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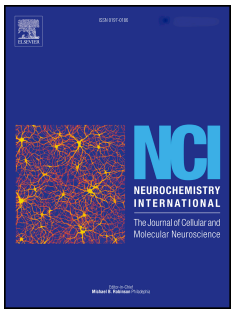

Mitochondrial permeability transition pore: Back to the drawing board

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Mitochondrial permeability transition pore: back to the drawing board

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Abstract

Current models theorizing on what the mitochondrial permeability transition (mPT) pore is made of, implicate the c-subunit rings of ATP synthase complex. However, two very recent studies, one on atomistic simulations and in the other disrupting all genes coding for the c subunit disproved those models. As a consequence of this, the structural elements of the pore remain unknown. The purpose of the present short-review is to i) briefly review the latest findings, ii) serve as an index for more comprehensive reviews regarding mPT specifics, iii) reiterate on the potential pitfalls while investigating mPT in conjunction to bioenergetics, and most importantly iv) suggest to those in search of mPT pore identity, to also look elsewhere.

Keywords: c-subunit ring; ATP synthase; dimers; bioenergetics

INTRODUCTION

The mitochondrial permeability transition (mPT) pore is a megachannel of the inner mitochondrial membrane with a diameter of 2-3 nm (Zoratti and Szabo, 1995) exhibiting a non-selective conductance of 1-1.3 nS (Kinnally et al., 1989), (Petronilli et al., 1989), allowing flux of metabolites with a molecular weight of up to 1.5 kDa (Haworth and Hunter, 1979), (Bernardi et al., 2015b). Mechanisms of induction, inhibition, regulation, implication in physiological and pathological states as well as historical perspectives of this phenomenon are outlined under "Index of reviews on mPT specifics". Mindful that mPT is not an *in vitro* artifact (Bernardi et al., 2006), its prolonged opening leads to mitochondrial demise causing cell death (Petronilli et al., 2001) and that this seems to be a final common pathway in major maladies of our times such as in heart disease (Halestrap and Pasdois, 2009), it is not surprising that the race for identifying its components –which could serve as targets amenable to pharmacological manipulation- is going strong. Indeed, the mPT modulator cyclophilin D, has been implicated in a number of pathologies involving mitochondrial dysfunction (Giorgio et al., 2010), such as in collagen VI diseases (Lampe and Bushby, 2005). The immense interest in establishing mPT as a pharmacological target can be better appreciated by the number and extent of commentaries on a recent study (Cung et al., 2015) recruiting 970 patients with myocardial infarction (MI) and assessing the effect of cyclosporine A administration, a cyclophilin D inhibitor and a universally-accepted negative effector of mPT (Waldmeier et al., 2003), (Giorgio et al., 2010). Cung and colleagues reported that "cyclosporine did not result in better clinical outcomes than those with placebo and did not prevent adverse left ventricular remodeling at 1 year", thus arguing against using cyclosporine A as pharmacological treatment for MI; notably, this study has been scrutinized in seven commentaries (Bernardi and Di Lisa, 2016), (Zografos and Katritsis, 2016), (Hausenloy and Yellon, 2015), (Linkermann et al., 2016), (Lim, 2015), (Santos-Gallego and Badimon, 2016), (Pottecher et al., 2016).

So far, the only two proteins verified as modulatory but not structural elements of the pore are cyclophilin D (Baines et al., 2005), (Nakagawa et al., 2005), (Basso et al., 2005), (Schinzel et al., 2005), and the adenine nucleotide translocase (ANT), isoform 1 (Doczi et al., 2016). Remarkably, there is

renewed interest in synthesizing ANT binding ligands based on the classical inhibitor, bongkrekic acid (Francais et al., 2011), (Okuda et al., 2012), (Matsumoto et al., 2015) which is also an inhibitor of mPT (Halestrap and Brenner, 2003), (Klingenberg, 2008). For a review regarding on what is not the mPT, the reader is referred to (Siemen and Ziemer, 2013). To this end, the c-subunit rings of the ATP synthase complex (Alavian et al., 2014), (Bonora et al., 2013) or the interface between ATP synthase dimers (Giorgio et al., 2013), (Carraro et al., 2014) have been recently proposed to form pore structure. In the following sections, the two papers published on atomistic simulations of the c-subunit rings and the other where all genes coding for the c subunit were disrupted, disproving the models involving the c-subunit ring of the ATP synthase complex as a structural element of the pore, are briefly reviewed. Furthermore, the present short-review also addresses a most recent work published by the group of Pinton, showing that dissociation of ATP synthase dimers promotes mPT, albeit in a manner involving the c-subunit ring (Bonora et al., 2017).

