the channel activity. We, therefore investigated how pathological events might alter the oligomeric state of ATP synthase, its assembly, and the F₁/F₀ stoichiometric ratio. The membrane-embedded c-ring is part of the ATP synthase F₀ subcomplex. Tight interaction between F₁ and F₀ subcomplexes, and efficient chemo-mechanical coupling, have been suggested to be crucial for efficient ATP synthesis [24].
To determine if excitotoxicity produces dissociation of $F_1$ from $F_0$ and induces any changes in ATP synthase oligomeric state in a pathophysiological setting, we exposed primary hippocampal and cortical neurons to glutamate toxicity. After 18 h of glutamate exposure, mitochondria were isolated and $F_1$ and $F_0$ components were analyzed by immunoblotting. The levels of $F_1$ subunits alpha, beta, gamma and OSCP were significantly reduced compared to those of vehicle-exposed neurons in both hippocampal (Fig. 3A) and cortical neurons (Supplementary Fig. S3A). In contrast, the level of c-subunit was not decreased, suggesting a marked change in $F_1$/c stoichiometry that enhanced the level of “free” c-rings not complexed with $F_1$. 
High levels of free c-ring in mitochondrial membranes could increase the probability of c-ring channel opening and predispose neurons to death in a voltage dependent manner. To determine if dissociation of F₁ from F₀ is associated with the onset of mPTP-induced cell death, we used CsA, the well-known inhibitor of CypD that prevents mPTP opening by binding within the peripheral stalk [23]. We found that CsA inhibits the reduction of F₁ subunit levels and therefore prevents changes in F₁/c stoichiometry under glutamate excitotoxic conditions (Fig. 3A), presumably by preventing conformational changes within the ATP synthase that result in the separation of F₁ from c-ring. CsA also prevents cell death under these conditions as observed in studies of lactate dehydrogenase (LDH) release (LDH assay, Fig. 3B) and neuronal death measured by LDH release into the medium (Fig. 3C). However, it failed to prevent cell death measured by LDH release after glutamate exposure (Fig. 3C). This failure to prevent cell death measured by LDH release after glutamate exposure (Fig. 3C) suggests that CsA protects against dissociation of F₁ from F₀ whereas the proteasome inhibitor only prevents the degradation of F₁/c subunits without preventing their dissociation from F₀.

To study the changes in the oligomeric state of mitochondrial ATP synthase under conditions of glutamate toxicity, we analyzed glutamate-treated neuronal mitochondria with Blue Native Page (BNP). We found that glutamate-induced breakdown of ATP synthase dimers into monomers (Supplementary Fig. S3B, left panel), which is in agreement with a recent report [25]. We also observed an overall increase of free c-subunit levels in mitochondrial membranes in digitonin or n-dodecyl β-D-maltoside (DDM) solubilized mitochondria under conditions of glutamate toxicity, whereas the level of free, uncomplexed F₁ was reduced (Supplementary Fig. S3B), consistent with the SDS PAGE studies.

These data suggest that reduced F₁/c stoichiometric ratio and increased amount of free c-ring dissociated from F₁ are observed under glutamate excitotoxic conditions in which CsA-sensitive mPTP opening occurs.

To investigate further the role of free c-ring in the onset of mPTP, we measured provoked cell death in primary hippocampal neurons (Fig. 3E–G) and HEK 293 cells (Fig. 3H, I) after overexpressing c-subunit. We co-expressed GFP with the c-subunit since they have a high co-expression level, therefore indicating that GFP transfected cells represent c-subunit transfected cells. Cell death was measured only in GFP-expressing cells (Fig. 3E–I). After confirming that overexpressed c-subunit co-localizes with mitochondria in primary hippocampal cultures (Supplementary Fig. S3C), we determined that overexpression of c-subunit alone under these conditions does not induce death, consistent with our previous studies [26] but that it aggravates glutamate-induced cell death (Fig. 3E–G), supporting its role in mPTP. In contrast, reduction of c-subunit levels by shRNA prevents cell death under glutamate excitotoxic conditions (Fig. 3E–G), suggesting that F₁/F₀ dissociation and the change in F₁/F₀ stoichiometry precedes the cell death.

Similarly, overexpression of c-subunit in HEK 293 cells markedly increased H₂O₂-induced cell death (Fig. 3H, I). Immunoblot analysis of mitochondria isolated from c-subunit-overexpressing HEK 293 cells revealed that c-subunit is only partially assembled with the ATP synthase complex (Fig. 3J). A substantial amount of free c-ring uncomplexed with F₁ is present in mitochondrial membranes (Fig. 3J), the band at ~242 kDa position is free c-subunit complex, the band at ~720 kDa position is ATP synthase. Taken together, the data show that the stoichiometric change in F₁/c ratio precedes cell death in response to stimuli that induce mPT, but that c-subunit expression in the absence of a death stimulus does not depolarize the inner membrane.

Death of glutamate-treated neurons was additionally assessed by staining with Annexin V and propidium iodide (PID) to differentiate apoptotic cell death from the total, as previously described [27]. Annexin V and PID staining of hippocampal neurons are shown in (Supplementary Fig. S3D–F). These data suggest that apoptotic death comprises at least 50% of the total death. The initial events in mPT before cell death include mitochondrial inner membrane depolarization. A decrease of the inner membrane potential was observed under glutamate excitotoxic conditions (Supplementary Fig. S3G, H), and this was inhibited by CsA, confirming the role of loss of mitochondrial membrane potential in glutamate excitotoxicity in mPTP-induced neuronal death as described previously [28, 29].

