Techniques to Investigate Neuronal Mitochondrial Function and its Pharmacological Modulation

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ABSTRACT

Mitochondria are central regulators of neuronal homeostasis and survival, and increasingly viewed as a drug target in several acute and chronic neurological disorders, e.g. stroke, Alzheimer's, Parkinson's, and Huntington's diseases. Frequent working hypotheses aim to establish whether and how chemical or genetic lesions affect mitochondrial function in neurons, and whether this can be rescued by pharmacological treatments. However, the generic designation 'mitochondrial function' actually encompasses a wide spectrum of individual activities, too numerous to be fully quantified by any single available technique. This review aims to provide a broad perspective on the roles played by neuronal mitochondria, and addresses multiple techniques that can be used to derive instructive functional indicators. These include measurements of mitochondrial respiration, ATP production, membrane potential, calcium handling, biogenesis, dynamic movement as well as fusion and fission. Technique descriptions are preceded by a summary of mitochondrial physiology and pharmacological tools required for functional modulation and parameter determination. Hopefully, these will assist researchers interested in testing mitochondria as a drug target in neurological disease models.

1. Introduction

1.1. Neuronal mitochondria: a vital and dynamic asset

Neurons are highly specialized and polarized cells with large energy requirements. Excitability demands a continuous maintenance of steep ion gradients, which consumes vast amounts of ATP primarily provided by oxidative phosphorylation, thus rendering neurons critically dependent on mitochondria and continuous oxygen supply [1]. Mitochondria assist neurotransmission and synaptic plasticity via multiple roles beyond ATP production. They regulate spatiotemporal patterns of intracellular calcium ($[Ca^{2+}]_i$) signalling [2] and move purposefully to meet variable demands across neuronal soma, axons, dendrites and synaptic sites [3]. Throughout the lifespan of their post-mitotic hosts, neuronal mitochondria

continuously regenerate through a biogenesis process assisted by nuclear and mitochondrial gene products [4]. Moreover, mitochondria undergo fission and fusion cycles that modulate their number, size and content, allowing functional segregation and adaptation to different neuronal compartments and microenvironments [5,6], and likely assist mitochondrial quality control mechanisms [7]. Furthermore, mitochondria govern reactive oxygen species (ROS) formation and signalling, assist steroid and heme biosynthesis, and several other metabolic pathways, being also central regulators of apoptosis and neuronal survival [8]. Interestingly, recent evidence suggest that all extant eukaryotes possess mitochondria, some in the form of reduced homologues, e.g. hydrogenosomes and mitosomes. So far, the single common metabolic function identified across all homologues is iron-sulfur cluster assembly, clearly one of the most fundamental mitochondrial functions [9,10]. Focusing on humans, the frequent neurological phenotype linked to mitochondria gene mutations [11], and the association of mitochondrial dysfunction with acute and chronic neurological disorders [12,13], strongly emphasize how much neuronal health depends on fully functional mitochondria, and why these are targets for neuroprotection.

1.2. The value of representative functional indicators

When investigating neuronal mitochondria under (patho)-physiological conditions or pharmacological interventions one should consider representative functional indicators. Indeed, from the experimental standpoint, comprehensively quantifying the full spectrum of mitochondrial activities while searching for dysfunctions is tantamount to impossible. Hopefully, scientific progress will prove the previous sentence wrong. A Pubmed search (www.ncbi.nlm.nih.gov/pubmed) for "mitochondrial function" OR "mitochondrial dysfunction" AND "neurons" yields over 1,000 publications during the last decade, exceeding 7,000 if searching without "neurons". Indeed, two frequent working hypothesis in neuroscience and others fields are: (*i*) disease *x* affects mitochondrial function; and (*ii*) drug *y* induces/improves mitochondrial dysfunction. In some cases, sequencing the mitochondrial genome or nuclear-encoded mitochondrial genes objectively answers the first hypothesis, e.g. by finding loss-of-function mutations in critical genes. In others cases, mutations may be found but it will remain unknown whether these have functional consequences. Still, in most cases where the first, and particularly, the second hypothesis are formulated, one must rely on functional indicators. Thus, it becomes critical to consider: (*i*) which functional indicators; (*ii*) what do they mean; and (*iii*) how are they quantified. The present review addresses these issues in the context of neuronal mitochondria.

The next section summarizes mitochondria physiological and pharmacological principles, related to subsequently described techniques. Hopefully, these will provide the grounds for those interested in testing mitochondria as a drug target in neurological disease models.

2. Mitochondrial physiology and pharmacology

2.1. Respiratory chain, proton motive force, ATP synthesis, and proton leaks

Mitochondria exhibit a core matrix surrounded by two membranes, the inner one containing the respiratory chain (Figure 1, *A*). This chain combines the sequential activity of enzyme complexes, namely complex I (NADH-ubiquinone oxidoreductase), II (succinate-ubiquinone oxidoreductase), III (ubiquinol-cytochrome *c* oxidoreductase), and IV (cytochrome *c* oxidase). Significantly, complex II contains succinate dehydrogenase, a nuclear-encoded Krebs cycle enzyme. Electrons from NADH or $FADH_2$ enter the chain at Complex I or II, respectively, being sequentially shuttled down their electrochemical potential to complex III (via ubiquinone, a.k.a. coenzyme Q_{10}), and then IV (via cytochrome *c*) where O_2 is consumed

as the final electron acceptor. This electron flux drives proton (H^+) efflux towards the intermembrane space via H^+ pumps in complex I, III and IV, generating an electrochemical H^+ gradient ($\Delta\mu_{H+}$) expressed in electrical potential units (mV) as the *proton motive force (Δp)*. At 37ºC, $\Delta p = \Delta\psi_m - 60\Delta pH$ (where $\Delta\psi_m$ is the mitochondrial membrane potential, and ΔpH the H^+ gradient). Δp is the primary bioenergetic parameter controlling mitochondrial ATP synthesis, while $\Delta\psi_m$ regulates mitochondrial Ca^{2+} uptake and ROS generation. Under most conditions $\Delta\psi_m$ is the dominant component (~80%) of Δp. Thus, $\Delta\psi_m$ and Δp are often used interchangeably when referring to the common driving force for key mitochondria functions [14,15].