ATOMISTIC SIMULATIONS ARGUE AGAINST THE c-SUBUNIT RING OF THE ATP SYNTHASE FORMING THE mPT PORE

The group of Jonas (Alavian et al., 2014) showed that the mPT pore forms within the c-subunit ring of the ATP synthase complex when the ring dissociates from the catalytic domain. The concept that the c-subunits ring form a structural element of the pore has been proposed earlier by the group of Pinton (Bonora et al., 2013). Both studies implied that the lumen of the c-subunits ring form the mPT pore.

This claim has been scrutinized by the group of Faraldo-Gomez (Zhou et al., 2017), by calculating ion conductance and selectivity in two c-subunit rings of different lumen widths, based on all-atom molecular dynamics simulations. The number of the c-subunits per ring (determining the size of the lumen) varies depending on the species (Watt et al., 2010), however, mPT is a phenomenon encountered in almost all species (Azzolin et al., 2010), except in *Artemia franciscana* (Menze et al., 2005) (Konrad et al., 2011), (Konrad et al., 2012) and perhaps all crustaceans (Chen et al., 1974), (Tsokos et al., 1983). Thus, the choice of the c-subunit ring for atomistic simulations regarding mPT pore should bear no relevance. Because the lumen of the c-subunit rings is normally not an aqueous pore (Oberfeld et al., 2006), Zhou and colleagues also quantified the probability that it may become hydrated, and thus potentially conducting.

Zhou and colleagues calculated the free-energy gain (or cost) associated with hydrating an otherwise, highly hydrophobic, lipid-plugged c-ring lumen, and reported that a dehydrated, non-conducting lumen is more probable to exist than a conducting lumen, by multiple orders of magnitude (Zhou et al., 2017). They further estimated that if the c-ring lumen were to be filled with water, it would exhibit a resting conductance of 2.5 pS for K^+ and 116 pS for Cl^-. Such values are not only inconsistent with the purported mPT conductance of 1-1.3 nS (Kinnally et al., 1989), (Petronilli et al., 1989) but also the anticipated lack of ion selectivity. Finally, because Alavian and colleagues reported that the c-subunit rings become the mPT pore upon dissociation of the F_1 sector of the ATP synthase complex (Alavian et al., 2014), Zhou and colleagues performed atomistic simulation on a particularly large c-subunit ring (largest known to date), that from the bacterium *Bacillus pseudofirmus*, exhibiting a c_{13}-ring. Again, calculations yielded anion selectivity, albeit a slightly greater conductance, but still no preference for hydration.

In aggregate, the simulations were clear: the c-subunit ring, if correctly folded and assembled, cannot form the mPT pore, even if it detached from the F_1 sector of the ATP synthase complex (Zhou et al., 2017).

Nevertheless, no matter how categorical the results of the simulations were, they are still only simulations, and in fact, the authors admitted that their study does not rule out a yet-to-be-discovered molecular structure consisting of c-subunits, distinct from that of the c-ring that matches the properties of the mPT pore, despite no evidence of such an alternative structure. The definite answer regarding the possibility of the c-subunit rings forming the mPT pore came from Walker laboratory.