We have previously studied the detachment of F₁ subcomplex from c-ring in response to Ca²⁺ treatment in isolated mitochondria by immunoprecipitation [3]. Our studies revealed that upon Ca²⁺-induced mitochondrial swelling the release of c-ring oligomers occurred, which was inhibited by CsA and ADP treatment. Furthermore, as expected, detachment of F₁ from c-subunit was not observed in mitochondria from CypD KO animals [3], suggesting that CypD-mediated Ca²⁺ binding to F₁ destabilizes ATP synthase causing detachment of F₁ from c-subunit, which initiates mPTP.

Our findings suggest that F₁ constitutes an important inactivation gate of the ACLC (Fig. 3K) and small changes in the F₁/c stoichiometry alter inner membrane permeability after cell death stimulus by increasing the open probability of ACLC and enhancing the risk of neuronal death.

**ATP synthase is required for calcium-induced osmotic changes underlying permeability transition**

Our studies so far suggest that free c-ring dissociated from F₁ is responsible for the large conductance channel activity associated with pathological PT. Pathological PT is associated with water accumulation in the mitochondrial matrix after a high calcium load. Therefore, to determine if free c-subunit is required for calcium-dependent matrix swelling, we used CRISPR-Cas9 to knockdown c-subunit expression in mouse embryonic stem cells (ESCs). We successfully deleted five out of six alleles of the three genes (ATPSG1, ATPSG2 and ATPSG3) encoding ATP synthase c-subunit. The remaining one ATPSG1 allele resulted in 15 percent expression of c-subunit in mitochondrial inner membranes (Fig. 4A). Blue Native PAGE confirmed the existence of ATP synthase.
synthase dimers, monomers, as well as free c-ring in WT ESCs (Fig. 4B). In contrast, c-subunit KD cells failed to form ATP synthase dimers, had low monomer content and no free c-ring (Fig. 4B). Interestingly, the free c-ring is present in native mitochondrial membranes waiting to be assembled with F1, but it is completely absent in KD, which had severely reduced ATP synthase levels. In contrast, free F1 is present in KD mitochondria but it is absent in WT (Fig. 4B). ESCs in general have low coupled oxygen consumption [30]. Nevertheless, we show that oxygen consumption and membrane potential of c-subunit KD mitochondria were reduced compared to WT, confirming the contribution of the c-subunit to the ESCs mitochondrial leak and the inability of
addition of Ca\textsuperscript{2+} isolated KD mitochondria are partially swollen at rest and that the mPTP was fi
mitochondria c-subunit gene KD decreases mPTP-like channel activity of normal and pathological mitochondrial osmotic regulation.

The mPTP was first characterized as an abnormal swelling of mitochondria upon high calcium overload [34]. Later, it was shown by electrophysiological recordings that the calcium-induced mitochondrial swelling was due to the opening of a high conductance, non-selective channel of the mitochondrial inner membrane, named the "mitochondrial megachannel", "multiconductance channel" or mPTP [12, 20]. The mPTP is a \(~1.5\) nS conductance channel that is activated by voltage and Ca\textsuperscript{2+} and inhibited by CsA. If c-ring forms the largest conductance channel of the mitochondrial inner membrane, then its depletion should eliminate the large-conduc-
tance, Ca\textsuperscript{2+} and CsA-sensitive activity of the inner membrane. We set out to determine whether the decrease of free c-subunit and the overall reduction of ATP synthase level would result in significant changes in channel activity.

We used mitoplasts (mitochondrial inner membrane preparations lacking the outer membrane) isolated from WT and c-subunit KD ESCs for single-channel analysis. Patch-clamp recordings of WT mitoplasts demonstrated similar channel activity to that described previously for mPTP [3, 12], with the peak conductance of 3.3 nS and average conductance of 300 pS in the presence of Ca\textsuperscript{2+} and sensitivity to inhibition by CsA (Fig. 4H, J, L, Supplementary Fig. 54B). KD mitoplasts demonstrated channel activity of smaller conductance (~300 pS) that was not sensitive to Ca\textsuperscript{2+} and CsA (Fig. 4I, K, L). The voltage ramp recordings showed that channel activity of WT mitochondria not pre-exposed to Ca\textsuperscript{2+} was activated by Ca\textsuperscript{2+} addition and enhanced at negative potentials (Fig. 4I). In contrast, the activity of the KD mitochondria had a reduced sensitivity to voltage and Ca\textsuperscript{2+} (Fig. 4K, L). Our data suggest that c-subunit depletion eliminates the major contributor to Ca\textsuperscript{2+} and CsA-sensitive large conductance channel (mPTP-like) activity of the inner membrane. The smaller, ~300 pS conductance activity found in KD mitoplasts may be related to ANT (Supplementary Fig. 54C). Our data are in agreement with the recent patch-clamp analysis of mitoplasts isolated from the complete c-subunit knockout HAP1-A12 cells first studied in [35]. A small (~300 pS) channel was still found to be present in the c-subunit knockout mitoplasts [36]. This activity was sensitive to bongkrekic acid, suggesting the role of ANT in at least some of the 300 pS conductance activity. These findings suggest that the c-ring is the largest conductance channel of the inner mitochondrial membrane contributing to Ca\textsuperscript{2+} and CsA-sensitive mPTP, however ANT also may contribute to lower conductance single ion channel activity of mitochondrial membranes.