ATP synthase (complex V or F_0F_1 ATPase) closes the proton circuit established by respiratory chain activity. In analogy to hydroelectric dams accumulating upstream water and channelling it through turbines, mitochondria accumulate protons in the intermembrane space and channel them through ATP synthase generating ATP, which is exported to the cytosol via the adenine nucleotide translocase (ANT). Meaningfully, mitochondria may reverse ATP synthase, consuming rather than generating ATP. While transient ATP synthase reversal allows Δp maintenance, crucial for mitochondrial activity, unrestrained reversal may lead to cellular ATP depletion [16].

H^+ leaks across the inner mitochondrial membrane decrease the coupling efficiency between O_2 consumption and ATP synthesis. This 'uncoupling' plays a physiological role in limiting Δp, otherwise increasing electrons dwell time in complex I and III where they leak into oxygen forming superoxide, which in excess may have deleterious effects [17]. Mitochondrial H^+ leaks include an inducible and a basal component, which is insensitive to known activators and inhibitors [18]. Inducible H^+ leaks involve uncoupling proteins (e.g. UCP1; [19]) and the ANT fatty-acid-dependent H^+ leak. Basal H^+ leaks may occur at the ANT-phospholipid interface, since they increase with ANT content but are independent from known ANT functions [20].

2.2. Mitochondrial calcium handling in neurons

Mitochondria are central to neuronal Ca^{2+} homeostasis (Figure 1, *right*). In addition to energizing non-mitochondrial Ca^{2+}-handling mechanisms, e.g. plasmalemmal and endoplasmic reticulum (ER) Ca^{2+}-ATPases, mitochondria are $[Ca^{2+}]_i$ modulators and high capacity Ca^{2+} storage systems. The latter being particularly relevant in neurons, excitable cells capable of sudden and extensive $[Ca^{2+}]_i$ increases, where mitochondria Ca^{2+} sequestration prevents cytosolic Ca^{2+} overload and shapes feedback inhibition of Ca^{2+} transients [1,21]. Mitochondria regulate $[Ca^{2+}]_i$ via uptake and release, controlling cytoplasmic Ca^{2+} microdomains, and shaping frequency/propagation of Ca^{2+} signals. The regulation is reciprocal since mitochondrial Ca^{2+} uptake plays key metabolic roles, tuning substrate uptake, Krebs cycle activity and ATP synthesis, and influences mitochondrial motility and morphology [22,23]. Mitochondria start accumulating Ca^{2+} when the neighbouring concentration rises above the set point (~0.5 μM) at which uptake is balanced by efflux [24]. The mitochondrial Ca^{2+} uniporter and the Na^+/Ca^{2+} (3:1) exchanger provide the main matrix Ca^{2+} uptake and efflux pathways, respectively [25,26]. Above the set point, increasing uniporter activity drives Ca^{2+} to the matrix where buffering occurs via Ca^{2+} phosphate complexes. These are reversible complexes, and when plasmalemmal Ca^{2+} pumps restore $[Ca^{2+}]_i$ below the set point, mitochondria gradually release Ca^{2+} back to the cytosol [27].

Ca^{2+} accumulation is driven by $\Delta\psi_m$ in competition with ATP production. If oxidative phosphorylation decreases, glycolysis may increase via the Pasteur effect, albeit much less prominently in neurons than in glycogen containing cells. Upon ATP synthase reversal, Ca^{2+} accumulation may also be driven by glycolytic ATP hydrolysis. Still, mitochondria have a

limited Ca^{2+} buffering capacity, and when exceeded it precipitates mitochondrial permeability transition (mPT). This consists of non-specific pore opening at the inner mitochondrial membrane, rendering it permeable to ions and molecules < 1.5 KDa. With the exception of transient pore openings, which may play physiological roles, unrestrained mPT has catastrophic consequences for mitochondria and their hosts. Thus, mPT is currently a subject of intense research in neuronal injury and a drug target in neuroprotection [28-30].

2.3. Mitochondrial biogenesis and dynamics

Biogenesis is critical to replenish mitochondria throughout neuronal lifespan (Figure 1, *B*). Damaged mitochondria are degraded by mitophagy (mitochondria autophagy), a controlled process preventing the release of apoptotic factors that might lead to neuronal death. In addition, biogenesis increases to meet metabolic demands or compensate for mitochondrial dysfunction [4,6,31]. Mitochondria possess their own DNA (mtDNA), each cell containing several thousand copies of these small circular genomes that hold testimony to a remarkable evolutionary background [32,33]. Nevertheless, the mitochondrial genome is now insufficient for independent replication. Moreover, only a few proteins are synthesized within mitochondria, which must import about 1,000 different nuclear-encoded proteins [34]. Thus, for mitochondria to proliferate, i.e., undergo biogenesis, a concerted action must take place between nuclear and mitochondrial genes. Several transcription factors play an important role in mitochondrial biogenesis, most notably, mitochondria transcription factor A (mtTFA), mitochondrial transcription specificity factors, nuclear respiratory factors (NRF-1 and NRF-2), and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), the latter possibly exerting master regulatory roles [35]. Mitochondrial biogenesis may be altered in neurological disorders, thus being a putative therapeutic target [4].

Neuronal mitochondria are highly dynamic, undergoing fission, fusion and movement along neuronal processes (Figure 1, *B,C*). Fission-fusion cycles allow mixing and asymmetric segregation of mitochondrial contents, including mtDNA, which may compensate or propagate mutations. In addition to functional segregation, fission-fusion cycles regulate mitochondria number, size and morphology, whereas movement displaces mitochondria to sites in need, which in human motor neurons may exceed one meter distance. In combination, fission-fusion and movement ensure efficient management and distribution of limited mitochondrial resources across vast neuritic networks with variable spatiotemporal demands.

Mitochondrial fission and fusion are membrane-remodelling events assisted by evolutionarily conserved dynamin-related GTPases (Figure 1, *B*). Fission involves recruitment of cytosolic DRP-1 and membrane bound Fis-1, whereas fusion of the outer and inner membranes is assisted by mitofusins (Mfn-1 and Mfn-2) and optic atrophy-1 (OPA-1), respectively. Significantly, Mfn-2 mutation is linked to the peripheral neuropathy Charcot-Marie-Tooth type 2A, OPA-1 mutation causes hereditary optic nerve degeneration and progressive blindness, and DRP-1 mutation has been associated with abnormal brain development [36,37].

