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Review

Latest assessment methods for mitochondrial homeostasis in cognitive diseases

Wei You[1, 2, #], Yue Li[1, #], Kaixi Liu[1], Xinning Mi[1], Yitong Li[1], Xiangyang Guo[1, 3, 4, *], Zhengqian Li[1, 3, 4, *]

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Abstract

Mitochondria play an essential role in neural function, such as supporting normal energy metabolism, regulating reactive oxygen species, buffering physiological calcium loads, and maintaining the balance of morphology, subcellular distribution, and overall health through mitochondrial dynamics. Given the recent technological advances in the assessment of mitochondrial structure and functions, mitochondrial dysfunction has been regarded as the early and key pathophysiological mechanism of cognitive disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, mild cognitive impairment, and postoperative cognitive dysfunction. This review will focus on the recent advances in mitochondrial medicine and research methodology in the field of cognitive sciences, from the perspectives of energy metabolism, oxidative stress, calcium homeostasis, and mitochondrial dynamics (including fission-fusion, transport, and mitophagy).

Key Words: cognitive disorders; mitochondrial dysfunction; mitochondrial energy metabolism; mitochondrial dynamics; mitochondrial transport; mitophagy; mitochondrial biogenesis; oxidative stress; calcium homeostasis

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Introduction

The mitochondria are double-membrane organelles forming a complex network in neurons. They mainly supply adenosine triphosphate (ATP) to cells, so the maintenance of mitochondrial structure and physiological function is essential for cellular growth and development (Liesa and Shirihai, 2013). Mitochondria are highly dynamic organelles that undergo constant fission, fusion, transport, biogenesis, and degradation, to maintain the balance of morphology and subcellular distribution, which is referred to as mitochondrial dynamics (Giacomello et al., 2020). Neurons have a high demand for ATP, especially at the presynaptic and postsynaptic terminals, active growth cones, and axonal branches, to support several crucial functions; thus, mitochondrial transport from the soma to their destinations is essential to support normal energy metabolism (Sheng and Cai, 2012). In addition, mitochondria have a high capacity to sequester calcium and can buffer physiological calcium loads in neurons (Werth and Thayer, 1994). Under extracellular or intracellular stress, damaged mitochondria can give rise to many abnormalities including the destruction of energy metabolism, oxidative stress, calcium homeostasis, mitochondrial dynamics, and mitochondrial biogenesis, and cause synaptic dysfunction, neural death, and cognitive decline (Behzadfar et al., 2017; Manfredini et al., 2019).

In many diseases based on cognitive disorders, mitochondrial dysfunction has been regarded as an early and prominent feature of the disease and plays an important role in the pathological processes. For example, impaired energy metabolism of mitochondria typically precedes the clinical onset of Alzheimer's disease (AD) and increased oxidative stress, disturbed mitochondrial genomic homeostasis, abnormal mitochondrial dynamics, mitochondrial axonal trafficking deficits, impaired mitochondrial biogenesis, impaired mitophagy, and impaired mitochondrial proteostasis also take part in the AD pathogenesis (Wang et al., 2020b; Wei et al., 2022). Besides, mitochondrial dysfunction also plays an important role in Parkinson's disease (PD), Huntington's disease (HD), mild cognitive impairment, postoperative cognitive dysfunction, sepsis-associated encephalopathy, age-associated cognitive decline, vascular dementia, human immunodeficiency virus-1 associated neurocognitive disorders and diabetes-associated cognitive impairment (Netto et al., 2018; Manfredini et al., 2019; Han et al., 2020; Olesen et al., 2020; Chandra et al., 2021; Malpartida et al., 2021; Liu et al., 2022a; Sun et al., 2022; Yang et al., 2022). Therefore, it is important to assess mitochondrial function, and using effective approaches to assess mitochondrial function is crucial for studying mitochondrial dysfunction in diseases. This review is aimed to address methods currently used in the mitochondrial function assessment and the implications of mitochondrial dysfunction in the pathogenesis of cognitive disorders.

Search Strategy

A computer-based online search of the PubMed database was performed to retrieve articles published up to May 30, 2023. The key words included cognitive disorders, assessment, mitochondrial energy metabolism, ATP, oxygen consumption rate, tricarboxylic acid, mitochondrial respiratory chain, mitochondrial membrane potential, reactive oxygen species (ROS), oxidative stress, calcium, mitochondrial permeability transition pore, mitochondrial dynamics, mitochondrial fission, mitochondrial fusion, posttranslational modifications, dynamin-related protein 1 (Drp1), mitochondrion morphology, mitochondrial transport, mitophagy, mitochondrial biogenesis, circulating cell-free mitochondrial DNA, and ascorbate peroxidase proximity labeling. The results were further screened by title and abstract base on their relevance to the subject matter.

Mitochondrial Energy Metabolism

It is well known that the most important function of mitochondria is ATP production through the tricarboxylic acid (TCA) cycle and oxidative

[1]Department of Anesthesiology, Peking University Third Hospital, Beijing, China; [2]Peking University Third Clinical Medical College, Beijing, China; [3]Beijing Center of Quality Control and Improvement on Clinical Anesthesia, Beijing, China; [4]Anesthesia and Perioperative Medicine Branch of China International Exchange and Promotive Association for Medical and Health Care (CPAM), Beijing, China

***Correspondence to:** Zhengqian Li, MD, PhD, zhengqianli@hsc.pku.edu.cn; Xiangyang Guo, MD, puthmzk@hsc.pku.edu.cn.

https://orcid.org/0000-0002-5694-2174 (Xiangyang Guo); https://orcid.org/0000-0001-6758-0685 (Zhengqian Li)

#Both authors contributed equally to this work.

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phosphorylation (**Figure 1**). The human brain needs high energy to continuously support healthy neuronal activity and cognition, and ATP is the most important energy source (Liesa and Shirihai, 2013). In AD, mitochondrial dysfunction affects mitochondrial bioenergetics and disrupts the electron transport chain (ETC) and ATP production, causing impairments in attention, short-term memory, and damage to fine motor speed (Rhein et al., 2009; Behzadfar et al., 2017). In other neurodegenerative diseases, including PD and HD, neuropathological conditions can also lead to decreased ATP levels and increased glycolytic production of ATP, and conversely alleviating energy deficiency may have neuroprotective effects (Tang, 2020). Furthermore, ATP is a widespread signaling molecule in the brain, and abnormal release in signals is involved in the development of AD (Madeira et al., 2021). In addition, the circulating ATP is a strong inflammatory signaling molecule and participates in the neuroinflammatory response through activation of tumor necrosis factor-α, interleukin-6, and interleukin-1β, which may cause cognitive decline in cerebral dysfunction of sepsis (Zhang et al., 2019b).

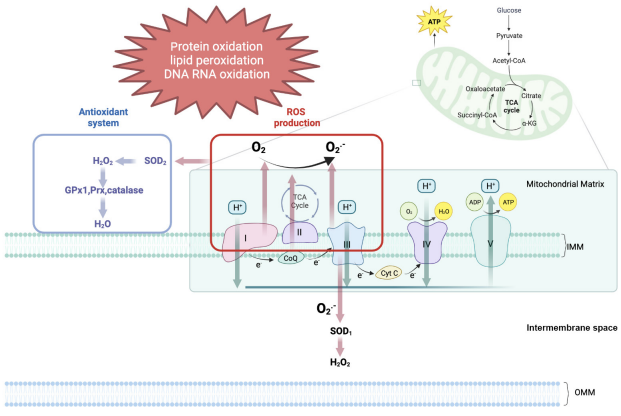

Figure 1 | **Mitochondrial energy metabolism and oxidative stress.**
Glycolysis metabolizes glucose to pyruvate which produces acetyl-CoA in the mitochondrial matrix for the TCA cycle. The TCA cycle can oxidize nutrients after a series of reactions to generate reducing equivalents for oxidative phosphorylation, including ETC composed of four complexes (CI–CIV) and F0F1-ATPase (CV) in the IMM. Complex I, III, and IV pump H^+ from the mitochondrial matrix to the intermembrane space along with the transport of electrons, forming MMP. The flux of H^+ back into the mitochondrial matrix is mostly mediated by F0F1-ATPase and drives ADP to synthesize ATP. $O_2^{\cdot-}$ is the primary ROS mainly generated through the leaking of superoxide at complexes I and III. $O_2^{\cdot-}$ is dismutated to H_2O_2 by SOD2 and to GPx and catalase in the matrix and SOD1 in the intermembrane space. Created with BioRender.com. ADP: Adenosine diphosphate; ATP: adenosine triphosphate; ETC: electron transport chain; GPx: glutathione peroxidase; H_2O_2: hydrogen peroxide; IMM: inner mitochondrial membrane; MMP: mitochondrial membrane potential; $O_2^{\cdot-}$: superoxide anion; SOD1/2: superoxide dismutase 1/2; TCA: tricarboxylic acid.

Assessment of overall energy metabolism

Mitochondrial respiration is the set of metabolic reactions that store large amounts of energy in the form of ATP. Hence, we can measure ATP to detect energy homeostasis. In addition, mitochondria consume 90% of the oxygen through ECT system (Acin-Perez et al., 2020), so measuring the oxygen consumption rate (OCR) can directly assess the ATP production of mitochondria and mitochondrial oxidative function.

ATP

The assessment of ATP mainly includes biochemical and biophysical approaches, such as nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC), and bioluminescence assay methods (Ley-Ngardigal and Bertolin, 2022), respectively. Proton and phosphorus NMR spectroscopy can be used to quantitatively assess the ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) simultaneously, based on the structural differences between H and P atoms. This method is simple, comprehensive, and has perfect linearity in a certain range. Nevertheless, it has a low temporal resolution as it requires approximately 14 hours to complete the detection of a specific spectrum and requires significant amounts of biological materials to provide reliable readouts (Lian et al., 2016). HPLC is another common method, which is used to rapidly extract ATP, ADP, and AMP in cell or tissue samples and also has the advantage of high sensitivity, automation, and reproducibility (García-Tardón and Guigas, 2018). Nevertheless, these methods do not provide sufficient spatiotemporal information. The use of genetically encoded ATP biosensors can solve this problem. Among them, fluorescent ATP sensors based on luciferin-luciferase reaction are commonly used, such as ATeam (Yoshida et al., 2017) and Rh6G-ACFPN (Ren et al., 2020) that can measure real-time ATP production at the single-cell level and subcellular regions including the mitochondria, cytoplasm, and nucleus (Kitajima et al., 2020). Furthermore, different indices related to ATP can be measured by combining different types of probes. For example, novel fluorescent polymer probes can simultaneously detect mitochondrial temperature and ATP fluctuation (Qiao et al., 2018). In addition, some mitochondria-targeted probes have been recently improved, including a self-assembled DNA nanostructure as a Förster resonance energy transfer nanoflare (Cai et al., 2021), mitochondria-targeted nanoscale Zeolitic Imidazole Framework-90 (Deng et al., 2017), and mitochondria-targeting single-layered graphene quantum dots (Liu et al., 2018). These new probes have higher selectivity and efficiency and are expected to promote a more accurate assessment of mitochondria.