DISCUSSION

Here we show that the highly purified human ATP synthase c-ring forms a large multi-conductance voltage-gated ion channel that undergoes inactivation by purified F\textsubscript{1} application. We also find that a series of events occurs in primary hippocampal neurons.
during glutamate excitotoxic treatment (intracellular Ca\(^{2+}\) elevation): A Ca\(^{2+}\)-induced conformational change in ATP synthase leads to dissociation of ATP synthase dimers, dissociation of ATP synthase F\(_1\) subcomplex from F\(_{0}\), and a change in the F\(_1/c\) stoichiometric ratio, predisposing neurons to cell death. Proteolysis of F\(_1\) occurs over time, but is not required for cell death. Inhibition of proteolysis prevents F\(_1\) loss, but does not prevent cell death associated with F\(_1\) detachment suggesting that F\(_1\) dissociation is non-reversible in severe pathological conditions leading to cell death. These findings highlight the importance of F\(_1/c\) subunit stoichiometry in regulating ACLC activity and confirm the role of F\(_1\) in forming a gate of the channel. Interestingly, an age-
Fig. 4 ATP synthase is required for normal mitochondrial cristae morphology and large-conductance mPTP-like channel activity. A Representative western blot of WT and c-subunit KD mitochondria. Group data show that only 15% c-subunit is expressed in KD. (n = 4, ***P < 0.0001, unpaired t-test). B Immunoblot after non-denaturing Blue Native Page (representative of n = 3). Free c-subunit is readily discernible in WT but not in KD mitochondria. C EM images of WT and KD mitochondria before and after treatment with ionomycin (mouse ES cells (ESCs) were treated with 1 μM ionomycin for 20 min before they were fixed for further EM analysis). Swelling (electron lucency) observed in WT ESC mitochondria is not present in c-subunit KD ESCs mitochondria. D Group data of the total number of mitochondrial cristae divided by the area of mitochondria (n = 34 mitochondria for WT, n = 39 for KD, (**P < 0.0001), unpaired t-test). E Group data showing electron lucencies before and after treatment with ionomycin, (***P < 0.0004, n = 11 micrographs for WT, n = 11 for WT + ionomycin, n = 15, for KD, n = 15, for KD + ionomycin). F c-subunit KD ESC isolated mitochondria do not swell after Ca2+ addition. Representative traces for WT and KD isolated mitochondrial swelling, monitored upon Ca2+ (0.5 mM) addition in the presence or absence of CsA. Ca2+-sensitive swelling observed in WT mitochondria is not present in c-subunit KD mitochondria. The application of the membrane permeabilizing agent, alamethicin (10 μM), revealed that KD mitochondria were otherwise intact and capable of swelling. G Histogram for group data representing the Ca2+-induced swelling rate. Data show mean ± SEM, n = 3; ***P = 0.0002. One-Way ANOVA with multiple comparison test. H Representative patch-clamp recordings of WT and KD mitoplasts, pre-treated with Ca2+ (1 mM) at –50 mV holding voltage. J, K Representative recordings of WT and KD mitochondria during continuous ramp voltage clamp from −100 mV to +100 mV in the presence or absence of Ca2+ (1 mM). L Group data for peak conductances of mitoplasts, pre-treated with 1 mM Ca2+ in the presence or absence of CsA (5 μM). The Ca2+ and CsA-sensitive activity of WT mitoplasts is absent in c-subunit KD mitoplast recordings. A paired t-test was used to compare WT or KD recordings with and without CsA (5 μM). Unpaired t-test was used to compare WT and KD recordings (n = 15 for WT, n = 9 for KD, n = 6 paired patches for WT + CsA, n = 7 paired patches for KD + CsA ***P < 0.0001).

dependent decrease of F1 content with respect to that observed for Fo has been reported for rat brain and heart mitochondria [37]. Reduced expression levels of F1 subunits and age-dependent dissociation of ATP synthase dimers have also been reported for synaptic mitochondria of the Alzheimer’s disease 5xFAD model mouse [38].

As a more direct experiment of free (uncomplexed with F1) c-ring-induced cell death, we performed c-subunit overexpression in neurons undergoing glutamate excitotoxicity and HEK 293 cells undergoing oxidant-induced death. Our data show that c-subunit overexpression aggravates glutamate-induced cell death in primary hippocampal neurons (Fig. 3E–G) and in H2O2-induced cell death in HEK 293 cells (Fig. 3H, I), while c-subunit KD by shRNA in neurons undergoing glutamate excitotoxicity prevents death. As we show in Fig. 3J, c-subunit is only partially assembled in the ATP synthase complex upon its overexpression in HEK293 cells and it is therefore also present in the mitochondrial membranes as a free c-ring implying that c-ring uncomplexed with F1, places c subunit at risk for death. We suggest that in cells with a reduced F1/c-ring ratio, the increased amount of free c-ring increases the probability of ACLC opening in a voltage dependent manner in response to stimuli that induce mPT, whereas without mPT stimuli, there is no evidence for membrane depolarization and therefore a low probability of voltage dependent mPTP opening (Fig. 3E, G).

