Perspective

Amyloid pores in mitochondrial membranes

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Neurodegenerative diseases of the amyloid type include common conditions such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis. Despite the fact that the phenotypes of these neuropathic maladies differ widely, ranging from cognitive to motor and psychotic disturbances, they are all characterized by the pathological accumulation and deposition in the central nervous system of well-ordered protein aggregates known as amyloid fibrils. Accumulating evidence indicates that rather than the end-stage mature fibrils, however, it is the smaller, metastable intermediate forms (known as oligomers) of the aggregated protein and peptides which represent the most neurotoxic species (Chiti and Dobson, 2017). One suggested mechanism for such toxicity appears to involve the ability of oligomers to interact with plasma membranes whilst inducing cell leakage (Surguchov et al., 2017). However, contemporary work increasingly points to mitochondria, and hence mitochondrial membranes, as preferential targets for the pathogenic action of oligomers in the neuronal cell (Ghio et al., 2016).

In recent years, we henceforth focused our attention on the consequences of the interaction between membrane-active oligomeric aggregates of amyloidogenic proteins involved in the major brain neurodegenerative diseases, like α-synuclein (α-syn), tau and amyloid-β (Aβ), with the unique double-membrane of mitochondria. We were initially intrigued by the finding that lipid vesicles bearing mito-mimetic membranes were more susceptible than other membrane types to permeation by aggregated forms of $A\beta_{1-42}$, α-syn and tau (Camilleri et al., 2013). The mito-mimetic membranes were enriched with the unique mitochondrial signature phosospholipid cardiolipin (CL). Thus, membranes with a 15% CL content, which are similar to the mitochondrial inner membrane, displayed a two-fold higher permeabilization level than equivalent membranes lacking CL. Motivated by these findings, we wanted to investigate membranes of proper mitochondria organelles, so we proceeded to examine how these oligomeric aggregates affected mitochondria freshly isolated from SH-SY5Y neuroblastoma cells. Since our attention was focused on aggregate-induced membrane damage, we looked at three key indicators of permeabilization of the outer and/or inner mitochondrial membranes (OMM/IMM), namely: swelling of mitochondria, efflux of cytochrome c (cyto c) from the mitochondrial inter-membrane space, and a decrease in the proton gradient across the IMM (i.e., the mitochondrial membrane potential, $\Delta\phi m$). Indeed, all three amyloid proteins/peptides, as pre-aggregated soluble oligomers, triggered a combination of robust mitochondrial swelling, cyto c release and lowered the $\Delta\phi m$ (Camilleri et al., 2013, 2020; Ghio et al., 2019). Pharmacological inhibitors of essential outer and inner membrane components of the mitochondrial permeability transition pore complex, as well as the antioxidant N-acetylcysteine, failed to suppress the mitotoxic effects induced by the oligomers. On the other hand, incubation of the oligomers, prior to application to mitochondria, with small-molecule compounds known to have powerful anti-amyloidogenic properties, such as the diphenylpyrazole "anle138b" [3-(1,3-benzodioxol-5-yl)-5-(3-bromophenyl)-1H-pyrazole] or the flavonoid morin, strongly inhibited the oligomer-induced mitochondrial membrane damage (Camilleri et al., 2013, 2020; Ghio et al., 2019). Together, the results presented above indicate that all three amyloid proteins in the oligomeric form trigger mitochondrial membrane toxicity, potentially through direct association of the oligomers with mitochondria.