NMR and HPLC allow the direct quantification of total ATP levels in tissues and cellular extracts. Especially in complex biological samples and in vivo, NMR has the advantage of simultaneously determining the concentration of ATP and other molecules, despite being relatively time consuming and requiring expensive instruments. Nevertheless, they are not real-time analyses and cannot provide spatial information because the mutual revulsion between ADP and ATP occurs in seconds. By contrast, genetically encoded ATP biosensors can detect ATP levels in living cells in a real-time manner and provide sufficient spatiotemporal resolution at the subcellular level including the mitochondria and cytoplasm.

OCR (i.e., respirometry)

The assessment of OCR mainly includes Clark electrodes (commercially available oxygen electrodes) (Silva and Oliveira, 2018) and Seahorse XF extracellular flux Analyzer (Seahorse Bioscience, Agilent Technologies, Santa Clara, CA, USA) (Zhang et al., 2019a). Using Clark electrodes is a classical method to measure OCR and its results are reliable, but it requires a large quantity of purified mitochondria and is relatively time-consuming (Ribeiro et al., 2015; Silva and Oliveira, 2018). The Seahorse XF Analyzer can measure real-time OCR in living cells and is label-free and non-invasive, and it also allows compounds or reagents to be automatically added to determine bioenergetics parameters and compare multiple samples simultaneously (Ribeiro et al., 2015). OCR can directly be linked to ATP production, so it can be used to measure the efficiency of mitochondrial ATP production indirectly, and it is convenient and less expensive than NMR and HPLC. Furthermore, it allows the analysis of intact samples without prior intervention, which can decrease the external disturbance to tissues or cells. However, it cannot provide sufficient spatiotemporal information.

Assessment of TCA and mitochondrial respiratory chain

The TCA cycle is a central hub of cellular metabolism, oxidizing nutrients to generate reducing equivalents like reduced nicotinamide adenine dinucleotide and flavine adenine dinucleotide for oxidative phosphorylation and critical metabolites for biosynthetic reactions (Arnold et al., 2022). Oxidative phosphorylation includes the ETC composed of four complexes (CI–CIV) and F0F1-ATPase (CV), and they are embedded in the inner mitochondrial membrane (IMM) together (**Figure 1**). Therefore, we can measure relevant enzymatic activities and intermediates to assess TCA and the mitochondrial respiratory chain.

TCA

The assessment of TCA includes detecting specific enzymatic activities by spectrophotometric assays (Naseri et al., 2015) and its intermediates by gas chromatography-mass spectrometry (GC-MS) (Andersen et al., 2021) or NMR-based metabolomics analysis (Gao et al., 2019). Generally, spectrophotometric assays can measure the related enzymatic activities including pyruvate dehydrogenase complex, citrate synthase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase complex, succinyl thiokinase, succinate dehydrogenase fumarase, and malate dehydrogenase, and are simple to carry out and useful (Naseri et al., 2015). GC-MS and NMR are generally the preferred analytical methods for metabolomics analysis, and they are comprehensive, simultaneous, and systematic (Lou et al., 2018). They can be used to measure TCA cycle intermediates including lactate, pyruvate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate, β-hydroxybutyrate, and acetoacetate (Pawlosky et al., 2017).

Mitochondrial respiratory chain

The assessment of the mitochondrial respiratory chain mainly includes monitoring specific enzymatic activities by spectrophotometric assays (Frazier et al., 2020) and the expression of subunit proteins of Complex I–V by western blotting (Lou et al., 2016) or two-dimensional difference gel electrophoresis (Chou et al., 2011). The activities of Complex I–IV can be assayed by monitoring the absorbance at specific wavelengths because of the redox products. Complex V can be measured by enzyme cascade, which eventually links ATP synthase activity to NADH oxidation, and assays the change in absorbance at specific wavelengths (Gauba et al., 2021). This method is simple and useful and can provide information on the maximal activities and obtain reproducible results (Barrientos et al., 2009). Western blotting and difference gel electrophoresis are the commonly used techniques in proteomics, as they have high resolution, sensitivity, and reproducibility (Mishra et al., 2017). In addition, a native gel electrophoresis–fluorimetric scan—approach has been used to measure the absolute content of Complex I and catalytic turnover, and is useful for any biochemical characterization of Complex I (Ansari et al., 2021).

ATP production can indicate the overall energy metabolism of the mitochondria, but the assessment of TCA and mitochondrial respiratory chain can provide more detailed information about every portion of mitochondrial energy metabolism and glucose metabolism in the brain. The fundamental metabolic deficits along with mitochondrial dysfunction might facilitate the pathogenesis of cognitive disorders. At present, therapies have focused on mitochondrial electron transport complexes, including partial inhibition of Complex I, and have been exploited as a novel strategy for delaying the onset

of neurodegenerative diseases such as AD (Trushina et al., 2022).

Mitochondrial membrane potential

Complexes I, III, and IV pump H^+ from the mitochondrial matrix to the intermembrane space along with the transport of electrons, forming mitochondrial membrane potential (MMP; ΔΨm) (Liu et al., 2021a). It is necessary for mitochondrial energy metabolism that mitochondria maintain stable levels of MMP, and it plays an important role in the mitochondrial division and mitochondrial quality control process (Cho et al., 2019). Hence, MMP is an important indicator to assess whether mitochondria are healthy. In addition, MMP decreases obviously under the overproduction of ROS or other stress in cognitive disorders, and scavenging ROS can restore the MMP and improve cognitive decline in cerebral dysfunction caused by alcohol and PD (Jin et al., 2019; Ma et al., 2021).

Membrane-permeant cationic fluorescent probes are commonly used tools to assess MMP, including 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) (Martín-Montañez et al., 2017); tetramethyl rhodamine methyl and ethyl esters (TMRM and TMRE, respectively); (Wang et al., 2020a) and rhodamine-123 (Rh123) (Shao et al., 2021). According to the level of MMP, JC-1 will form aggregates or monomers that emit different fluorescence at specific wavelengths; thus, it allows dual-color and ratiometric assessment of MMP and is slowly permeant and very photosensitive (Martín-Montañez et al., 2017). TMRM and TMRE have the lowest mitochondrial binding and ETC inhibition and fast equilibration, and are often used in both matrix-quenching and non-quenching modes (Nicholls and Ward, 2000). Rh123 is slowly permeant, and its plasma membrane redistribution can be ignored in short-term experiments that are often used in matrix-quenching mode (Nicholls and Ward, 2000). MMP is generated by the serial reduction of electrons through the effective working of ETC and is significant to ATP production, so it is often used to assess whether mitochondria are healthy. These fluorescent probes have become important tools to directly measure the MMP and provide visualization information, which can be chosen according to different conditions as mentioned above. The mitochondrial energy metabolism targets and assessment methods are summarized in **Table 1**.

Reactive Oxygen Species and Oxidative Stress

Mitochondria contribute approximately 90% of the cellular ROS (Balaban et al., 2005). ROS are unavoidable byproducts produced mainly by the leaking of superoxide at complexes I and III of the mitochondrial respiratory chain (Balaban et al., 2005). When ROS and antioxidants are imbalanced in cells, the overabundance of ROS will cause severe oxidative damage (Li et al., 2021a). Generally, oxidative stress is present and always increased in the course of cognitive disorders. ROS overproduction and oxidative stress can impair different types of molecules, including DNA, lipids, and proteins, and further cause mitochondrial damage, neuroinflammation, and cognitive impairment in AD, HD, and PD (Li et al., 2021b; O'Regan et al., 2021; Vaillant-Beuchot et al., 2021; Chen et al., 2023; Wakatsuki and Araki, 2023; Xu et al., 2023). Excessive lipid peroxidation is the trigger for ferroptosis and neuronal cell death, and antioxidants are significant therapeutic directions delaying the progression of neurodegenerative diseases (Angelova et al., 2021). The overproduction of ROS can also promote defective mitochondrial mitophagy, which will cause accumulation of damaged mitochondria and contribute to cognitive disorders (Pradeepkiran and Reddy, 2020).

ROS

A series of fluorescent probes are widely used to measure ROS in cytosolic and mitochondrial ROS directly. Hydroethidine (HE) (Harland et al., 2020) and mitochondria-targeted HE (mito-HE or mitoSOX red) (Fernandez et al., 2019) are mainly used to detect superoxide anion ($O_2^{\bullet-}$). However, these probes have a high autoxidation rate and can form both non-specific oxidation products such as ethidium and the $O_2^{\bullet-}$-specific product 2-hydroxyethidium, which have spectral overlap in fluorescence spectra and make it difficult to differentiate the contribution of non-specific oxidation and $O_2^{\bullet-}$-dependent oxidation to the overall fluorescence (Murphy et al., 2022). The union of HE-based probes and liquid chromatography-tandem mass spectrometry (LC-MS/MS) can overcome the limitation and can improve the specificity (Xiao and Meierhofer, 2019). Besides, Amplex Red (Lopes et al., 2022) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Behzadfar et al., 2017) are commonly used to assess hydrogen peroxide (H_2O_2). Amplex Red is a highly specific and sensitive probe based on the horseradish peroxidase-oxidizing substrates, but it can be interfered with by other horseradish peroxidase substrates and by $O_2^{\bullet-}$, which can inactivate horseradish peroxidase (Murphy et al., 2022). DCFH-DA is cell-permeable and easy to use, and can respond to enhanced peroxidase activity; however, it can also lead to artifactual amplification of the fluorescence signal via a redox-cycling mechanism by generating the intermediate radical, DCF semiquinone anion radical ($DCF^{\bullet-}$) (Kalyanaraman et al., 2012). Genetically encoded fluorescent protein sensors have provided major advances for H_2O_2 detection, and have high sensitivity and specificity, such as HyPer series and roGFP2-based probes (Pak et al., 2020). These probes are ratiometric and can change the overall fluorescence of the probe depending on its oxidation status (Pak et al., 2020).