MITOCHONDRIA HARBORING VESTIGIAL ATP SYNTHASE COMPLEXES DEVOID OF c-SUBUNIT RINGS EXHIBIT THE mPT

The group of Walker deleted the expression of c subunits in HAP1-A12 cells (He et al., 2017), by disrupting *ATP5G1*, *ATP5G2*, and *ATP5G3* genes, all coding for identical copies of the c subunit protein (Dyer and Walker, 1993), (Yan et al., 1994). The mitochondria of these cells exhibited a vestigial ATP synthase entailing intact F_1-catalytic and peripheral stalk domains and supernumerary subunits e, f, and g, but lacked a c-subunit ring as well as subunits ATP6 and ATP8. A cyclosporin A-sensitive mPT could be observed in both control cells and in those lacking c-subunit proteins as tested by three different, but classical mPT pore-opening protocols evoking an elevation in Ca^{2+} concentration. There was not even a statistically-significant difference in the amounts of Ca^{2+} load required for mPT opening in either cell type. These results could be interpreted in only one way: the c-subunits ring is not an essential, let alone structural component of the mPT pore. However, it must be noted that 'pore' *per se*, in the sense of electrophysiological detection of a conductance exhibiting voltage-sensing properties, was not addressed. Thus, it remains possible that the increase in cytosolic calcium detected in the Walker study during mPT protocols could still be due to a low-conductance pore, known to mediate Ca^{2+}-induced Ca^{2+} release events (Ichas et al., 1997). Nonetheless, for reasons explained in (He et al., 2017) subunits DAPIT and 6.8PL of the ATP synthase complex could also not be part of the mPT event. Finally, because these vestigial ATP synthase complexes were mostly monomeric, the fact that there was no measurable difference in mPT pore opening between cell types casts doubt on another mPT model substantiated by ATP synthase dimers (Giorgio et al., 2013), further unsupported by the recent results from the Pinton group (Bonora et al., 2017), see under "DISSOCIATION OF ATP SYNTHASE DIMERS PROMOTES mPT OPENING".

It is to be noted that the concept of c-subunit rings forming the mPT pore has been criticized before (Chinopoulos and Szabadkai, 2013), (Chinopoulos and Szabadkai, 2014), (Halestrap, 2014), prior to the results published by the groups of Faraldo-Gomez and Walker: there, it is reviewed that although reconstitution of c subunits in lipid bilayers yields a voltage-sensitive pore mediating a Ca^{2+}-regulated channel (McGeoch et al., 2000), in that study Ca^{2+} was inhibiting its conductance, a phenomenon which is at odds with the trait of mPT pore opening by Ca^{2+}. Furthermore, the current-voltage plot of the c-subunit pore exhibit outward rectification, a finding which is incompatible with a non-selective mPT channel exhibiting a 1-1.3 nS conductance allowing flux of metabolites with a molecular weight of up to 1,500 Da, i.e. there is no possibility for rectification.

DISSOCIATION OF ATP SYNTHASE DIMERS PROMOTES mPT OPENING

Criticism on the concept of ATP synthase dimers as an mPT pore model also appears in the literature (Szabadkai and Chinopoulos, 2013): there, the proposal of mPT pore appearing in-between ATP synthase dimers is scrutinized from the point of view that this locus is normally occupied by membrane lipids; formation of a pore dictates that at the dimerization interface of a membrane protein dimer, two hydrophobic surfaces should be able to provide a hydrophilic lining while the pore is assembled in order to allow ion flow. Upon pore closure (note that mPT is reversible (Malkevitch et al., 1997), (Petronilli et al., 1994)) these surfaces should regain their hydrophobicity in order to allow membrane lipids interaction. To date, there is no such evidence that this can occur within the ATP synthase dimer. On the contrary, a very recent report by the group of Pinton, showed that stabilization of ATP synthase dimers *disfavors* mPT opening; by the same token, destabilization of the dimers *triggers* mPT, but in a most unexpected twist, this effect required correctly folded c-subunit rings (Bonora et al., 2017). In this work, the authors argued that because different genetic interventions favoring ATP synthase dimers stabilization limited mPT opening, this implies that "the dissociation of F_1F_O ATP synthase dimers is a cause, not a consequence, of mPT"; however, the semantics of this claim are not clear to this author, and dimers dissociation maybe a consequence, not a cause of mitochondrial reversible swelling (Petronilli et al., 1994). Clarification whether dimers dissociation is a cause or a consequence of mPT, is eagerly awaited;

to this end, dimers/monomers ratio quantification by *i.e.* the pore-forming peptide alamethicin and establishment of a protocol quantifying the same ratio online (with a sufficiently rapid acquisition time of the measured variable) during mPT, would assist in shedding light on this debate.

