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MITOCHONDRIA-ENDOPLASMIC RETICULUM CONTACTS IN NEURONAL CELLS: FROM PHYSIOLOGY TO THERAPEUTICS

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Mitochondria-Endoplasmic Reticulum contacts in neuronal cells: From physiology to therapeutics

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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"Non temete i momenti difficili, il meglio viene da lì"

Prof.ssa Rita Levi-Montalcini

"Above all, don't fear difficult moments, the best things come from them"

Prof. Rita Levi-Montalcini

ABSTRACT

Mitochondria and the endoplasmic reticulum (ER) are intracellular organelles that play vital physiological functions. Mitochondria are key players in energy production through adenosine triphosphate (ATP) production and calcium (Ca^{2+}) buffering, while the ER is involved in protein and lipid synthesis along with Ca^{2+} signalling in the cell. In the last 10 years scientists have realised the importance of intracellular organelle communication as a pivotal process for physiological functions. Among these interactions, mitochondria and ER functionally and structurally interact with each other forming mitochondria-ER contact sites (MERCS). Importantly, these structures oversee a variety of pathways including intracellular Ca^{2+} signalling. Indeed, ER to mitochondria Ca^{2+} shuttling has been shown to impact on mitochondrial respiration and bioenergetics. On the other hand, sustained increase in Ca^{2+} signalling between these two organelles can cause activation of apoptosis mediators leading to cell death. In Alzheimer´s disease (AD), cerebral hypometabolism, mitochondrial dysfunction, and functional and structural upregulation of ER to mitochondria apposition appear as early events in disease pathogenesis. Despite over 30 years of studies, the causes of AD are essentially unknown and only two symptomatic drugs have been approved for treatment, which means that AD leads to decline of quality of life and ultimately death.

In this thesis, using human brain biopsies from idiopathic normal pressure hydrocephalus (iNPH) patients, mouse models of AD and cellular models, we investigated the role of mitochondria and MERCS in synapses and exocytotic mechanism and their role in the development of pathology in AD. Additionally, we have set up a high throughput screen (HTS) to find potential modulators of mitochondrial function with the overarching aim to find drugs to target neurodegeneration.

In **PAPER I**, for the first time we have shown the presence of several organelle contact sites in human brain material and we have confirmed the presence of MERCS in human synapses. In this study we have also shown that patients suffering from dementia have more MERCS compared to non-demented patients. Furthermore, we have shown correlation of soluble Aβ levels, thought to be one of the initiators of AD, and MERCS number in iNPH patients.

In **PAPER II**, through knockdown of Mitofusin 2 (Mfn2) in SH-SY5Y cells, a negative regulator of MERCS, we have detected substantial increased juxtaposition between ER and mitochondria. Upon Mfn2 knockdown, we have observed decreased levels of cytoplasmic vesicle and increased vesicle release upon cellular depolarization. Furthermore, we have shown that this mechanism was dependent on IP3Rs activity, an important channel for Ca^{2+} transfer from ER to mitochondria.

In **PAPER III** we have characterised *in vitro* a novel knock-in model of AD, the App^{NL-F} model, which overcomes the problem of overexpressing amyloid precursor protein (APP). We have shown that embryonic cells derived from App^{NL-F} mice are capable of secreting levels of Aβ similar to adult brains, causing bioenergetics impairments, movement abnormalities along

neurites and increased MERCS functions. Furthermore, these cells seem to be more susceptible to cell death upon inhibition of mitochondrial respiration compared to WT cells.

In **PAPER IV**, we have assessed whether the other pathological protein in AD, tau, impacts on mitochondrial function and MERCS using the pure tauopathy model P301s. We detected that before tau pathology onset, at 22 days post-natal, animals displayed mitochondrial respiration dysfunctions and increase in MERCS. This pathology was sustained throughout mice life up to 10 months of age.

In **PAPER V**, setting up a HTS platform evaluating mitochondrial enhancers, we have found luteolin, a natural compound from the flavonoid family, to be capable of increasing ATP production *in vitro* in SH-SY5Y cells and primary cortical neurons, and *ex vivo* in isolated mitochondria and synaptosomes. The ATP increase shown was due to increased ER to mitochondria juxtaposition and Ca^{2+} transfer. We have further tested luteolin in Huntington's disease mutations bearing primary cortical neurons and *C.elegans*, showing improvement in respiration *in vitro* and recovery in movement *in vivo*.

In conclusion, this thesis has contributed to expand the knowledge on the role of mitochondria and MERCS in synapses and in exocytotic mechanisms. We have further shown that MERCS and bioenergetics dysfunction occur early during the pathogenic development of disease in tau and amyloid AD models. We have also provided a platform for the study of drugs in neuronal cells, revealing luteolin as a promising enhancer of mitochondrial function.

SINTESI

I mitocondri e il reticolo endoplasmatico (ER) sono organelli intracellulari che hanno importanti funzioni per la salute della cellula. Il primo organello media la produzione di ATP e il buffering del calcio mentre il secondo la sintesi delle proteine, dei lipidi e il buffering del calcio. Negli ultimi dieci anni si è iniziato ad apprezzare come questi organelli non agiscono indipendentemente nella cellula, ma interagiscono tra di loro strutturalmente e funzionalmente in siti di contatto tra i mitochondri e l'ER, chiamati MERCS. Di particolar importanza è il ruolo di queste strutture subcellulari nelle vie di segnalazione intracellulari dipendenti dal calcio (Ca^{2+}). Infatti, è stato dimostrato che il flusso di Ca^{2+} tra ER e mitochondri è in grado di attivare la respirazione mitocondriale. D'altra parte, se questo flusso di Ca^{2+} rimanendo costante può attivare meccanismi apoptotici nella cellula, causando la morte cellulare. Nella malattia di Alzheimer (AD), una forma di demenza senile, ipometabolismo cerebrale, disfunzione mitocondriale e incrementato strutturale e funzionale di MERCS sembrano essere tratti distintivi del decorso della malattia. Tutt'oggi i meccanismi che determinano la malattia sono ancora sconosciuti. Solo due farmaci sono stati approvati per mediare i sintomi, ma nessuna cura per bloccare la malattia è stata trovata nonostate gli ultimi 20 anni di studi.

In questa tesi, usando biopsie del cervello di pazienti affetti da idrocefalo normoteso (iNPH), modelli murini di Alzheimer e modelli cellulari, abbiamo cercato di capire come i mitochondri e particolarmente MERCS, siano importanti nella fisiologia sinaptica, esocitosi e nello sviluppo della patologia neuronale nelle malattie neurodegenerative come l'Alzheimer. Inoltre, abbiamo creato una piattaforma cellulare per lo studio di farmaci che potrebbero potenziare l´attività mitocondriale.

Nello specifico, nel **PAPER I** abbiamo osservato per la prima volta siti di contatto tra i mitocondri e altri organelli in boipsie del cervello e abbiamo appurato la presenza dei MERCS nelle sinapsi umane. Abbiamo inoltre visto che pazienti affetti da varie demenze dimostrano un numero elevato di MERCS rispetto a pazienti non affetti da demenza e notato come il numero di MERCS si correla con i livelli di β-amiloide, una delle possibili cause scatenanti dell'Alzheimer, in pazienti con iNPH.

Nel **PAPER II**, abbiamo artificialmente incrementato i MERCS attraverso il knockdown della proteina Mfn2, un regolatore negativo dei contatti. In questo studio abbiamo visto che aumentando il numero di questi contatti, si osserva un decremento nei livelli di vescicole citoplasmatiche e incremento del rilascio di queste vescicole. Inoltre, abbiamo visto che questo meccanismo è dipendente dall'attivita del recettore dell'inositolo trifosfato (IP3R), importante per il trasferimento di Ca^{2+} tra ER e mitocondri.

Nel **PAPER III**, abbiamo caratterizzato *in vitro* un nuovo modello knock-in di AD, chiamato *App*$^{NL-F}$, caratterizzato dall´ assenza di sovraespressione della Proteina precorritrice della beta-amiloide (APP), che appunto genera β-amiloide. Abbiamo osservato che cellule embrionali *in vitro* sono in grado di secernere β-amiloide come nei cervelli adulti, causando un'alterazione dei mitocondri, alterazioni bioenergetiche e di trasporto lungo dendriti e assoni, e dei MERCS.

Inoltre, queste cellule sono piu suscettibili a danni quando inibitori della respirazione mitochondriale sono usati, rispetto alle cellule sane.

Nel **PAPER IV**, abbiamo considerato come l'altra proteina resposabile dell´Alzheimer, tau, danneggi i mitochondri e MERCS usando il modello murino di taupatia pura P301s. Abbiamo visto che prima che la patologia dovuta a tau si sviluppasse, sin da 22 giorni le cavie dimostrino già prolemi nella respirazione mitocondriale e incremento nel numero di MERCS. Questa patologia è sostenuta durante l'invecchiamento delle cavie fino a 10 mesi.

Nel **PAPER V,** creando una piattaforma cellulare per lo studio di farmaci mirati ai mitocondri, abbiamo scoperto che la luteolina, un composto naturale della famiglia dei flavonoidi, è in grado di aumentare fino al venti percento l´ATP cellulare e attività mitocondriale *in vitro* in cellule neuroblastoma, cellule corticali primarie ed *ex vivo* in mitocondri isolati e sinaptosomi. Questo incremento in ATP è dovuto all'aumento in numero di MERCS e trasfer di Ca^{2+} tra i due organelli. Abbiamo testato la luteolina in cellue derivanti dal modello murino e c.elegans della malattia di Huntington, riportando miglioramenti nelle respirazione mitochondriale *in vitro* e miglioramento nel movimento *in vivo*.

In sintesi, questa tesi ha contribuito a sviluppare la conoscienza del ruolo dei mitochondri e MERCS nelle sinapsi e nei meccanimsi esocitotici, nella disfunzione precoce nei modelli tau e beta amilodide di questi organelli. Abbiamo inoltre fornito una piattaforma per lo studio di farmaci in cellule neuronali con la scoperta della luteolina come promettente mediatore di potenziamento funzione mitocondriale.

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I. Nuno Santos Leal, **Giacomo Dentoni**, Bernadette Schreiner, Olli-Pekka Kämäräinen, Nelli Partanen, Sanna-Kaisa Herukka, Anne M Koivisto, Mikko Hiltunen, Tuomas Rauramaa, Ville Leinonen, Maria Ankarcrona
Alterations in mitochondria-endoplasmic reticulum connectivity in human brain biopsies from idiopathic normal pressure hydrocephalus patients
Acta Neuropathologica Communications (2018) 6:102

II. **Giacomo Dentoni,** Luana Naia, Maria Ankarcrona
Increased mitochondria-endoplasmic reticulum contacts result in elevated exocytosis depending on inositol 1,4,5-trisphosphate receptor function in human neuroblastoma cells
Manuscript

III. **Giacomo Dentoni***, Luana Naia*, Nuno Santos Leal, Per Nilsson, Maria Ankarcrona
***In vitro* characterization of mitochondrial function and mitochondria-ER contact sites in primary cortical neurons derived from the *App*$^{NL-F}$ mouse model**
Manuscript

IV. **Giacomo Dentoni***, Ania Goncalves*, Stephane Duvezin-Caubet, Nuno Santos Leal, Severine Deforges, Anne Devin, Maria Ankarcrona, Sandrine Pouvreau
Early mitochondrial and mitochondria-endoplasmic reticulum contact sites dysfunction in the P301s tauopathy model
Manuscript

V. Luana Naia*, Catarina M. Pinho*, **Giacomo Dentoni**, Jianping Liu, Nuno Santos Leal, Duarte M. S. Ferreira, Bernadette Schreiner, Riccardo Filadi, Lígia Fão, Niamh M. C. Connolly, Pontus Forsell, Gunnar Nordvall, Makoto Shimozawa, Elisa Greotti, Emy Basso, Pierre Theurey, Anna Gioran, Alvin Joselin, Marie Arsenian-Henriksson, Per Nilsson, A. Cristina Rego, Jorge L. Ruas, David Park, Daniele Bano, Paola Pizzo, Jochen H. M. Prehn, Maria Ankarcrona
Neuronal cell-based high-throughput screen for enhancers of mitochondrial function reveals luteolin as a modulator of mitochondria-endoplasmic reticulum coupling
BMC Biology (2021) 19:57