It has been suggested that amyloid and antimicrobial peptides share a common mechanism of membrane permeabilization involving the nucleation-dependent formation of stable membrane pores (Last and Miranker, 2013). Given the enhanced vulnerability we had observed of mitochondrial membranes to permeation by different amyloid peptides, we turned our attention to the possibility that amyloid oligomers might spontaneously insert and punch ion-permeable openings in mitochondrial membranes. We have to date reported that, under physiologic conditions, oligomeric preparations of α-syn and tau efficiently formed nanopores with channel-like properties in CL-containing planar lipid bilayer membranes (BLM) reflecting the phospholipid composition of the IMM and mitochondrial contact sites. The latter are domains where the OMM and IMM are in close apposition to each other, thereby allowing diffusion of CL from the mitochondrial inner to outer membranes. A detailed characterization of the α-syn and tau mitochondrial pores was carried out using single-channel electrophysiology. Results showed a number of similar electrophysiological properties between these two types of pores. Both α-syn and tau pores exhibited stable, well-defined conductance states ranging in the hundreds of pS (100–1200 pS), reflecting ring-like structures with a pore diameter of a few nanometers. Furthermore, consistent with the features of amyloid peptide channels in simple binary BLMs described previously (Azimov and Kagan, 2015), the α-syn and tau oligomer pores in mito-mimetic bilayers were voltage-independent. One difference between the two pore types was that while the (larger) tau pores showed no preference for either cations or anions, the α-syn pores demonstrated selectivity for anions such as Cl^-. With regards to amyloid-β, we have additional data indicating that the $A\beta_{1-42}$ peptide can also form channels in a mito-mimetic BLM environment (**Figure 1**). The Aβ channels exhibit conductance states of 400–1200 pS, voltage-independence and non-selectivity for ions. In addition, the electrophysiological data allowed us to hypothesize models which would account for the multiple conductance states generated by the oligomeric pores. Essentially, these would reflect a multimeric aggregate complex undergoing either, (i) addition/removal of a fixed subunit with corresponding changes in the pore diameter, or (ii) supramolecular assembly into mono-, di-, or tetrameric structures.

Collectively, our data therefore prompts us to contemplate a "mitocentric" view for the pathophysiological role of prefibrillar oligomeric species of amyloid proteins in neurodegenerative diseases. The abundance of mitochondrial organelles in neuronal bodies and, especially, at synaptic terminals would presuppose the facile accessibility of mitochondrial membranes to intracellular cytosolic amyloid aggregates of α-syn, tau and amyloid-β. One can thus envisage direct piercing of mitochondria through a common pore-forming mechanism targeting specialized mitochondrial membrane domains enriched in CL (for instance, outer membrane contact sites and the IMM). A major conceptual advance in this regards was made when we studied the permeabilization of mitochondrial membranes by oligomeric aggregates prepared from the N-terminal domain of the Escherichia coli HypF (HypF-N) protein. HypF-N, which has no link with any human neurodegenerative disease, has proved an invaluable model system for understanding the fundamental mechanisms behind the aberrant self-assembly and toxicity of misfolded proteins. One particularly useful feature of HypF-N is that aggregation can be directed into two alternate oligomeric states, referred to as "type A" and "type B," which, despite their similar size and morphology, are significantly toxic and non-toxic, respectively, to cultured primary neurons and in brains of animal models. In fact, the type A HypF-N oligomers have been shown to possess remarkably similar behavior to the prefibrillar oligomers involved in the pathogenesis of neurodegenerative proteinopathies, including Aβ and α-syn (Chiti and Dobson, 2017). Notably, in our experiments, the type A HypF-N oligomers, which are characterized by a greater extent of solvent-exposed hydrophobicity, induced electrical recordings in planar mito-mimetic BLM displaying multi-level channel conductances with open/close step-current transitions (Farrugia et al., 2020). This reflected what we had observed in the electrophysiological studies with α-syn, tau and Aβ. Moreover, type A HypF-N oligomers released fluorophore from liposomes possessing CL-rich membranes as well as cyto c from isolated mitochondria, whilst lowering the $\Delta\phi m$ and causing