Overall, the mPT pore is not structured by the c-subunit rings, and perhaps not formed in-between ATP synthase dimers. Having said that, the possibility of mPT forming by any of the intramembrane ATP synthase subunits b, e, f, and/or g, assisted by the peripheral stalk subunits in a manner integrated by OSCP, is viable; the details of this proposal are reviewed in (Giorgio et al., 2017b). Furthermore, the mechanisms envisioned in that review are in accord with the recent discovery that the Ca^{2+} trigger site of mPT is T163 of β subunit of the ATP synthase (Giorgio et al., 2017a).

INDEX OF REVIEWS ON mPT SPECIFICS

Prior to the reports by the groups of Faraldo-Gomez (Zhou et al., 2017) and Walker (He et al., 2017), consensus was that the mystery shrouding mPT pore structure was solved, albeit with remaining open questions (Bernardi, 2013). A large number of reviews on mPT appeared in the literature regarding historical perspective, induction, inhibition, regulation, channel characteristics, structural models and role in pathophysiology. Despite the fact that the results presented by (Zhou et al., 2017) and (He et al., 2017) invalidates the vast majority of this body or work, as for any other 'journey' *i.e.* the quest for identifying mPT pore structure, it is imperative to know where one came from. Thus, an index of reviews regarding mPT specifics is outlined below that reflects my solemn opinion as being a 'palatable' amount of literature for delving into this niche of scientific knowledge.

Foremost, the most recent and exhaustively comprehensive review on mPT prior to the reports of (Zhou et al., 2017) and (He et al., 2017), is that by Bernardi (Bernardi et al., 2015b). This review follows a previous Physiological Reviews article on the same topic (Bernardi, 1999). Before that, the most comprehensive review on mPT is that by Zoratti and Szabo (Zoratti and Szabo, 1995), since the characterization of mPT by Hunter and Haworth (Hunter et al., 1976), (Hunter and Haworth, 1979a), (Haworth and Hunter, 1979), (Hunter and Haworth, 1979b), (Haworth and Hunter, 1980). For an exhaustive non-mPT review focusing on mitochondrial channels, the reader is referred to (Szabo and Zoratti, 2014). For more concise reviews on mPT, the reader is referred to (Biasutto et al., 2016), (Bernardi et al., 2015a) and (Bernardi and Di Lisa, 2015), and for a thematic indexing of mPT in regard to electrophysiological properties, activators/inhibitors proposed models and regulatory modules, see (Beutner et al., 2016). A review regarding small molecule mPT inhibitors is that by (Sileikyte and Forte, 2016), while (Zulian et al., 2016) discuss mPT as a pharmacological target. For work regarding alternative mPT models the reader is referred to (Bernardi and Forte, 2015), (Shanmughapriya et al., 2015), and (Varanyuwatana and Halestrap, 2012). For a physiological role of the mPT, the reader is referred to (Mnatsakanyan et al., 2017). Finally, for a historical perspective of the mPT, the review by (Halestrap and Richardson, 2015) is recommended.

Besides the fact that the above reviews allow for a thorough characterization of mPT function but not structure, there are some reports/reviews that deserve special merit: in mitochondria lacking c-subunit rings, can another protein assume the task of mediating mPT? Is there a possibility that more than one protein can have the same role? This has been proposed by Zoratti et al (Zoratti et al., 2005). On the same line of thought, mPT has been proposed to be mediated by different proteins exhibiting proline(s) that are amenable to *cis-trans* isomerization by cyclophilin D (an intramitochondrial peptidyl-prolyl cis-trans isomerase), caught transiting through the inner mitochondrial membrane; this hypothesis has been put forwarded by the group of Lemasters (He and Lemasters, 2002), (Kim et al., 2003). An alternative possibility relies on the concept that poly(3-hydroxybutyrate) and polyphosphate (PHB/polyP) form channels in mitochondria (Pavlov et al., 2005) with properties that resemble native mPT (Elustondo et al., 2016), (Solesio et al., 2016). Mindful of the latter studies by the group of Pavlov, together with those showing that depletion of polyP from mitochondria prevents them from undergoing mPT (Abramov et al., 2007) while adding PHB to cells induces a cyclosporin A-dependent loss of mitochondrial membrane

potential (Elustondo et al., 2013) the possibility that PHB/polyP may be eventually credited as the mPT is increasing.

