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ATP synthase c-subunit ring as the channel of mitochondrial permeability transition: Regulator of metabolism in development and degeneration

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Abstract

The mitochondrial permeability transition pore (mPTP) or mitochondrial megachannel is arguably one of the most mysterious phenomena in biology today. mPTP has been at the center of ongoing extensive scientific research for the last several decades. In this review we will discuss recent advances in the field that enhance our understanding of the molecular composition of mPTP, its regulatory mechanisms and its pathophysiological role. We will describe our recent findings on the role of ATP synthase c-subunit ring as a central player in mitochondrial permeability transition and as an important metabolic regulator during development and in degenerative diseases.

Introduction

The mitochondrial permeability transition (mPT) was discovered in the 1970s. It was first characterized as an abnormal swelling of mitochondria upon high calcium overload [1-4]. Later, it was shown by electrophysiological recordings that mitochondrial matrix swelling and subsequent rupture of the mitochondrial outer membrane were due to the opening of a high conductance, non-selective channel of the mitochondrial inner membrane, named the “mitochondrial megachannel” by Zoratti’s group or “multiconductance channel” by Kinnally’s group [5-10]. For decades, mPTP was assumed to be a cell death channel that caused irreversible, apoptosis-inducing changes in mitochondria, rather than a pore having any physiological function [11, 12]. Nevertheless, we now know that the mPTP is a pivotal physiological channel, which regulates Ca^{2+} signaling, [13-15] intracellular Ca^{2+} homeostasis [16-23] and ATP production efficiency [24-30]. The mechanism of how mPTP converts from a physiological to a pathological channel remains mysterious. Open conformations of mPTP with both low and high conductance states have been reported during electrophysiological recordings of isolated mitochondria [5-8, 10, 18]. A flickering channel corresponding to a low conductance conformation has been reported to have

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Disclosures

None.

different subconductance levels with a ~300 pS peak conductance, while the large conductance state has a peak conductance of ~1200-1800 pS [5-8]. The low conductance openings were suggested to be brief and reversible and perhaps to contribute to the physiological function of mPTP, while the prolonged openings of the high conductance state may induce irreversible depolarization of the inner membrane, mitochondrial swelling and subsequent cell death [6, 13-15, 31-33].

Nevertheless, the exact molecular mechanism of mPTP opening, the difference between low and high conductance states and the molecular mechanism of conversion between these states remains unknown. The mPT is a complex phenomenon that occurs during a combination of events that also disrupt mitochondrial structure and function. These changes include, but are not limited to, depletion of adenine nucleotides, elevated mitochondrial matrix Ca^{2+} levels, increases in inorganic phosphate and reactive oxygen species, dissipation of mitochondrial inner membrane potential and matrix swelling [11, 32-36]. The combination of these changes induces mPTP opening and downstream cell death mechanisms.

The molecular composition of mPTP: ATP synthase c-subunit ring as the inner membrane leak channel

The molecular identity of mPTP is still controversial and is a subject of intensive scientific debate. Different mitochondrial proteins, such as the adenine nucleotide translocator (ANT) [37], the voltage-dependent anion channel (VDAC) [38, 39], the phosphate carrier (PiC) [40] and the translocator protein (TSPO) [41-43] have been suggested previously to play a direct role in mPTP formation. Further studies have shown that these proteins regulate channel behavior rather than constitute the pore of the mitochondrial permeability transition (mPTP) [44-47]. Mitochondrial F_OF_1 ATP synthase was suggested to be a key player in mPTP formation according to several recent reports [28, 30, 48-57]. The peptidyl-prolyl cis-trans isomerase cyclophilin D (CypD) is a well-known regulator of mPTP [58, 59]. Its interaction with ATP synthase OSCP subunit was described by Bernardi's lab [60], leading them to hypothesize that ATP synthase is involved in mPTP formation. Our lab independently built a hypothetical model of the involvement of ATP synthase in mPT based on our studies in which we reported interaction of the anti-cell death protein Bcl-xL with the ATP synthase β subunit [27, 28].

Multidisciplinary studies performed by Bernardi's group suggested that the channel is located between ATP synthase dimers with the direct involvement of F_O subunits e and g [48-52]. Yeast mutant strains lacking subunits e and g, subunits that are necessary for dimer formation [61, 62], displayed a striking resistance to mPTP opening suggesting at first glance that ATP synthase dimerization is required for pore formation *in situ* [49, 50]. The Arg-8 residue of subunit e was found to be crucial for stabilizing the ATP synthase dimers and for formation of full conductance mitochondrial megachannels in yeast [51]. Furthermore, channel formation was observed in detergent-solubilized tetramers and dimers but not in monomers of F_1F_O ATP synthase purified from bovine and porcine hearts [48, 52]. Nevertheless, our studies have recently shown that ATP synthase monomer produces a

high multi-conductance channel activity with the biophysical characteristics of mPTP, suggesting that the channel is not located between e and g subunits and ATP synthase dimerization is not required for its activity [30].