Our data implicate c-subunit induced membrane conductance changes in the onset of cell death. It, therefore, seemed prudent to independently assess the recent findings suggesting that ATP synthase c-subunit is not required for mPT [35]. We created c-subunit CRISPR KD embryonic stem cells (with 85% depleted c-subunit) to independently characterize the morphology and single-channel activity of KD mitochondria. The BNP analysis of KD mitochondria suggests that there is a low amount of ATP synthase monomer and no dimers or free c-ring present. Electron micrographs demonstrate the severe change in normal cristae morphology and matrix swelling of the KD cells. These results were not surprising since the ATP synthase dimers are known to be crucial for normal cristae morphology [31, 32]. Although in WT ESCs, Ca2+ treatment caused typical changes in electron lucency and spectrophotometric changes in isolated mitochondria, no responses were found in the KD ESCs or isolated mitochondria after Ca2+ addition. These findings suggest that c-subunit KD cells have a different mitochondrial phenotype: There are baseline disturbances in mitochondrial inner membrane morphology due to the lack of ATP synthase dimers and in osmotic regulation of matrix volume, allowing us to suggest that ATP synthase and perhaps its c-subunit channel are required for matrix osmotic regulation. It is therefore likely that loss of ATP synthase is associated with disruption of ion gradients across the inner mitochondrial membrane. Such gradients are essential for the normal mitochondrial K+ cycle, which consists mainly of influx and efflux pathways for K+, H+ and associated anions between the matrix and the intermembrane space. The K+ cycle is known to be mediated by passive diffusion (“K+ leak”), mitochondrial K+/H+ antipporter and mitochondrial ATP-sensitive K+ channel (mitoKATP) [39, 40]. The mitochondrial K+ cycle has been reported to be crucial for mitochondrial matrix volume homeostasis, preventing excess matrix swelling and maintaining the structural integrity of mitochondria [39, 40]. We also show that, despite the baseline osmotic changes, CypD and Ca2+-induced mitochondrial swelling do not occur, suggesting absence of evoked mPTP opening in c-subunit KD cells.

Our patch-clamp recordings also reveal that c-subunit KD eliminates the large conductance, Ca2+- and CsA-sensitive mPTP-like openings present in WT. Our data suggest that although the ALCCL may not be the only contributor to mPT under conditions of Ca2+ overload and cell stress, it is most likely the largest conductance contributor to CypD-regulated mPT.

Nevertheless, the HAPI-A12 cells with complete knockout (KO) of the ATP synthase c-subunit were recently shown to have no change in the sensitivity of the mPT to calcium during calcium regulation at maximum capacity (CRC) experiments, allowing the authors to conclude that c-subunit is not required for mPT [35]. The CRC experiments, however, only indicate a loss of membrane potential and not mPT-induced swelling. In fact, when swelling was studied in c-subunit knockout HAPI-A12 cells, there was a marked loss of normal mPT-induced swelling behavior [41].

The role of ANT in mPT formation was studied in Ant-triple-null (Ant1, Ant2, Ant4) and quadruple-null mice (Ant1, Ant2, Ant4, Ppif). The mPT opening was inhibited in mouse embryonic fibroblasts (MEFs) of Ant-triple-null mice but not in liver cells, which required also the genetic deletion of Ppif (CypD), suggesting that ANT may constitute a pore of mPT in MEFs but not in liver cells [21]. Another interpretation of these findings is that ANT is an important regulator of mPT, rather than a pore-forming component, possibly by maintaining the physiological balance of adenine nucleotides between the matrix and cytosol. If complete deletion of ANT leads to accumulation of the ATP/ADP pool in the matrix, then this will inhibit mPT channel activity, which would be observed as an inability to depolarize the inner membrane in a calcium-dependent manner, coupled to reduced channel activity as it has been reported for ANT depleted cells [21]. Our findings do not rule out the possibility of the existence of more than one pore of mPT in mitochondrial inner membranes. Nevertheless, our data suggest that the c-ring is the largest conductance channel of the inner mitochondrial membrane contributing to Ca2+ and CsA-sensitive mPT.
Another reason for the controversy surrounding the idea of c-ring as the pore of mPT is the hydrophobic nature of the c-ring pore-lining residues. Different types of densities were found to occupy the c-ring lumen in recent structures of ATP synthases from bacteria to eukaryotes. In some studies, the c-ring lumen has been reported to be filled with detergents, lipids or quinones \[42–46\], which are predicted to preclude ion conductance. c-ring has also been shown to be filled with a 40 amino acid-long alpha-helical protein instead of lipids in the cryo-EM structure of porcine ATP synthase tetramer \[14\]. 6.8PL proteolipid subunit of ATP synthase was assigned to this density although the most recent high-resolution structure of dimeric ATP synthase reports a different location for 6.8PL and suggests that the c-ring lumen is occupied by phospholipids, as suggested previously \[46\]. Interestingly, in both of these structures, the C-terminal tail of subunit e interacts with the densities occupying the c-ring \[11, 14, 46\]. A similar curved region of a presumed Fo protein interacting with the detergent micelles of the c-ring cavity was reported earlier for the bovine ATP synthase \[1\]. Based on this structure the “death finger” model was suggested \[47\], which proposed that the curved region of Fo (recently identified as subunit e \[14\]) may play an important role in removing the lipid plug from the c-ring.