Mitochondria use adaptor proteins to ride molecular motors, possibly paying an ATP fare to accelerate along cytoskeletal tracks (Figure 1, *C*). They use microtubules for long distance fast axonal transport, and shift to actin microfilaments for short distances in pre- and postsynaptic endings. Typically, kinesin motors drive mitochondria anterogradely, towards the (+) end of microtubules in growth cones, whereas dynein motors mediate retrograde transport. Miro, Milton and syntabulin are adaptor proteins for kinesins, whereas dynactin adapts mitochondria to dyneins [3,13,38]. Miro is also a Ca^{2+} sensor mediating motility in low $[Ca^{2+}]_i$ environments and arresting mitochondria in high $[Ca^{2+}]_i$ sites in need of buffering such as active synapses [39,40]. Mitochondria also follow increasing ADP gradients, moving towards ATP impoverished sites [38]. Interestingly, molecular motors remain functional at

relatively low $[ATP]_i$ sites [3], but inhibition of mitochondrial ATP synthesis with oligomycin arrested trafficking in forebrain [41] but not cerebellar neurons [42], which might be explained by differences in glycolytic ATP availability or $[Ca^{2+}]_i$ levels. Additional modulation of mitochondrial function is described below.

2.4. Pharmacological modulation of mitochondrial function

Several pharmacological tools assist the study of mitochondrial function. However, these are not widely applicable across different preparations and techniques. Useful tools in isolated mitochondria may not reach effective concentrations within intact cells, sometimes requiring microinjection or membrane permeabilization, e.g. with digitonin or saponin. Also, unpredicted/non-selective drug effects on extra-mitochondrial targets often complicate *in situ* data interpretation. On the other hand, useless drugs for isolated mitochondria experiments may be quite helpful *in situ* (e.g. modulation of trafficking and other dynamic properties).

2.4.1. Respiratory Complexes and ATP Synthase Inhibitors

In intact neurons, inhibition of any respiratory complex impairs the chain. Complex I inhibition limits NAD^+ availability, compromising succinate synthesis for alternative feeding of complex II. Conversely, complex II inhibition arrests the Krebs cycle, limiting complex I substrate [14]. Because complex III and IV act downstream of electron entry sites, their inhibition also halts the respiratory chain (Figure 1, *A*). Still, complex I-III inhibition can be elegantly bypassed in intact neurons by using TMPD (2,3,5,6-tetramethyl-*p*-phenylenediamine)/ascorbate, which donates electrons to complex IV, allowing H^+ extrusion and Δp recovery [8]. To bypass complex I inhibition in intact neurons, one possibility is methyl-succinate, a cell-permeable analogue of a complex II substrate [43].

Complex I inhibitors are numerous, being chiefly represented by the semi-quinone antagonist rotenone and by MPP^+, the toxic metabolite from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine commonly used to induce Parkinson-like neurodegeneration [44,45]. *Complex II* inhibitors primarily include the competitive malonate and the irreversible 3-nitropropionic acid (3-NP), both acting on succinate dehydrogenase. Notably, 3-NP is frequently used to simulate Huntington's disease neurodegeneration [46]. *Complex III* is inhibited by myxothiazol and antimycin A, respectively, upstream and downstream to the ubisemiquinone site. Because at that site electrons can be transferred to oxygen, blocking downstream electron flow with antimycin A is a useful strategy to increase mitochondrial superoxide formation in neurons [47,48]. *Complex IV* inhibitors like cyanide and azide inhibit electron transfer to oxygen, inducing chemical hypoxia in mechanistic studies of hypoxic neurodegeneration [49].

Mitochondrial ATP synthase contains a proton-translocating membrane-embedded domain (F_0), and a catalytic domain (F_1). Protons extruded by complex I, III and IV re-enter the matrix via F_0 and energize a mechanical rotary mechanism linked to ATP synthesis via F_1 (Figure 1, *A*). Numerous natural and synthetic compounds inhibit ATP synthase (for a comprehensive review see Ref. [50]). The macrolide oligomycin is commonly used in experiments with neurons. Direct ATP synthase inhibition can be achieved by blocking F_0 proton-flux with oligomycin, thus preventing ATP synthesis/hydrolysis. In neurons, excess oligomycin may also inhibit plasmalemmal Na^+/K^+ ATPase [51]. Oligomycin typically increases $Δψ_m$ (hyperpolarization; but see oligomycin null-point test in Section 4), and allows the study of other $Δψ_m$-related functions, e.g. Ca^{2+} buffering, ROS production, independently of ATP synthesis/hydrolysis. Cell survival in the presence of oligomycin will primarily depend on glycolytic capacity, which may vary significantly across neuronal types [14]. F_1 domain inhibition also directly arrests mitochondrial ATP synthase. The endogenous inhibitor protein IF_1 may prevent ATP depletion when neuronal insults reverse ATP synthase [16].

Significantly, some mitochondrial markers like rhodamine-6G and structurally related lipophilic cations block the F_1 domain when in excess concentration, compromising interpretation of functional assays [52,53]. Indirect F_0F_1 ATP synthase inhibition occurs upon mitochondrial uncoupling, namely by drugs decreasing the H^+ gradient required for ATP generation (e.g. protonophores; see below).

2.4.2. Ionophores, Inhibitors of mPT, Ca^{2+} Uniporter and other Transporters

Ionophores are central to the study of highly polarized membranes, such as that of neurons and their mitochondria. Ionophores modify the ion permeability of lipid bilayers, typically lacking selectivity towards the multiple membranes in a cell [54]. Some ionophores form relatively non-selective cationic channels (e.g. gramicidin), whereas others are selective mobile carriers catalyzing the electrogenic uniport of a single ion [e.g. H^+ (protonophores), K^+ (valinomycin), or Ca^{2+} (ferutinin)], or the electroneutral antiport/exchange of two different ions [e.g. K^+/H^+ (nigericin), $Ca^{2+}/2H^+$ (ionomycin or calcimycin)] [14,55].