If the channel resides within the ATP synthase monomer, the likely location is the membrane embedded c-subunit ring, considering its transmembrane pore-like cavity. Several studies have suggested that the c-subunit comprises the pore [28, 30, 53-57]. The chloroform extract of isolated rat liver mitochondria was reported to have a channel activity similar to the mPTP channel seen in patch-clamp recordings of native mitoplasts [56]. The analysis of biochemical composition of the chloroform-extracted material revealed that it contains a low molecular weight protein, identified to be the ATP synthase c-subunit. The chloroform extract also contained the PHB/polyP/Ca^{2+} complex, which the authors at that time suggested to be the ion-conducting module of the mPTP [56]. In another study, phosphorylated and non-phosphorylated forms of c-subunit isolated from rat liver mitochondria by chloroform extraction were studied in lipid bilayer experiments [53]. Phosphorylated c-subunit was shown to form cation-selective channels with brief openings in artificial lipid membranes, while Ca^{2+}-induced dephosphorylation of c-subunit led to the formation of non-selective channels, suggesting that the phosphorylation status of c-subunit might determine activation of a structural/regulatory component of mPTP [53]. Genetic manipulation of c-subunit expression levels by siRNA in HeLa cells confirmed that c-subunit is required for mPTP-driven mitochondrial fragmentation and cell death triggered by cytosolic calcium overload and oxidative stress [54].

In our studies, affinity purified, reconstituted human c-subunit was demonstrated to form a large, voltage-sensitive channel in patch-clamp recordings, but its activity was not sensitive to well-known mPTP regulators, CsA and Ca^{2+}, since their binding sites were found to be located in ATP synthase F_1 subunits [28]. Depletion of the c-subunit attenuated Ca^{2+}-induced depolarization of the inner mitochondrial membrane while its overexpression sensitized primary hippocampal neurons to cell death. c-subunit was observed to undergo measurable conformational changes by expanding its size upon activation of the channel [28]. Additionally, mutations that loosen the packing of the c-subunit ring led to the formation of c-rings with a larger internal diameter and significantly increased conductance compared to WT [28] and these mutations sensitized cells to death. CypD/Ca^{2+}-mediated and CsA/ADP-inhibited dissociation of ATP synthase F_1 subunits from F_O was associated with mPT suggesting that unmasking of the c-subunit ring is required for initiation of channel conductance [28]. Further evidence that F_1 becomes uncoupled from F_O in the presence of high Ca^2 is that direct binding of Ca^{2+} to ATP synthase β subunit [63, 64] dissociates ATP hydrolysis from H^+ pumping in the presence of Ca^{2+} [65]. Interestingly, age-dependent decreases in F_1 content compared to FO, decreasing the F_1/F_O ratio, have been reported in rat brain and heart mitochondria [66, 67].

Nevertheless, the prominent role of the c-subunit ring in mPTP has been recently questioned in several studies [68-70]. One of the main reasons for this controversy is the hydrophobic nature of the c-subunit pore-lining residues. However, different kinds of densities are observed to occupy the c-subunit lumen in recent structures of F_1F_O ATP synthases from bacteria to eukaryotes. In some studies the c-subunit pore has been reported to be filled with

detergents or lipids [71-73], which are predicted to preclude ion conductance through its cavity, although partial occlusion of other known conducting channels with lipids has been reported [74]. Interestingly, c-subunit lumen was shown to be filled by a 40 amino acid-long alpha-helical protein instead of lipids in the recent cryo-EM structure of porcine ATP synthase tetramer [75]. 6.8PL proteolipid subunit of ATP synthase was assigned to this density although further studies are needed to fully reveal the identity of this protein. According to this structure, the C-termini of the four ATP synthase e subunits are bent toward the four c-subunit rings in each tetramer, which allows them to interact with the C-terminal ends of the 6.8PL helices occupying the central pore of the four c-rings [75]. It is possible that the interaction between subunits c, 6.8PL and e is important for keeping the c-subunit lumen sealed and the c-subunit channel closed. This will prevent dissipation of membrane potential under normal physiological conditions, decreasing the probability of channel opening and initiation of permeability transition.

A similar curved region of a presumed F_O protein interacting with the detergent micelles occupying the c-subunit cavity was reported earlier for the bovine mitochondrial ATP synthase [76]. Likewise, the recent high-resolution structure of the *in meso* crystallized c-ring from spinach chloroplasts revealed additional electron densities inside the c-rings, suggested to originate from isoprenoid quinones such as coenzyme Q in mitochondria and plastoquinone in chloroplasts [77]. Coenzyme Q and its analogues are electron carriers of bioenergetic chains, also hypothesized to serve as universal cofactors of ATP synthase, stabilizing the c-ring and preventing ion leakage through it [77]. Additional studies are required to verify the exact nature of the densities contained within the c-subunit pore [77].

In light of these recent findings we suggest a new “bent-pull” model of c-subunit channel gating (Figure 1a, b). ATP synthase consists of two subcomplexes, the hydrophilic F_1, which bears the catalytic nucleotide binding sites on the β subunits, and the membrane-embedded F_O [76, 78, 79]. Tight coupling between F_1 and F_O subcomplexes is required for highly efficient rotational catalysis and occurs through two stalks, the F_1 central stalk (consisting of γ, δ, ε subunits in mitochondria) and the F_O peripheral stalk (primarily consisting of OSCP, b, h, d subunits) [76, 78, 79]. OSCP connects F_O with F_1 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 [80-82]. During the mPT initiation step, mPTP modulators bind directly to different ATP synthase subunits: CypD binds to OSCP subunit [83-85] and Ca^{2+} binds to β subunit [63, 64] (Figure 1a). We suggest that these binding steps induce conformational changes in the ATP synthase peripheral stalk subunits, which then modify interactions of c-subunit with membrane embedded F_O subunits, including subunit e. The subsequent conformational changes in e subunit then pull the lipid “plug” or the proteins out from the c-subunit lumen to open the channel from the side facing the intermembrane space (Figure 1a). This model highlights both the importance of c-subunit as a pore forming component of mPTP [28, 30] and also the crucial role of ATP synthase subunits e and g in mPTP formation [48-51]. The conformational changes in ATP synthase due to the binding of mPTP inducers may also pull away F_1 from the mouth of the c-subunit pore to free the channel from the side facing the matrix. These concurrent events will open the pore. This model represents the reversible, brief openings of the mPTP channel, the type of openings that most likely occur under physiological conditions. We