Table 1 | **Assessment of mitochondrial energy metabolism**

Targets	Markers	Assessment methods	Advantages and limitations	References
Overall energy metabolism	ATP	NMR	Simple, comprehensive and good linearity; but time consuming and need enough materials	Ley-Ngardigal and Bertolin, 2022
		HPLC	Fast and high sensitivity	
		Genetically encoded ATP biosensors: Ateam, Rh6G-ACFPN	Provide spatial information and real-time	Yoshida et al., 2017; Ren et al., 2020
		Novel fluorescent polymer probes	Detect mitochondrial temperature and ATP fluctuation simultaneously	Qiao et al., 2018
		Mitochondria targeted probes: a self-assembled DNA nanostructure as a FRET nanoflare, mitochondria targeted nanoscale Zeolitic Imidazole Framework-90, mitochondria-targeting single-layered graphene quantum dots	Mitochondria-targeted	Deng et al., 2017; Liu et al., 2018; Cai et al., 2021
	OCR	Clark electrodes	Reliable; but requires large amounts of purified mitochondria and is relatively time consuming	Silva and Oliveira, 2018
		Seahorse XF extracellular flux Analyzer	Real-time, label-free, non-invasive, and allows adding compounds or reagents automatically and can compare multiple samples simultaneously	Zhang et al., 2019a
TCA	Enzymatic activities	Spectrophotometric assay	Simple and useful	Naseri et al., 2015
	Intermediates	GC-MS and NMR-based metabolomics analysis	Comprehensive, simultaneous, and systematic	Andersen et al., 2021
		NMR-based metabolomics analysis		Gao et al., 2019
Mitochondrial respiratory chain	Enzymatic activities	Spectrophotometric assay	Simple, useful, and reproducible, and can provide information on the maximal activities	Frazier et al., 2020
	Expression of subunits	Western blotting, 2D-DIGE	High resolution, sensitive, and reproducible	Chou et al., 2011; Lou et al., 2016
		Native gel electrophoresis-fluorimetry scan	Measures the absolute content of complex I and catalytic turnover	Ansari et al., 2021
MMP		JC-1	Allows dual-color and ratiometric assessment	Martín-Montañez et al., 2017
		TMRM and TMRE	Lowest mitochondrial binding and ETC inhibition, and fast equilibration	Wang et al., 2020a
		Rh123	Slowly permeant and plasma membrane redistribution, which can be ignored in short-term experiments	Shao et al., 2021

2D-DIGE: Two-dimensional difference gel electrophoresis; ATP: adenosine triphosphate; GC-MS: Gas chromatography-mass spectrometry; HPLC: high-performance liquid chromatography; JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; NMR: nuclear magnetic resonance; OCR: oxygen consumption rate; Rh123: rhodamine-123; TCA: tricarboxylic acid; TMRM and TMRE: tetramethyl rhodamine methyl and ethyl esters.

Oxidative damage markers

Mitochondrial DNA (mtDNA) lacks histone proteins and any major repair mechanisms, making it highly vulnerable to ROS. Therefore, we can measure the peroxidation products of nucleic acids, also including oxidized protein and lipids to assess oxidative damage.

Protein carbonyls and 3-nitrotyrosine are the markers of protein oxidation (Murphy et al., 2022). Protein carbonyls are formed by the oxidative cleavage of the protein backbone, lysine, and proline, and can be assessed by detecting the production of dinitrophenylhydrazone, which forms through derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine, by direct spectrophotometric measurement (Giustina et al., 2020), or enzyme linked immunosorbent assay (ELISA) (Buss et al., 1997), western blot after electrophoretic separation (Sorolla et al., 2010), and HPLC based on anti-dinitrophenylhydrazone antibodies (Lan et al., 2012). These methods are widely used and have high specificity and sensitivity. 3-Nitrotyrosine is the main product of tyrosine oxidation. It can be measured by ELISA (Yu et al., 2015), but it has low sensitivity and different affinity to antibodies for different nitrated proteins. By contrast, mass spectrometry coupled with gas chromatography (GC-MS, GC-MS/MS) or LC-MS/MS has accurate determination, but the presence of nitrite and acid conditions during protein precipitation and hydrolysis may nitrate tyrosine residues in the sample (Tsikas and Duncan, 2014). In addition, LC-MS/MS combined with stable isotopic dilution analysis provides a high sensitivity and high specificity multiplexed method for robust quantitation of protein glycation, oxidation, and nitration adduct analytes (Rabbani and Thornalley, 2020).

4-Hydroxynonenal, malondialdehyde, and F2-isoprostanes (F2-IsoPs) are markers of lipid peroxidation (Murphy et al., 2022). 4-Hydroxynonenal can be detected by HPLC directly or as a derivatized product with 2,4-dinitrophenylhydrazine or 1,3-cyclohexanedione by GC-MS (Williams et al., 2006), and by means of immunological techniques such as ELISA (Yu et al., 2015), using antibodies against the protein adducts formed by 4-hydroxynonenal. Malondialdehyde can be measured utilizing the thiobarbituric acid reactive substances, which produce a pink adduct complex, and can be quantified by spectrophotometry (Das et al., 2021) and fluorimetric assay (Perrotte et al., 2019). However, it is unspecific and prone to artifacts. F2-IsoPs are produced by the reaction of polyunsaturated fatty acids in membrane phospholipids with ROS and can be quantified by ELISA, which is susceptible to artifact, and GC–MS, and LC–MS/MS with appropriate internal standards, which is relatively accurate when compared with ELISA (Pharaoh et al., 2020).

8-Oxo-7,8-dihydro-2′-deoxyguanosine and 8-hydroxyguanosine are widely used markers of oxidative damage to DNA and RNA (Nunomura et al., 1999). They can be measured by HPLC with electrochemical detection (Isobe et al., 2010), LC-MS, ultra-performance liquid chromatography-tandem mass spectrometry analysis (Peña-Bautista et al., 2019), and ELISA (Yu et al., 2015), but ELISA methods are relatively less sensitive and specific, and LC-MS assays are more accurate than HPLC with electrochemical detection (Borowsky et al., 2013).

Antioxidant enzymes

The antioxidant systems in cytoplasm and mitochondria are different. Manganese superoxide dismutase (MnSOD or SOD2) and classic glutathione peroxidase (Gpx1) are the primary antioxidant defenses in mitochondria, and other enzymes include CuZn-superoxide dismutase (CuZnSOD or SOD1), catalase, and phospholipid hydroperoxide glutathione peroxidase (Gpx4) (**Figure 1**; Yant et al., 2003; Van Remmen et al., 2004).

The activities of SOD (including SOD1 and SOD2) and Gpx1/4 can be measured by spectrophotometry (Esposito et al., 2006). The spectrophotometry of SOD measurement mainly uses nitroblue tetrazolium method, cytochrome c method and water-soluble tetrazolium (WST). Compared with the previous two methods, the reaction product of WST-1 is a stable, water-soluble product overcoming the limitations of nitroblue tetrazolium assay, which are poor water solubility, easy to interact with xanthine oxidase and inhibition percentage less than 100%. WST-1 can be used to determine SOD activity by absorbance detection at a single time point, making it suitable for high-throughput measurements rather than cytochrome c that needs to continuously measure the absorbance value, making it relatively low sensitivity and not suitable for the detection of large quantities of samples (Peskin and Winterbourn, 2017). The quantitative detection of SOD and Gpx protein levels can be performed with ELISA, two-dimensional difference gel electrophoresis, western blotting (Porcellotti et al., 2015), and mass spectrometry (Fan et al., 2020). mRNA expression can be measured by quantitative reverse transcription-polymerase chain reaction (Hambright et al., 2017).

In the neurodegenerative disorders with cognitive impairment,the accumulation of the amyloid-beta peptide (Aβ) and the protein tau might lead to ROS overproduction or exhaustion of antioxidants, which become deleterious and cause oxidative stress. Fluorescent probes are an excellent approach to measuring ROS because they have high sensitivity and simplicity, and provide spatial resolution. However, it is important to pay attention to their main applications and limitations. Detecting oxidative damage and antioxidant enzymes can reflect systemic or tissue-specific oxidative stress and assess the redox state of the brain or specific tissues and cells. Here, we enumerate the common methods to assess the ROS and oxidative stress targets, which are summarized in **Table 2**. Further application and interpretation of these measurements can refer to the guidelines (Murphy et al., 2022).

Mitochondrial Calcium Monitoring

The mitochondrial uptake and extrusion of calcium is a fundamental biological process and plays an important role in cellular metabolism, signaling, and survival (Garbincius and Elrod, 2022). Mitochondrial calcium is mainly regulated by the mitochondria-associated endoplasmic reticulum membranes (MAMs), whose main components that participate in the Ca^{2+} communication include inositol-1,4,5-trisphosphate receptors, voltage-dependent anion channel (VDAC), molecular chaperone glucose-regulated protein 75, sigma-1 receptor, and binding immunoglobulin protein (Szabadkai et al., 2006; Hayashi and Su, 2007). At MAMs, microdomains of high Ca^{2+} are formed from the lumen of the endoplasmic reticulum directly on the mitochondrial surface at the close vicinity, which can be generated upon opening of the inositol-1,4,5-trisphosphate-gated channels and allow the mitochondria to uptake Ca^{2+} by rapidly using the low-affinity mitochondrial calcium uniporter (MCU) (**Figure 2**; Filippin et al., 2003). Mitochondrial calcium dysregulation may be a fundamental upstream mechanism in the pathogenesis of neurodegenerative diseases, causing synaptic dysfunction, excitotoxicity, and cell death via ionotropic glutamate receptors, nicotinic acetylcholine receptors, and voltage-gated calcium channels (Schrank et al., 2020). Excessive mitochondrial calcium influx caused by MAMs or MCU can cause mitochondrial dysfunction, including mitochondrial ROS accumulation, decreased MMP and ATP, increased neuronal death, and cognitive impairment in AD (Yang et al., 2008; Han et al., 2021; Lee et al., 2021).

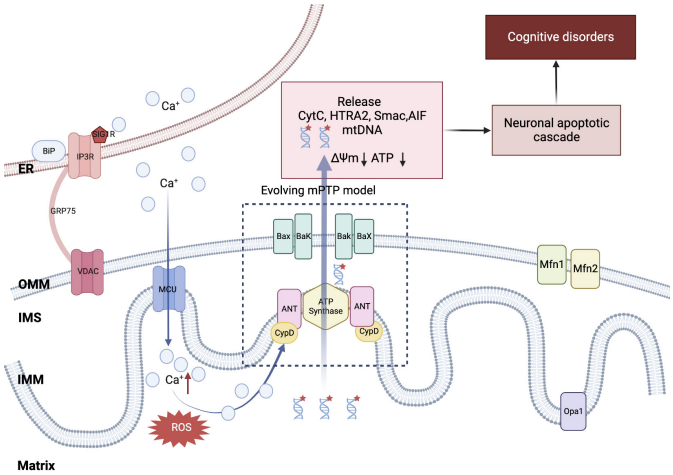

Figure 2 | **Mitochondrial calcium homeostasis and mPTP.**

Mitochondrial calcium is mainly regulated by MAMs, whose main components that participate in the Ca^{+} communication include IP3Rs, VDAC, grp75, Sig-1R, and BiP, and uptake $Ca2^{+}$ into the matrix via MCU. The accumulation of Ca^{+} in the mitochondrial matrix can trigger mPTP opening, which is composed of ANT, CypD, and ATP synthase in IMM, and Bax/Bak function in OMM. Persistent and widespread opening of the mPTP can cause ROS overproduction, depletion of ΔΨm and ATP, and release of apoptogenic proteins such as cyt c, HTRA2, Smac, and AIF, causing neuronal cell apoptotic cascade and cognitive disorders. In addition, the fragmented mtDNA can exit the mitochondria from mPTP forming ccf-mtDNA. Created with BioRender.com. AIF: Apoptosis-inducing factor; ANT: adenine nucleotide translocator; ATP: adenosine triphosphate; Bak: bcl-2 homologous antagonist-killer protein; Bax: bcl-2-associated x protein; BiP: binding immunoglobulin protein; ccf-mtDNA: circulating cell-free mitochondrial DNA; CypD: cyclophilin D; cyt c: cytochrome c; grp75: glucose-regulated protein 75; HTRA2: high-temperature requirement a serine peptidase 2; IMM: inner mitochondrial membrane; IMS: intermembrane space; IP3R: inositol trisphosphate receptor; MAMs: mitochondria-associated endoplasmic reticulum membranes; MCU: mitochondrial calcium uniporter; mPTP: mitochondrial permeability transition pore; OMM: outer mitochondrial membrane; ROS: reactive oxygen species; Sig-1R: sigma-1 receptor; Smac: second mitochondria-derived activator of caspases; VDAC: voltage-dependent anion channel; ΔΨm: mitochondrial membrane potential.