Now, based on the recent structures of ATP synthase, our electrophysiology findings and cell death studies in neurons, we suggest a new “bent-pull-twist” model for ACLC gating which highlights the importance of Fo gating. We suggest that during the mPT initiation step, mPTP modulators bind directly to different ATP synthase subunits: CypD binds to OSCP subunit \[23, 48, 49\] and Ca\(^{2+}\) binds to \(\beta\) subunit \[6, 50\] (Fig. 5). OSCP connects Fo with Fo through the peripheral stalk and is an important site of modulation of ATP synthase leak channel activity due to its interaction with different endogenous and pharmacological inducers of mPTP \[51, 52\]. We suggest that these binding steps induce conformational changes in the ATP synthase peripheral stalk subunits, which then modify interactions of c-subunit with the other Fo subunits. The subsequent conformational changes may then pull away Fo from the mouth of the c-ring pore to free the channel from the side facing the matrix. These conformational changes may also pull out or displace the lipid or other “plugs” from the c-ring lumen to free the channel from the side facing the intermembrane space. We suggest that the c-ring lumen expands during these concurrent events to facilitate channel opening (Fig. 5). We have reported earlier the expansion in the c-ring diameter during Ca\(^{2+}\) induced mPT \[3\] and it is supported here by the behavior of continuously increasing channel conductance of

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**Fig. 5** Proposed “bent-pull-twist” model of ACLC gating in physiological and pathological conditions. A Illustration of the c-ring diameter changes during the electrophysiology recordings, upon the application of voltage and Fo. We suggest that Fo application during c-ring recordings inactivates the channel due to the specific interactions between Fo and c-ring that induce twisting motions of monomers in a clockwise direction, to stabilize the ring, reduce the pore diameter and close the channel. B Reversible brief openings of ACLC in physiological conditions. C Non-reversible dissociation of Fo from Fo occurs during long-lasting openings of c-ring channel in severe pathology. For simplicity only ATP synthase monomer is shown. ATP synthase subunits are drawn as ribbon representations (modified PDB ID code: 6J5I) \[14\]. In B and C, red arrows indicate the path of ion flow through the channel. Closed and open conformations of the channel are noted.
the c-ring (Fig. 1D). According to cryo-EM structures of mammalian c-ring, the eight monomers are twisted clockwise [14]. Here we suggest that this corresponds to the narrow pore, closed channel conformation of ATP synthase. We speculate that during the above-mentioned conformational changes, c-subunit monomers twist counterclockwise, which widens the pore and induces the opening of the channel (Fig. 5A). F1 application during c-subunit recordings inactivates the channel since the specific interactions between F1 and c-ring [53, 54] induce twisting motions in c-subunit monomers in a clockwise direction, stabilizing the ring, reducing the pore diameter and closing the channel (Fig. 5A). The model in Fig. 5B represents the reversible, brief openings of the ACLC, the type of openings that most likely occur under physiological or sub-lethal conditions. We suggest that in severe pathology such as brain ischemia represented here by an extended period of glutamate toxicity, non-reversible dissociation of F1 from F0 occurs following long-lasting openings of ACLC (Fig. 5C). These events are accompanied by inability to repolarize the inner membrane. This marks the point of no return because marked matrix swelling as a result of prolonged pore opening triggers outer membrane rupture, release of cytochrome c and activation of downstream cell death pathways.

Our proposed model highlights the crucial role of c-ring as the main pore-forming component of mPTP [3, 4], the role of F1 as a gate of ACLC and the auxiliary roles of the other ATP synthase subunits, OSCP, e and g [5, 9, 10, 55]. Our findings and model suggest that the c-subunit channel is activated by voltage and gated by F1, from the side of the matrix. ACLC may possibly have a second gate (lipids or other plugs), from the side of the intermembrane space as suggested before [11, 47]. These plugs may be partially displaced or completely removed by voltage application (in vitro experiments) or due to membrane depolarization (in vivo) (Fig. 5). Further electrophysiological studies are needed to fully characterize the gating mechanism of ACLC.

Similarly to our current studies on excitotoxicity, we reported previously that levels of the ATP synthase F1 β subunit are low in aged DJ-1 deficient, Parkinson’s Disease (PD) mouse model brain and in patient-derived cell lines, while the c-subunit levels are normal, suggesting that mutant DJ-1-induced degenerative disease is associated with a reduction in F1/c ratio with resultant ACLC activation [56]. Likewise, we have recently reported a similar reduction in F1/c ratio in Fmr1 deficient mouse neurons [26]. Fmr1 encodes the Fragile X mental retardation protein (FMRP) which we find regulates the closure of the ACLC by increasing the translation rate of F1 β subunit, thus increasing the F1/c ratio, enhancing ATP synthase efficiency, resulting in synaptic growth [26, 57]. Reversible disassembly is a well-known occurrence of the vacuolar ATPase (V-ATPase) which has a remarkable similarity with ATP synthase. Interestingly, it was shown that c-ring isolated from yeast V-ATPase forms a large unitary conductance channel [58].

Occulsion of other known ion channels with lipids and a hypothetical gating mechanism, termed “lipid gating” has been reported recently [59]. Another possible mechanism of c-ring conductance was suggested to involve the Ca2+ /CypD-dependent association of polyhydroxybutyrate-polyphosphate complex with the c-ring [60].

In-depth structural and functional studies of ATP synthase are required to increase understanding of the conformational changes accompanying ACLC opening and to better characterize this mitochondrial “death” channel which is ironically located in the main energy-producing enzyme of the cell.

METHODS

Purification of c-subunit, F1, aββ, complex and c-subunit knock-down

The human ORF ATP-synthase c1 (ATP5G1) subunit construct tagged on the C terminus with Myc and DDK (Flag) was used (Origene Technologies).