Protonophores, most commonly FCCP or CCCP, increase H^+ permeability of the inner mitochondrial membrane, decreasing Δp and uncoupling respiration from ATP synthesis. Protonophores only partially mimic the endogenous uncoupling protein UCP-1 [56]. Indeed, UCP-1 operates selectively at the inner mitochondrial membrane where it provides a Δp-dependent endogenous H^+ leak, which does not compromise maximal ATP generation capacity since UCP-1 becomes almost inactive during state 3 respiration (see Section 3) [57]. Conversely, protonophores affect H^+ gradients across non-mitochondrial membranes, e.g. modifying plasmalemmal and synaptic vesicle transmembrane potentials that are particularly relevant for neuronal physiology. Some selectivity can be achieved by protonophore titration. Indeed, 0.25 μM FCCP selectively collapsed $\Delta\psi_m$ in cerebellar neurons, whereas 2.5 μM FCCP also collapsed plasma membrane potential ($\Delta\psi_p$), as evidenced by simultaneous recording of both potentials [54]. Still, 1 μM FCCP increases $\Delta\psi_p$, as shown by patch clamp recordings in hippocampal neurons [58]. Thus, $\Delta\psi_p$ collapse with 2.5 μM FCCP likely involves ATP depletion and changes in permeability to ions other than H^+. In neurons, the prevailing effect of low FCCP concentrations ('mild-uncoupling') is a decrease in maximal ATP generating capacity, whose consequences outweigh marginal reductions in matrix superoxide levels [59]. Moreover, by increasing mitochondrial H^+ conductance FCCP reverses ATP synthase, which consumes ATP to extrude H^+ in a futile attempt to recover Δp. This H^+ cycling will lead to cellular ATP depletion, delayed only by glycolytic ATP availability. Thus, in intact neurons, if the goal is to collapse $\Delta\psi_m$ without consuming cellular ATP, FCCP should be combined with oligomycin. Alternatively, the same goal can be achieved with a respiratory chain inhibitor (e.g. myxothiazol) plus oligomycin, with the advantage of avoiding FCCP-induced H^+ permeability of non-mitochondrial membranes [8].

Potassium ionophores are useful in isolated mitochondria experiments to equilibrate the transmembrane K^+ gradient with $\Delta\psi_m$ (valinomycin, K^+ uniport) or with ΔpH (nigericin, K^+/H^+ antiport; allowing Δp to be expressed solely as $\Delta\psi_m$) [60-62]. In neurons, valinomycin depolarizes and swells mitochondria, a property applied to study how mitochondria volume affects their trafficking in neurites [42,63]. Valinomycin and nigericin oppositely modulate neuronal $\Delta\psi_m$ and $\Delta\psi_p$. Valinomycin K^+ uniport decreases $\Delta\psi_m$ via K^+ influx, and increases $\Delta\psi_p$ via K^+ efflux. Conversely, nigericin K^+/H^+ antiport increases $\Delta\psi_m$ via compensatory response to ΔpH collapse, and decreases $\Delta\psi_p$ via plasmalemmal H^+ influx in exchange for K^+ efflux [54]. Moreover, > 1 μM nigericin allows extracellular Na^+ influx, further decreasing neuronal $\Delta\psi_p$ and short-circuiting the Na^+-K^+ ATPase [64].

Calcium ionophores, typically $Ca^{2+}/2H^+$ exchangers such as ionomycin and calcimycin (A23187), are frequently used in neurons for calibrating fluorescent Ca^{2+} probes, including Fura-2 and derivatives (a condition in which the non-fluorescent 4Br-calcimycin is preferred;

[55,65]). In isolated mitochondria, ionophore-induced $Ca^{2+}/2H^+$ exchange is partly compensated by Ca^{2+} reuptake and increased respiration-driven H^+ extrusion, leading to futile Ca^{2+} cycling and uncoupling [66]. *In situ*, effects of Ca^{2+} ionophores are more complex, influencing both $\Delta\psi_m$ and $\Delta\psi_p$, and also modifying plasmalemmal and ER ion exchange [54,55]. While neutral ionophores ($Ca^{2+}/2H^+$ exchangers) like calcimycin are frequently used to study Ca^{2+}-dependent neuronal cell death [67], electrogenic Ca^{2+} uniport with ferutinin may promote a more physiological model of mitochondria Ca^{2+} overload, involving Ca^{2+}-dependent and cyclosporin A sensitive mPT pore opening [55].

mPT inhibitors like cyclosporin A (CsA; [68]) interfere with cyclophilin-D (a mitochondrial matrix peptidyl-prolyl cis-trans isomerase; [69]), preventing its facilitatory effect on Ca^{2+}-triggered mPT. CsA also binds cytosolic cyclophilin-A, forming a complex that inhibits calcineurin and leads to immunosuppression. When studying neuronal mitochondria *in situ* it is relevant to discriminate CsA's effect on both targets. For this purpose one might compare experiments with CsA with those performed with tacrolimus (a.k.a. FK-506 or fujimycin), which inhibits calcineurin but not mPT [70]. Alternatively, non-immunosupressive CsA analogs like *N*-Me-Val-4-CsA, 6-Me-Ala-CsA, *N*-Me-5-isoleucine-CsA (NIM811), and D-3-MeAla-4-EtVal-CsA (Debio-25), retain cyclophilin-D and mPT inhibition but their complexes with cyclophilin-A fail to inhibit calcineurin [28,71]. Similarly, the unrelated mPT inhibitor sanglifehrin A does not inhibit calcineurin, binding both cyclophilin-D and cyclophilin-A at distinct sites from CsA [72]. The mPT pore is also inhibited by ATP and ADP, but not by their Mg^{2+} complexes, nor by other nucleotides that are poorly or not transported by the ANT (e.g. AMP, GDP or GTP). Interestingly, *ANT inhibitors* oppositely modulate mPT pore opening probability by trapping ANT in opposite conformations (carboxyatractyloside increases and bongkrekic acid decreases mPT Ca^{2+} sensitivity). Thus, despite recent knockout studies showing that ANT and VDAC (voltage dependent anion channel, a.k.a. porin) are not essential for mPT to occur, the ANT does exert a regulatory role [28,73-75].

Ca^{2+} uniporter inhibitors like ruthenium red (RuRed) inhibit Ca^{2+} uptake into isolated mitochondria. RuRed exhibits poor membrane permeability and selectivity, significantly affecting other Ca^{2+} channels (e.g. plasmalemmal L-type, and ER ryanodine-sensitive), which limits it usefulness in intact cells. Comparatively, the analog ruthenium 360 (Ru360) displayed increased potency, selectivity, and membrane permeability in isolated cardiac myocytes, suggesting that Ru360 might be used in intact cells [76]. Microinjected Ru360 was effective in neurons from hippocampal slice cultures, and higher concentrations were reported for counterbalancing illumination-dependent decomposition of the ruthenium complex [77]. More recently, in studies with cultured hippocampal neurons, Ru360 did not modify cytosolic Ca^{2+} responses to glutamate nor associated changes in mitochondria morphology or $\Delta\psi_m$. However, Ru360 was effective following plasmalemma permeabilization with saponin, thus arguing against Ru360 entering intact neurons [78]. Still, because Ru360 is rapidly oxidized by room air, and prolonged incubation periods are required to load intact cells, it is critical to renew Ru360 with freshly prepared solutions in order to ensure compound integrity in long-term experiments [43].