suggest that non-reversible dissociation of F_1 from F_O occurs during long-lasting openings of mPTP (Figure 1b). This marks the point of no return because swelling as a result of prolonged pore opening triggers outer membrane rupture, release of cytochrome c and activation of downstream cell death pathways in detrimental pathological conditions, such as during brain or heart ischemia or in neurodegenerative diseases.

Surprisingly, the complete knockout of the ATP synthase c-subunit from HAP1-A12 cells results in no change in sensitivity of the mPT to calcium as studied in calcium retention capacity (CRC) experiments of mitochondria, allowing the authors of this study to conclude that c-subunit and ATP synthase are not required for mPT [70]. The CRC experiments, however, may only indicate a loss of membrane potential and not mPT-induced swelling, which has not been studied in c-subunit knockout HAP1-A12 cells [70]. Swelling indicates an osmotic change induced by solute and water entering into the matrix. It is independent of a loss in mitochondrial membrane potential since, for example, it is known that experimental mitochondrial isolation where membrane potential is absent does not produce appreciable swelling. The driving force for the Ca^{2+} uptake in CRC experiments is the mitochondrial membrane potential and as long as the c-subunit knockout HAP1-A12 cells are able to maintain a membrane potential, by possible reversal of ANT or other mechanisms, they can have similar Ca^{2+} uptake compared with the control cells, as has been observed in [70].

Unlike the CRC, patch-clamp recordings are a direct measure of mPTP pore opening by the measurement of its channel activity. Patch-clamp recordings of these same c-subunit knockout mitoplasts (mitochondrial inner membrane preparations lacking the outer membrane) revealed that they lack the high conductance CsA-sensitive mPTP channel activity recorded in WT [57]. A small and CsA-sensitive channel was still found to be present in c-subunit knockout mitoplasts, and this channel was also bongkrekic acid-sensitive suggesting that the ANT could possibly contribute to small conductance inner mitochondrial membrane activity recorded in the absence of c-subunit [57]. In contrast to these findings, the channel activity recorded from mitochondrial inner membranes of wild-type and ANT-deficient yeast strains demonstrated the same conductance, ion selectivity and voltage dependence in both strains [9]. The recorded ion conductance was not sensitive to the specific ANT inhibitor carboxyatractyloside, suggesting that ANT is not responsible for mPTP activity in yeast [9].

The role of ANT in mPTP formation was further studied by using the *Ant*-triple-null mice and quadruple-null mice, for *Ant1*, *Ant2*, *Ant4* and *Ppif*. Genetic ablation of all three ANT isoforms inhibited mPTP opening in mouse embryonic fibroblasts (MEFs) of *Ant*-triple-null mice but not in liver cells. mPTP opening was completely inhibited only after genetic deletion of *Ant* and *Ppif* in the liver mitochondria, suggesting that ANT may constitute the main pore forming component of mPTP in MEFs while another, CypD-dependent protein is required for mPTP formation in liver cells [86]. In contrast to this implication, another interpretation suggests that inhibition of mPTP in MEFs does not necessarily mean that ANT itself is the pore-forming component in this cell type, but instead it could be a regulatory component by maintaining the physiological balance of adenine nucleotides between the matrix and cytosol. For example, if complete deletion of ANT leads to the accumulation of the ATP/ADP pool in the matrix, then this will completely inhibit mPTP

channel activity, which would be observed as an inability to depolarize the inner membrane in a calcium dependent manner, coupled to a decrease in channel activity in patch-clamp recordings of ANT depleted mitoplasts as was described in the ANT KO studies [86]. Future ANT/c-subunit double KO studies will be required to reach a conclusion. Based on these findings we suggest that ANT is an important regulator of mPTP in all cell types as has been reported for CypD [19, 32, 60, 87].

Physiological relevance of the ATP synthase leak channel in synaptic plasticity

We have reported earlier that mPTP channel openings serve a physiological function and contribute to synaptic plasticity in neurons [88, 89]. Recordings of mitochondrial ion channel activity inside living neuronal synapses demonstrate that presynaptic nerve stimulation results in Ca^{2+} dependent channel openings in the mitochondrial membranes [88, 89]. The study is consistent with the requirement for the opening of an inner membrane Ca^{2+} activated conductance to allow Ca^{2+} to flow between the matrix and the cytosol during and for a short time after high frequency presynaptic activity [89-91]. We reported that anti-apoptotic protein Bcl-xL regulates this activity, since application of the recombinant protein enhances mitochondrial channel activity, increases certain forms of short term presynaptic plasticity [92], and restores stimulus dependent postsynaptic depolarization and action potential firing from depleted synapses. Therefore, a Bcl-xL-regulated, Ca^{2+}-dependent conductance spanning the two mitochondrial membranes is required for short term plasticity of presynaptic neurotransmitter release. The Ca^{2+} release occurring coincident with this opening is required for synaptic strengthening [90, 91].