* contributed eqally

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Riccardo Filadi*, Nuno Santos Leal*, Bernadette Schreiner*, Alice Rossi, **Giacomo Dentoni**, Catarina Moreira Pinho, Birgitta Wiehager, Domenico Cieri, Tito Calì, Paola Pizzo, Maria Ankarcrona
TOM70 Sustains Cell Bioenergetics by Promoting IP3R3-Mediated Mitochondria Ca^{2+} Transfer
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II. Nuno Santos Leal, **Giacomo Dentoni**, Bernadette Schreiner, Luana Naia, Antonio Piras, Caroline Graff, Antonio Cattaneo, Giovanni Meli, Maho Hamasaki, Per Nilsson, Maria Ankarcrona
Amyloid β-Peptide Increases Mitochondria-Endoplasmic Reticulum Contact Altering Mitochondrial Function and Autophagosome Formation in Alzheimer's Disease-Related Models
Cells, 2020 Nov 28;9(12):2552

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
AD	Alzheimer´s disease
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ions
CL	Cardiolipin
CoQ10	Coenzyme Q 10
CSF	Cerebrospinal fluid
CTF	APP C terminal fragment
DMSO	Dimethyl sulfoxide
Drp1	Dynamin-related protein 1
EGCG	Epigallocatechin Gallate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERMES	Endoplasmic Reticulum and Mitochondria encounter structures
ETC	Electron transport chain
FAD	Familial Alzheimer´s disease
Fis1	Mitochondrial fission 1 protein
FRET	Förster (or Fluorescence) Resonance Energy Transfer
FTD	Frontotemporal dementia
GFP	Green fluorescent Protein
GSK-3β	Glycogen synthase kinase 3 β
GTP	Guanosine-5'-triphosphate
H^+	Hydrogen ions
HD	Huntington´s disease
HTS	High Throughput Screen
IMM	Inner mitochondrial membrane
IMS	Intramembrane space
iNPH	Idiopathic normal pressure hydrocephalus
IP3R	Inositol 1,4,5-trisphosphate receptor
KCl	Potassium Chloride
KD	Knockdown
KIF5	Kinesin family member 5
LBD	Lewy body dementia
LDH	Lactate dehydrogenase
LOAD	Late onset Alzheimer´s disease
MAM	Mitochondria associated ER membranes
MAPT	Microtubule associated protein tau
MCI	Mild cognitive impairment
MCU	Mitochondrial calcium uniporter
MERCS	Mitochondria ER contact sites
Mfn	Mitofusin
MOMP	Mitochondria outer membrane permeabilization
mPTP	Mitochondrial permeability transition pore

NFT	Neurofibrillary tangles
oAβ	Oligomeric amyloid beta
OCR	Oxidative consumption rate
OMM	Outer mitochondrial membrane
Opa1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
P22	22 days Post-natal
PC	Phosphatidylcholines
PD	Parkinson´s disease
PDH	Pyruvate dehydrogenase
PE	Phosphatidylethanolamines
Pi	Inorganic phosphate
PLA	Proximity ligation assay
PMCA	Plasma membrane Ca^{2+} ATPase
PS	Phosphatidylserine
PS1 & 2	Presenilin 1 and 2
PTPIP51	Protein tyrosine phosphatase interacting protein 51
ROS	Reactive oxygen species
RyR	Ryanodine receptors
SERCA	Sarcoplasmic Ca^{2+}-ATPase
Sig1R	Sigma-1 receptor
SNARE	Soluble N-ethylmaleimide–sensitive factor attachment protein receptor
SV	Synaptic vesicle
TCA	Tricarboxylic acid cycle
TEM	Transmission electron microscopy
TIM	Translocase of the mitochondria inner membrane
TMRM	Tetramethylrhodamine methyl ester
TOM	translocase of the outer mitochondrial membrane
vAD	Vascular Alzheimer's disease
VAPB	Vesicle-associated membrane protein-associated protein B
VDAC	Voltage-dependent anion-selective channel
WT	Wild type
XeC	Xestospongin C
$\Delta\Psi_m$	Mitochondrial membrane potential

1 INTRODUCTION

”*Over the long term, symbiosis is more useful than parasitism. More fun, too. Ask any mitochondria*.” Larry Wall

This quote sums up perfectly how I would like to start my thesis, by looking into what compelled mitochondria to enter a foreign cell and establish a symbiotic relationship, and why they are so important for cellular and particularly neuronal physiology. I will further assess mitochondria´s own intracellular symbiotic relationship with other organelles such as the endoplasmic reticulum (ER) and how this interaction is affected in Alzheimer´s disease (AD) pathology. Lastly, I will investigate potential therapeutic strategies to tackle mitochondria dysfunction along with mitochondria interaction with the ER.

1.1 Mitochondria

1.1.1 Mitochondria and the cell, an endosymbiotic beginning

Thus far two competing theories have been put forward on the origin of mitochondria. The first one claims that the host cell acquiring the proto-mitochondrion was an anaerobic nucleus-bearing cell, a pre-eukaryote capable of engulfing the ancestral mitochondrion actively via phagocytosis. This first theory claims that the initial symbiotic benefit might have been due to the mitochondrion´s ability to detoxify oxygen for the cell. On the other hand, the second theory claims that the host that acquired the mitochondrion was a prokaryote and the proto-mitochondrion was able to live with or without oxygen. What might have compelled the ancestral mitochondrion to seek refuge in the host cell could be the host´s production of hydrogen ions (H^+) to be used by the mitochondrion as an indirect source of energy [1-3]. Which one among these two theories is correct remains a mystery and likely will remain such, as most of these hypothesis stem from comparative studies on modern bacteria and single cell

organisms. Regardless of their origins, the proto-mitochondria entering or being phagocytosed by the cell established a mutually favourable relationship with the host cell, referred to as endosymbiosis.

1.1.2 Mitochondria, the powerhouse of the cell

Mitochondria are double membrane-bound organelles found in the cytoplasm. Two separate membranes make up mitochondria: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). IMM is an impermeable membrane, characterised by the presence of membrane invaginations called *cristae* which significantly increase IMM surface area compared to OMM. These membranes compartmentalise mitochondria into two distinct environments: the intermembrane space (IMS) and the matrix [4, 5], depicted in Figure 1.

Mitochondria in the matrix contain numerous enzymes involved in the tricarboxylic acid (TCA) cycle as well as several copies of the mitochondrial genome, which is made up of 16 569 base pairs. Mitochondrial DNA contains 37 genes that encode for certain electron transport chain (ETC) proteins as well as the ribosomal and transfer RNAs needed for translation, but it is dependent on the nucleus for the majority of its protein supply. [6, 7]. The translocase of the outer mitochondrial membrane (TOM) complex and translocase of the mitochondria inner membrane (TIM) complex form pores in the respective membranes allowing nuclear encoded proteins to be transported into this organelle, shown in Figure 1 [8].

Cells produce energy in the form of adenosine triphosphate (ATP) by sequentially breaking down biomolecules such as sugars, fatty acids, and amino acids. [9]. In normoxia, glucose is broken down into pyruvate through glycolysis and shuttled into the mitochondrial matrix through IMM resident mitochondrial pyruvate carriers [10]. In the matrix, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase (PDH), thus entering the TCA cycle [11]. In this process enzymes generate electron donors (NADH and $FADH_2$), which give electrons to IMM-resident proteins of the electron transport chain (ETC), mediating the process called

oxidative phosphorylation (OXPHOS). The ETC consists of four protein complexes (I–IV), which through sequential redox reactions undergo conformational changes, resulting in the final donation of two electrons to ½ O_2 molecule, the final electron acceptor of oxidative phosphorylation. The proton gradient generated by complexes (I, III, and IV) which pump H^+ from the matrix to the IMS, is used advantageously by the ATP synthase, complex V, through coupling movement of H^+ with the ionic gradient and mechanical turbine-like movement of its elements, which in turns drives phosphorylation of ADP + Pi to ATP [12]. The shuttling of H^+ mediated by the complexes, the final integration of two H^+ into one O_2 molecule and the consequent establishment of the proton gradient across the IMM generates a characteristic negative potential in the matrix, called mitochondrial membrane potential ($\Delta\Psi_m$), depicted in Figure 1 [13]. This potential varies from cells and tissues (100–140 mV) and isolated mitochondria (180–220 mV), which is advantageously used in mitochondrial research to label mitochondria with positively charged fluorescent probes, such as tetramethylrhodamine methyl ester (TMRM) [14].

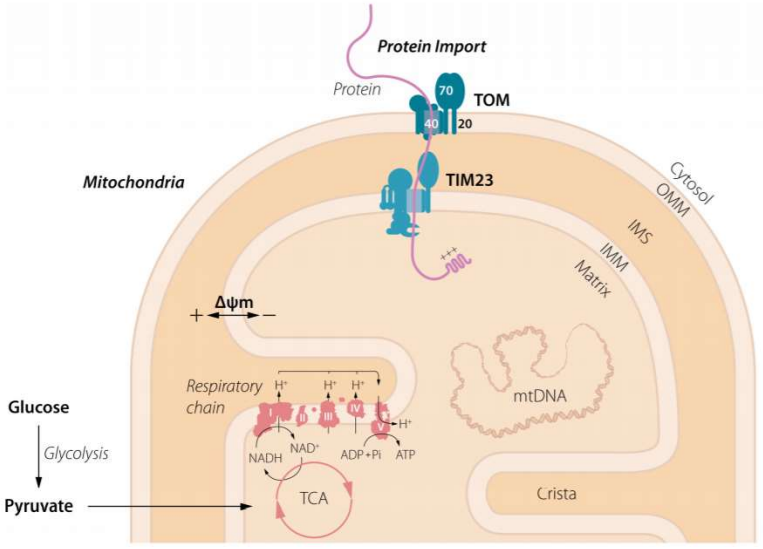

Figure 1 **Mitochondrial structure and function**. Mitochondria are responsible for a wide range of biological activities. Mitochondria have a specific DNA that is different from that found in the nucleus. However, most of mitochondrial proteins need to be imported into mitochondria. The TOM and TIM complexes direct proteins with an N-terminal mitochondria targeting sequence (MTS) to the matrix. Several of these proteins include the respiratory chain complexes, which use electron transfer, provided by the breakdown of glucose generating electron donors (NADH and $FADH_2$), to produce an electrochemical gradient, generating a mitochondrial membrane potential ($\Delta\Psi_m$), that drives the synthesis of the majority of cellular ATP.

1.1.3 Mitochondria and their calcium fluxes

Calcium ions (Ca^{2+}) are fundamental and universal signalling molecules mediating several signalling pathways. Cytoplasmic concentration of Ca^{2+} in resting cells is around 100 nM; nonetheless, different cellular signals including membrane depolarization, extracellular signalling molecules, or intracellular messengers raise the concentration of this ion from 100 nM up to 3 μM [15]. Mitochondria are pivotal regulators of Ca^{2+} dynamics in the cell by sequestering and releasing Ca^{2+}. Early studies on isolated mitochondria showed that these organelles take up a large quantity of Ca^{2+} at unphysiological concentrations (10–100 μM) [16, 17]. However, in intact cells, Ca^{2+} cytoplasmic concentration of above 3 μM triggers mitochondrial Ca^{2+} uptake [18, 19]. The inconsistency of these results, claiming different Ca^{2+} uptake by mitochondrial Ca^{2+} transporters, was solved once Rizzuto et al. reported that microdomains of high Ca^{2+} between the mitochondria and the ER, were responsible for creating high concentration within these Ca^{2+} hotspots [20], which will be discussed thoroughly in section 1.2.5. These observations explain the discrepancy between different concentrations of Ca^{2+} needed for its uptake in isolated mitochondria and in intact cells. In this organelle, Ca^{2+} is accumulated in the matrix; therefore, Ca^{2+} needs to cross both OMM and IMM.

In the OMM the voltage-dependent anion channel 1 (VDAC1), in its high conductance state allows for passage of small peptides including respiratory substrates, small peptides and molecules such as ATP, while in its low-conductance state, the channel transports only cations such as Ca^{2+}, K^+ and Na^+ [21].

The IMM is more impermeable than OMM, allowing free diffusion exclusively to O_2, CO_2, and water molecules, due to it phospholipid composition. In fact, cardiolipin (CL) is a distinct dimeric phospholipid found almost entirely in the IMM and constituting up a barrier for molecules to freely diffuse across the IMM [6, 22]. Specific transporters are required for metabolites and ions exchange across the IMM, requiring highly selective Ca^{2+} channels to

allow timely and appropriate influx of Ca^{2+} to the matrix. Several proteins have been identified in the IMM to mediate Ca^{2+} influx, presenting different kinetics. Pathways for Ca^{2+} influx include: mitochondrial Ca^{2+} uniporter (MCU) complex [23, 24], RaM [23], mitochondrial ryanodine receptor type1 (mRyR1) [24], the canonical short transient receptor potential channel 3 (TRPC3) [25], Ca^{2+}-selective conductance (mCa) 1 and 2 [26], I_{MiCa} [27], Coenzyme Q10 [28], uncoupling proteins (UCPs) 2 and 3 [29], and Letm1 [30].