The mitochondrial Na^+/Ca^{2+} exchanger is inhibited by CGP-37157 (7-chloro-3,5-dihydro-5-phenyl-1H-4,1-benzothiazepine-2-one). Caution is advised when using CGP-37157 in intact neurons. At concentrations required for measurable effects on mitochondrial Ca^{2+} buffering, CGP-37157 directly inhibits voltage-gated Ca^{2+} channels preventing depolarization-induced Ca^{2+} influx [79]. CGP-37157 may also inhibit neuronal NMDA receptors [80], and some plasmalemmal Na^+/Ca^{2+} exchanger isoforms expressed in neurons [81] (Figure 1, *right*).

2.4.3. Disrupting mtDNA, Protein Synthesis, and Trafficking Cytoskeleton
Mitochondrial DNA is selectively disrupted in eukaryotic cells exposed to ethidium bromide

(EtBr), chronically producing ρ^0 cells without a functional respiratory chain and auxotrophic for uridine and pyruvate. ρ^0 cells can be repopulated with mitochondria from other cells originating cytoplasmic hybrids (cybrids) [82,83]. Although EtBr preferentially intercalates mitochondrial double-stranded DNA, mutagenic effects on nuclear DNA cannot be excluded thus prompting development of alternative strategies, e.g. targeted restriction endonucleases [84]. In neurons, EtBr mtDNA disruption was used to investigate the mitochondrial role in axonogenesis [85]. Alternatively to EtBr, the toxic mitochondrial dye rhodamine 6-G also prevents mtDNA transmission. Rhodamine 6-G treated embryonic stem cells can be rescued by fusion with cytoplasts, containing wild-type or mutated mtDNA, and differentiated into neurons for functional studies [86,87].

Mitochondrial protein synthesis is selectively inhibited with chloramphenicol, previously used for studying how mitochondrial translation impacts neuronal differentiation [88]. Conversely, cycloheximide reversibly inhibits cytosolic protein synthesis in neurons, whereas puromycin inhibits both cytosolic and mitochondrial protein synthesis [89,90].

Mitochondrial trafficking cytoskeleton may be selectively disrupted to investigate neuronal mitochondrial motility. Neuronal microtubules are depolymerized by nocodazole or vinblastine, whereas actin filaments are depolymerized or disorganized by latrunculin or cytochalasin [91,92]. Taxol and nocodozale, respectively, stabilizing and disrupting neuronal microtubules, were shown to depolarize mitochondria and evoke Ca^{2+} release. CsA blocked these effects on mitochondria, suggesting that taxol and nocodazole induce mPT [93]. Cytochalasin and nocodazole, respectively, disrupting actin filaments and microtubules, differentially affected the stochastic and directed modes of mitochondrial movement in respiratory neurons [94].

2.4.4. Additional Tools
RNA interference allows modulation of *in situ* mitochondrial function, e.g. preventing expression of fusion/fission related proteins [95,96], or interfering with mitochondrial protein import [97,98]. Moreover, multiple drugs affecting neuronal function can be applied to the study of *in situ* mitochondria, e.g. tetrodotoxin or veratridin to decrease or increase neuronal activity, respectively [99]. In addition to the experimental modulation addressed in this review, mitochondria are primary or secondary targets for several therapeutic molecules [100,101] and gene-therapy [102].

3. Oxygen consumption and ATP production

Mitochondrial oxygen consumption ('respiration') is controlled by cellular ATP turnover ('respiratory control'), and directly proportional to H^+ current across the inner mitochondrial membrane. This H^+ current comprises H^+ flow through ATP synthase and H^+ leaks. Importantly, respiration driving H^+ leaks changes in a 'non-ohmic' manner, i.e., increases disproportionately to Δp at high membrane potential [14]. In neurons, ATP is primarily produced by mitochondria when compared with glycolysis, and mostly spent in energizing plasmalemmal Na^+/K^+- and Ca^{2+}-ATPases in order to maintain ion gradients and thus excitability [1]. Monitoring of mitochondrial respiratory rates with oxygen electrodes provides valuable quantitative information on: (*i*) mitochondrial ATP synthesis; (*ii*) magnitude of the H^+ leak, i.e., uncoupling; and (*iii*) spare respiratory capacity, i.e., the mitochondrial ability to fulfil increased ATP demand.

The respiratory control ratio (RCR) is a classical parameter indicating the coupling efficiency of oxidative phosphorylation. RCR is calculated by dividing the respiration rate when ATP synthesis is maximal (*state 3*) by the respiration rate without ATP synthesis, driven solely by H^+ leaks (*state 4*). For isolated mitochondria suspended in cytosolic-like

buffer (high K^+), containing phosphate and substrate, the maximal ADP-evoked respiration provides the state 3 rate. Subsequent ATP synthase inhibition with oligomycin provides the state 4 rate. In intact neurons, respiration can be assessed by monitoring downstream oxygen levels in the buffer perfusing coverslip-attached cells [103,104], or by using microplate-based respirometry [105,106].

In situ neuronal mitochondria typically respire in '*state 3½*' [107], between state 3 and 4, with submaximal ATP synthesis. Oligomycin places mitochondria in state 4, whereas FCCP induces maximal uncoupled respiration. Full mitochondrial complex I and III inhibition with rotenone and myxothiazol, respectively, provides non-mitochondrial respiration that should be subtracted from all measurements. The difference between basal (state 3½) and oligomycin respiration (state 4) is an approximate measure of basal ATP turnover. The difference from FCCP respiration indicates spare respiratory capacity. With the proviso that FCCP respiration is not limited by ATP synthase activity, but represents maximal substrate oxidation capacity, comparing FCCP respiration with that reached during neuronal challenges, such as NMDA receptor activation, allows estimates of whether a lack of spare ATP generating capacity limits neuronal survival [108,109]. In practice, oligomycin respiration underestimates basal ATP turnover because ATP synthase inhibition increases Δp and, consequently, the non-ohmic conductance, thus overestimating H^+ leaks in state 3½ [110]. Assessing the variable impact of non-ohmic H^+ conductance on coupling efficiency is feasible in isolated mitochondria, namely with the systems approach 'modular kinetic analysis' [111]. Simultaneous oxygen consumption and $\Delta\psi_m$ measurements while substrate oxidation is titrated with FCCP, and state 4 respiration titrated with complex inhibitor (rotenone or malonate for complex I or II substrates, respectively), allows coupling efficiency calculations at any chosen $\Delta\psi_m$ value [62], thus accounting for the non-ohmic conductance and possibly identifying functional differences between control and test mitochondria.