Bcl-xL could also mediate an important metabolic change brought on by high frequency activity. This was supported by the finding that injection of Bcl-xL or ATP into the presynapse caused synaptic strengthening beyond the pre-stimulation level. Such plasticity of neurotransmitter release after intense activity prevents neurotransmitter depletion during high frequency firing, is crucial to brain development [93] and is required for long term changes in synaptic plasticity that underlie memory formation and learning [94, 95].

Mitochondrial plasticity is linked to structural and functional synaptic remodeling. Overexpressed Bcl-xL in isolated neurons localizes mostly to mitochondria but also to synaptic vesicle membranes [96, 97]. Neurons overexpressing Bcl-xL have increased numbers of presynaptic vesicle clusters [96], synapses and mitochondria. The largest mitochondria reside close to the expanded vesicle pools [96]. Bcl-xL coordinates with the mitochondrial fission protein, Drp-1 and the biogenesis factor PGC-1alpha to achieve mitochondrial positioning, but fusion is not required, suggesting that mitochondrial growth at a more proximal location [98, 99], followed by mitochondrial fission, supplies the enlarged synapses with newly minted mitochondria [100, 101]. Bcl-xL and Drp-1 work together directly on synaptic vesicles to bring about activity dependent vesicle pool refilling within the first few minutes after high frequency action potential firing [97]. ATP production is markedly increased by Bcl-xL overexpression in the same neurons where synapses are

larger and vesicle recycling is enhanced [27], suggesting that Bcl-xL also affects metabolism to enhance synaptic growth.

Despite the increased activity of neurons overexpressing Bcl-xL, oxygen consumption is decreased [27]. This paradox was resolved by the finding that Bcl-xL is bound directly to the ATP synthase β subunit [27, 102], where it increases ATP synthesis enzymatic rate and reduces inner mitochondrial membrane leak, enhancing the efficiency of ATP production by the ATP synthase (Figure 2). In contrast to overexpression, depletion of Bcl-xL causes instability of mitochondrial membrane potential and energy wasting, consistent with a leak that dissipates the membrane potential [102]. Bcl-xL KO causes embryonic lethality just before E12.5 [103], but conditional KO mice lacking Bcl-xL in neuronal progenitors survive, albeit with a highly malformed cortex and severe loss of neurite growth and synaptogenesis [102].

These findings led us to assume that the likely place for the Bcl-xL regulated mitochondrial inner membrane leak is the ATP synthase itself, since the Bcl-xL inhibitor ABT-737 [27], and the even more selective Bcl-xL inhibitor WEHI-539, induce inner membrane leak formation during patch clamp recordings or during measurements of mitochondrial membrane potential [104].

ATP synthase leak channel modulates cardiac and neuronal development

If Bcl-xL and perhaps other pro-survival molecules enhance the function of the ATP synthase by enhancing coupling in oxidative phosphorylation, this suggests that ATP synthase leak channel activity or open probability may be graded to adjust metabolism even in healthy cells. Relative leak closure could occur progressively during embryonic development and during the process of synaptogenesis. Bcl-xL expression increases rapidly during these developmental stages in the early embryo from E8.5-E11.5, at the time that the heart and brain are forming. The fact that embryos die at this stage in the absence of Bcl-xL makes a striking argument for the critical role of ATP synthase leak closure as causative of developmental maturation.

Much previous evidence supports the notion that metabolic changes are crucial to different developmental periods in the embryo [105]. Very early (preimplantation) embryos produce mitochondrial ATP and TCA products [106, 107]. They also regulate development by balancing redox state to manage oxidant load [106]. Just before implantation, glucose use is turned on by low ATP/ADP ratios. Subsequently both glycolysis and mitochondrial oxidative phosphorylation increase.

After the morula stage, glucose uptake continues to increase. Glucose transporters increase on the cell membranes and there is increase in lactate production and a decrease in pyruvate uptake [108]. During the subsequent blastocyst stage where the embryo forms a cavity, glucose uptake is dramatically increased, followed by an increase in oxidative phosphorylation and an increase in pyruvate use again. Mitochondrial oxidative metabolism is required during the early post-implantation period, despite the evidence that the environment is relatively hypoxic. The rapid growth of the embryo during this stage of

development and the requirement for reducing equivalents and NAD make a convincing case for aerobic glycolysis plus TCA use as the primary form of metabolism during this period.

This Warburg-style metabolism of developing embryos at the post-implantation stage is also observed in highly proliferating cancer cells [105, 109-114]. The Warburg effect [115] is characterized by increased glycolytic activity despite the presence of adequate environmental oxygen concentration. Even though ATP generation from carbon sources is less efficient compared with oxidative phosphorylation, aerobic glycolysis at this developmental period efficiently meets demands for the production of macromolecules by the TCA cycle, useful to facilitate cell proliferation [105, 116-118].