The most studied among these influx channels and the primary Ca^{2+}-influx channel of IMM is MCU. MCU was characterised 10 years ago, showing high Ca^{2+} selectivity and low Ca^{2+} affinity (K_d ~ 20-30 μM) [31-33]. Interestingly, no Ca^{2+}-sensing domains have been found in the MCU channel structure and MCU complex subunits mediate Ca^{2+} influx through several regulatory mechanism [34]. MCU is a component of the MCU complex, that comprises the mitochondrial Ca^{2+} uptake machinery, a macromolecular complex consisting of: the MCU channel itself, MCUb the endogenous dominant negative subunit of the MCU complex, EMRE required for Ca^{2+} channelling activity and overseeing the attachment of other Ca^{2+}-sensing elements, MICU1 and MICU2 [35]. Each Ca^{2+} transported into the matrix results in influx of two positive charges into matrix, decreasing $\Delta\Psi_m$ and being energetically unfavourable for the cell [36] Ca^{2+} import into mitochondria is fundamental for activation of TCA cycle-related enzymes such as pyruvate dehydrogenase phosphatase (PDP) [37], isocitrate dehydrogenase (IDH) [38, 39], oxoglutarate dehydrogenase (OGDH) [38, 39] and directly modulates ATP synthase activity [40]. Taken all together this results in increased ATP production, therefore Ca^{2+} import into the matrix is ultimately energetically favourable [37].

Equally important to influx mechanisms is also efflux of Ca^{2+} from the matrix, as Ca^{2+} signalling in mitochondria seems to follow an oscillatory and flux nature rather than stationary. Mitochondrial Ca^{2+} efflux mechanisms are mediated by Na^+ dependent transport through the mitochondrial Na^+–Ca^{2+} exchanger (mNCX), being greatly expressed in excitable cells including neurons [41]. Another mechanism of Ca^{2+} efflux through the mitochondrial

H^+/Ca^{2+} exchanger (HCX) has also been described [30], however mainly being involved in non-excitable cells. In addition, the mitochondria permeability transition pore (mPTP) can also serve as a rapid Ca^{2+} efflux mechanism [42]; however, whether this channel is indispensable for physiological Ca^{2+} efflux still remains up for debate [43].

1.1.4 Mitochondria and cell death

As discussed in the previous section, Ca^{2+} has a pro-survival role by enhancing mitochondrial bioenergetics; however, on the other hand, mitochondrial Ca^{2+} uptake capacity is limited and uncontrolled upregulated Ca^{2+} in the mitochondrial matrix triggers cell death through apoptosis or necrosis. Apoptosis is a finely tuned and fundamental process of cell elimination leading to non-inflammatory dismantling of cells. In contrast, cellular and organellar swelling, plasma membrane rupture, and cellular material overflowing into the extracellular space are all features of necrosis [44, 45]. The main difference between the two processes is that necrosis is an ATP independent process characterized by mitochondrial dysfunction while apoptosis requires mitochondrial viability [46]. In the intrinsic pathway of apoptosis, cellular stressors such as DNA damage, lead to the activation of effector apoptotic molecules Bak and Bax, which form pores in the OMM, resulting in mitochondrial outer membrane permeabilization (MOMP). These pores allow for release of IMS proteins, such as cytochrome *c*, shown in Figure 4 Apoptosis panel. This protein mediates shuttling of electrons between complex III to IV and normally resides in outer part of IMM with a small portion of this protein available for mobilization [47]. Released cytochrome *c* interacts in the cytosol with apoptosis protease activating factor 1 (APAF-1) and ATP forming a complex called “apoptosome” activating procaspase-9, and consequently activating effector caspases (caspases-3, caspase-6, and caspase-7) resulting in cleavage of proteins and DNA fragmentation by endonucleases [48]. Ca^{2+} accumulation in the matrix of mitochondria can

trigger membrane permeabilization by facilitating the opening of the mitochondrial permeability transition pore (mPTP). mPTP is a non-selective, poorly characterised, high-conductance channel that activates upon oxidative stress, Ca^{2+} toxicity, increased ADP levels and ischemia/reperfusion injury. Activation of this channel results in mitochondrial depolarization, inhibition of ATP synthesis and mitochondrial swelling, often leading to OMM rupture [49]. While mPTP opening is often associated with necrosis rather than apoptosis, this view is quite reductive. In fact, cytochrome *c* can be released upon transient mitochondrial swelling, initiating the apoptotic cascade and several interactions have been observed between apoptotic mediators and mPTP [50]. Additionally to these effects, mitochondria themselves are structurally altered during apoptosis, leading to cristae remodelling and mitochondrial fragmentation at early stages of cell death [51].

1.1.5 Balancing mitochondrial network: fusion and fission

Mitochondria were originally viewed as bean-shaped cytoplasmic organelles, and many illustrations depict them as such for simplicity. However, mitochondria are highly dynamic structures, by fusing and dividing they form a dynamic interconnecting network across the cell [52]. Fusion between two mitochondria occurs when two adjacent mitochondria join, while during fission the mitochondrial membrane is pinched and a mitochondrion is split into two new ones [53]. Fusion and fission mechanisms in the cells are maintained in balance in order to retain a stable and healthy population of mitochondria [54].

The mitochondrial fusion machinery consists of three GTPases: mitofusin (Mfn) 1, Mfn2 in the OMM and optic atrophy 1 (Opa1) in the IMM [55]. Mfn´s N-terminal is a GTP-binding domain, while its C-terminal transmembrane domain is anchored to the OMM. OMM fusion is carried out by heterodimeric or homodimeric interactions between adjacent Mfns, through their hydrophobic heptad repeats domains, while GTP breakdown provides the energy

necessary to bring the two membranes together [56], depicted in upper panel of Figure 2. Opa1 also contains a GTPase domain and is anchored at N-terminal transmembrane domain to the IMM. This protein is responsible for the fusion of IMM once the OMMs of two mitochondria have fused together [57]. Homomeric interactions between Opa1 on adjacent membranes results in IMM fusion, upon GTP hydrolysis [58], shown in upper panel of Figure 2. Opa1 also regulates cristae structure independently of fusion, preventing cytochrome *c* release by regulating the opening of mitochondrial cristae [59]. Mfn1, Mfn2, and Opa1 knockouts are embryonically lethal, hence all these proteins are necessary component of mitochondrial dynamics and development [60-62].

Fission is initiated by recruitment and oligomerization of cytoplasmic protein dynamin-related protein 1 (Drp1), a dynamin-like GTPase, at the OMM forming spiral structures pinching the organelle, shown in lower panel of Figure 2 [63]. Other components of the fission machinery include four Drp1 receptors: Fis1, Mff, MiD49, and MiD51. A consensus has not been reached on the role of these receptors in Drp1-dependent fission, with fission mechanisms likely being cell-type specific [64-67]. Mitochondrial elongation is observed in Drp1 knockdown cells [68] while Drp1 knockouts are embryonically lethal [69]. Notably, Drp1 drives peroxisome fission, hence genetical manipulation of this protein may result in extra-mitochondrial effects [70].

Physiologically, these mechanisms are fundamental for mitochondrial health. Fusion contributes to the retention of critical material and allows mitochondria to cope with stressors by mixing the contents of partially damaged mitochondria, maximizing oxidative capacity in response to toxic stress [71]. Fission is necessary to generate new mitochondria; however, it also works as a quality control mechanism allowing for removal of damaged mitochondria. Although fission can initiate mitophagy, the selective process of mitochondrial degradation by autophagy, it is not sufficient to carry the process through [72].

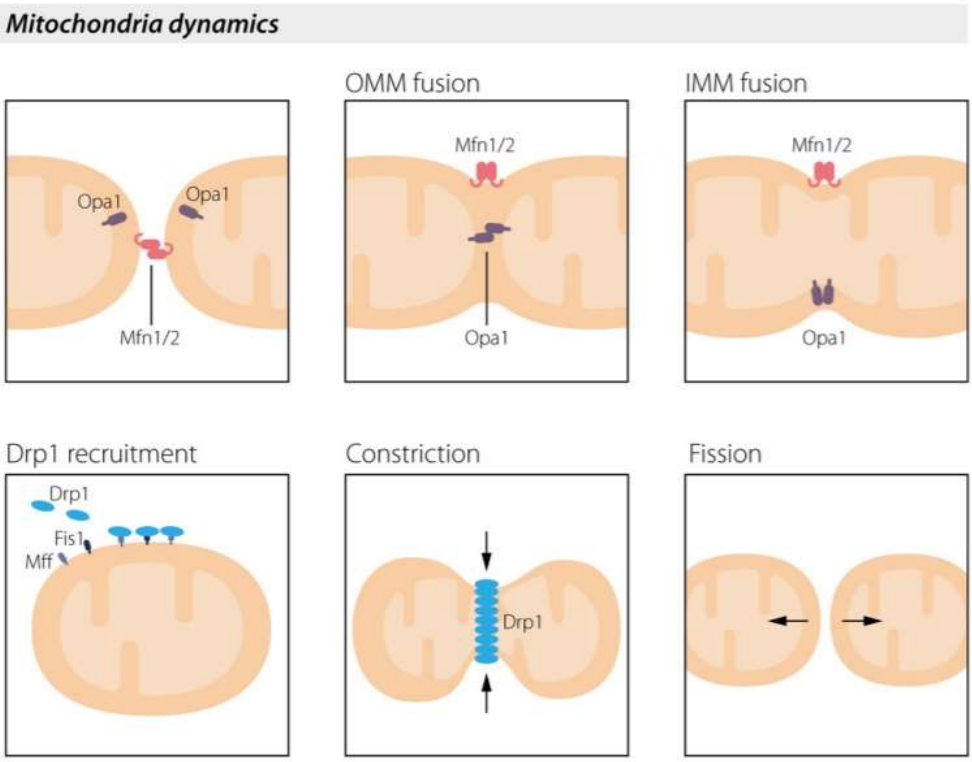

Figure 2 **Mitochondrial dynamics in mammalian cells.** The mitochondrial fusion process consists of two stages. OMM fusion is mediated by Mfn1 and Mfn2 homodymeric or heterodymeic interactions. Completion of fusion process is achieved through Opa1 with IMM fusion. In fission, the cytosolic Drp1 protein is recruited to the OMM by several receptor proteins, including Fis1 and Mff, resulting in oligomerization into ring-like structures that constrict the mitochondrial membrane, leading to generation of two distinct mitochondria.

1.1.6 Mitochondria as ROS production hubs

ETC activity is linked to the production of reactive oxygen species (ROS), due to mitochondria easy accessibility to oxygen. Indeed, mitochondria contribute to 90% of cellular ROS [73]. ETC complexes I and III are thought to contribute to most of the ROS production in the cell [74, 75]. Unpaired electrons in these complexes interact with O_2 molecules generating ROS in the form of superoxide ions ($O_2^{\bullet -}$) which are later converted in more complex ROS species, namely hydrogen peroxide (H_2O_2) and hydroxyl ions (OH^-) [76]. Mitochondria have developed protective mechanisms to counteract ROS deleterious effects such as lipid peroxidation [77] , DNA damage [78] and protein oxidation [79], by developing a highly efficient antioxidant system. On the other hand, rapid fluctuations in mitochondrial ROS are associated with several physiological pathways, making ROS ideal molecules for mediating processes such as inositol

trisphosphate-triggered Ca^{2+}signalling [80], activation of ER Ca^{2+} chnnels [81] and cell proliferation[82].

1.1.7 Neuronal mitochondria, why are they different?

The brain makes up just 2% of the body mass but requires 20% of the oxygen consumption via OXPHOS [83]. Neurons demand quite large amounts of energy to maintain synaptic activity and excitability. Most of the energy consumption goes towards ionic gradients maintenance through the Na^{+}-K^{+}-ATPases and Ca^{2+}-ATPases covering about 60–80% of ATP consumption in the central nervous system [84, 85]. Due to the limited capacity of neurons for glycolysis, they are highly dependent on OXPHOS and oxygen supply, presenting up to 1.5 fold decrease in glycolytic enzymes compared to astrocytes [86, 87]. During neuronal development neurons shift their metabolism from aerobic glycolysis to OXPHOS, with downregulation of transcriptional activators of glycolysis and enhancement in transcription of TCA enzymes and ETC complexes [88]. Nevertheless, it has been reported that several enzymes involved in glycolysis are specifically enriched in synaptosomes and blocking glycolysis inhibits endocytosis in the synapse [89, 90]. Hence, while glycolysis might not be the preferential pathway used by neurons to produce ATP, it is nonetheless needed in certain physiological mechanism in the synapse. Neurons can also use lactate dehydrogenase-1 to convert lactate into pyruvate, which can be provided by astrocytes through anaerobic glycolysis, and intercellular shuttling of these substrates [91]. Therefore, neuronal function and survival are highly dependent on optimal functioning of mitochondria.