Measuring neuronal ATP levels may provide information on mitochondrial ATP synthesis. In such measurements one should consider that: (*i*) glycolysis also contributes for cellular ATP and may be increased by Pasteur effect masking mitochondrial dysfunction [112]; (*ii*) presence of non-neuronal cells, e.g. astrocytes, influences global ATP measurements [113]; and (*iii*) ATP synthase reversal causes mitochondria to consume rather than generate ATP. The ATP content of neuronal populations can be quantified at specific time-points using luciferin-luciferase luminescence or HPLC techniques, which allow additional measurements [114-116]. Single-neuron dynamic changes in $[ATP]_i$ can be monitored indirectly via changes in $[Mg^{2+}]_i$. Cytosolic Mg^{2+} normally binds ATP and is released upon ATP hydrolysis, causing free $[Mg^{2+}]_i$ to inversely correlate with $[ATP]_i$. This can be assessed by real-time fluorescence microscopy using Mg^{2+}-sensitive indicators like Magnesium Green (K_d Mg^{2+} ~1 mM; K_d Ca^{2+} ~6 μM) or Mag-Fura-2 (K_d Mg^{2+} ~2 mM; K_d Ca^{2+} ~25 μM). Simultaneous recordings with a spectrally distinct and selective Ca^{2+} indicator, e.g. Magnesium Green + Fura-2, allow correction for $[Ca^{2+}]_i$ changes [117,118]. Also, real-time monitoring of intracellular ATP or ATP:ADP ratio may be performed with genetically encoded reporters [119-121]. Furthermore, qualitative information on whether neuronal mitochondria are generating or consuming ATP can be inferred from the 'oligomycin null-point test' [122] using $\Delta\psi_m$-sensitive probes, as addressed below.

4. Mitochondrial and plasma membrane potentials

Mitochondrial membrane potential ($\Delta\psi_m$) measurements follow the Nernstian distribution of lipophilic cations, which cross membranes and re-equilibrate in response to changes in electrochemical potential gradients. Isolated mitochondria $\Delta\psi_m$ is traditionally assessed with

tetraphenylphosphonium (TPP$^+$) and electrode-based detection, whereas *in situ* $\Delta\psi_m$ measurements frequently use fluorescent detection of rhodamine derived probes, most notably, tetramethylrhodamine methyl or ethyl ester (TMRM$^+$ or TMRE$^+$, respectively) and rhodamine-123 (Rh123) [123]. Interpreting fluorescence changes in neurons loaded with these probes is far from trivial, but observing three important principles minimises misinterpretations. First, lipophilic cations are $\Delta\psi_m$-'sensitive', not $\Delta\psi_m$-'specific'. They partition across plasma and mitochondrial membranes and respond to $\Delta\psi_p$ and $\Delta\psi_m$ changes (Figure 1, *bottom right*: TMRM$^+$). Second, redistribution is much faster across the mitochondrial vs. plasma membrane due to surface/volume differences. Third, when matrix probe reaches an aggregation threshold, fluorescence is quenched becoming nonlinear with concentration [15,54].

Quench vs. non-quench (or equilibrium) are two different experimental approaches with $\Delta\psi_m$-sensitive probes. Their interpretation varies significantly, and probe titration is essential to ensure the desired approach. *In equilibrium conditions*, using low probe concentrations to avoid matrix quenching, whole-cell fluorescence originates mostly (~75%) from polarized mitochondria (for $\Delta\psi_m$ ~150mV and 1% matrix/cytoplasm volume; [15]). Under these conditions, whole-cell fluorescence is theoretically related to $\Delta\psi_m$. Still, variation in matrix/cytoplasm volume or in $\Delta\psi_p$ may influence differences among cells. *In quench mode*, resting whole-cell fluorescence does not reflect $\Delta\psi_m$ because matrix probe aggregation blunts concentration differences. Nevertheless, because mitochondrial hyperpolarization or depolarization drives matrix probe influx or efflux, respectively, these changes in $\Delta\psi_m$ can be transiently detected in cytosolic or whole-cell fluorescence [8,15].

Rh123 is less permeable than TMRM$^+$, thus equilibrating slowly across the plasma membrane. Hence, short-term experiments using Rh123 in quench mode allow dynamic $\Delta\psi_m$ recording with less interference from $\Delta\psi_p$ [122,124,125]. For TMRM$^+$, the ambiguity in relating changes in fluorescence to $\Delta\psi_m$ or $\Delta\psi_p$ can be circumvented via simultaneous recordings with an anionic $\Delta\psi_p$ probe, and with the assistance of computer algorithms [54]. Simultaneous $\Delta\psi_m/\Delta\psi_p$ and $[Ca^{2+}]_i$ measurements are feasible in intact neurons. Stepwise extracellular K$^+$:Na$^+$ replacement and the Goldman equation allow $\Delta\psi_p$ probe calibration, whereas maximal and minimal (0 Ca^{2+}:EGTA) ratiometric signals with ionophore allow $[Ca^{2+}]_i$ probe calibration [54,126].

The oligomycin null-point test distinguishes neurons with competent (ATP-producing) mitochondria from those with damaged (ATP-consuming) mitochondria. With sufficient glycolytic ATP, damaged mitochondria can hold $\Delta\psi_m$ by ATP synthase reversal and load efficiently with $\Delta\psi_m$-sensitive probes. Oligomycin hyperpolarizes ATP-producing mitochondria by preventing F$_0$ H$^+$ re-entry. Conversely, oligomycin depolarizes ATP-consuming mitochondria by preventing F$_0$ H$^+$ extrusion. Under quench conditions, hyperpolarization decreases whole-cell fluorescence by driving cytoplasmic probe to the matrix quenching environment. Depolarization increases whole-cell fuorescence as the probe exits the matrix towards the cytosol. Terminating experiments with full $\Delta\psi_m$ collapse by FCCP provides positive control for quenching conditions [122,127,128].