It has been proposed that the shift during embryonic development to aerobic glycolysis sustains the dramatic increase in body mass observed during the stages just prior to terminal differentiation of cardiac and nervous system cells [111]. Aerobic glycolysis, however, may also be required for signaling, regulating differentiation spatially and temporally even after proliferation has ceased [105, 110, 119]. We suggest that the harboring of a leak channel within the ATP synthase enables cells to quickly shift between oxidative phosphorylation and aerobic glycolysis by placing mitochondrial ATP synthase dependent ATP production “off-line” to increase glycolysis and TCA cycle-dependent biosynthesis. How much off-line will depend on the species, the presence of regulators such as Bcl-xL and crucially, the ratio of F_1 subunits to F_O. The evidence for this is that the c-subunit leak relatively “closes” during the period of central nervous system and cardiac development between E8.5 and E11.5 [120, 121]. In the heart, it was found that mitochondria are normally depolarized and certain electron complexes are relatively quiescent at E9.5, but that by E11.5, the ATP synthase begins producing ATP and the membrane potential becomes properly hyperpolarized [120]. These dates coincide with the onset of increased oxygen consumption and cardiac muscle contraction, suggesting that oxidative phosphorylation becomes more active at the time of inner membrane leak closure [120]. Indeed the mPTP inhibitor CsA hastens cardiac development and depletion of the main activator of the mPTP, CypD, causes cardiac myocyte differentiation to shift earlier [48, 60, 87, 122-124]. These developmental studies suggest that closure of the ATP synthase (c-subunit) leak by developmentally timed ATP synthase assembly (of F_O with F_1) [27, 102] could control development of the heart between E8.5-E11.5 (Figure 2).

In the developing mammalian nervous system there is an increase in oxidative enzymes associated with downregulation of MYC during neuronal differentiation [121]. Acid production (from lactate) but also oxygen consumption are high *in vitro* in explanted cells at E8.5 but by E10.5 as nervous system cells begin to differentiate and the neural tube forms, both acid production and oxygen consumption shift down dramatically [121].

Immunoblotting at this time (E10.5) demonstrates an upregulation of fully assembled Complex IV and V but it is clear from RNA sequencing of the explants comparing E8.5 to E10.5 that all three isoforms of ATP synthase c-subunit are downregulated by E10.5 [121]. This suggests that a change in stoichiometry of the ATP synthase, favoring fully assembled ATP synthase over uncomplexed (free) c-subunit, is causative of a reduction in both glycolysis and oxygen consumption and increases the coupling of oxidative

phosphorylation. A similar change in ATP synthase stoichiometry was suggested previously in a large study of the flexibility of cancer cells upon switching to different metabolic states under varied environmental conditions [125].

These studies highlight that changes in metabolic phenotype during development are complex. In the pre-differentiation period, when cells are moving rapidly into position, they upregulate glycolytic enzymes and mitochondrial metabolism so that TCA cycle may be used for anabolism [124, 126-128]. Most importantly, upregulation of glycolytic and mitochondrial pathways at this time balances the NAD^+/NADH ratio. Mitochondria are therefore not silent when glycolytic metabolism is favored [129] but are required for TCA cycle anabolic pathways to regenerate adequate supplies of reducing equivalents and NAD^+ as well as intermediates for biosynthetic pathways. During differentiation, however, oxidative phosphorylation increases, the stoichiometry of the ATP synthase changes to favor fully formed ATP synthase, and molecules like Bcl-xL close the leak for proper cardiac and nervous system development. We argue that, during the pre-differentiation period, the ATP synthase (c-subunit) leak is required for anaplerosis and maintenance of the NAD^+/NADH ratio. If the inner membrane leak is too low, mitochondrial inner membrane potential builds up and NADH levels become high, feeding back to shut down TCA cycle function. To continue to run TCA and provide anaplerotic intermediates, NAD^+ must be remade from the activity of the NADH dehydrogenase. As Vander Heiden argues in his recent review, “continued nutrient oxidation [for the purpose of biosynthesis] requires cycling of NADH back to NAD^+, which necessitates transfer of electrons to an electron acceptor such as oxygen” [118].

ATP synthase leak closure regulates synaptic development and cancer cell growth

An even further decrease in mitochondrial inner membrane leak occurs again later in nervous system development when neurons begin to make contact with other neuronal partners to form mature synaptic connections. This period of synaptogenesis begins to occur just after E10 in the rodent and continues exuberantly during the early postnatal period up to the time of weaning. We find that ATP synthase leak closure during synaptic development heralds the onset of mature synapse formation.

The time of formation of mature synapses is accompanied by a decrease in glycolytic enzymes and an increase in use of oxidative phosphorylation for ATP production. In contrast to the normal developmental metabolic changes in brain, by examining the metabolism of an autism spectrum disorder known as Fragile X (FX) Syndrome, we discovered a persistent glycolytic phenotype [130]. Fragile X Syndrome is caused by a CGG repeat sequence that forms in the X-linked gene Fmr1 which codes for FMRP [131, 132]. The repeat sequence causes the gene for FMRP to become hypermethylated and inactivated [133]. This results in complete absence of the FMRP protein, severe intellectual disability and features of autism including neuronal hyperexcitability, sensory misperceptions and seizures [134-138]. The disorder is modeled in the mouse and in drosophila by Fmr1 gene deletion. Striking cellular features of the disorder in all animals during development include high protein synthesis

rates [139], marked abnormalities of synaptic phenotype [135] and decreases in brain connectivity [140].