1.1.8 Moving mitochondria to the periphery and back, a neuronal problem

Neurons are highly polarized cells with different compartments exhibiting different energy demands. Mitochondria are tightly packed in the soma and form a dynamic network throughout the neuronal compartments [92]. Neurons face challenges which are not present in other cells, as their complex morphology requires them to maintain energy homeostasis throughout their processes. Another challenge is that mitochondria biogenesis occurs mainly in the soma, thus neurons need to effectively transport these organelles to the periphery [93].

Mitochondria display complex pattern of movement, moving bidirectionally along axons, halting their progress, docking and changing direction. Mitochondria move along microtubules anterogradely or retrogradely through interactions with molecular motors [94]. The kinesin family members, particularly KIF5 are the main meditators of neuronal mitochondrial anterograde movement [95]. Other players in mitochondrial movement are adaptor proteins which mediate the attachment of mitochondria to kinesis such as Miro1 and Miro2. This protein contains a Rho-GTPase comprising two GTPase domains and two Ca^{2+} binding domains [96]. Other adaptor proteins are Trak1 and Trak2 [97]. In hippocampal neurons mitochondrial transport is mediated by Miro-Trak complex driving anterograde transport [96]. Indeed, knockdown of either adaptor protein impairs mitochondrial trafficking [97]. Retrograde transport mechanisms are less understood. It is particularly unclear which adaptor proteins are involved; however, studies show that retrograde movement is mediated by dynein [98] and is fundamental for removal of damaged mitochondria from synapses [99]. Interestingly, mitophagy has also been described to occur in distal axons [100]. Docking mitochondria near synapses and in axons is fundamental for synaptic health, and syntaphilin has been identified as a mediator of mitochondrial anchoring in axons [101]. Mitochondria are recruited to pre-synaptic terminals, by sensing intracellular Ca^{2+} rises triggered by voltage gated Ca^{2+} channels (VGCC) in synaptic boutons during continuous synaptic activity. Mitochondria disengagement from microtubules at active synapses occurs via Ca^{2+} dependent Miro sensing pathway [102-

104]. Indeed, high Ca^{2+} induces conformational change in Miro deactivating the KIF5-Trak-Miro complex. The exact logistics through which this complex disassembles remains debated [102, 104].

1.1.9 Mitochondria at the synapse: why are they there?

In the neuronal periphery, synaptic vesicle dynamics presynaptically and plasticity mechanisms postsynaptically require tight regulation of Ca^{2+} and supply of ATP [90, 105-107]. Synaptic mitochondria are located in a highly energy-demanding domain and need to orchestrate such functions spatially and temporally. Furthermore, synaptic mitochondria appear to have specialised functions and distinct protein enrichment compared to non-synaptic ones. Indeed, synaptic mitochondria display increased ETC complexes levels and activities, increase of some TCA enzymes and decreased ROS scavenging proteins [108-110]. Hence, there appears to be a distinct function of mitochondria located in distal parts of the cell compared to somatic/axonal mitochondria. These organelles are maintained in peripheral regions of the axons where ATP demand is high, particularly near the active sites where synaptic vesicles (SV) are released [111]. Mitochondria in boutons with high synaptic activity exhibit structural features adapted for intense metabolic requirements, compared to terminals with lower activity, such as higher crista membrane surface and lamellar cristae along with higher levels of the ETC protein cytochrome *c* [112]. 3-dimensional (3D) reconstruction of hippocampal synaptosomes revealed correlation between the numbers of SVs with total mitochondrial volume in selected presynaptic terminals [113].

Synaptic vesicle release requires constant supply of ATP, as it involves several energy demanding steps, including mobilization of SV close to the release site [114], exocytosis, loading neurotransmitter to SV and recycling SV mechanisms [115]. Several studies have shown mitochondrial bioenergetics' role at the synapse, and OXPHOS appears to be of vital

importance for synaptic transmission. Indeed, inhibition of ETC in neurons impaired vesicle dynamics and decreased levels of synaptic ATP [116-119]. Mitochondrially derived ATP in the presynaptic terminal is also needed for proper functioning of the Ca^{2+} ATPases, such as PMCA, with complex V inhibitor oligomycin treatment inducing impairment in Ca^{2+} clearance [120]. Accurate distribution and location of mitochondria near or around the presynaptic terminal have been shown to ATP-dependently affect SV release [121] [114] [122]. Mitochondria and ATP provision seem to be fundamental during high frequency synaptic release [90, 123]. Hence, appropriate energy balance in synaptic terminals is ideal to optimise presynaptic terminal dynamics and presynaptic activity, summarised in Figure 3.

Similarly, Ca^{2+} is of fundamental importance in the presynaptic terminal, depicted in Figure 3. Upon action potential arrival at presynaptic boutons, large amounts of Ca^{2+} flow through VGCC and increase cytosolic Ca^{2+} levels. This results in SV fusion events elicited by synaptotagmin, a Ca^{2+} sensitive protein in SV, which induces fusion between SV membranes and plasma membrane through the soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) complex [124], see Figure 3. Presynaptic mitochondria can buffer cytosolic Ca^{2+} rises, diminishing SV release probability and enhancing neurotransmission recovery. Indeed, sustained electrical stimulation results in elevation of mitochondrial Ca^{2+} transients [120, 125]. Impaired Ca^{2+} mitochondrial buffering results in prolonged cytosolic Ca^{2+} peaks after stimulation, depleting SV pool and upregulating synaptic depression [126-128]. On the other hand, mitochondria can release Ca^{2+} buffered from the cytoplasm after a depolarizing stimulus, increasing the probability of SV release [126, 129]. From this conflicting evidence, mitochondria seem to act either as enhancers or dimmers of SV release. Ca^{2+} role in presynaptic mechanisms is further complicated by its function in SV recycling via endocytosis [130]. Hence, the function of mitochondria in the presynaptic terminal has both negative and positive effects on synaptic activity and has synapse-specific and complex variations. Lastly, presynaptic Ca^{2+} peaks seem to function as a signal to increase ATP production, as previously

mentioned in section 1.1.3, as Ca^{2+} is capable of activating TCA cycle-related enzymes and ATP synthesis [37], as depicted in Figure 3. Ca^{2+} spikes in sensory neuronal cultures result in increased ATP production, likely coupling depolarization to increased ATP requirement in the synapse [131].

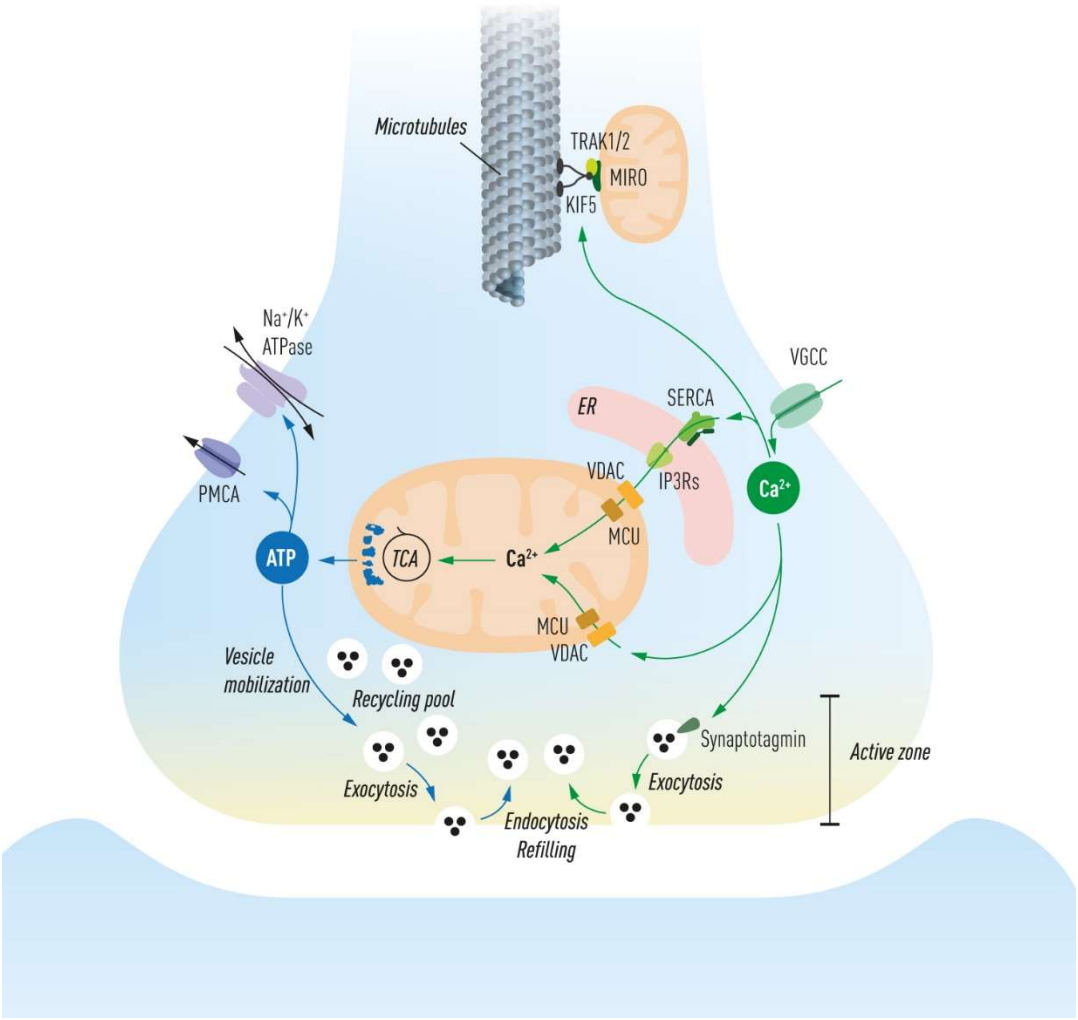

Figure 3 **Mitochondria in presynaptic terminals.** On the right highlighted in **green** we can appreciate mitochondria and MERCS Ca^{2+} mediated mechanism in the synapse, including from top to bottom, disassociation of Miro-Trak1/2-KIF5 complex leading to detachment of mitochondria near synaptic terminals, Ca^{2+} sequestration through MERCS and activating TCA cycle and ETC complexes in IMM, calcium dynamics overseen by mitochondria can activate Ca^{2+} sensitive synaptic protein synaptotagmin leading to exocytosis and Ca^{2+}plays a role in endocytosis and neurotransmitter refilling. On the left in **blue** we can appreciate mitochondrial role in ATP production through OXPHOS in IMM, leading to activation of plasma pumps that mediate ionic balance in presynaptic terminals such as the plasma membrane Ca^{2+} ATPase (PMCA) and Na^{+}/K^{+}-ATPase, furthermore ATP is fundamental for vesicle mobilization near the active zone, exocytosis and endocytosis and refilling of SV.

1.2. Mitochondria-ER contact sites

1.2.1 Contactology, a new field for organelle interaction

Long gone are the days where we thought of the cellular organelle as a singular and static organism, where metabolites were thought to transfer from one compartment to the other mainly through diffusion, active transport, or vesicular transport. The interaction of organelles is revealing a fascinating new field in cellular biology and increasing cellular physiological and pathological mechanism have been ascribed to these contacts. This new field of study has been granted a term of its own, ”contactology” coined by Csordás, Weaver and Hajnóczky [132]. Membrane contact sites are locations of close juxtaposition intracellularly where the membranes of two organelles come into contact. They present tethering regulators of proximity between two membranes, contain a specific lipidome/proteome that is different from the rest of the other membranes and, importantly, lack fusion events between the two membranes [133]. Several contacts have been described between several organelles; however, the best studied organelle contacts all involve the ER. This organelle forms contact with plasma membrane, the Golgi apparatus, vacuole/lysosome, and mitochondria mediating Ca^{2+} homeostasis and lipid shuttling. Additionally, ER interactions with vacuole/lysosome and mitochondria have been shown to mediate microautophagy and autophagosome formation, respectively[134].