The construct for c-subunit was expressed in HEK 293 cells and purified using the EZ view Red ANTI-FLAG M2 Affinity Gel (Sigma), according to the manufacturer’s protocol.

Endogenous c-subunit knockdown (for ATP5G1 gene) was performed using a plasmid, expressing short hairpin RNAs (shRNA) (Origene Technologies).

The sequence of human ATP synthase c-subunit corresponding to mature protein was cloned into the bacterial pEX-1 expression vector (BlueHeron Biotech). DNA codon optimization strategy was used to increase the expression level of protein in E. coli cells (Supplementary Fig. S1C). The protein was tagged with six histidine residues at its C-terminus to facilitate purification (Ni-NTA).

F1 ATPase was purified from HEK 293 cell mitochondria according to a previously published protocol [61]. The α6β3 complex (G. stea thermophilus) was purified according to previously published protocols [62].

Proteoliposome preparation

Proteoliposomes were prepared according to published protocols [3]. Briefly, 50 mg of phosphatidylcholine (Sigma) or phosphatidylcholine, phosphatidylethanolamine and cardiolipin (3:3:1 ratio) (Avanti) mixture was dissolved in 1 mL of chloroform. A thin lipid film was formed on a glass surface by evaporating the chloroform. Liposomes were formed by the reconstitution of the lipid in rehydration buffer containing 250 mM KCl, 5 mM HEPES, and 0.1 mM EDTA. Then, 20 μg of recombinant c-subunit protein was added to 100 μL of the liposome mixture (~2 mg of lipids, final), and the samples were vortexed twice. For recording, proteoliposomes were dehydrated in a recording chamber and rehydrated in a recording solution (120 mM KCl, 8 mM NaCl, 0.5 mM EGTA, 10 mM HEPES (pH 7.4)) in the presence of 25 mM glutamate. We suggest that the c-subunit channel is activated by voltage and gated from the side of the matrix. ACLC may possibly have a second gate (lipids or other plugs), from the side of the intermembrane space as suggested before [11, 47]. These plugs may be partially displaced or completely removed by voltage application (in vitro experiments) or due to membrane depolarization (in vivo) (Fig. 5). Further electrophysiological studies are needed to fully characterize the gating mechanism of ACLC.

Generation of c-subunit knockdown cells via CRISPR/Cas9

Two guide RNA target sites specifically designed for each ATP synthase c-subunit gene (ATP5G1, 2, 3) for mouse embryonic stem cells (ESCs) were selected using the CRISPOR website (https://tefor.net/portfolio/crispor). The selected sequences were: ATP5g1T: CACCGACCATTTGTCTCTGGC, ATP5g1B: AAACGGCGAGAAGAAGAAAGTGTC, ATP5g2T: CACCGTGCTTCAGAGAAGGCTTC, ATP5g2B: AAACAGGAACCCTTCTCTCAAGCAC; ATP5g3T: CACCGAACAGCTGCTGCTTCAGTGA; ATP5g3B: AAACGCCAGAGACAGAAAAGGGTGC; ATP5g1T: CACCGCACCCTTTTCTGTCTCTGGC, ATP5g1B: AAACGGCGAGAAGAAGAAAGTGTC, ATP5g2T: CACCGTGCTTCAGAGAAGGCTTC, ATP5g2B: AAACAGGAACCCTTCTCTCAAGCAC; ATP5g3T: CACCGAACAGCTGCTGCTTCAGTGA; ATP5g3B: AAACGCCAGAGACAGAAAAGGGTGC. Oligos for these sequences were annealed and ligated into the px330 plasmid (Addgene) that was cut with the BbsI restriction enzyme. Cas9/sgRNA expressing plasmids were electroporated into mouse ES cells via standard techniques (Manipulating the Mouse Embryo: A Laboratory Manual, 3rd ed. (Nagy et al., eds.) 2003: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The efficiency of knockdown was verified by Western blot analysis using antibodies against the c-subunit (Abcam, Cat. No. 181243).

Mitochondrial isolation from primary neuronal cultures, HEK 293 cells, and mouse ES cells

Mitochondria from primary hippocampal and cortical neurons, HEK 293 cells and mouse ES cells were isolated using the Qproteome Mitochondria isolation kit (Qiagen, Cat. No. 37612). In brief, cells were transferred to ice-cold isolation buffer, supplemented with 1x Halt protease inhibitor. Cells were minced, homogenized with a Dounce homogenizer, and centrifuged at 6000 × g for 60 min at 4 °C; the pellet containing mitochondria was washed in isolation buffer and pelleted by centrifugation at 6000 × g. Feeder cells were depleted before mitochondrial isolation from mouse ES cells. Protein concentration was determined by the BCA method using BSA as a standard. Isolated mitochondria were used immediately or stored at −80 °C until use.