Our studies of young FMRP-lacking mice at the time of normally high expression levels of FMRP (P10), confirmed that body temperature and lactate production were elevated, consistent with an aerobic glycolytic phenotype. Indeed, closer studies of mitochondria isolated from the brains of the KO animals using modular kinetic techniques [141] identified an inner membrane leak that caused enhanced oxygen consumption in a depolarization-dependent manner [130]. The leak was reminiscent of the futile cycling of the membrane potential we had identified previously in neurons depleted of Bcl-xL [102]. We found that the likely cause of the leak was enhanced depolarization-dependent opening of the mPTP channel [130]. The closure of this leak with CoQ_{10} on P9 acutely induced changes in morphology and density of dendritic spines in *Fmr1* KO mouse forebrain suggesting that the mPTP regulates synapse maturation and development and that an open pore maintains the spines in an immature state [130]. It is known that $Fmr1^{yY}$ synaptoneurosomes are unable to synthesize proteins in response to stimulation, which leads to significantly reduced number of synapses in *Fmr1* KO mice in the first few weeks of life [142]. Additionally, mitochondria were shown to be necessary for stimulus-induced synaptic protein translation and synaptic plasticity [143, 144]. Consistent with these findings, we reported significantly lower rates of protein synthesis in the 10-day-old *Fmr1* KO mouse forebrain during synaptogenesis, which was restored after closing the pathological mitochondrial leak channel by CoQ_{10} in *Fmr1* mutant mice [130]. This suggests that increasing the efficiency of oxidative phosphorylation due to mPTP leak closure in neurons of developing brain is important for meeting the substantial protein synthesis needs and metabolic demands during synaptogenesis.

The timing of synthesis of proteins involved in oxidative phosphorylation, in particular the timing of the synthesis of ATP synthase F_1 catalytic domain subunits, is crucial to mitochondrial biogenesis during growth and development. Cuezva lab working in cancer cells and our lab working in synapses found that the mechanism regulating the accretion of ATP synthase β subunit during cellular proliferation and synapse formation, respectively, is controlled at the level of mRNA translation [145]. Cuezva lab described that the translation of ATP synthase β subunit is dependent on the 3'UTR of the transcript and it is being regulated by the common mechanism both in the fetal liver and cancer cells. The mRNAs that accumulate in the fetal liver and cancer cells are in a translation-repressed state. The fetal liver [146] and cancer cells [147] contain a set of proteins, the 3′UTR β-F_1-ATPase mRNA-binding proteins (3′βFBPs): p129, p89, p61, p59, p54, and p51 that specifically bind the 3'UTR of the mRNA. This interaction prevents mRNA translation at the ribosome leading to decreased expression of ATP synthase β subunit both during fetal development and in liver carcinogenesis.

The expression of ATP synthase β subunit regulates the assembly of F_1. It was demonstrated that inactivation of the *ATP2* gene expression, coding for the catalytic subunit β, by RNA interference in the green alga *Chlamydomonas reinhardtii* impaired the assembly of full ATP synthase [148].

Successful translation of the β subunit mRNA to produce fully formed ATP synthase is also dependent on mechanisms that participate in ATP synthase β subunit mRNA localization to sites of mRNA translation [145]. In synapses, this is crucial since the site of synaptic mRNA translation is often very distant from the nucleus. An RNA binding particle (ribonuclear particle, RNP) containing several different RNA binding proteins and ATP synthase β subunit mRNA has been described. Most likely, based on studies in yeast and mammalian liver, this particle is bound to ribosomes and localized to the mitochondrial outer membrane at the site of local translation of β subunit in response to mitochondrial metabolic signals [149-151]. Based on this information we suggest that timed increased in expression of ATP synthase β subunit leads to an increase in the F_1/F_O ratio, which increases the level of assembled ATP synthase and closes the c-subunit leak. The closure of c-subunit leak enhances efficient ATP synthesis to support neuronal differentiation and synapse maturation (Figure 2).

Stoichiometry change in ATP synthase F_1 subunits to F_O as a critical cause of neurodegenerative disease

Mitochondrial dysfunction has been widely implicated in neurodegenerative disease, but we and others are increasingly focused on changes in function and in regulation of the ATP synthase. Alzheimer's Disease (AD) is one of the most commonly occurring neurodegenerative diseases, accounting for up to 80% of all dementia cases. It is characterized by progressive cognitive and motor deterioration, and affects memory, attention, speech and behavior. Over many pre-clinical and symptomatic (clinical) years, AD pathology causes synaptic dysfunction followed by the death of brain cells, leading to significant tissue shrinkage [152, 153]. Oxidative damage and redox imbalance produce stress that is most severe for mitochondria, where most reactive oxygen species are produced [154, 155]. It was noticed in human pathological samples of early AD that mitochondrial components suffered oxidative damage, impairing their function [156-160]. The α subunit of the mitochondrial ATP-synthase was recognized as the most common lipoxidized protein in the entorhinal cortex of all AD cases at stages I/II of the disease [161]. Cyclophilin D (CypD) knock out mice have a reduced propensity toward mitochondrial permeability transition (mPT) and crossing the CypD−/− mice with an AD model mouse reduced mPT, enhanced calcium handling by neuronal mitochondria and protected the mice from loss of function in tasks of learning and memory [162, 163]. A decrease in levels of the oligomycin sensitivity conferring protein (OSCP) subunit of the F_1F_O-ATP synthase was attributed to binding of the aggregated protein amyloid beta (Aβ) with OSCP in the brains of AD individuals and in an AD mouse model [164]. Decreases in OSCP levels were pronounced in neuronal mitochondria [164]. The loss of OSCP led to reduced ATP production, elevated oxidative stress and activated mPT and these abnormalities were alleviated in the mouse by resupply of OSCP [164]. When Aβ enters mitochondria, cyclin-dependent kinase-1 (Cdk1) is activated and cyclin B1 is stabilized [165]. The Cyclin B1–Cdk1 complex in the mitochondria phosphorylates Bcl-xL, leading to its dissociation from the β subunit of F_1F_O–ATP synthase. This inhibits ATP synthase activity and causes mitochondrial depolarization [165] that activates mPT.