1.2.2 The endoplasmic reticulum (ER)

The ER is a single membrane-bound organelle consisting of a continuous bilayer membrane system that includes flat sheets and tubular structures expanding from the nuclear envelope all the way to the periphery of the cell. There are two main kinds of ER: rough (RER) and smooth (SER). RER presents ribosomes on its cytosolic membrane surface, is involved in synthesising proteins and is found mainly around the nucleus [135]. SER is devoid of ribosomes and this organelle is found in peripheral parts of the cell and is mainly involved in lipid synthesis and

Ca^{2+} storage [136]. In mammalians, the ER has been found in virtually all components of neurons including soma, dendrites, axons and synaptic boutons [137], which plays a critical role in physiological processes in the periphery including SV release probability [138, 139].

1.2.3 Mitochondria-ER contact sites

Mitochondria need to be in contact with other organelles, including the ER, to modulate their function within the cell. Lipid raft-like domains in the ER called mitochondria-associated ER membranes (MAM) can interact with the (OMM). MAM is an insoluble lipid-raft, biochemically different from the pure ER and pure mitochondria [140]. About 20% of the mitochondrial surface is closely apposed (10- to 30-nm distance) to MAM, forming mitochondria-ER contact sites (MERCS) [141]. MERCS were first observed on electron micrographs of rat liver in the 1950s [142]. These subcellular regions, received more attention when subcellular fractions enriched in MAM, were firstly characterised as the fraction X, by Jean Vance in the early 1990s [140]. Since then, the field has developed considerably and great efforts by the scientific community has been made to characterise the mechanisms linking ER and mitochondria contact sites.

1.2.4 Linking the two membranes: MERCS tethers and contact regulators

Distance between ER and mitochondria (10 to 30 nm) allows for proteinaceous tethers to be formed. Proteolytic cleavage has been shown to untether the two organelles, validating the presence of protein bridges between the two structures [143]. In yeast, the composition of the tethering structure linking the two membranes has been thoroughly characterised containing OMM proteins Mdm34 and Mdm10, cytosolic protein Mdm12, and ER transmembrane protein Mmm1 forming together as a complex the ER-mitochondria encounter structure (ERMES)

[144]. Thus far, no ERMES homologues have been identified in mammals, and the other proteins involved in MERCS juxtaposition have been identified by assessing proximity and MERCS functional alteration upon manipulation of the studied proteins, such as Ca^{2+} shuttling between ER and mitochondria [145]. Mammalian proteins that have thus far been identified include:

Phosphofurin acid cluster sorting protein 2 (PACS2) was identified as the first mediator of ER to mitochondria juxtaposition in mammalian cells. PACS2 is not a MERCS tether *per se* as it is a cytoplasmic protein, hence it might indirectly couple ER to mitochondria. Nevertheless, knockdown of this protein decreases MERCS and Ca^{2+} shuttling from ER to mitochondria and was used in earlier studies of MERCS modulation [146].

Homodimeric and heterodimeric interactions between **Mfn1** and **Mfn2,** proteins involved in mitochondrial fusion, see section 1.1.5, were firstly described as a scaffolding bridge between ER and mitochondria [147]. Several studies demonstrated that deletion of Mfn2 gene caused decreasing mitochondria-ER interaction in several models including in cell lines [148, 149], brain [150] and neurons [151]. However, this view has been challenged by several studies, including our **PAPER II**, suggesting that Mfn2 knockdown increases MERCS apposition and Ca^{2+} shuttling between the two organelles, hinting to this protein being a negative regulator of contact formation [152-155]. These discrepancies between different studies may be due to various cell models and culturing methods used, different compensatory mechanism from loss or lack of Mfn2 and methods used to assess ER to mitochondria proximity, which have been thoroughly reviewed by Filadi et al. [156]. Regardless of its role, Mfn2 manipulation has been used and characterised as a method to modify ER-mitochondria proximity.

Another pair of proteins working as *bona fide* tethers are OMM protein **tyrosine phosphatase interacting protein 51 (PTPIP51)** and ER protein **vesicle-associated membrane protein associated protein B (VAPB)**. Indeed, knockdown of either protein results in decreased

apposition between the two organelles and overexpression yields opposite effects [157, 158]. Importantly, knockdown of either protein abolishes ER to mitochondria Ca^{2+} shuttling, while overexpression induces increased functional coupling between the two organelles [144, 158].

PDZD8, an ER transmembrane protein, has been identified through a modelling screening approach, whereby synaptotagmin-like mitochondrial-lipid-binding (SMP) domain homology between ERMES proteins and mammalian proteins was assessed. Indeed, PDZD8 SMP domain shows substantial homology to ERMES ER protein Mmm1. PDZD8 knockout or knockdown revealed substantial downregulation of MERCS apposition and Ca^{2+}shuttling in cell lines and neurons, shaping dendritic Ca^{2+} fluxes. PDZD8 mitochondrial tethering counterpart remains undetected [159].

IP3R-Grp75-VDAC bridge is a trimer comprised by inositol 1,4,5-triphosphate receptor (IP3R), glucose-regulated protein 75 (Grp75) and VDAC. Grp75 is an ER chaperone allowing close apposition between IP3R, a major ER Ca^{2+} release channel, to the OMM and VDAC [160], hence constituting the main route of Ca^{2+} transfer from ER to mitochondria. Earlier studies showed that deletion of IP3Rs does not result in changes in proximity between the two organelles [143], suggesting that their apposition to VDAC may have a purely functional role between ER and mitochondria. However, more recently, studies on IP3Rs-deficient (triple KO) DT40 cells were carried out whereby individual IP3R isoforms were rescued. From this study it appears that each IP3Rs restored ER-mitochondria proximity and Ca^{2+} shuttling in triple KO cells, suggestive of their structural role in MERCS apposition [161].

Other less studied tethering proteins have been identified in mammalian cells, including B cell receptor-associated protein31 (BAP31) and Fis1 [162], FATE1 [163] , transglutaminase type 2 (TG2) [164], and FK506 binding protein 8 (FKBP8) [165].

While several approaches have been used to assess tethering mediators of ER and mitochondria juxtaposition, limited studies have specifically targeted the proteome of these structures. To

date, three proteomic studies have been carried out on mouse brain fractions enriched in MERCS, and between 1000-2400 proteins have been identified [166-168]. A comparative study between previously mentioned datasets revealed 648 common proteins present in mouse brain MERCS enriched fraction, including previously mentioned MERCS proteins including PACS, Mfn2, VAPB, IP3R1 and all VDAC isoforms [169]. Interestingly, when comparing the proteome of MERCS-enriched fraction from brain to the proteome from other tissues and cells, such as testis, liver and NG108-15 cell line, only 18 proteins were shown to be common within these databases [169], suggesting a tissue-specific enrichment of proteins at MERCS [170].

1.2.5 MERCS functions: a physiological subcellular hub

Several proteins have been identified at MERCS working as functional rather than structural mediators of juxtaposition between the two membranes, indeed these proteins play diverse physiological roles in this subcellular domain, summarised in Figure 4.

ER to mitochondria Ca^{2+} shuttling

Functionally, MERCS have a pivotal role in Ca^{2+} shuttling from ER to mitochondria generating high Ca^{2+} hotspots (Ca^{2+}>10μM) at the interface between the two membranes, allowing the activation of low affinity calcium transporters MCU and avoiding overall cellular increase in Ca^{2+} [20]. Ca^{2+} import from ER to mitochondria is carried out through the IP3R-Grp75-VDAC bridge and has a central bioenergetic function by activating TCA cycle dehydrogenases and bosting ATP production [171, 172], depicted in Figure 4, which seems to be the preferential method for boosting metabolism compared to cytosolic calcium influx [173]. Ryanodine receptor (RyR) together with IP3R are the major extrusion channels in ER, RyR has also been localised at MERCS and, similarly to IP3R, it mediates Ca^{2+} shuttling to mitochondria; however, most of the studies thus far on RyR have been carried out in muscle cells [174]. Under- or over-shuttling of this ion and activation of these channels can have deleterious

effects. Decreased Ca^{2+} shuttling leads to a bioenergetics crisis while pathologically high levels of Ca^{2+} can promote mitophagy and apoptosis [175]. Ca^{2+} responses can be modulated by several proteins in this subcellular area. MERCS resident protein sigma-1 receptor (Sig1R) modulates IP3Rs activity through ankyrin 2 (Ank2) interaction [176]. Upon ER Ca^{2+} depletion and ER stress, Sig1R increases Ca^{2+} shuttling to mitochondria resulting in enhanced ATP production [177]. We have also shown that TOM70 acts as an ER-Ca^{2+} regulator by interacting with IP3R3 and thus mediating Ca^{2+} shuttling at MERCS, controlling bioenergetics, cell proliferation and autophagy [178]. The sarco/endoplasmic reticulum (SR/ER) Ca^{2+} ATPase (SERCA) which replenishes intracellular ER Ca^{2+} stores, has also been shown to control ER–mitochondria Ca^{2+} transfer by attenuating mitochondrial Ca^{2+} uptake during continuous Ca^{2+} release at MERCS [179]. ER Ca^{2+} modulators found at MERCS controlling SERCA activity include calnexin (CNX)[180], calreticulin [181], ERp57 [182] and the thioredoxin-related transmembrane protein (TMX1) [183].

Phospholipid shuttling, cholesterol, and lipid metabolism

MAM is a lipid raft domain enriched in cholesterol and sphingolipid as compared to the bulk of the ER, helping to stabilize protein enrichment in these membranes [184]. MERCS are also involved in the inter-organelle shuttling of phospholipids by facilitating their exchange between the two organelles [140]. Indeed, phosphatidylserine (PS) is synthetized at MAM and then transported to mitochondria OMM where it is converted into phosphatidylethanolamine (PE) [140] through a triple contact site formed with MAM and IMM [185]. PE can either stay in the IMM or be trafficked back to the ER [186]. This can then be further modified in the ER to phosphatidylcholine (PC). Furthermore, MERCS play a vital role in the metabolism of other lipids, including cholesterol and triglycerides [184]. Triglycerides and sterol esters are the main components of lipid droplets [187], which have been shown to be generated close to the ER and mitochondria interface [188, 189]. For these reasons, lipid droplet formation has been used as an indirect way to assess MERCS function [190, 191].

Initiation of autophagosome formation

Autophagosome formation has been identified as yet another function ascribed to MERCS [192, 193]. Overexpression of tethering protein VAPB or PTPIP51 increases ER-mitochondria contacts, impairing autophagosome formation; while downregulation of these proteins loosens contacts, upregulating autophagosome formation [194]. It is interesting to note that in MERCS where autophagosome biosynthesis occurs, distance between the two membranes is further spaced out (50 nm), compared to MERCS mediating Ca^{2+} and lipid transfer (10-30nm), to accommodate autophagosome formation [195]. Due to both its phospholipid exchange and formation of autophagosome membranes, MERCS appears to be an area of active membrane assembly.

Apoptosis and mitochondrial fission

Although increased apposition between the two organelles is fundamental for bioenergetics, sustained increased connectivity makes mitochondria prone to Ca^{2+} overloading, ensuing mPTP activation [143]. Furthermore, ER tethering protein BAP31 interacts with its mitochondrial partner Fis1,which together create a platform for the activation of procaspase 8 [162]; in fact, depletion of Fis1 has been shown to delay apoptosis [196].

Drp1 mediates mitochondrial division by being recruited at MERCS [197], by ER resident inverted formin 2 (INF2) [198]. This process requires polymerization of actin near constriction sites and ER to mitochondria Ca^{2+} shuttling [199]. Interestingly, Drp1 is recruited to mitochondria by apoptotic mediator Bax during apoptosis at MERCS [200]. Bax and Drp1 colocalization at MERCS coordinate the activation of MOMP, hence mechanistically linking mitochondria fragmentation to apoptosis.

ROS production and inflammasome formation

ER–mitochondria communication is also crucial for redox signalling between the two organelles. ER to mitochondria Ca^{2+} shuttling can increase ROS production in mitochondria generating MERCS ROS nanodomains [201]. Indeed, ROS can influence activity of ER Ca^{2+} channels such as RyR and IP3Rs [202], thus establishing a bidirectional communication at MERCS.

ROS production at MERCS has also been shown to induce the assembly and activation of the inflammasome [203], a protein complex formed by nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3), which translocates to MERCS upon activation, triggering, together with other proteins, the release of pro-inflammatory cytokines [204].