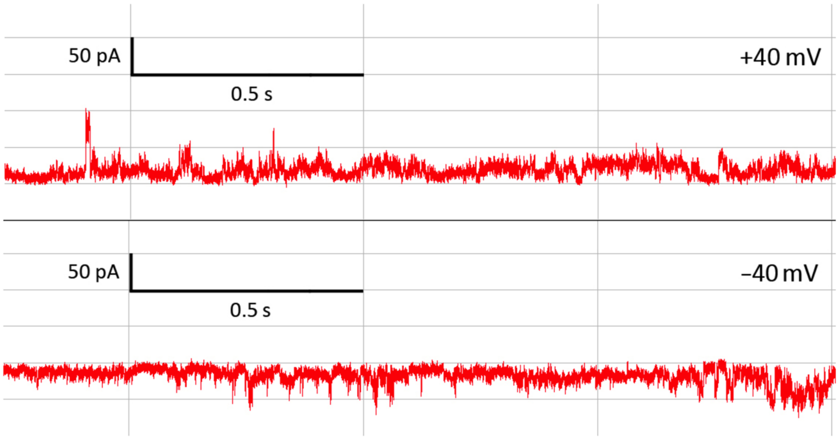

Figure 1 | A$\beta_{1–42}$ oligomers incorporate into mito-mimetic lipid bilayers as pores.
Representative current vs. time traces, obtained using single-channel electrophysiology, of Aβ_{42} oligomers in planar BLM formed from a CL-rich (15% CL) lipid composition (Ghio et al., 2019) showing "burst-like" and "spiky" behavior. Oligomers were added to the cis compartment to a final concentration of 0.5 µM. Bilayers were formed using the painting method, under voltage-clamp conditions of +40 mV (upper panel) and –40 mV (lower panel). Membrane capacitance was monitored continuously to ensure stability of the BLM. Symmetrical electrolyte buffer solutions were used in the cis and trans chambers (250/250 mM KCl, 10 mM MOPS/Tris, pH 7.2). Aβ: Amyloid-β; BLM: bilayer membrane; CL: cardiolipin.

changes in mitochondrial volume (Farrugia et al., 2020). Conversely, the type B HypF-N oligomers manifested no deleterious effects to mitochondrial function, or channel-like activity in BLM.

In this context, taken together, our recent studies raise the exciting possibility that pore-forming oligomeric assemblies of proteins involved in neurodegenerative diseases of the amyloid type may represent a new class of "mitochondrial porins." Water molecules as well as most ion types would be able to pass through the membrane-spanning amyloid porins, resulting in dysregulation of mitochondrial ionic homeostasis and potentially activating a cascade of events leading to mitochondrial swelling, dissipation of the $\Delta\phi$m, a decrease in ATP synthesis, cyto c release and ultimately activation of neuronal/synaptic apoptosis. Importantly, lipid membrane domains with a high CL content would represent a particularly vulnerable locus of action for the pathogenic amyloid pores. Such a mechanism would be strikingly reminiscent of OMM permeabilization by the proteins Bax, Bak and Bid, which represent pro-apoptotic members of the Bcl-2 family. For example, truncated Bid translocates to mitochondria to preferentially associate with CL in outer mitochondrial contact site membranes, subsequently activating Bax pore formation (Luo et al., 2014). Natural cytotoxins like Naja oxiana cobra venom cardiotoxins also bind to CL in the OMM to form toroidal pores which disrupt mitochondrial structural integrity and function (Li et al., 2020). Further, there are several known instances of bacterial porins incorporating into mitochondrial membranes and forming high conductance pores. To mention two examples, the p34 subunit of the vacuolating toxin VacA released by the gram-negative bacterium Helicobacter pylori, which is essential for microbial virulence, forms ion-conducting oligomeric pores in IMM-like bilayers with anion selectivity (Domanska et al., 2010); whilst the trimeric porin PorB from Neisseria gonorrhoeae incorporates into the IMM forming high conductance pores that would dissipate the $\Delta\phi$m in a mere 0.8 ms (Kozjak-Pavlovic et al., 2009).

In conclusion, the well-known affinity of misfolded amyloid oligomers to lipid bilayers should now be extended to specifically include mitochondrial membranes, particularly those specialized domains enriched in CL. Certainly, it is plausible that pathogenic ion-conducting pores may be formed in both plasma and mitochondrial membranes concurrently, depending largely on whether the toxic amyloid entities are located exogenously or intracellularly, respectively. Further, it is fascinating to consider that the molecular mechanism of poration of mitochondrial membranes discussed here for amyloidogenic peptides, is also shared by a diverse array of other proteins, including the pro-apoptotic Bcl-2 family members, antimicrobial peptides and natural cytotoxins. In order to bolster this hypothesis, future work will require that structural data be obtained regarding the physical nature of these amyloid nanopores in mitochondrial membranes. Another useful approach would involve combining measurement of electrical conductivity across the planar BLM with real-time fluorescence monitoring of the association of the oligomeric complexes with the membrane. Ultimately, an important implication of the amyloid "mitochondrial porin" mechanism is that the conducting activity of embedded pores could be blocked using small-molecule compounds. This might represent a novel and promising therapeutic strategy for patients with neurodegenerative proteinopathies that should be investigated further.

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