We have now found that loss of the ATP synthase β subunit occurs in a Parkinson's Disease (PD) mouse model [166]. Rare mutations in the gene encoding DJ-1 are found in about 1% of familial cases of PD. DJ-1 is a peptidase C56 family protein with known and uncharacterized cellular functions [167]. DJ-1 mutant animals show increased sensitivity to neuronal toxins, and in different species DJ-1 is required for normal life span, motor function, and neuronal resistance to oxidative damage [168-170]. Defects in DJ-1 alter mitochondrial morphology and function [171]. DJ-1 translocates to mitochondria from the cytosol in response to mitochondrial stress [172-174], suggesting that DJ-1 may assist Parkin and PINK1 in protein trafficking. DJ-1 also regulates mitochondrial metabolism: DJ-1 mutant cells have impaired ATP production and abnormal respiration [170]. The mutant mitochondria are sensitive to mPT [175] and DJ-1 deficient mitochondria demonstrate a leaky mitochondrial inner membrane [166, 170]. We found that the leak of DJ −/− mitochondria is produced by the ATP synthase; WT DJ-1 binds tightly to the β subunit causing leak closure in mitochondrial recordings (Figure 2) [166]. Mutant DJ-1 fails to close the leak despite persistent binding to the β subunit. The abnormalities of the ATP synthase in DJ-1−/− animals are associated with reduced enzymatic function, reduced ATP levels and impaired neurite extension in isolated dopaminergic neurons, suggesting that normal ATP synthase function is critical for growth and targeting of dopaminergic neuron endings [166]. Levels of the ATP synthase β subunit are low in aged DJ-1−/− mouse brain and in patient cell lines even though levels of the c-subunit are normal, suggesting that this degenerative disease is associated with a reduction in F_1/F_O ratio with resultant c-subunit leak channel formation.

Concluding remarks

Here we discussed some current concepts and controversies in the field of mitochondrial pathophysiology and the key role of ATP synthase c-subunit in forming a mitochondrial inner membrane leak channel, which has physiological as well as pathological functions in cell metabolism, in embryonic and synaptic development and during aging-related degenerative diseases. We have proposed a new model of mPTP formation, which describes that brief, physiological openings of ATP synthase leak channel are due to the reversible conformational changes in ATP synthase structure. We have also suggested a model of long-lasting pathological openings of mPTP; these persistent openings under pathological conditions result in non-reversible dissociation of ATP synthase F_1 subcomplex from F_O. While our models are supported by structural and functional findings and corroborative findings in disease models, further studies are needed to understand mPTP structure and the molecular mechanisms of its regulation in health and disease.

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Highlights

- ATP synthase c-subunit is the leak channel of mitochondrial permeability transition.
- c-subunit channel is the master regulator of cell metabolism in embryonic and synaptic development.
- F_1/F_O stoichiometry change is critical for c-subunit regulatory function.
- Non-reversible dissociation of ATP synthase F_1 from F_O triggers age-related ischemic and degenerative diseases.