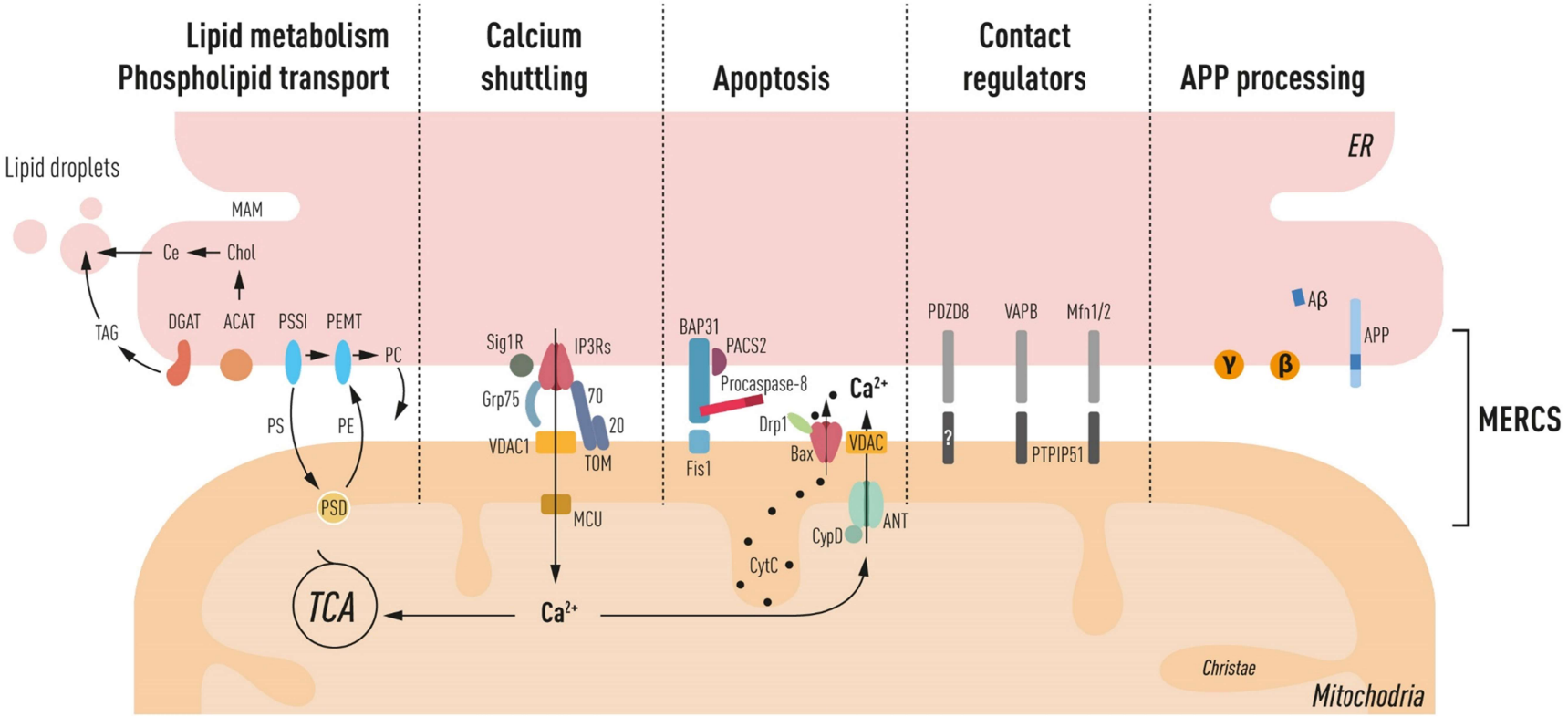

Figure 4 **Schematic representation of processes ascribed at MERCS. Lipid metabolism and phospholipid transport** PS phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; shuttling occurs through MAM, OMM and IMM triple contact sites and PSSI, PSD and PEMT mediate their synthesis, respectively. Furthermore, synthesis of cholesteryl esters (Ce) through ACAT and triglycerides (TAG) through DGAT occurs at MAM, which are the main components of lipid droplets, generated close to the ER and mitochondria interface. **Ca^{2+} shuttling** between ER and mitochondria is mediated by IP3Rs, Grp75 mediating close juxtaposition of IP3Rs to VDAC in OMM and MCU in IMM facilitates Ca^{2+} entry into matrix leading to TCA cycle activation. Sig1R and TOM70 mediate IP3Rs activity. **Apoptosis** PACS-2, a multifunctional sorting protein, by localizing to MAM regulates BAP31 interaction with Fis1, mediating a platform for procaspase-8 activation. Increased Ca^{2+} in the matrix can lead to activation of the mPTP (CyD, ANT and VDAC). Bax has also been located at MERCS and upon apoptosis induction it leads to the formation of pores and cytochrome *c* (CytC) release. **Contact regulators** several proteins have been shown to have a role in mediating ER to mitochondria apposition including PDZD8, VAPB-PTPIP51 and Mfn2 a negative regulator of MERCS **APP processing** Amyloid precursor protein (APP) has been located at MAM along with the APP cleaving machinery (β and γ-secretases).

1.2.6 MERCS in the periphery: synaptic MERCS

Very limited studies exist on the role of MERCS in neurons and synapses. Previous work in our laboratory has shown the presence of MERCS in synaptosomal preparations and throughout the neuronal arborisation [205]. A 3D transmission electron micrograph rendering study using Focused Ion Beam milling combined with Scanning Electron Microscopy (FIB-SEM) assessed the distribution of these contacts in mouse neurons, revealing their presence in all compartments of the cell including both pre- and post-synaptic terminals [137]. Similarly, we have also shown the presence of MERCS in the neuronal periphery in human brain biopsies, see **PAPER I** [206]. However, very limited studies have assessed the role of ER-mitochondria contacts at the presynaptic terminal. One of the first studies hinting at a relationship between the two structures in synapses, reported that modulating presynaptic colocalization of mitochondria and ER affected presynaptic activity and neuronal firing [207]. However, this study used actin-disrupting drugs to affect MERCS, hence unspecific effects could have ensued as actin polymerisation is a key player in synaptic vesicle release [208]. Furthermore, more recent evidence suggests that altering proximity between ER and mitochondria, through VAPB and PTPIP51 knockdown dampens vesicle release and postsynaptic spine formation [159]. This study also showed that increased activity results in upregulated MERCS apposition [159]. No mechanism has been found in these studies through which MERCS could affect exocytosis. In **PAPER II**, we studied exocytosis in a neuroblastoma cell model presenting increased MERCS and reported enhanced vesicle release, which was dependent on amplified ER to mitochondria communication and IP3Rs activity.

1.2.7 MERCS and neurodegeneration

MERCS play a vital role in a variety of cellular and neuronal mechanisms. Therefore, it is not surprising that MERCS dysfunction has been reported in a variety of neurodegenerative diseases. Although it is unclear whether MERCS actively participate in disease onset, several

lines of evidence point to MERCS and pathologically related proteins located at MERCS as playing a significant role in these disorders [141]. In amyotrophic lateral sclerosis (ALS), a neuromuscular neurodegenerative disorder that causes gradual loss of motor neurons, overexpression of TDP-43 one of the thought initiators of disease, dampens ER to mitochondria apposition via the VAPB-PTPIP51 tethering complex [158]. In Parkinson's disease (PD) dopaminergic neuronal loss and cytosolic α-synuclein aggregates are observed in patients brains; α-synuclein has been localized at MERCS and its mutant forms disrupt ER to mitochondria apposition [209]. In Huntington´s disease (HD), an autosomal dominant neurodegenerative disorder, is characterised by nuclear accumulation of mutated Huntingtin protein (Htt); Htt disrupts ER-mitochondrial interactions, resulting in dampening of Ca^{2+} signalling, in the striatum of HD mutant mice [210]. In Alzheimer's disease (AD) pathology, a neurodegenerative disordered of unknown aetiology, thoroughly described in the next section, MERCS have received particular attention by the scientific community with evidence pointing to increased ER to mitochondria apposition contributing to AD pathogenesis, see section 1.3.6.

1.3 Alzheimer´s disease

AD is the most common cause of dementia and a fatal neurodegenerative condition. The presence of intracellular neurofibrillary tangles (NFT), extracellular amyloid plaques, and widespread synaptic loss characterises AD brains. AD aetiology is unknown; however, the major risk factor for developing the disease is advancing age [211].

1.3.1 AD pathology

Familial AD (FAD) accounts for less than 1% of diagnosed cases and is caused by inheritance of autosomal mutations in three major genes: amyloid precursor protein (APP), presenilin (PS) 1 and 2. Most AD cases are sporadic, late-onset AD (LOAD), which still lack a clear genetic or environmental component [211]. The protein apolipoprotein E (APOE) is an established genetic risk factor for LOAD; indeed, high frequency of the *APOE4* allele is found in patients

with AD than in the general population [212]. Genome-wide association study (GWAS) report single nucleotide polymorphisms (SNPs) in the AD population, reporting genes involved in immune response and inflammation (TREM2), endocytosis and endosome trafficking (SORL1) and synapse function and endocytosis (PICALM) [213]. Similarities in progression of the disease have been observed in FAD and LOAD [214]; however, FAD pathology is more extensive [215-217]. Both LOAD and FAD cases have been shown to be rather heterogeneous, probably due to old age-related comorbidities which alter progression of the disease [214].

1.3.2 Amyloid hypothesis

Amyloid plaques comprise dense extracellular accumulations of amyloid β-peptide (Aβ), a peptide of variable length (37-43 amino acids). Amyloid precursor protein (APP) is a transmembrane protein that is metabolized in two opposing pathways: the amyloidogenic pathway and the non-amyloidogenic pathway, shown in Figure 5. The amyloidogenic pathway involves cleavage of APP by β- and γ-secretases on either side of the Aβ sequence to yield the Aβ peptide. This pathway is over-activated in familial AD due to missense mutations in APP and in PS1 and 2 [218], and amyloid plaque formation is believed to occur due to either overproduction of all species of Aβ (Swedish mutation KM670/671NL), overproduction of $A\beta_{42}$ (Beyreuther/Iberian mutation I716F) or increased fibrillation and impaired clearance of Aβ (Arctic mutation E693G) [219-221]. Whether plaques are directly toxic to surrounding cells remains controversial. Aβ oligomers (oAβ) appear to cause cognitive deficits, as mice injected with these oligomers scored consistently lower than wild type mice in behavioural tasks, presenting impairment in working memory [222]. oAβ have been shown to induce electrophysiological changes in neurons [223], disrupting synaptic function [224] and Ca^{2+} mediated excitotoxic cascades [225]. oAβ is therefore accepted to be directly toxic to brain cells and has been commonly used to model AD *in vitro*. Several mouse models have been developed based on the amyloid hypothesis, according to which Aβ is an upstream event in the pathogenesis of AD. Mouse models overexpressing human mutant APP develop a

neuropathology that shows similar histopathological hallmarks found in the human brain, such as amyloid plaques formation, along with cognitive impairment [226]. The novel *App*$^{NL-F}$ mouse model uses a knock-in approach, in which the Aβ sequence is humanized and the Swedish (NL) and the Beyreuther/Iberian (F) mutations are introduced into the *App* mouse gene. This approach circumvents APP overexpression artifacts and allows physiological expression of APP while altering $A\beta_{40}$ and $A\beta_{42}$ expression in the brain towards pathological ratios [227].

From the data presented, Aβ seems to have a robust negative role in neuronal pathogenesis of AD. The amyloid hypothesis assumes that oAβ induces several pathological pathways leading to tau phosphorylation and aggregation, synapses dysfunction and neuronal death, presenting as pathological hallmarks of Aβ plaques, NFTs, synapse loss and neurodegeneration, leading to cognitive impairment and dementia [228]. This hypothesis however fails to explain:

- the presence of Aβ deposition in people with normal cognitive abilities [229, 230];
- the reason why not all Down Syndrome patients develop AD pathogenesis regardless of triplication of APP gene [231];
- the lack of correlation between amyloid plaques deposition and progression of dementia, as shown in Figure 5 [232];
- why eliminating tau from APP/PS1/tau mice protects against harmful effects of Aβ accumulation, including synapse and neuronal loss [233];
- failure of clinical trials targeting amyloid such as bapineuzumab, solanezumab, and gantenerumab, and why they did not provide recovery in clinical trials [234-236].

While these observations do not categorically disprove amyloid hypothesis, they suggest that other players might have an additional role in AD progression.