A major source of artefacts with $\Delta\psi_m$-sensitive probes is their efficient extrusion by the multi-drug resistance (MDR) pump, P-glycoprotein, expressed by some cells-lines used as "neuronal models". To avoid erroneous interpretations of decreased $\Delta\psi_m$ in such cells, one should test whether MDR inhibitors like verapamil or CsA significantly increase probe-loading efficiency. Conversely, to avoid erroneous interpretations of mPT inhibition when CsA increases probe fluorescence, one should test whether verapamil is devoid of the same effect [129], and/or use other mPT inhibitors (see Section 2.4.2). Note that, in neurons, Ca^{2+} channel blockade by verapamil may confound data interpretation.

5. Mitochondrial-dependent calcium handling

Ca^{2+}-buffering capacity is a frequently assessed mitochondrial function. A high capacity affords protection from Ca^{2+}-induced damage, e.g. following excitotoxic Ca^{2+} elevation. Conversely, a decreased capacity suggests mitochondrial dysfunction. Measurements in isolated mitochondria typically monitor how much Ca^{2+} (nmol/mg protein) the population can buffer before generalized mPT. Ca^{2+}-sensitive electrodes or fluorescent probes, e.g. Calcium-Green, allow continuous extra-mitochondrial [Ca^{2+}] monitoring. Mitochondria concentration, buffer composition, notably in phosphate and adenine nucleotides, and the mode of Ca^{2+} addition, repetitive bolus vs. continuous infusion, significantly impact maximal Ca^{2+} buffering capacity [104,130]. Non-mitochondrial Ca^{2+}-handling mechanisms and glycolytic ATP in intact neurons complicate assessment of *in situ* mitochondrial Ca^{2+}-buffering [127].

Mitochondrial-dependent Ca^{2+}-handling, i.e., without glycolytic ATP interference, can be assessed by replacing glucose with 2-deoxy-D-glucose plus pyruvate as mitochondria substrate. This is particularly relevant in cell-lines where abundant glycolysis obscures mitochondrial Ca^{2+}-handling [65]. Fluorescent Ca^{2+}-probes carrying a delocalized positive charge, e.g. rhod-2 and analogues, accumulate preferentially in mitochondria but require appropriate measures to minimize extra-mitochondrial signal [8]. In addition, genetically encoded mitochondria-targeted Ca^{2+}-sensors provide elegant ways to monitor mitochondrial Ca^{2+}-handling in intact cells, including *in vivo*. Novel aequorin and GFP-based Ca^{2+} sensors are not without limitations, but there have been improvements in selective organelle targeting, [Ca^{2+}]$_i$ detection range, and imaging technology to deal with low photon yield and fluorescent signal/noise ratios [131,132]. Importantly, dynamic measurements with mitochondrial matrix Ca^{2+} probes report changes in free (not total) Ca^{2+} levels. Due to formation of Ca^{2+}-phosphate complexes, free Ca^{2+} is buffered at ~ 0.2–5 μM in spite of continuous increase in total matrix Ca^{2+} [24,27]. Inducing Ca^{2+} release with FCCP, and monitoring the area under the curve with a low affinity ratiometric Ca^{2+}-probe, estimates total Ca^{2+} buffered by neuronal mitochondria during glutamate receptor activation [133]. Recently, we proposed a strategy for comparisons of *in situ* mitochondrial Ca^{2+}-buffering capacity, using neurons and astrocytes from different brain regions, with calibration for different rates of [Ca^{2+}]$_i$ elevation and mitochondrial content [126].

In situ evaluation of mPT in neurons has been performed by the calcein/cobalt-quenching technique [93,134]. Cells loaded with sufficient calcein-AM display both mitochondrial and cytosolic fluorescence. However, the latter is selectively quenched with cobalt (Co^{2+}), which does not permeate an intact inner mitochondrial membrane. Induction of mPT releases mitochondrial entrapped calcein, detected by mitochondrial fluorescence decay [135]. With Co^{2+} in the cytosol, mitochondria fluorescence decay may also be due to Co^{2+} influx and calcein quenching [136]. Different cell-types exhibit different Co^{2+}permeability. In neurons, Co^{2+} uptake occurs via active AMPA/kainate receptors, thus varying with receptor density and neuronal activity. Also, single neurons display incomplete co-localization of calcein and Δψ$_m$-sensitive probes, so their combination is recommended to identify calcein-loaded mitochondria [134]. Co^{2+} microinjection may overcome permeability issues, but high cytosolic Co^{2+} may be problematic. Although Co^{2+} is not transported by the Ca^{2+} uniporter, it inhibits Ca^{2+} uptake and decreases mitochondria respiration [135]. An alternative technique, described in non-neuronal cells, combines two fluorescent probes to discriminate polarized from depolarized (possibly via mPT) mitochondria within the same cell. The two probes, Mitotracker Green (MTG) and TMRM$^+$, accumulate electrophoretically into mitochondria, but MTG establishes covalent bonds becoming retained after depolarization. By imaging fluorescence resonance energy transfer (FRET) between MTG and TMRM$^+$, depolarized

mitochondria (MTG only) were identified against a background of hundreds of polarized (MTG + TMRM$^+$) mitochondria [137].

6. Biogenesis, trafficking, fusion and fission

Mitochondrial biogenesis indicators include mtDNA copy number, mitochondria mass/volume, transcription factors (e.g. PGC-1α, mtTFA, NRF-1 and NRF-2), and mitochondrial proteins (e.g. cytochrome oxidase and ATP synthase subunits) [35]. Biogenesis has been assessed in populations of cultured neurons and brain tissue, namely in the context of diabetic neuropathy or stroke, using hyperglycaemic or hypoxic-ischemic injury, respectively [31,138]. Moreover, differential biogenesis between single neurons and within subneuronal compartments can be investigated by incorporating deoxyuridine derivatives into newly synthesized mtDNA [139].

Labelling mitochondria with fluorescent probes, e.g. lipophilic cations or genetically encoded mitochondrially-targeted proteins [140-142], allows monitoring and quantification of mitochondria dynamics using video-microscopy and digital image processing techniques, respectively. In any case, care should be taken to minimize mitochondrial toxicity [143]. Mitochondrial fractional occupancy measurements ($\Sigma_{\text{mitochondria length}}$ /$\Sigma_{\text{neurites length}}$, using skeletonized images; [99]) provide information on mitochondrial biogenesis. Trafficking information can be derived from time-lapse image acquisition and processing, most often requiring manual mitochondria tracking [3], although some measurements can be automated, e.g. using an 'optical flow' method [144]. Several analysis strategies have been described, notably, 'single particle analysis', 'dwell areas' visited by mitochondria in a given time interval, and 'kymographs', i.e., time-projections of fluorescence intensities across neuritic lines, where slopes are proportional to speed, and direction can be inferred (see [38]). Levels of motor or adaptor proteins and their association with mitochondria provide additional trafficking-related information [145,146]. Fusion and fission can be estimated from the number and size of mitochondrial particles [95], as well as by quantifying levels of fusion and fission related GTPases [96,138,147].