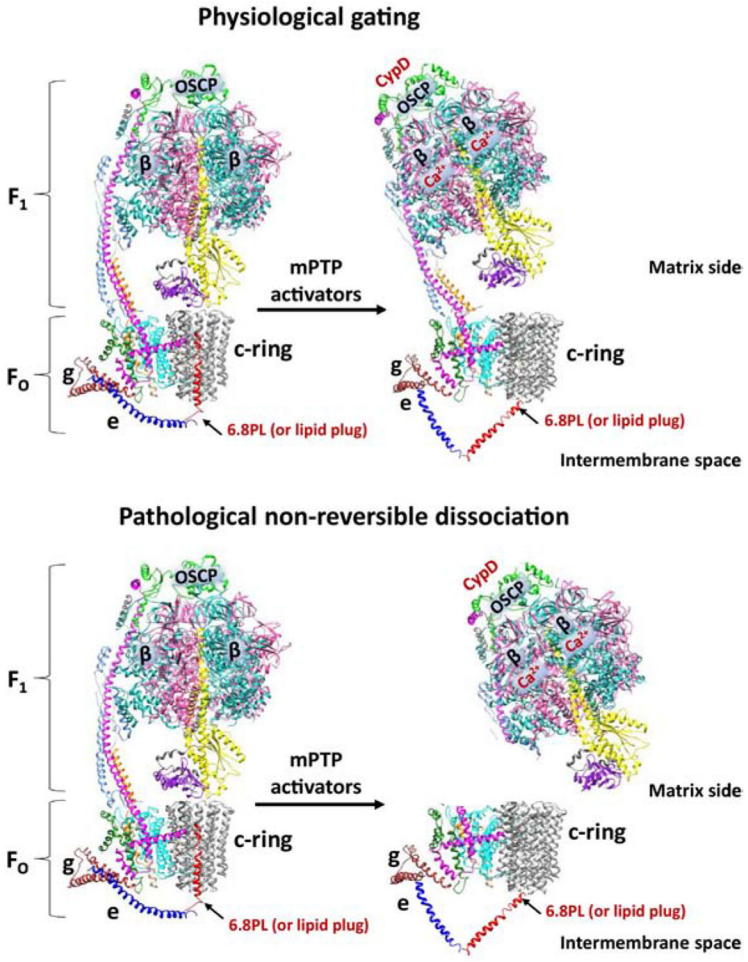

Figure 1. Proposed "bent-pull" model of F_1F_O ATP synthase c-subunit channel gating in physiological and pathological conditions.

A. Reversible brief openings of ATP synthase channel. mPTP inducing agents, CypD and Ca^{2+} bind to the OSCP and β subunits, respectively, inducing conformational changes in ATP synthase peripheral stalk subunits then modifying interactions of membrane embedded F_O subunits, including subunit e, with the lipid or protein (6.8PL) "plug" occupying c-subunit. The subsequent conformational changes in e subunit then pull this "plug" out from the c-subunit lumen to open the channel from the side facing the intermembrane space. These conformational changes also pull away F_1 from the mouth of the c-subunit pore to open the channel from the side facing the matrix. ATP synthase subunits are drawn as ribbon representations (modified PDB ID code: 6J5I [75]). **B.** Non-reversible dissociation of F_1 from F_O occurring during long-lasting openings of c-subunit channel in pathology. F_1 dissociates from F_O, which marks the point of no return in cell metabolism, since swelling as a result of prolonged pore opening triggers outer membrane rupture in detrimental pathological conditions, such as in brain or heart ischemia or in neurodegenerative diseases. For simplicity only ATP synthase monomer is shown, even though the ATP synthase is present as a dimer in native mitochondrial inner membrane. ATP synthase subunits are drawn as ribbon representations (modified PDB ID code: 6J5I [75]).

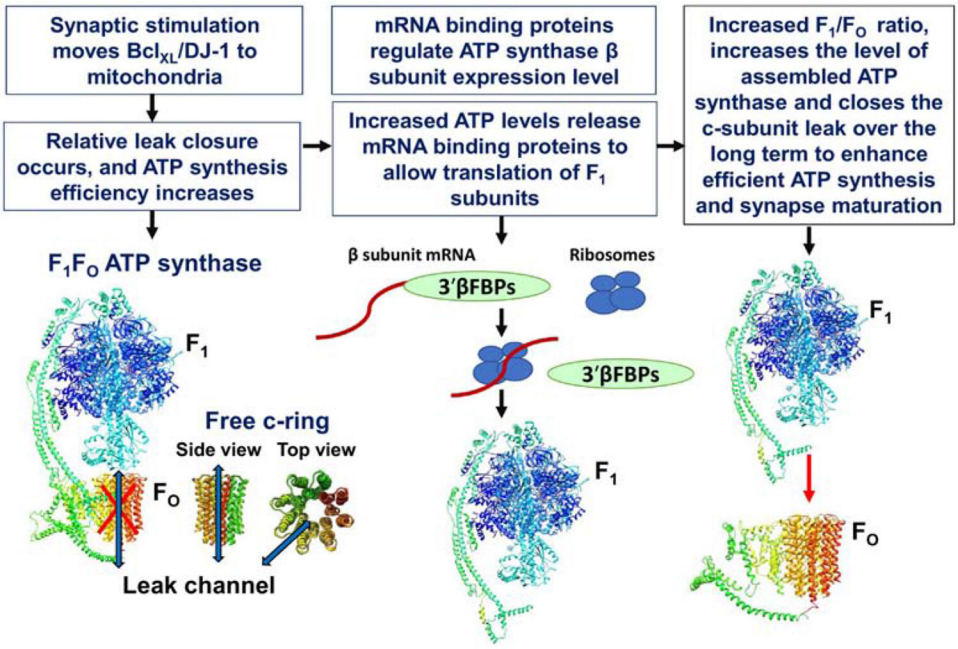

Fig. 2. Ratio of ATP synthase F_1:F_O is determined by Bcl-xL and DJ-1.

In response to synaptic stimulation, Bcl-xL and DJ-1 move to mitochondria to close the leak. ATP production by mitochondria causes the removal of the 3′UTR β-F_1-ATPase mRNA-binding proteins (3′βFBPs): p129, p89, p61, p59, p54, and p51, from the 3'UTR of the mRNA of F_1 β subunit. This allows F_1 synthesis which leads to a long-lasting change in F_1/F_O ATP synthase ratio by reducing the level of free, uncomplexed c-subunits in mitochondria; this further decreases the probability of leak opening. DJ-1 and Bcl-xL are required for adjusting F_1/F_O ATP synthase ratio and neurite outgrowth in dopaminergic neurons. Closure of the leak supports neuronal differentiation and synapse maturation. ATP synthase subunits are drawn as ribbon representations (modified PDB ID code: 6J5I [75]).