Calcium

Measurements of Ca^{2+} imaging mainly include fluorescent dyes and genetically encoded calcium indicators (GECIs) based on bioluminescence and fluorescent proteins (Pendin et al., 2015). Fluorescent dyes include Fura-2FF-AM (Hamilton et al., 2016), Fluo-3-AM (Qu et al., 2021), Fluo-4-AM, and Rhod2-AM (Stoica et al., 2014). These fluorescent dyes are commercially available and easy and fast to load in the cells, but they are difficult to confine in the mitochondrial matrix even with the AM technique, and have strong signal contamination caused by cytosol (Fernandez-Sanz et al., 2019). Bioluminescence-based GECIs mainly use aequorin (AEQ), which is the organelle-targeted engineered Ca^{2+} indicator, such as mitochondria-targeted photoprotein AEQ (MIT-AEQ) (Dematteis et al., 2020), wild type AEQ, and mutant AEQ (Cieri et al., 2018). They have a wide dynamic range, marginal interference with endogenous Ca^{2+} buffering proteins, low sensitivity to pH, and high signal-to-noise ratio, but they have a low amount of light and the irreversibility of the Ca^{2+} triggered reaction (Bonora et al., 2013). Fluorescent-based GECIs include Cameleons such as Yellow Cameleon 3.6 (Calvo-Rodriguez et al., 2020), GECOs such as R-GECO1 (Jadiya et al., 2019), low-affinity red

Table 2 | **Assessment of ROS and oxidative stress**

Targets	Markers	Assessment methods	Advantages and limitations	References
ROS	O_2•−	Fluorescent probes: mitoSOX red, HE	High autoxidation rate	Fernandez et al., 2019; Harland et al., 2020
		Union of HE-based probes and LC-MS/MS	Solves spectral overlap and improves specificity	Xiao and Meierhofer, 2019
	H_2O_2	Amplex Red	Highly specific and sensitive; but can be interfered with by other HRP substrates and $O_2^{\bullet-}$	Lopes et al., 2022
		DCFH-DA	Cell-permeable and easy to use; but leads to an artifactual amplification of the fluorescence signal via $DCF^{\bullet-}$	Behzadfar et al., 2017
		Genetically encoded fluorescent protein sensors: Hyper series, rogfp2-based probes	High sensitivity, high specificity, and allows ratiometric analysis	Pak et al., 2020
Oxidative damage	Protein oxidation			
	Protein carbonyls	Spectrophotometric measurement, ELISA, western blot, HPLC	High specificity and sensitivity	Buss et al., 1997; Sorolla et al., 2010; Lan et al., 2012; Giustina et al., 2020
	3-NT	ELISA	Low sensitivity	Yu et al., 2015
		GC-MS, GC-MS/MS, LC-MS/MS	Accurate determination; but nitrite and acid conditions during protein precipitation and hydrolysis may nitrate the tyrosine residues	Tsikas and Duncan, 2014
		LC-MS/MS combined with stable isotopic dilution analysis	High sensitivity, high specificity, and multiplexed quantitation	Rabbani and Thornalley, 2020
	Lipid peroxidation			
	HNE	HPLC, GC-MS, ELISA	Commonly used	Williams et al., 2006; Yu et al., 2015
	MDA	TBARS, spectrophotometric assay, fluorimetric assay	Unspecific and prone to artifacts	Perrotte et al., 2019; Das et al., 2021
	F2-isops	ELISA	Susceptible to artefact	Pharaoh et al., 2020
		LC-MS/MS	Relatively accurate	
	DNA and RNA oxidation			
	8OHdG, 8OHG	HPLC-ECD, LC-MS, UPLC-MS/MS, ELISA	ELISA is less sensitive and specific; LC-MS is more accurate than HPLC-ECD	Isobe et al., 2010; Yu et al., 2015; Peña-Bautista et al., 2019
Antioxidant enzymes: SOD1/2, Gpx1/4	Enzyme activities	Spectrophotometer	WST-1 has apparent simplicity and less limitation, and it is suitable for high-throughput measurements	Esposito et al., 2006
	Protein levels	ELISA, 2D-DIGE, western blot, mass spectrometry	Commonly used	Porcellotti et al., 2015; Fan et al., 2020
	mRNA expression	RT-qPCR	Commonly used	Hambright et al., 2017

DCFH-DA: 2′,7′-Dichlorofluorescein diacetate; F2-isops: F2-isoprostanes; GC-MS, GC-MS/MS: mass spectrometry coupled with gas chromatography; Gpx: glutathione peroxidase; HE: hydroethidine; HNE: 4-hydroxynonenal; HPLC: high-performance liquid chromatography; HPLC-ECD: HPLC with electrochemical detection; HRP: horseradish peroxidase; LC-MS/MS: liquid chromatography-tandem mass spectrometry; MDA: malondialdehyde; RT-qPCR: quantitative reverse transcription-polymerase chain reaction; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances; UPLC-MS/MS: ultra-performance liquid chromatography-tandem mass spectrometry analysis; WST-1: Water-soluble tetrazolium; 2D-DIGE: two-dimensional difference gel electrophoresis; 3-NT: 3-nitrotyrosine; 8OHdG: 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8OHG: 8-hydroxyguanosine.

fluorescent genetically encoded Ca^{2+} indicators for optical imaging (Wu et al., 2014) and jRGECO1 (Dana et al., 2016), and others include GCaMPs (Nakai et al., 2001), pericams (Nagai et al., 2001), and calcium-measuring organelle-entrapped protein indicators (Ooi et al., 2021). Their fluorescent reaction is reversible, and they have high fluorescence emission, Ca^{2+} affinity, and spatial and temporal visualization of subcellular Ca^{2+} dynamics (Chen et al., 2013). Besides, mitochondrial Ca^{2+} uptake capacity can be indicated by a decrease in the external Ca^{2+} concentration with a miniature Ca^{2+}-selective electrode (Hamilton et al., 2016).

Only detecting calcium-channel proteins like inositol-1,4,5-trisphosphate receptors, VDAC, and MCU might not sufficiently illustrate whether mitochondrial Ca^{2+} uptake is increased, so Ca^{2+} imaging in the cell and mitochondria remain significant. Fluorescent dyes and GECIs can detect Ca^{2+} dynamics in living cells with sufficient sensitivity and spatiotemporal accuracy. GECIs can be especially targeted to organelles or cytoplasmic domains selectively by fusing specific targeting signals to the GECI sequence and allowing the control of their expression by tissue-specific or inducible promoters (Bonora et al., 2013). They are expected to monitor mitochondrial Ca^{2+} in live animals.

Mitochondrial permeability transition pore

Mitochondrial permeability transition pore (mPTP) is composed of adenine nucleotide translocator, cyclophilin D, mitochondrial inorganic phosphate carrier, ATP synthase in IMM, and bcl-2-associated X (Bax)/bcl-2 homologous antagonist-killer protein (Bak) function in the outer mitochondrial membrane, mediating the permeability of IMM (Bonora et al., 2022). Typically, the accumulation of Ca^{2+} in the mitochondrial matrix can trigger mPTP opening, through probability binding to the F1 portion of ATP synthase (Giorgio et al., 2017). Persistent and widespread opening of the mPTP can cause mitochondrial damage such as depletion of NADH and MMP, decreased ATP, ROS overproduction, mitochondrial depolarization, mitochondrial swelling, and inhibition of the TCA cycle, and contribute to cognitive decline in HD and AD (Quintanilla et al., 2013; Pérez et al., 2018b; Kim et al., 2021). In addition, the opening of the mPTP can release intermembrane proteins with apoptogenic potential to amplify apoptotic mechanisms, such as cytochrome c, high-temperature requirement a serine protease A2, a second mitochondria-derived activator of caspases (Smac/DIABLO), and apoptosis-inducing factor, causing neuronal cell apoptotic cascade, and also contribute to cognitive disorders (**Figure 2**) in HD and AD (Goffredo et al., 2005; Yu et al., 2010).

The assessments of mPTP include mitochondrial swelling assay, calcium retention capacity/swelling, calcein-cobalt assay, and indirect measurement by MMP or mitochondrial respiration. The classic method of mPTP is mitochondrial swelling assay, which detects changes in the diffraction/absorption of light measured at 540 nm because of the increase in mitochondrial matrix volume (Pellman et al., 2015). Calcium retention capacity/swelling assay is an improved version of the classic mitochondrial swelling assay, and can measure Ca^{2+} uptake before mitochondrial swelling, obtain total Ca^{2+} capacity and the rate of Ca^{2+} uptake, and increase the amount of data (Mendoza and Karch, 2022). The calcein-cobalt assay can be used to measure mPTP opening in living cells under many pathological conditions and in a wide range of cell types, and can quantify by assessing mitochondrial network integrity using a fluorescence microscope (Bonora et al., 2016). mPTP opening can also be measured indirectly through the dissipation of MMP, and elevated mitochondrial oxygen consumption (Gautier et al., 2012). In all the cases mentioned above, it is necessary to validate that the observed changes are directly caused by mPTP opening. The most commonly used compound inhibiting mPTP opening is cyclosporine A, which works by inhibiting cyclophilin D, as a “gold standard” to confirm mPTP opening (Bonora et al., 2016; Mendoza and Karch, 2022).

The mitochondrial swelling assay is the classical method for mPTP monitoring using isolated mitochondria. The mPTP may exist in low and high-conductance modes (Bonora et al., 2016), and the low-conductance state has limited permeability, which only permits the diffusion of small ions such as H^+ and Ca^{2+}, and does not trigger detectable mitochondrial swelling. Therefore, the mitochondrial swelling assay may not be exact when mPTP is in a low-conductance state. Calcein-cobalt assay can detect mPTP opening in living cells, which can avoid isolating mitochondria and increase physiological relevance, and has more advantages to provide visualization of mitochondria. The mitochondrial calcium and mPTP targets and assessment methods are summarized in **Table 3**.