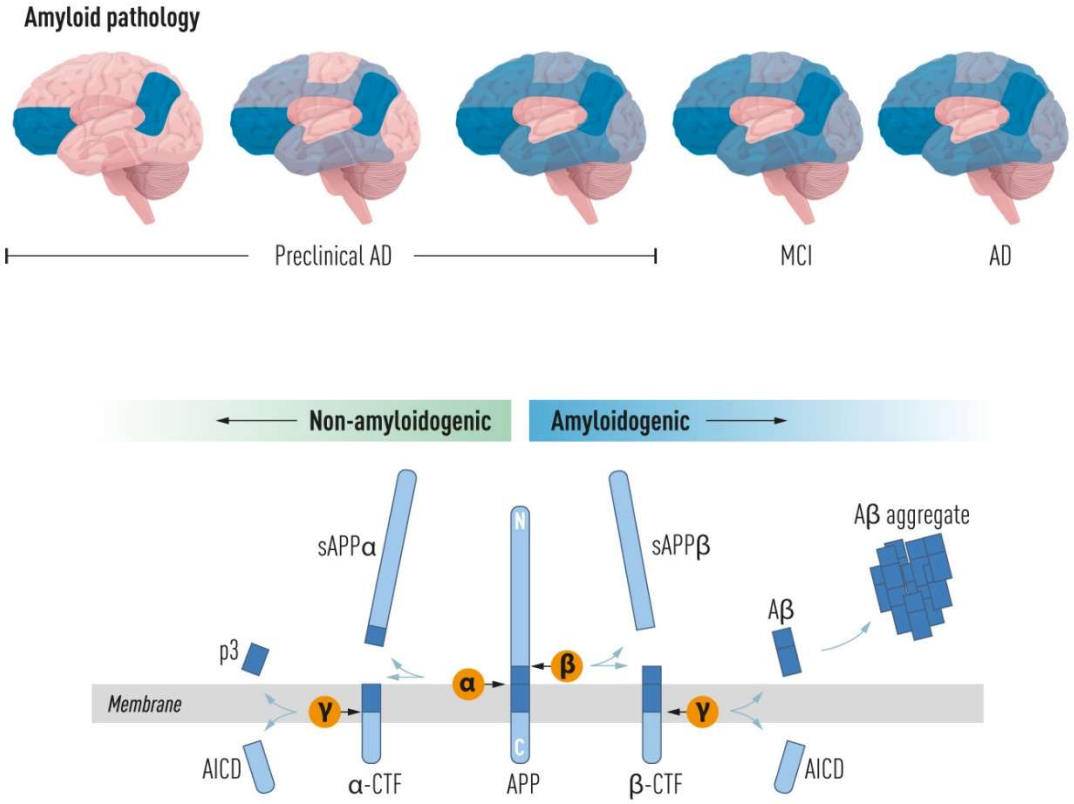

Figure 5 **Amyloid pathology in AD** Upper panner depicts spread of amyloid pathology in AD brains from preclinical AD to mild cognitive impairment (MCI) to AD. Bottom panel depicts mechanism of amyloid precursor protein (APP) cleavage by the amylogenic and the non-amylogenic pathway. In the non-amylogenic pathway on the left ,APP is cleaved by α-secretase within the Aβ sequence of APP (in dark blue) generating sAPPα and α-CTF which is further cleaved in p3 and AICD by γ-secretase. In the amyloidogenic pathway , APP is cut into sAPPβ and β-CTF by β-secretase which is further cleaved into Aβ and AICD by γ-secretase. Aβ is prone to aggregation leading to generation of Aβ-oligomers and amyloid-plaques extracellularly.

1.3.3 Tau, the bullet or the trigger in AD pathology?

NFTs are formed from paired helical filaments (PHF) made up by abnormally phosphorylated and aggregated microtubule-associated protein tau, also referred to as its gene name microtubule associated protein tau (MAPT). Tau is largely expressed in axons and it is critical for axonal development, axonal organelle and vesicle transport and for establishing neuronal polarity [237]. Tau contains a microtubule binding basic proline-rich region (155–242), which

can be abundantly phosphorylated [238]. Post-translational modification of tau through serine/threonine phosphorylation affects its binding affinity to microtubules, depicted in Figure 6 [239].

Tauopathies are a category of progressive neurodegenerative pathologies, including AD, that are characterised by the deposition of misfolded and hyperphosphorylated tau protein aggregates. AD is characterised by abnormal hyperphosphorylation and aggregation of tau and several abnormally hyperphosphorylated tau sites have been identified in AD, including AT8 Phospho-Tau (Ser202, Thr205) epitope [240]. This impairs tau microtubule stabilisation and protein trafficking to the synapse in active neurons, resulting in synaptic dysfunction and neuronal death, see Figure 6 [241]. Thus far, studies have not identified mutations in the MAPT gene in AD. Tau pathology in AD follows a specific temporal and anatomical pattern, starting from a few projecting cells in the entorhinal cortex in the hippocampus in prodromal AD leading to the spreading through limbic and neocortical areas in moderate-late stages of the disease, via established neuroanatomical connections, categorised in Braak staging, shown in Figure 6 [242, 243].

To recapitulate AD-tauopathy-like aspects of the disease, cDNA containing human mutant frontotemporal dementia with parkinsonism-17 (FTDP-17) tau has been introduced in mouse genome. P301 codon is affected by a two base transition resulting in amino acid substitution in the MAPT gene, causing P301S and P301L mutations [244]. P301S mutation adds an extra phosphorylation site to the protein, making it more prone to form insoluble fibrils. These mice show early hippocampal synaptic pathology, significant cognitive decline, and widespread tau aggregates [245, 246].

Similar problems discussed for the amyloid hypothesis arise with the role of tau in AD pathology, namely:

- Aβ has been shown to amplify pre-existing tau pathology [247, 248] and activate Glycogen synthase kinase 3 beta (GSK-3β), a major tau kinase mediating phosphorylation of tau in most serine and threonine residues [249]. Hence, it appears unclear whether tau can be an initiator for AD or requires Aβ to exert its cytotoxic effects.
- Phosphorylated tau in neurons during mammalian hibernation mediates neuronal plasticity, suggesting that tau phosphorylation might be a physiological mechanism independent from pathology [250].
- Similarly to trials applying the amyloid hypothesis, Tideglusib targeting GSK-3β, did not show any significant clinical benefit in a phase II trial [251].

It is therefore likely that tau and Aβ might work as pathological feedback loop which, together with other factors, might affect AD pathology progression.

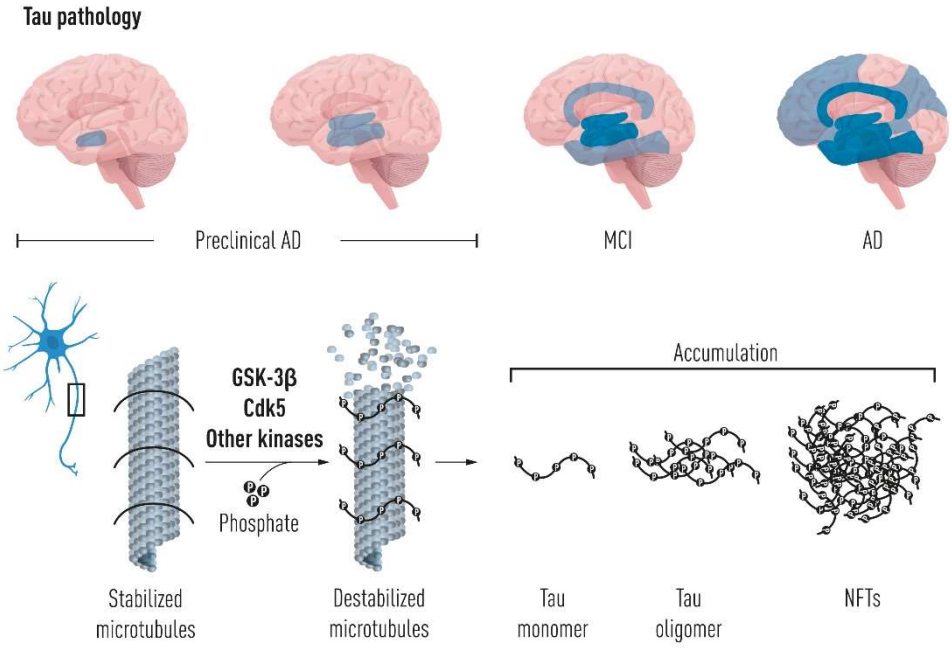

Figure 6 **Tau pathology in AD** Upper panner depicts spread of tau pathology in AD brains from preclinical AD to mild cognitive impairment (MCI) to AD. Bottom panel depicts mechanism of tau aggregation. Tau stabilizes axonal microtubules, ensuing proper axonal transport. Overactivation of kinases in AD such as GSK3β and Cdk5 induces phosphorylation and conformational changes in tau, which detaches from microtubules and leads to destabilization of microtubules. Phosphorylated tau can then aggregate generating monomers, oligomers and leading to intracellular NFTs formation.

1.3.4. Synaptic dysfunction

Synaptic dysfunction is an early feature of AD pathogenesis and greatly correlates with progression of dementia [252-254]. Synaptic proteins are severely downregulated in human AD cases [255]. Synaptic loss is seen in normal ageing; however, in AD synaptic demise seems to be exacerbated [256, 257]. This suggests that in this disorder additional mechanisms may play a role in synaptic loss. Synapse loss is increased in the vicinities of Aβ plaques in post-mortem brain tissue [258] and mouse models [259]. Tangle pathology is highly correlated to the amount of synaptic demise in AD brains [260]**.** Furthermore, neurons containing NFTs are characterized by synaptic deficits and lower levels of presynaptic proteins and mRNA [261, 262]. Therefore, tackling synaptic dysfunction early and understanding the underlying pathological mechanisms may be of fundamental importance to reverse or block the progression of the disease.

Mounting evidence supports the idea that impairment in presynaptic terminals may be a critical event in the development of AD pathogenesis. Indeed, proteomic studies point to the presynaptic terminal proteome being substantially more affected than the postsynaptic one in AD *post-mortem* brain tissue [263, 264]. Several lines of evidence *in vitro* and *in vivo* support this idea. Aβ has been shown to either enhance or inhibit vesicle release at hippocampal excitatory synapses [265, 266]. These contradictory findings were reconciled by a study that found that nanomolar incubation of Aβ causes a temporary increase in vesicular transmitter release, accompanied by a delayed decrease in the synaptic vesicular pool. [267]. Overexpression of human tau results in impairment in neurotransmitter release, presynaptic deformities and reduced synaptic vesicle pool [268, 269]. Additionally, tau binds to synaptic vesicles decreasing their mobility and release [270]. Presenilins, being ER Ca^{2+} leak channels, also control synaptic mechanisms through Ca^{2+} homeostasis. Increased synaptic release has been shown in APP/PS1, PS1 and 3xTg AD mutant mice due to enhanced ER Ca^{2+} release in presynaptic terminals [138, 271, 272]. From these data, it appears that presynaptic terminals

might present heightened vulnerability to AD pathology with presynaptic failure being a pivotal mechanism in AD pathogenesis [273].

1.3.5 Mitochondria in AD pathology

Substantial evidence points to mitochondrial dysregulation being an early event in AD pathology and having a substantial pathogenic role in animal and cell models of AD [274].

Metabolism Cerebral hypometabolism has been reported in patients with mild cognitive impairment (MCI) and AD [275, 276]. Aβ is also deposited in mitochondria resulting in dampened mitochondrial respiration [277-279]. Aβ reduces complex IV activity in AD cell models and *App*$^{Swe/Lon}$ mouse mutants [280, 281]. APP processing has been ascribed to mitochondria, which can in turn contribute to mitochondrial dysfunction [282, 283]. In neurons' periphery, synaptic mitochondria seem to be more susceptible to Aβ compared to non-synaptic mitochondria, displaying early dysfunctional activity and Ca^{2+} handling [284, 285]. Similarly, in tauopathy models, transgenic P301L mice show severe mitochondrial impairment, including dysfunction specific in complex I and V and impaired ATP synthesis [286].

Mitochondrial dynamics Changes in mitochondria morphology have been shown in AD-post mortem brains, along with changes in fusion and fission proteins expression [287, 288]. Decreased mitochondrial content and swelled, fragmented mitochondria were observed near amyloid plaques *in vivo* [289]. Furthermore, fission to fusion balance was altered in APP overexpressing models, resulting in fragmented mitochondria and perinuclear accumulation of mitochondria [290]. Aβ and phosphorylated tau interact with Drp1 leading to excessive mitochondrial fragmentation [291, 292].

Mitochondrial movement Trafficking of mitochondria to the synapse is vastly impaired in AD models and decreased number of presynaptic mitochondria has been observed in AD brains

[293]. Indeed, Aβ has been shown to impair mitochondrial trafficking and anterograde movement *in vivo* and *in vitro* [294, 295]. As previously mentioned, tau has a pivotal role in microtubule assembly and allows proper transport of organelles to peripheral regions of the cell. Phosphorylated tau inhibits mitochondrial transport by increasing the inter-microtubule distance [296] and resulting in perinuclear clumping [297]. Lack of mitochondria in the presynaptic terminal and perinuclear distribution of mitochondria is a likely cause of synaptic dysfunction [298].