BIOENERGETIC PITFALLS IN SEARCH OF THE mPT

Upon opening of the mPT pore, swelling of the mitochondria is an invariable event. Thus, the most direct way of testing for mPT in isolated mitochondria is by following mitochondrial light scatter (Gotterer et al., 1961), while for *in situ* mitochondria, time-lapse of microscopic visualization of mito-targeted fluorophores is warranted (Doczi et al., 2011), (Doczi et al., 2016). In addition, the method of quenching mitochondrially-trapped calcein by exogenously added cobalt is also widely used (Petronilli et al., 1998), however, the materials are too toxic for some cell types (Doczi et al., 2016). Of course, electron microscopic evaluation of mitochondria is also suitable for evaluating mPT (Berman et al., 2000), (Arrazola and Inestrosa, 2015), but this is an end-point assay. A few additional protocols exist in the literature, however, all of them rely on measuring bioenergetic properties such as the mitochondrial membrane potential ($\Delta\Psi m$). Opening of the mPT is invariably associated with loss of $\Delta\Psi m$, but this relationship is not obligatory true if in reverse, *i.e.* loss of $\Delta\Psi m$ does not always mean mPT opening. Unfortunately, the 'assessment' of mPT by voltage-sensitive probes is widespread in the literature, which is entirely wrong and misleading. Furthermore, apart from this easy-to-avoid mistake, there are a number of bioenergetic considerations that need to be born in mind in mPT-evoking experiments, reviewed by (Chinopoulos and Adam-Vizi, 2012), and reiterated below.

It has been demonstrated that cyclophilin D binds to the lateral stalk of the ATP synthase complex in a phosphate-dependent manner (Giorgio et al., 2009), and by doing so, it masks an mPT inhibitory side for P_i (Basso et al., 2008). Subsequently, the binding of cyclophilin D to the ATP synthase complex was shown to be responsible for the slowing in ATP synthesis or hydrolysis rate, depending on the prevailing $\Delta\Psi m$ (Chinopoulos et al., 2011), ultimately regulating matrix adenine nucleotide levels. Mindful of the inherent connection of cyclophilin D, its binding to the ATP synthase complex, phosphate and adenine nucleotides concentration as well as the role of $\Delta\Psi m$ on mPT (Bernardi et al., 2015b), it can be easily envisaged that most –if not all- bioenergetic parameters will *indirectly* affect mPT open probability, apart from their known *direct* effect(s), reviewed elsewhere (Bernardi et al., 2015b). For example, in mitochondria where $\Delta\Psi m$ is sufficiently diminished and therefore, ATP synthase operates in reverse, genetic ablation of cyclophilin D or its inhibition by cyclosporin A should result in acceleration of proton pumping outside the matrix at the expense of ATP hydrolysis, antagonizing a further drop in membrane potential and promoting high matrix phosphate levels, both decreasing mPT open probability. This and other confounding factors such as i) the binding of phosphate on non-catalytic sites on the ATP synthase and how it is affected by pH and Mg^{2+}, inorganic anions, adenine nucleotides, the protonmotive force, and ii) the 'Mg-ADP block' hypothesis, reviewed by (Chinopoulos and Adam-Vizi, 2012), should be contemplated and appropriate controls must be ensured in mPT-evoking experiments.

Relevant to the association of ATP synthase operation (direct or indirect) to the mPT, it is noteworthy that reports linking the two entities appeared in the literature as early as 1989 (Novgorodov et al., 1989), (Azarashvili et al., 2002), (Shchepina et al., 2002). In particular, the effect of the well-known ATP synthase inhibitor, oligomycin, on mPT as described by the group of Skulachev (Shchepina et al., 2002) deserve special attention: there, it was reported that oligomycin but not aurovertin B suppressed the TNF-induced apoptosis –an mPT opening event- in a manner unrelated to inhibition of ATP synthesis/hydrolysis by the ATP synthase complex, or to downstream effects on $\Delta\Psi m$. This finding implies that the OSCP subunit (the binding protein for oligomycin but not aurovertin) exerts some role on mPT pore opening, a concept actively investigated by the group of Bernardi (Giorgio et al., 2017a), (Giorgio et al., 2017b)

mPT PORE STRUCTURE: LOOK ELSEWHERE

Mindful of the (Zhou et al., 2017), (He et al., 2017) and (Bonora et al., 2017) reports, the case of c-subunits rings and most likely the membrane space in-between ATP synthase dimers as structural elements of the mPT pore, is closed. Having said that, the obvious question arises, where to look from here? In my opinion, there are the following leads:

A) As pointed out in (He et al., 2017), subunits e, f, and g of the ATP synthase complex could still be examined as structural elements of the pore, as each is predicted to exhibit a single transmembrane α-helix, thus being capable of participating in a pore structure. Such an experimental protocol could follow the same gene disruption strategy as for the c-subunit employed by (He et al., 2017). This proposal is entertained in more detail by a recent review by Bernardi's group (Giorgio et al., 2017b).

B) Among thousands of publications regarding the mPT, it is evident that both cyclophilin D and the ANT are modulatory, but not structural elements of the pore (Bernardi et al., 2015b) (Doczi et al., 2016). Thus, common binding partners for these two proteins should be sought. Inexorably, this is more easily said than done, and such attempts have been made in the past, reviewed in (Bernardi et al., 2015b). However, novel technological advances regarding protein-protein interactions, proteomic detection and identification methods, as well as bioinformatics tools (Guan and Kiss-Toth, 2008) (Lievens et al., 2009), (Speth et al., 2014), (Kotlyar et al., 2015), (You et al., 2015), (Keskin et al., 2016), could help to reduce or eliminate false-positive binding proteins and assist in identifying the real partners forming the mPT pore. To this end, the fact that the total mitochondrial proteome is known and continuously refined (Calvo et al., 2016) should also assist in the search of protein(s) as mPT pore structural elements.

C) In addition to latest improvements in proteomic tools, recent advances in gene disruption technology – especially of high-throughput format- may play a crucial role in mPT pore identification: specifically, a brute-force silencing strategy using libraries of siRNA (Kumar et al., 2003), or the CRISPR-Cas9 System (Wade, 2015) for each and every gene coding for mitochondrially targeted peptides may provide 'hard' leads regarding pore identity; a similar approach appears in the literature, where a high-throughput screening relying on cDNA overexpression identified the phosphate carrier as a regulator of cytochrome c release, attributing this to mPT pore opening (Alcala et al., 2008).

D) It is possible that the assumption of both cyclophilin D and the ANT binding to the putative mPT pore structural element is invalid, and their modulations are somehow 'transduced' through the embedding inner mitochondrial membrane, perhaps through specific membrane lipids. The effect of the lipidome on membrane-embedded protein transduction is a very new, and scarcely researched field (Vitrac et al., 2015), however, it is easy to envisage that membrane lipids do play a significant –perhaps deterministic- role in events such cyclophilin D and/or ANT modulation of mPT pore opening. Relevant to this, the well-known ANT inhibitor, bongkrekic acid, which is also an mPT inhibitor, does not inhibit ANT activity in mitochondria of *Artemia franciscana* (Konrad et al., 2011), an organism that does not exhibit the mPT (Menze et al., 2005), while the heterologous expression of Artemia ANT in yeasts restores bongkrekic acid sensitivity (Wysocka-Kapcinska et al., 2013). Thus, the differential effect of bongkrekic acid on the same ANT but expressed in *Artemia vs* yeasts maybe due to the lipid environment in which the ANT is embedded. Indeed, mitochondria of *Artemia franciscana* harbor a very different lipidome than those found in other organisms, including yeasts (Chen et al., 2016). Thus, the lipidome composition of the inner mitochondrial membrane and how it is affected by known mPT modulatory mechanisms may hold the key to identifying mPT pore structure.

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> c-subunit ring was proposed to form mPT structure, disproved by two recent studies

> these two studies are reviewed

> bioenergetic pitfalls regarding mPT protocols are reiterated

> alternative approaches for identifying mPT structure are reviewed