Table 3 | **Assessment of calcium and mPTP**

Targets	Markers	Assessment methods	Advantages and limitations	References
Calcium	Ca^{2+} imaging	Fluorescent dyes: Fura-2FF-AM, Fluo-3-AM, Fluo-4-AM, Rhod2-AM	Commercially available and easy and fast to load in the cells, but difficult to confine in the mitochondrial matrix, and has strong signal contamination	Stoica et al., 2014; Hamilton et al., 2016; Qu et al., 2021
		Bioluminescence-based GECIs: MIT-AEQ, wt AEQ, mutant AEQ	Wide dynamic range, marginal interference with endogenous Ca^{2+} buffering proteins, low sensitivity to pH and high signal-to-noise ratio, but has low amount of light, and the Ca^{2+} triggered reaction is irreversible	Cieri et al., 2018; Dematteis et al., 2020
		Fluorescent-based GECIs: Cameleons: YC3.6; GECOs: R-GECO1, LAR-GECO, jRGECO1; GGaMPs; Pericams; CEPIA	Fluorescent reaction is reversible, high fluorescence emission and Ca^{2+} affinity, and spatial and temporal visualization	Nagai et al., 2001; Nakai et al., 2001; Wu et al., 2014; Dana et al., 2016; Jadiya et al., 2019; Calvo-Rodriguez et al., 2020; Ooi et al., 2021
	Ca^{2+} uptake capacity	Miniature Ca^{2+}-selective electrode	Detect mitochondrial Ca^{2+} uptake capacity timely	Hamilton et al., 2016
mPTP		Mitochondrial swelling assay	Typical method	Pellman et al., 2015
		CRC/swelling	Measure Ca^{2+} uptake prior to mitochondrial swelling, and obtain total Ca^{2+} capacity	Mendoza and Karch, 2022
		Calcein-cobalt assay	Can be used under many pathological conditions and in a wide range of cell types	Bonora et al., 2016
		By MMP or mitochondrial respiration	Indirect measurement	Gautier et al., 2012

AEQ: Aequorin; CEPIA: calcium-measuring organelle-entrapped protein indicators; CRC: calcium retention capacity; GECIs: genetically encoded calcium indicators; LAR-GECO: low-affinity red fluorescent genetically encoded Ca^{2+} indicators for optical imaging; MIT-AEQ: mitochondria-targeted photoprotein aequorin; mPTP: mitochondrial permeability transition pore; YC3.6: Yellow Cameleon 3.6; ΔΨm: mitochondrial membrane potential.

Mitochondrial Dynamics

Mitochondria are highly dynamic organelles and undergo constant fission, fusion, and transport, to maintain the balance of morphology and subcellular distribution, which is referred to as mitochondrial dynamics (**Figure 3**) (Giacomello et al., 2020). In this section of the review, we mainly describe mitochondrial fission-fusion. In the progress of cognitive disorder conditions such as AD, PD, and HD, abnormal mitochondrial dynamics are key factors in the early stages (Manczak and Reddy, 2012; Geng et al., 2019; Hu et al., 2021). An imbalance in the fission-fusion (generally increased fission and decreased fusion) can cause increased mitochondrial numbers and circularity, and reduced mitochondrial length, meaning mitochondrial fragmentation in AD (Reddy et al., 2021). Mitochondrial fragmentation can cause mitochondrial damage including dissipation of MMP, ROS overproduction, enhancement of oxidative stress, loss of mtDNA, impaired transport of mitochondria, and increase mitophagy and autophagy. Further, it can cause neuroinflammation, synaptic degeneration, energetic failure in neurons, and cognitive disorders in neuronal degeneration (Zhang et al., 2020; Ishikawa et al., 2021). It is the activation of Drp1 or interaction of Drp1 with its adaptors, especially mitochondrial fission 1 protein, and depletion of optic atrophy-1 (OPA1) caused by pathological proteins such as tau, Aβ, Parkin, and the serine-threonine kinase phosphatase and tensin-induced putative kinase (PINK1) that plays an important role in the pathological progress (Manczak and Reddy, 2012; Narendra et al., 2012). Fusion proteins including OPA1 and mitofusin-2 (Mfn2) are neuroprotective factors, whose upregulation will rescue the progress caused by mitochondrial fragmentation in neurodegenerative diseases (Harland et al., 2020; Batista et al., 2021).

Expression of genes related to mitochondrial fission and fusion

The processes of fusion and fission are tightly regulated by several mitochondria-shaping proteins. Fission is mainly regulated by Drp1 and its adaptors—fission 1, mitochondrial fission factor, and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51)—and fusion is mainly regulated by mitofusin-1 (Mfn1), Mfn2, and OPA1 (**Figure 3**; Wang et al., 2009). Hence, we can measure the content of these proteins to detect fusion and fission. These methods include the proteins that can be measured by western blot analysis (Abulizi et al., 2021), immunofluorescence analysis, and quantitative immunofluorescence analysis (Kandimalla et al., 2016), or the mRNA that can be measured by quantitative reverse transcription-polymerase chain reaction (Kandimalla et al., 2016). Because DRP1 is a dynamin-related GTPase, we can also measure GTPase Drp1 enzymatic activity using a calorimetric kit (Manczak and Reddy, 2015).

Posttranslational modifications of mitochondrial fission factor Drp1

The overexpression of Drp1 alone does not lead to mitochondrial fragmentation, because the activation status of Drp1 depends on several posttranslational modifications (PTMs), including phosphorylation, ubiquitination, SUMO modification (SUMOylation), S-nitrosylation, and O-linked-N-acetyl-glucosamine glycosylation (O-GlcNAcylation). Here, we summarize the effects of these PTMs on the activation or inhibition of Drp1 and their roles in cognitive disorders.

Phosphorylation is the best characterized PTM of Drp1, which can activate or inhibit Drp1 depending on the specific position, such as Ser-616, Ser-637, Ser-656, Ser-40, Ser-44, Ser-579, Ser-585, and Ser-592; among them Ser-616 and Ser-637 are the representative sites (Qi et al., 2019). The phosphorylation of Ser-616 is an activating event for Drp1, which can stimulate mitochondrial fission, and contribute to neuronal injury and cognitive deficits in HD and chronic ethanol exposure-induced cognitive impairment (Liu et al., 2022a, b). The phosphorylation of Ser-637 by glucagon-like peptide-1 can mitigate mitochondrial fragmentation and improve the Aβ-induced energy failure, ROS overproduction, MMP collapse, and cell toxicity via the cyclic adenosine monophosphate/protein kinase A pathway in AD (Xie et al., 2021). The association of protein kinase A and dual-specificity anchoring protein 1 can phosphorylate Drp1 at Ser-656, which can decrease the Drp1 activity and downregulate mitochondrial fragmentation and reduce dendrite retraction and apoptosis in neurons caused by Aβ in AD (Banerjee et al., 2021). In addition, the phosphorylation of Ser-40, Ser-44, Ser-579, Ser-585, and Ser-592 can also activate Drp1 and damage neurons in neurodegenerative disease (Qi et al., 2019).

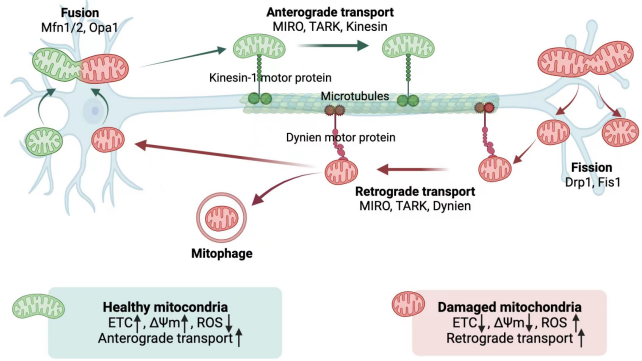

Figure 3 | **Neuronal mitochondrial dynamics.**
Mitochondrial dynamics in neurons is the dynamic equilibrium of mitochondrial fusion and fission, transport, and mitophagy. Healthy mitochondria are transported along the axon from the soma to synapses through the anterograde axonal transport system (composed of the kinesin-1 family, MIRO, and TRAK) to support normal energy metabolism. Damaged mitochondria can undergo fission driven by Drp1 and Fis1, and are taken back to the soma through the retrograde axonal transport machinery (composed of MIRO, TRAK, and the molecular motor dynein), where they can be restored through fusion with healthy mitochondria driven by Mfn and OPA1 or degraded by mitophagy. If the balance is broken, neurons may be damaged and cause cognitive disorders. Created with BioRender.com. Drp1: Dynamin-related protein 1; ETC: electron transport chain; Fis1: mitochondrial fission 1; Kinesin: kinesin heavy chain 5A-C; Mfn1/2: mitofusin1/2; MIRO: mitochondrial Rho GTPase; OPA1: optic dominant atrophy 1; ROS: reactive oxygen species; TRAK: trafficking kinesin protein; ΔΨm: mitochondrial membrane potential.

Ubiquitination is another type of post-translational modification that influences Drp1 function. Parkin is an E3 ubiquitin ligase that can ubiquitinylate Drp1, leading to proteasomal degradation pathogenic mutation or knockdown of Parkin inhibits the degradation of Drp1, leading to mitochondrial fragmentation and mitochondrial dysfunction, which is the potential mechanism of HD (Wang et al., 2011).

SUMOylation is another important mechanism for the functional regulation of Drp1 that usually alters the subcellular localization of proteins or protects them from ubiquitin-triggered destruction (Serasinghe and Chipuk, 2017). SUMOylation of Drp1 can inhibit cytochrome c release and cell apoptosis, but deSUMOylation of Drp1 increases Drp1 stability on the outer mitochondrial membrane, stimulating mitochondrial fission in neurons (Prudent et al.,

2015). Therefore, this suggests that the SUMOylation of Drp1 may have a neuroprotective effect. Suppressing Drp1 deSUMOylation can decrease mitochondrial fragmentation and memory loss caused by sevoflurane, improving cognitive dysfunction in sevoflurane-induced cognitive dysfunction (Zheng et al., 2020).

Nitric oxide functions as a signaling molecule, but the excessive generation of reactive nitrogen species can cause S-nitrosylation of proteins. The nitric oxide-mediated S-nitrosylation of Drp1 at Cys-644 within the GED domain can activate Drp1 and trigger mitochondrial fission, synaptic loss, and neuronal damage, and it may be the prominent pathological feature of AD and HD (Cho et al., 2009; Haun et al., 2013). S-nitrosylation can also inhibit insulin and Aβ catabolism, and cause hyperglycemia and Aβ accumulation, which may increase the risk of transformation from metabolic syndrome and type 2 diabetes mellitus to AD (Akhtar et al., 2016).