Swelling assay

To initiate mitochondrial swelling by Ca2+ uptake, freshly isolated mitochondria were suspended in 120 mM KCl, 5 mM succrose, 5 mM KH2PO4, 0.1 mM EGTA, 20 MOPS (pH 7.2) in the presence of 5 mM malate and 5 mM pyruvate as substrates, in the presence or absence of CsA (1 μM). A membrane-permeabilizing nonspecific agent, amelaminich (10 μM) was used. The selected sequences were: ATP5g1T: CACCGACCATTTGTCTCTGGC, ATP5g1B: AAACGGCGAGAAGAAGAAAGTGTC, ATP5g2T: CACCGTGCTTCAGAGAAGGCTTC, ATP5g2B: AAACAGGAACCCTTCTCTCAAGCAC; ATP5g3T: CACCGAACAGCTGCTGCTTCAGTGA; ATP5g3B: AAACGCCAGAGACAGAAAAGGGTGC. Oligos for these sequences were annealed and ligated into the px330 plasmid (Addgene) that was cut with the BbsI restriction enzyme. Cas9/sgRNA expressing plasmids were electroporated into mouse ES cells via standard techniques (Manipulating the Mouse Embryo: A Laboratory Manual, 3rd ed. (Nagy et al., eds.) 2003: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The efficiency of knockdown was verified by Western blot analysis using antibodies against the c-subunit (Abcam, Cat. No. 181243).
used to determine if mitochondria were capable of swelling. The swelling was recorded as the decrease of the density of the mitochondrial matrix at 540 nm with a UV/Vis spectrophotometer after adding 0.5 mM Ca²⁺ into the medium.

**Mitochondrial oxygen consumption and membrane potential measurement in isolated mitochondria**

Mitochondria (0.2 mg) isolated from mouse embryonic stem cells were added to 1 ml of respiration buffer [200 mM sucrose, 25 mM KCl, 2 mM K₂HPO₄, 5 mM HEPES-KOH (pH 7.2), 5 mM MgCl₂, 0.2 mg/µL BSA]. Oxygen consumption and mitochondrial membrane potential were measured simultaneously [63]. Oxygen consumption was measured using a Clark-type electrode (Oxymeter, Hansatech, UK) with Complex II-dependent substrate (10 mM succinate in the presence of 5 µM rotenone) at 32 °C. Membrane potential was determined using an ion-sensitive electrode selective for the lipophilic cation, tetraphenylphosphonium (TPP⁺) [World Precision Instruments, Sarasota, FL] and calculated using the Nernst equation as previously described [57]. ADP (200 µM) was added to initiate ADP-dependent respiration.

**Electrophysiology**

The patch-clamp recordings of mitoplasts and ATP synthase c-ring reconstituted liposomes were performed by forming a giga-ohm seal in intracellular solution (10 mM Hepes, pH 7.3, 120 mM KCl, 8 mM NaCl, 0.5 mM EGTA) using an Axopatch 200B amplifier (Axon Instruments) at room temperature (22–25 °C). Recording electrodes were pulled from borosilicate glass capillaries (WPI) with a final resistance in the range of ~50 MΩ. Signals were filtered at 5 kHz using the amplifier circuitry.

Planar lipid bilayer recordings were performed in intracellular solution by using the α-L-phosphatidycholine (Sigma) or α-L-phosphatidylcholine and cardiolipin (Avanti) in a 3:1 ratio for forming the bilayer membrane. ePatch amplifier (Elements) was used for lipid bilayer recordings. ATP (1 mM, final concentration), bongkrekic acid (BA, 5 µM), purified F₁ (5 µg, final concentration) and αβ₃, complex (5 µg) were added into the bath during the recordings without perfusion.

pCLAMP-10 software was used for electrophysiology data acquisition and analysis (Molecular Devices). All current measurements were adjusted for the holding voltage assuming a linear current-voltage relationship. The resulting conductances are expressed in pS according to the equation for the holding voltage assuming a linear current-voltage relationship: The equation as previously described [64]. In brief, data were calculated by subtracting the baseline electrode leak current. Group data were quantified in terms of conductance and probability of channel opening, where NPo is the number of open channels ("level" in pCLAMP) times the probability of channel opening at each level. All population data were expressed as mean ± SEM.

**Primary cultures of rat hippocampal neurons**

Primary rat hippocampal neurons were prepared from rat feti (Sprague-Dawley, day 18 of gestation; Harlan, Indianapolis, IN) as described previously [3] with modifications specific for this study. After isolation of hippocampi from prenatal brains, neurons were dissociated and seeded (0.2 x10⁶ cells/cm²) in planar conditions medium with 5% FBS. After 2 h incubation, cells were maintained in Neurobasal medium supplemented with B-27, glutamine, and antibiotics (Invitrogen Gibco life technologies, Carlsbad, CA). Neurons were grown at 37 °C in 5% CO₂ and 20% O₂ in a humidified incubator and treated at DIV 20–21. Glutamate treatment: glutamate (monosodium glutamate, 20 µM, final concentration), (Sigma-Aldrich, St. Louis, MO) was freshly made in sterile PBS as an aqueous solution and added to the cell culture medium as described in relevant figure legends. The vehicle group for glutamate experiments is treated with sterile PBS. Cyclosporine A (CsA) treatment: CsA (Cell signaling) was prepared in sterile ethanol and added to the cell culture medium (100 mM, final concentration). Proteasome inhibitor (PI) treatment: MG-132 (ApexBio, Houston, TX) was prepared in DMSO and added to the cell culture medium (0.1 µM, final concentration).

**Viability assay**

Lactate dehydrogenase assay: The level of cytotoxicity in primary hippocampal neurons was assayed by measuring leakage of LDH using an in vitro toxicity assay kit (Sigma-Aldrich) as previously described [64]. In brief, data were calculated by finding the activity of LDH leaked into the medium by damaged cells/total LDH activity in the culture (cells plus medium). The culture media and lysed cells were collected after the treatment of neurons as described in the relevant figure legends. The LDH assay mixture was made according to the manufacturer’s protocol and added to each sample. After 20 min incubation, the reaction was terminated by adding 1 N HCl. LDH activity was spectrophotometrically measured with a VICTOR multilabel reader (PerkinElmer, Waltham, MA, USA) with absorbance set at 490 nm.