7. Other techniques and concluding remarks

In addition to the parameters and techniques addressed in this review, multiple others assist the study of neuronal mitochondrial function. E.g. Changes in NAD(P)H and FADH$_2$ autofluorescence monitor *mitochondria redox state* in isolated suspensions, neurons or brain slices [124,148,149]; Oxidation of dichlorofluorescein, dihydroethidine or its mitochondrially-targeted derivative MitoSox estimate neuronal ROS formation, with some limitations related to pH sensitivity and $\Delta\psi_m$-dependent probe localization [59,107,118]; Apoptotic protein release, e.g. cytochrome *c*, signals mitochondria-dependent apoptosis [150]; Sequential histochemical staining for cytochrome c oxidase and succinate dehydrogenase activities identifies neurons with dysfunctional mitochondria, where mtDNA mutations can be probed via laser microdissection and polymerase chain reaction [151,152].

The study of neuronal mitochondria is clearly enriched with many complementary experimental approaches, allowing researchers to test disease-induced mitochondrial dysfunction and protective pharmacological strategies. Given the wide variety of possible research subjects and experimental models, choosing a single 'best' technique is a utopian endeavour. Nevertheless, given their central roles in mitochondrial activity, $\Delta\psi_m$, Ca^{2+}-buffering and the highly informative respiration measurements are good choices both for isolated and *in situ* mitochondria. The researcher interested in additional events will certainly

appreciate the alluring mitochondrial dynamics.

Acknowledgments

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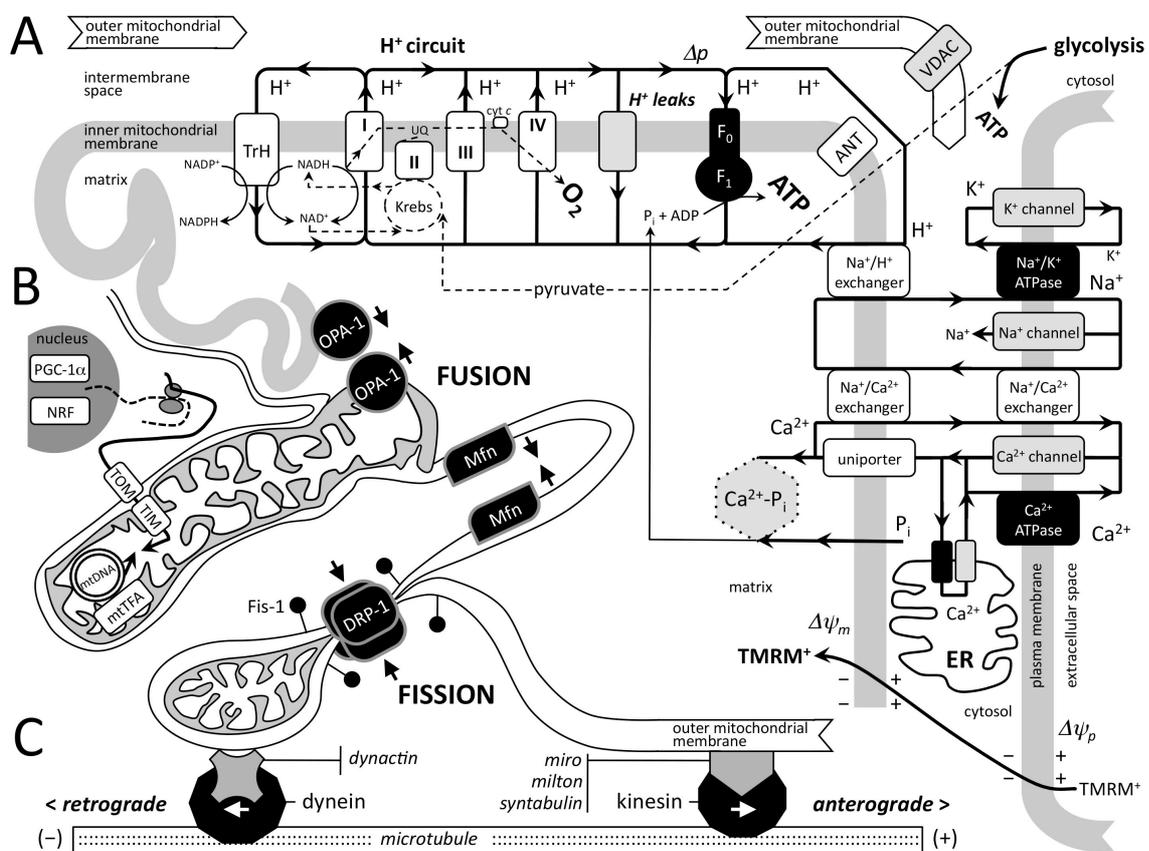
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Figure 1. Mitochondrial bioenergetics and dynamics.

A-(*left to right*), Respiratory chain and proton circuit. Transhydrogenase (TrH), complexes (I-IV), ubiquinone (UQ) and cytochrome *c* (cyt *c*), H^+ leaks and F_0F_1 ATPase generating ATP. Adenine nucleotide translocase (ANT) and voltage dependent anion channel (VDAC), at the inner (*grey*) and outer (*white*) mitochondrial membranes, respectively. Glycolysis (*top right*) feeds mitochondria via pyruvate (*dashed lines*).

A-(*top-right to bottom*), Simplified circuits for Ca^{2+} and other ions across mitochondrial, endoplasmic reticulum (ER) and plasma membranes. Grey hexagon depicts calcium phosphate complexes (Ca^{2+}-P_i). $TMRM^+$ (*bottom right*) distribution influenced by $\Delta\psi_m$ and $\Delta\psi_p$.

B, Biogenesis, transcription factors and protein import via outer and inner membrane translocases (TOM and TIM). Fusion (outer and inner membranes) and fission via respective GTPases. ***C***, Bi-directional microtubule-based transport, motors and adaptor proteins. See text for further details.