O-GlcNAcylation of the serine and threonine residues is a dynamic post-translational modification between O-GlcNAcase and O-GlcNAc transferase, and the effects on Drp1 function are reciprocal between O-GlcNAcylation and phosphorylation because their sites are reduplicative (Cheng and Hart, 2001). Recent research has shown that the O-GlcNAcylation of Drp1 is upregulated at Thr-585 and Thr-586, and induces mitochondrial fragmentation in AD mice (Park et al., 2021).

PTMs of fusion proteins

The PTMs of fusion proteins mainly include ubiquitinylation of Mfn1/2 and proteolytic cleavage of OPA1. Here, we summarize the effects of these PTMs on Mfn1/2 and OPA1 and their roles in cognitive disorders.

Ubiquitinylation by Parkin is the characterized post-translational modification of Mfn1/2, which can lead to their degradation and facilitate mitophagy, and its inhibition may lead to the accumulation of defective mitochondria and contribute to PD pathogenesis (Gegg et al., 2010). Proteolytic cleavage is the major regulatory mechanism for OPA1 function to regulate mitochondrial fusion (Ishihara et al., 2006). OPA1 variants have been categorized as long (L) and short (S) isoforms. The L-isoforms are generally fusion-competent, and can be cleaved by the IM peptidase (OMA1) and presenilin-associated rhomboid-like at S1 or the i-AAA protease (Ymel1) at S2 and form S-isoforms to stimulate the membrane fusion activity of OPA1 (Ishihara et al., 2006; Mishra et al., 2014). The co-expression of L and S-OPA1 isoforms is functional, but when L-OPA1 are processed by stress-induced peptidase OMA1 into short isoforms completely, they will inhibit fusion, trigger mitochondrial fragmentation, and disrupt mitochondrial cristae (Anand et al., 2014).

PTMs of fission and fusion proteins can be measured by western blotting. In particular, we should note that the level of PTMs of Drp1 may be directly related to the functional state of Drp1 molecules, rather than solely dependent on the expression level of Drp1. The activation of different positions of the special PTM may stand for the opposite status of Drp1. Similarly, proteolytic cleavage of OPA1 has not been studied clearly enough, including the function of different isoforms, and increased expression of OPA1 alone might not indicate increased fusion. Hence, combining the analysis of mitochondrial morphology and the expression of Drp1 and fusion proteins may be the most reliable measurement to evaluate mitochondrial fission and fusion.

Mitochondrion morphology

Mitochondria are highly dynamic organelles and assume different shapes that will affect their function during mitochondrial fusion and fission processes (**Figure 3**; Chen and Chan, 2009). Mitochondria are usually tubular in shape under normal conditions and can provide ATP for cells (Wasilewski and Scorrano, 2009). Mitochondrial shapes change from tubular to donut or blob forms because of increased mitochondrial ROS and calcium influx, closely related to cognitive decline (Ahmad et al., 2013). The percentages of tubular- or donut-shaped mitochondrial morphology are related to age and menopause-related decline in working memory by influencing synaptic transmission (Hara et al., 2014).

The qualitative assessment of mitochondrial morphology can use different types of transmission electron microscopy (TEM), which have been widely used to observe the different morphologies of mitochondria. The fluorescent microscope can be used to observe the mitochondria in living cells, but its spatial resolution is limited to 200 nm (Liu et al., 2021b). Recently, super-resolution fluorescence microscopes have surpassed the resolution limits, such as stimulated emission (Huang et al., 2013), structured illumination microscopy (Guo et al., 2018), and stochastic optical reconstruction microscopy (Rust et al., 2006), all of which can observe the mitochondria at the nanometer level for ultra-structural imaging. The quantitative assessment includes measuring the fission positions of mitochondria using structured illumination microscopy. Peripheral fission (< 25% from a tip) enables damaged material of mitochondria to be shed into smaller mitochondria destined for mitophagy, while midzone fission (within the central 50%) leads to biogenesis (Kleele et al., 2021). The other important mitochondrial parameters under TEM or fluorescence microscopy include two-dimensional evaluation indicators such as major axis; minor axis; aspect ratio (major axis/minor axis), which reflects the length-to-width ratio; surface area; perimeter; Feret's diameter (the longest distance between any two points within a given mitochondrion) (**Figure 4**); form factor (perimeter^2/4π·surface area), which reflects complexity and branching; circularity (4π·surface area/perimeter^2), and roundness (4·surface area/π·major axis^2), which are two-dimensional indices of sphericity; and mitochondrial density (the number of mitochondria counted per unit area of tissue section) (Abbade et al., 2020; Zhang et al., 2022). The same methods can be used to calculate the area, perimeter, and density of the vacuoles in the mitochondria, which reflect the remodeling of IMM into many separate vesicular matrix compartments (Zhang et al., 2022). Two-dimension analyses can comprehensively measure mitochondrial morphology and provide detailed indicators in an optical cross-section, but it is difficult to choose a representative plane, especially for thick cells, and we may need to collect amounts of planes to reduce the errors (Chaudhry et al., 2020). Three-dimensional evaluation indicators can be acquired through three-dimensional reconstruction of mitochondria or fluorescence microscopy, including mitochondrial volume, surface area and sphericity (36π·volune^2/surface area^3) (Chaudhry et al., 2020; Zhang et al., 2022). Three-dimensional analyses overcome the limitations of two-dimension and can provide maps of mitochondria in cells, making the measurement more accurate, but it is relatively more complex to acquire and process images. Three-dimensional reconstruction of mitochondria can acquire mitochondrial volume (Zhang et al., 2022). Mitochondria can be divided into different categories such as fragmented, intermediate, and tubular according to their lengths (Yan and Zhao, 2020), and into classes I–IV according to the loss of cristae and matrix (Vaillant-Beuchot et al., 2021).

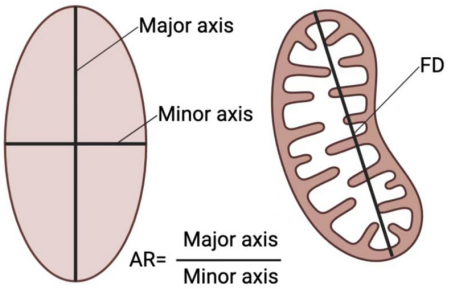

Figure 4 | **Methods for quantifying mitochondrial AR and FD on TEM images.** The diagram on the left shows the methods of quantifying mitochondrial AR that is the ratio of major axis to minor axis. The diagram on the right shows the method of quantifying mitochondrial FD that is the longest distance between any two points within a given mitochondrion. Created with BioRender.com. AR: Aspect ratio; FD: Feret's diameter; TEM: transmission electron microscopy.

The assessment of mitochondrial morphology is an important part of mitochondrial dynamics, especially ultrastructural imaging. Conventional visible light microscopy and TEM cannot image the complex internal structure of mitochondria or observe the mitochondria in living cells at the same time, so the change of instantaneous mitochondrial dynamics may be ignored. The progress of super-resolution fluorescence microscopes is expected to break the limits. They can provide super-resolution imaging of live cells for a long time and are compatible with commonly used fluorescent probes allowing multi-label observation. Through high-speed continuous recording and long-term observation of the transient changes of mitochondria in neurons, it will provide new insights into mitochondrial biology in health and disease. The mitochondrial fission and fusion and morphology targets and assessment methods are summarized in **Table 4**.

Mitochondrial Transport

Neurons have high demands for ATP to support many important functions, so they need to transport mitochondria from the soma to their destinations to support normal energy metabolism. Neuronal mitochondria undergo bidirectional transport along neuronal processes between the soma to distal axonal and dendritic regions along the neuronal microtubules, mainly including anterograde and retrograde transport (**Figure 3**; Misgeld et al., 2007). Mitochondrial transport and distribution are directly correlated with synaptic activity, and they tend to accumulate at areas of ATP depletion and elevated intracellular Ca^{2+} levels such as synapses, producing ATP and buffering Ca^{2+} to maintain neuronal functions (Werth and Thayer, 1994; Wang and Schwarz, 2009). Mitochondrial transport is also regulated by microtubule-associated proteins, especially tau, whose overexpression can selectively inhibit kinesin-driven anterograde transport and cause mitochondrial accumulation in the soma and decrease in synaptic terminals, and it is also significant in AD (Kopeikina et al., 2011). Tau undergoes transformation from soluble assembles into insoluble filaments, leading to mitochondrial failure, synaptic failure, and neurodegeneration by affecting mitochondrial transport, morphology and bioenergetics. (Pérez et al., 2018a). Except for AD, defective axonal transport is also found in HD and PD. In these diseases, abnormal transport can also be caused by the destruction of kinesin and dynein of neurons through more complex pathways such as the activation of glycogen synthase kinase 3-beta by presenilin-1 and N-methyl-D-aspartic acid receptor (Pigino et al., 2003; Decker et al., 2010).

Mitochondrial distribution

Mitochondrial transport can be assessed by observing the percentage of mitochondrial distribution of soma, axons, and other regions in neurons by using TEM similar to that described above (Pickett et al., 2018).