**Cell death assay**

Annexin V and Propidium iodide (PI): Apoptotic or total dead cells were stained with Annexin V or PI, respectively, as previously described [27]. After treatment of neurons expressing GFP, GFP plus c-subunit shRNA or GFP plus human c-subunit constructs with glutamate for 18 h, 5 µl/ml Annexin V or 0.5 µM PID (Thermo Fisher Scientific, Waltham, MA) was added into the culture medium for 30 min at 37 °C in the dark. Micrographs were taken using a Zeiss Axiovert A1 microscope (Zeiss, OberKochen, Germany) using a consistent exposure time. The number of PI-positive neurons or Annexin V fluorescence densitometry per cell was analyzed using AxioVision 4.9. The H₂O₂-induced cell death in HEK 293 cells was measured with PI after transfection cells with constructs expressing GFP or GFP and human c-subunit constructs. Cell death was measured 24 h after a 30 min exposure to 1 mM H₂O₂.

**Measurement of mitochondrial membrane potential in primary hippocampal neurons**

Mitochondrial membrane potential (ΔΨm) was measured using the fluorescent lipophilic cationic dye tetramethylrhodamine methyl ester (TMRM, Invitrogen, Thermofisher, Waltham, MA), which accumulates within mitochondria in a potential-dependent manner [64, 65]. Primary hippocampal neurons were treated with glutamate (20 µM) for 6 h and TMRM intensity was measured to assess the mitochondrial membrane potential. Primary hippocampal neurons were stained with 5 nM TMRM for 30 min at 37 °C in the dark. Cells were pre-incubated with CsA (100 nM, 30 min) before TMRM was added. Images were taken using a Zeiss Axiovert A1 microscope and TMRM fluorescence densitometry was analyzed using AxioVision 4.9.

**Immunocytochemistry**

Primary hippocampal neurons were transfected with Mitochondria-GFP (Invitrogen) at DIV14, and fixed in 10% buffered formalin at DIV 20–22. Then, samples were incubated with anti-Flag (1:500 dilution, OriGene, Rockville, MD) overnight at 4 °C. Cells were washed and incubated with Alexa-568 antibody (1:200 dilution, Invitrogen, Molecular Probes, Carlsbad, CA) for 1 h at room temperature and mounted on glass slides. Images were taken with Zeiss LSM 710 confocal scanning microscope (Zeiss, OberKochen, Germany) and processed using ZEN software (Carl Zeiss Microscopy GmbH, Jena, Germany).

**Electron microscopy**

Mouse ES cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 1 h. Buffer rinsed cells were scraped in 1% gelatin and spun down in 2% agar. Chilled blocks were trimmed and postfixed in 1% osmium tetroxide for 1 h. The samples went through three times in sodium cacodylate rinse buffer and postfixed in 1% osmium tetroxide for 1 h. Samples were then rinsed and stained in aqueous 2% uranyl acetate for 1 h followed by rinsing, dehydrating in an ethanol series, infiltrated with Embed 812 (Electron Microscopy Sciences) resin, and then baked overnight at 60 °C. Hardened blocks were cut using a Leica UltraCut UC7. Sections (60 nm) were collected in formvar-carbon-coated nickel grids and contrast stained with 2% uranyl acetate and lead citrate. They were viewed using a FEI Tecnai Biotwin TEM at 80 Kv. Images were taken on a Morada CCD using iTEM (Olympus) software.

**Blue and clear native page electrophoresis**

For Blue Native Page (BNP) electrophoresis, protein complexes from 20 µg of mitochondria (per lane) were separated on a Bis-Tris 3–12% Native gels. Samples were solubilized on ice for 20 min with 4 µg digitonin/µg protein or 2.5 µg n-dodecyl β-D-maltoside (DDM)/µg protein. After separation, the protein complexes were wet-transferred onto a polyvinylidene fluoride
Antibodies
Antibodies used in all experiments were obtained from commercial sources.

Statistical analysis
Data in graphs are shown as mean±SEM. For comparisons involving two groups, paired or unpaired Student t tests (2-tailed) were used, and exact P values are provided in the figure legends. For multiple comparisons, Prism (V6.02; GraphPad) was used to calculate the normality of data using the D’Agostino–Pearson omnibus or the Kolmogorov–Smirnov normality tests. Parametric data were analyzed using standard or repeated-measures ANOVA with Dunnnett’s or Tukey’s multiple comparison test whereas nonparametric data were analyzed using Kruskal–Wallis with Dunn’s multiple comparison test and multiplicity adjusted (“exact”) P values are reported. In some cases, data were normalized to control data before analysis. Unless noted, other comparisons were not significant.

DATA AVAILABILITY
All constructs will be made available to the scientific community upon request.

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AUTHOR CONTRIBUTIONS
Conceptualization, NM and EAJ; Methodology, NM, EAJ, RJL, JW and EVP; Investigation, NM, HP, JW, XH, MCL, ML, PM, BM, MG, EAJ; Formal Analysis, NM, HP, JW, XH, EAJ; Writing – Original Draft, NM and EAJ; Writing – Review & Editing, NM, EAJ and EVP; Funding Acquisition, NM, EAJ and EVP; Project Administration and Supervision, NM and EAJ.

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COMPETING INTERESTS
The authors declare no competing interests.

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