Table 4 | **Assessment of mitochondrial fission and fusion and morphology**

Targets	Markers	Assessment methods	Significance	References
Mitochondrial fission and fusion	Regulators of fission and fusion: Drp1, FIS1, Mff, MiD49, MiD51, Mfn1, Mfn2, OPA1	Proteins: western blot, immunofluorescence analysis, quantitative immunofluorescence analysis	Commonly used	Kandimalla et al., 2016; Abulizi et al., 2021
		mRNA: qRT-PCR		
	GTPase Drp1 enzymatic activity	Calorimetric kit	Commonly used	Manczak and Reddy, 2015
	Posttranslational modifications of Drp1	Western blot		
	Phosphorylation at Ser-616, Ser-40, Ser-44, Ser-579, Ser-585, and Ser-592		Activate Drp1, stimulate mitochondrial fragmentation and harmful for neurons	Qi et al., 2019; Liu et al., 2022a, b
	Phosphorylation at Ser-637 and Ser-656		Decrease Drp1 activity, mitigate mitochondrial fragmentation and neuroprotective effect	Banerjee et al., 2021; Xie et al., 2021
	Ubiquitination		Inhibition can lead to mitochondrial fragmentation and neural damage	Wang et al., 2011
	SUMOylation		Mitigate mitochondrial fragmentation and neuroprotective effect	Zheng et al., 2020
	S-nitrosylation		Activate Drp1, stimulate mitochondrial fragmentation and harmful for neurons	Cho et al., 2009; Haun et al., 2013
	O-glcNAcylation		Induce mitochondria fragmentation	Park et al., 2021
	Posttranslational modifications of fusion proteins	Western blot		
	Ubiquitinylation of Mfn1/2		Facilitate mitophagy	Gegg et al., 2010
	Proteolytic cleavage of OPA1		Co-expression of L and S-OPA1 isoforms is functional, but L-OPA1 that are processed into short isoforms completely will inhibit fusion, trigger mitochondrial fragmentation, and disrupt mitochondrial cristae	Anand et al., 2014
Mitochondrion morphology		Qualitative assessment: TEM	High-resolution images, but unavailable in live-cell imaging	
		Fluorescent microscope	Observe the mitochondria in living cells, but spatial resolution is limited to 200 nm	Liu et al., 2021b
		Super-resolution fluorescence microscopes: STED, SIM, STORM	Observe the mitochondria at the nanometer level in living cells	Rust et al., 2006; Huang et al., 2013, 2018
		Quantitative assessment: fission positions of mitochondria	Peripheral fission separates damaged material for mitophagy, while midzone fission leads to biogenesis	Kleele et al., 2021
		2D evaluation indicators	Comprehensive and can provide detailed indicators; but difficult to choose a representative plane	Abbade et al., 2020; Zhang et al., 2022
		Major axis and minor axis		
		AR	(Major axis/minor axis); reflect the length-to-width ratio	
		Surface area		
		Perimeter		
		FD	The longest distance between any two points	
		FF	(perimeter^2)/(4π·surface area); reflect complexity and branching	
		Circularity	(4π·surface area)/(perimeter^2)	
		Roundness	(4·surface area)/(π·major axis^2); two-dimensional indexes of sphericity	
		Mitochondrial density	The number of mitochondria counted per unit area of tissue section	
		Area, perimeter, and density of vacuoles	Reflect the remodeling of IMM into many separate vesicular matrix compartments	Zhang et al., 2022
		3D evaluation indicators	Can overcome the limitations of 2D, but is relatively more complex to acquire and process images	Chaudhry et al., 2020; Zhang et al., 2022
		Mitochondrial volume	Number of voxels	
		Surface area	Estimate of area on surface	
		Sphericity	(36π·volume^2)/(surface area^3)	

AR: Aspect ratio; Drp1: dynamin-related protein 1; FD: Feret's diameter; FF: form factor; Fis1: mitochondrial fission 1; L and S-OPA1: long and short isoforms of OPA1; Mff: mitochondrial fission factor; Mfn1/2: mitofusin1/2; MiD49 and MiD51: mitochondrial dynamics proteins of 49 and 51 kDa; O-GlcNAcylation: O-linked-N-acetyl-glucosamine glycosylation; OPA1: optic dominant atrophy 1; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; SIM: structured illumination microscopy; STED: stimulated emission deletion; STORM: stochastic optical reconstruction microscopy; SUMOylation: SUMO modification; TEM: transmission electron microscopy.

Mitochondrial transport velocity

Mitochondrial transport velocity is mainly measured by the kymograph generation technique. Briefly, time-lapse images of labeled neuronal mitochondria by targeted fluorescent proteins or mitochondrial dyes are collected using laser-scanning confocal microscopy, to make kymographs and line traces to determine the transport direction and velocity of mitochondria (Kiryu-Seo et al., 2010). A microfluidic culture platform is usually used to isolate and purify axons, somata, and dendrites and study axonal transport through the embedded microgrooves that can define transport direction (Taylor et al., 2005). Besides, "optical flow" can be used to measure instantaneous velocity vectors from a pair of images in individual mitochondrion or populations and can combine other bioenergetic parameters like MMP while minimizing photodynamic oxidative artifacts evoked by fluorescence excitation (Gerencser and Nicholls, 2008). In image processing, mitochondria are often tracked manually or automatedly. Research has shown that the automated tracking tools dramatically underestimate track length, mitochondrial displacement, and movement duration, while overestimating mitochondrial velocity generally, and are suitable for assessing relative transport differences between experimental groups (Bros et al., 2015).

At present, stable assessment methods of mitochondrial transport are limited. Detecting mitochondrial distribution of soma and axon by using TEM is convenient, reliable, and efficient, but it cannot provide real-time information and live-cell imaging. However, fluorescence microscopy to detect mitochondrial transport velocity is a more reliable strategy that can provide visualization information and other bioenergetic parameters by using relative probes. Especially, microfluidic culture platform can probe axons independently from cell bodies, which provide a highly adaptable system to culture various models of neural injury, and is more ideally suited for high-resolution axonal transport studies in living cells. Combining a microfluidic culture platform and optical microscopy can provide great convenience for mitochondrial transport research. The mitochondrial transport targets and assessment methods are summarized in **Table 5**.

Table 5 | **Assessment of mitochondrial transport**

Targets	Assessment methods	Advantages and limitations	References
Mitochondrial distribution	TEM	Commonly used	Pickett et al., 2018
Transport velocity	Kymograph generation technique	Measure the transport direction and velocity	Kiryu-Seo et al., 2010
	Microfluidic culture platform	Isolate and purify axons, somata, and dendrites	Taylor et al., 2005
	Optical flow	Combine other bioenergetic parameters and decrease photodynamic oxidative artifacts	Gerencser and Nicholls, 2008

TEM: Transmission electron microscopy.

Mitophagy

In cells, three main types of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy (Cuervo and Dice, 1996). Mitophagy is a form of macroautophagy that has been identified to participate in the turnover of damaged organelles and can eliminate damaged or superfluous mitochondria through selective autophagic degradation to maintain mitochondrial quality control (**Figure 3**; Gatica et al., 2018). Mitophagy is mainly mediated by ubiquitin, which is initiated by PINK1 and E3 ubiquitin ligase Parkin, and they are identified as causal genes for autosomal recessive juvenile parkinsonism (Valente et al., 2004). Decreased mitophagy will cause the accumulation of damaged mitochondria and increase oxidative damage and cellular energy deficits, contributing to synaptic dysfunction and cognitive deficits, which is a hallmark of diseases of cognitive disorders, including AD, PD, HD, and postoperative cognitive dysfunction. Increased mitophagy will improve the pathological process by bolstering mitochondrial health (Fang et al., 2019; Ahmed et al., 2021; Franco-Iborra et al., 2021; Jiang et al., 2022).

Traditional methods

Traditional methods usually rely on TEM, which can visualize mitochondria that are surrounded or engulfed by autophagic membranes and recognize mitochondria remnants within autophagosomes, but the obvious limitations include hardly recognizing mitochondria of the relatively late stages of mitophagy and difficult quantification assessment because the samples are only a small fraction of cells or tissues (Klionsky et al., 2016). Another method is the analysis of mitochondrial protein turnover rates (Kim et al., 2012). Nevertheless, this method cannot easily distinguish between different cell types within a tissue and assess whether these proteins are degraded by intrinsic mitochondrial proteases or whole mitochondria that are degraded following lysosomal delivery (Sun et al., 2015).

Fluorescent-based strategies

Fluorescent-based strategies can provide a more robust assessment. These approaches often label mitochondria by autophagy marker microtubule-associated protein light chain 3 (LC3), along with fluorescent proteins such as GFP-LC3 (Kim and Lemasters, 2011) or other mitochondrial fluorescent markers, such as TMRM and MFFR (Kim and Lemasters, 2011), mitochondrial-targeted RFP (Rambold et al., 2011), MitoTracker dyes (Dagda et al., 2009), and flag epitope-tagged Par (Kawajiri et al., 2010) and visualize colocalization of mitochondria enclosed in GFP-LC3-labeled autophagosomes. However, this approach has a relatively high false positive rate because LC3 proteins may aggregate in an autophagy-independent manner and easily incorporate into intracellular protein aggregates (Kuma et al., 2007). Mitochondrial-targeted monomeric Keima protein (mt-Keima) (Roca-Agujetas et al., 2021) is another fluorescent molecule, which is resistant to lysosomal degradation allowing for the mitophagy signal to be integrated, and has pH-dependent fluorescent properties that can be easily targeted to the mitochondrial matrix, making it widely applicable (Sun et al., 2017). Rosella is also a fluorescent pH-biosensor targeting mitochondria and can monitor mitophagy (Rosado et al., 2008).

Mitophagy is central to neural function and survival, and defective mitophagy leads to cellular dysfunction, contributing to age-predisposed neurodegeneration, which is the potential therapeutic target in cognitive disorders (Lizama and Chu, 2021). At present, standard methods to monitor mitophagy are limited. Although Traditional methods can directly detect mitophagy, they have low accuracy and quantification is challenging. Fluorescent-based strategies are relatively more reliable, and can provide live-cell imaging. Furthermore, we can acquire more mitochondrial parameters simultaneously by using different mitochondrial fluorescent markers to label LC3. Ratiometric fluorescent probes like mt-Keima and Rosella can also easily visualize and quantify mitophagy in living tissues, and can potentially provide substantially higher-resolution images with super-resolution microscopes. The mitophagy targets and assessment methods are summarized in **Table 6**.

Table 6 | **Assessment of mitophagy**

Targets	Assessment methods	Advantages and limitations	References
Mitophagy	Traditional methods: TEM	Difficult to recognize mitochondria in the relatively late stages of mitophagy; difficult quantification	Klionsky et al., 2016
	Mitochondrial protein turnover rates	Difficult to distinguish different cell types and degradation sites	Kim et al., 2012
	Fluorescent-based strategies: GFP-LC3, mitochondrial-targeted RFP, mitotracker dyes, flag epitope-tagged PINK1	Visualize colocalization of mitochondria; but high false positive rate	Dagda et al., 2009; Kawajiri et al., 2010; Kim and Lemasters, 2011; Rambold et al., 2011
	mt-Keima	Resistant to lysosomal degradation and pH-dependent fluorescent properties	Roca-Agujetas et al., 2021
	Rosella	pH-dependent fluorescent properties	Rosado et al., 2008

mt-Keima: Mitochondrial-targeted monomeric Keima protein; PINK1: serine-threonine kinase phosphatase and tensin-induced putative kinase; TEM: transmission electron microscopy.

Mitochondrial Genomic Homeostasis

Neurons are high-energy-demanding cells and need a sufficient number of mitochondria to produce ATP and buffer Ca^{2+} (Wang and Schwarz, 2009); therefore, mitochondrial biogenesis is required to maintain a healthy mitochondrial pool within the cell. The mitochondrial proteome is under dual genomic control, with 13 proteins encoded by mtDNA and the remaining over 1000 mitochondrial resident proteins originating from the nuclear genome (Rath et al., 2021), so constant communication between the nucleus and mitochondria is essential. Because mtDNA lacks the protection of histone proteins, it is highly vulnerable to ROS or other stressors and becomes fragmented (Xian et al., 2022). In many diseases of cognitive disorders such as AD, PD, HD, and postoperative cognitive dysfunction, mitochondrial biogenesis is downregulated, leading to a decrease in mtDNA levels and respiratory function, and increased neuronal death (Sheng et al., 2012; Dölle et al., 2016; Ho et al., 2022; Liu et al., 2022c). Impaired mitochondrial biogenesis is always associated with mitochondrial dysfunction, participating in the pathogenesis of neurodegenerative disorders, and it is expected to be a potential novel therapeutic target (Golpich et al., 2017). Peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α) is also the broad regulator of ROS metabolism and can promote the expressions of ROS-detoxifying enzymes, including Gpx1 and SOD1/2, and uncoupling proteins, protecting neural cells from oxidative stress in chronic cerebral hypoperfusion (Han et al., 2020). In addition, nuclear respiratory factor 2 (NRF-2) can also activate antioxidant systems and decrease neuroinflammation, improving cognitive decline in AD (Wang et al., 2022).

Assessment of mitochondrial biogenesis

Mitochondrial biogenesis is a set of molecular processes that ultimately result in the replication of mitochondria, which is mainly regulated by the PGC-1α-NRF-1/2-mitochondrial transcription factor A (TFAM) pathway (Cardanho-Ramos and Morais, 2021). PGC-1α is the master regulator of mitochondrial biogenesis in neurons, activating NRF-1 and NRF-2 that bind to the promoter region of many mitochondrial genes, including TFAM that can drive the replication and transcription of mtDNA (Jornayvaz and Shulman, 2010). Therefore, we can assess important regulators or mitochondrial content and synthesis to evaluate mitochondrial biogenesis.

Regulators of mitochondrial biogenesis

The regulators of mitochondrial biogenesis such as PGC-1α, NRF1/2, and TFAM are widely used as markers of mitochondrial biogenesis (Kandimalla et al., 2016; Manfredini et al., 2019). The expression of mRNA or protein can be measured using quantitative reverse transcription-polymerase chain reaction, western blotting, immunofluorescence analysis, and quantitative immunofluorescence analysis (Manczak and Reddy, 2015; Kandimalla et al., 2016; Manfredini et al., 2019). Nevertheless, some researchers consider the regulators perhaps do not determine biogenesis. For example, adenosine monophosphate-activated protein kinase can activate PGC-1α presumably

to increase the potential for aerobic energy production under the cellular energetic stress, simultaneously downregulating translation through inhibition of the mammalian target of rapamycin; hence, it would be hard to predict whether mitochondrial proteins are produced (Miller and Hamilton, 2012).

Mitochondrial content and synthesis

mtDNA copy number is a widely used marker in mitochondrial biogenesis by real-time quantitative polymerase chain reaction (Chen et al., 2021). The mitochondrial genome to nuclear genome ratio (Mt/N) is a convenient way to measure mtDNA content in a cell (Malik et al., 2011), and it may be the potential biomarker of mitochondrial dysfunction (Malik and Czajka, 2013). In addition, measuring mitochondrial protein synthesis rates with isotopic tracers can also assess mitochondrial biogenesis (Reid et al., 2020). Similarly, the rate of mtDNA replication can be used to monitor mitochondrial biogenesis, and using nucleotide analogs 5-bromo-2-deoxyuridine and 5-ethynyl-2′-deoxyuridine to label mtDNA, together with a tyramide signal amplification protocol, can visualize and quantify newly synthesized mtDNA in individual cells (Lentz et al., 2010).

Though the regulators of mitochondrial biogenesis are widely used to assess mitochondrial biogenesis, they are not enough to determine mitochondrial biogenesis by themselves as previously mentioned. Mitochondrial content is determined by mitochondrial biogenesis and degradation, and mitochondrial biogenesis is reflective of the processes of synthesis. When mitochondrial content decreases or remains unchanged, it might be difficult to estimate if mitochondrial biogenesis is decreased because mitochondrial degradation may play a major role. Relatively, detecting mitochondrial synthesis might be more reliable, but it is more complex. In addition, the association of mitochondrial content and mitophagy measurements may make it more accurate.

Circulating cell-free mitochondrial DNA

When neurons are damaged, their mitochondria become stressed and open the mPTP through MCU, increasing calcium uptake into the mitochondria. The increased calcium triggers VDAC oligomerization, and the fragmented mtDNA cleaved by the flap endonuclease 1 will exit mitochondria via mPTP- and VDAC-dependent channels (**Figure 2**; Xian et al., 2022). When the mtDNA is released into the bloodstream or cerebrospinal fluid, they form circulating cell-free mtDNA (ccf-mtDNA) (Lowes et al., 2020). ccf-mtDNA is a systemic inflammatory mediator and is related to pro-inflammatory cytokines including granulocyte-macrophage colony-stimulating factor, interleukin-2, interleukin-4, and interleukin-6, and may activate downstream systemic inflammation, which may promote the progression of PD (Borsche et al., 2020). The ccf-mtDNA levels were reduced in the cerebrospinal fluid of preclinical AD, which supports that mtDNA depletion may be a characteristic pathophysiological indicator of neurodegeneration (Podlesniy et al., 2013). In neurological diseases like AD and PD ccf-mtDNA is associated with cognitive impairment and can be the biomarker for the early process of neurodegeneration and guide treatment (Gambardella et al., 2019).

The assessment of ccf-mtDNA is similar to mtDNA and can also be measured by quantitative polymerase chain reaction (Pyle et al., 2015). Droplet digital polymerase chain reaction can also be used (Trifunov et al., 2021), which is a refinement of conventional PCR with higher sensitivity, specificity, reproducibility, and efficacy (Kojabad et al., 2021). The mitochondrial genomic homeostasis targets and assessment methods are summarized in **Table 7**.

Table 7 | **Assessment of mitochondrial genomic homeostasis**

Targets	Markers	Assessment methods	Advantages and limitations	References
Mitochondrial biogenesis	Regulators of mitochondrial biogenesis: PGC-1α, NRF1/2, TFAM	Proteins: western blotting, immunofluorescence analysis, quantitative immunofluorescence analysis	Regulators perhaps do not determine biogenesis	Manczak and Reddy, 2015; Kandimalla et al., 2016; Manfredini et al., 2019
		mRNA: qRT-PCR		
	Mitochondrial content: mtDNA copy number	qPCR		Chen et al., 2021
	Mt/N	qPCR	Convenient; potential biomarker of mitochondrial dysfunction	Malik et al., 2011
	Mitochondrial synthesis: protein synthesis rates	Isotopic tracers	Commonly used	Reid et al., 2020
	Rate of mtDNA replication	BrdU and EdU labels in mtDNA	Visualize and quantify newly synthesized mtDNA in individual cells	Lentz et al., 2010
ccf-mtDNA		qPCR	Commonly used	Pyle et al., 2015
		ddPCR	high sensitivity, specificity, reproducibility, and efficacy	Trifunov et al., 2021

BrdU: Nucleotide analogs 5-bromo-2-deoxyuridine; ccf-mtDNA: circulating cell-free mitochondrial DNA; ddPCR: droplet digital polymerase chain reaction; EdU: 5-ethynyl-2'-deoxyuridine; Mt/N: mitochondrial genome to nuclear genome ratio; NRF-1/2: nuclear respiratory factor 1/2; PGC-1α: peroxisome proliferator-activated receptor γ coactivator 1 α; qPCR: real-time quantitative polymerase chain reaction; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; TFAM: mitochondrial transcription factor A.

Proteomic Mapping of Mitochondria

Ascorbate peroxidase (APEX) proximity labeling is a powerful tool for exploring protein interactions in living cells. In the presence of H_2O_2, APEX oxidizes biotin-phenol to the short-lived (< 1 ms) phenoxyl radical, which can react with electron-rich amino acids such as Tyr, Trp, His, and Cys within a radius of 20 nm. Compared with horseradish peroxidase, APEX is not affected by protein localization and can maintain activity in all cellular compartments (Rhee et al., 2013). Therefore, it has been widely used in mapping spatial mitochondrial proteomics (Marx, 2015). Using APEX proximity labeling, Ting's lab identified 137 mitochondrial outer membrane proteins and 634 endoplasmic reticulum outer membrane proteins. Among them, 94 proteins with distribution in both were found through comparative analysis of proteome (Hung et al., 2017). In other words, these proteins may be distributed in MAMs (Hung et al., 2017). Chung et al. (2017) identified 225 proteins in the immediate vicinity of alpha-synuclein in living neurons using the APEX-based labeling method, including endocytosis and vesicular transport-related proteins, systematically revealing that abnormal aggregation of alpha-synuclein in neurons may be caused by multiple factors. Consequently, the emergence of APEX proximity labeling has provided a new research strategy for mitochondrial proteomics in neurodegenerative diseases.

Conclusion

Mitochondrial dysfunction has been considered the key mechanism in the progress of diseases of cognitive disorders. Therefore, it is important to detect mitochondrial dysfunction more accurately. Herein, we sum up common markers of mitochondrial dysfunction and relevant assessment methods, and briefly account for the meanings of cognitive disorders of these targets, including energy metabolism, oxidative stress, calcium homeostasis, mitochondrial dynamics (including fission-fusion, transport, and mitophagy), and mitochondrial genomic homeostasis. It is worth noting that we did not discover direct evidence to prove that mitochondrial dysfunction is specific in different pathological pathways of cognitive disorders. In acute brain damage such as ischemic stroke, hypoxic-ischemic brain injury, and traumatic brain injury, mitochondria are usually involved in ROS generation, oxidative stress, ECT dysfunction, and abnormal mitochondrial dynamics (Cheng et al., 2012; Yang et al., 2018). We can use live cell imaging to capture real-time information about mitochondrial dysfunction. In chronic diseases of the nervous system such as AD, PD, and HD, mitochondria are responsible for excessive oxidative stress, reduced ATP levels, Ca^{2+} dyshomeostasis, altered mitochondrial DNA, abnormal PTMs of fission and fusion proteins, and defective mitophagy (Gegg et al., 2010; Shoshan-Barmatz et al., 2018; Qi et al., 2019; Monzio Compagnoni et al., 2020; Zhang et al., 2021). We can use APEX or other methods to capture the changes in proteins. Strictly speaking, mitochondrial dysfunction caused by acute and chronic brain injury is difficult to be distinguished because they may exhibit similar structural and functional impairments. Mitochondrial physiological function and morphology are integral, so when one is damaged, the other is also involved. We recommend that all of the above methods can be used to explore mitochondrial dysfunction in different pathological pathways of cognitive disorders, and the results may be related to special pathological pathways, sensitivity of the method, experiment cost, and degree of proficiency.

The holistic, comprehensive, and precise comprehension of mitochondrial dysfunction in specific diseases is difficult, and it prompts us to improve the existing methodology and instrumentation. Super-resolution techniques represented by structured illumination microscopy may provide a potential solution strategy, which allows fast, long-term, and super-resolution imaging (Huang et al., 2018), and greatly enhances the ability to locate subcellular structures and explore their interrelationships. Generally, it is expected to use advanced detection methods to illustrate the molecular mechanisms of mitochondrial dysfunction and their relationship with the diseases. We hope that this review can help researchers assess mitochondrial functions in different pathological conditions more conveniently, and facilitate the development and design of pharmacological therapies to target mitochondria in diseases or cognitive disorders.

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