Calcium Impaired mitochondrial Ca^{2+} handling has been reported in both tau and amyloid models of AD and fibroblasts derived from AD patients [299]. According to the Ca^{2+} hypothesis of AD, impairment in Ca^{2+} handling, due to increased Ca^{2+} influx into the cell along with mitochondria and ER dysfunction, leads to neuronal impairment [300]. Indeed, *in vitro* studies have reported that oAβ species induce substantial mitochondrial Ca^{2+} uptake [301]. Likewise *in vivo*, increased Ca^{2+} levels in neuronal mitochondria were observed in AD transgenic mice, due to oligomeric Aβ species and was abolished upon MCU inhibition [302]. Indeed, in AD, excessive mitochondrial Ca^{2+} uptake and Aβ cause mPTP opening, resulting in apoptosis [303], as explained in section 1.1.4.

Oxidative stress is one of the earliest pathological features in AD, often observed prior to amyloid deposition, and accumulation of ROS in mitochondria can trigger several pathological pathways [304]. Increase in ROS damage has been shown in the hippocampus and cortex of the brain during AD progression [305]. ROS derived from mitochondria can activate signaling pathways that alter APP or tau processing. Oxidative stress, for example, enhances the expression of β-secretase [306] and increases abnormal phosphorylation of tau through activation of GSK-3β [307], thus aggravating AD phenotype. On the other hand, both Aβ and phospho-tau promote ROS production [308-310]. Therefore, ROS generate a reciprocal cycle of damage promoting APP metabolism and tau phosphorylation while Aβ and phospho-tau

further induce ROS production, hence inducing synaptic dysfunction and inflammatory responses, leading to cell death [305].

Overall, these studies suggest that mitochondrial contribution to pathology is quite extensive and involves multiple pathways. The central role of these organelles in the development of the disease has spurred Swerdlow and Khan to propose the mitochondrial cascade hypothesis to explain LOAD development [311, 312]. This theory hypothesizes that mitochondrial dysfunction is the primary event that causes impaired Aβ metabolism, tau phosphorylation and synaptic loss. This is supported by studies showing that ETC inhibition results in upregulated tau phosphorylation and amylogenic pathway activation [307, 313]. Furthermore, introducing mitochondrial DNA of LOAD subjects in mitochondrial DNA deprived cells increases apoptotic mechanism such as cytochrome *c* release and caspase-3 activation [314], abnormal intracellular Ca^{2+} signalling [315] and over-secretion of Aβ peptides [314].

1.3.6 MERCS in AD pathology

MERCS dysfunction has been reported in a variety of disorder including cancer, diabetes, obesity and neurodegenerative diseases such as PD, HD, ALS and AD [316]. Seminal studies from Area-Gomez et al showed the presence of APP, γ-secretase components PS1 and PS2 in the MERCS enriched fraction of mouse brains [317]. Increased MERCS number was observed by us and others in familial AD fibroblasts and sporadic AD fibroblasts [190], primary neurons treated with Aβ [205], SH-SY5Y cells overexpressing APP [191], and hippocampus of App^{NL-F} and App^{NL-G-F} mice [318]. We have also recently shown that selective inhibition of oAβ, through scFvA13 antibody co-treatment, could revert increase in MERCS number [318]. Further evidence has been emerging connecting MERCS and AD: membrane cellular fractionation experiments on adult mouse brains reveal that Aβ is present in the MERCS-enriched fraction along with the APP processing machinery including γ- and β-secretase [317, 319], as shown in Figure 4. Unprocessed APP fragment C99, has been localised at MAM and

shown to increase MERCS activity and apposition [320]. Moreover, knockdown of contact regulator Mfn2 and consequent modulation of mitochondria-ER contacts was accompanied by decreased γ-secretase activity and Aβ production [154].

To date, limited studies have analysed the role of tau in ER-mitochondria contacts. Tau has been localized at both mitochondria and ER [321, 322]. Mice overexpressing P301L tau showed upregulated juxtaposition between the two organelles in spinal cord motor neurons [322]. Recently, truncated caspase 3-cleaved 2N4RΔC20 tau protein, induced fibrillation and seeding of wild type (WT) tau, resulted in increased ER to mitochondria proximity [321]. In **PAPER IV** we have thoroughly evaluated progression of MERCS pathology in the hippocampus of P301s animals.

Other players in AD seem to be involved in modulating contacts including PS [323] and APOE4 [324]. From this evidence, it appears that MERCS may play a substantial role in the progression of AD pathology, with most studies reporting increased ER to mitochondria contacts as a pathological phenotype in AD models. Thus far only two studies report a different trend, with decreased ER to mitochondria proximity in AD models. Lower MERCS apposition has been shown in transgenic rats overexpressing APP at 10 nm distance using a FRET system of proximity detection [325]. Accordingly, in post-mortem LOAD brain cortex using proximity ligation assay (PLA) a decreased number of ER to mitochondria apposition was detected in early to mid Braak stages [326]. These conflicting results might be due to less sensitive techniques used in these latter studies, which measure indirectly ER to mitochondria apposition, compared to aforementioned papers detecting increased ER to mitochondria proximity mostly using transmission electron microscopy (TEM).

1.4 Mitotherapeutics and neurodegenerative diseases

From the previous section, several lines of evidence point to AD pathogenic changes leading to mitochondrial vulnerability and mitochondria themselves playing a pivotal role in the progression of the disease. These observations suggest that mitochondrial dysfunction can be pharmaceutically targeted to stop the progression of neurodegeneration.

Mitochondria are affected by pharmacologic approaches acting on mitochondria directly or indirectly. Drugs can be targeted to mitochondria through lipophilic compounds driven by $\Delta\Psi_m$ [327]. As mitochondria in neurodegenerative disease tend to be depolarised, using mitochondrially targeted peptides could overcome these limitations [328]. Furthermore, liposome-based MITO-porters have also been suggested as possible ways to target compounds to mitochondria [329]. Additionally, drugs affecting upstream targets and signalling pathways to mitochondria, other organelles or nuclear factors can indirectly influence mitochondrial function.

Several classes of drugs, with different mode of action have been studied to tackle mitochondrial dysfunction, summarised in Figure 7. Antioxidants are perhaps the most studied compounds targeting this organelle and are believed to be effective in slowing down ROS mediated neurodegeneration. Coenzyme Q10 **(**CoQ10**)** was shown to prevent cognitive decline in a Streptozotocin -injected rat AD model, however the study was discontinued in humans due to low bioavailability in the brain [330, 331]. Newer generation antioxidants targeted to mitochondria such as MitoQ, has shown promising neuroprotective potential *in vitro* and *in vivo* [332, 333], and is currently being tested on MCI patients (https://clinicaltrials.gov/, NCT03514875). Compounds targeting $\Delta\Psi_m$ and inhibiting mPTP opening have also been studied, namely dimebon which inhibits opening of mPTP induced by Aβ as well as cell death [334, 335]. While some clinical studies showed improvement in mitochondrial health it was deemed overall ineffective in humans [336, 337]. Natural compounds improving bioenergetics

and overall mitochondrial health have also been used as mitotherapeutics, including resveratrol which promotes mitochondrial biogenesis restoring mitochondrial health in models of AD [338], and has shown to slow down cognitive decline and neuroinflammation in AD patients[339]. Among natural compounds in the flavonoid family, Epigallocatechin-3-Gallate (EGCG), a component of green tea, accumulates in mitochondrial matrix and improves mitochondrial functions [338, 340]. A clinical trial on ECGC oral treatment combined with cognitive training, showed improvement in cognitive function compared to patients undergoing cognitive training alone [341].

As MERCS are also emerging as viable target for neurodegeneration, recent evidence suggests that targeting ER to mitochondria apposition and function might be beneficial in neurodegeneration. Indeed, silencing studies or using artificial linkers altering ER-mitochondria tethering in mice models of obesity and type 2 diabetes [342], *Drosophila melanogaster* models of PD [343] and cellular models of reperfusion injury [344] have been shown to delay neurodegeneration and cell death. MERCS can also be modulated by compounds interacting directly with proteins located at MERCS and tethering proteins, altering either structure or function of MERCS [345]. Pharmacological modulators of the VAPB/PTPIP51 tethering complex have been identified and LDC-3, a small molecule inhibiting cytosolic dynein, was shown to increase the interaction between these tethering proteins [346]. Mfn2 agonists such as mini-peptides promote fusion in rat motor neurons [347], and could be also pharmaceutically re-directed to test alterations in ER to mitochondria proximity, Mfn2 being a negative regulator of MERCS. Molecules targeting IP3R-Grp75-VDAC Ca^{2+} shuttling complex have been identified, inhibiting or enhancing ER Ca^{2+} release or VDAC opening [345]. However, due the delicate MERCS-Ca^{2+} balance in mitochondria with over shuttling causing apoptosis and under shuttling causing bioenergetics failure, it might be a difficult pathway to specifically target. Recent evidence shows that modulating Ca^{2+} chaperones activity at MERCS with Pridopidine, a selective Sig1R agonist, prevents ER to

mitochondria disengagement and increases IP3R colocalization around mitochondria in an *in vitro* model of HD [348]. Importantly this drug was shown to have positive effect in a double-blinded clinical trial in early-stage HD patients [349]. Similarly in **PAPER V**, we have identified the flavonoid luteolin as having a role in increasing MERCS apposition and boosting ATP production in WT and HD primary cortical neurons [350].

A considerable amount of work has been done to investigate possible therapeutic strategies to target mitochondria. More recently, altering MERCS shows promising therapeutic potential for neurodegenerative diseases. In the future better understanding of mitochondria and MERCS physiology, together with improved drug delivery strategies, might lead to finding specific modulators of mitochondria and MERCS specific for the nervous system, avoiding unspecific peripheral side effects.

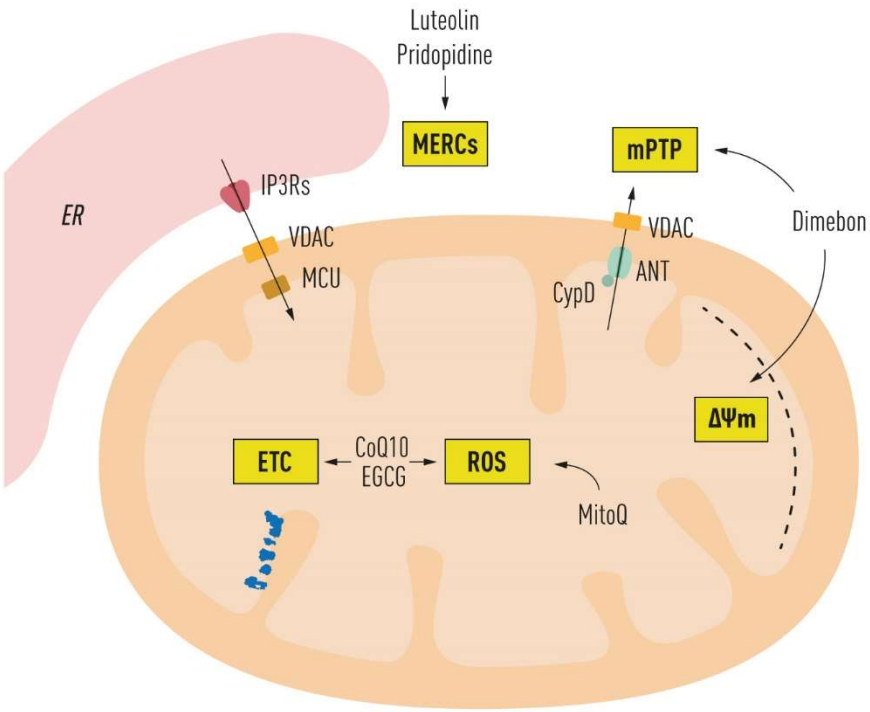

Figure 7 **Targeting mitochondria in neurodegeneration**. Several strategies have been used to rescue mitochondrial function including: mitochondrial targeted antioxidants (MitoQ, CoQ10,EGCG), enhancement of mitochondrial metabolism and electron transport chain (ETC) activity (CoQ10,EGCG), manipulation of mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial permeability transition pore (mPTP) opening (Dimebon), and altering mitochondria ER contacts (MERCS) apposition (Luteolin and Pridopidine).

2 RESEARCH AIMS

This thesis aimed to gain insight into the role of mitochondria and MERCS in physiology, AD pathology and potential therapeutic strategies tackling mitochondrial dysfunction in neurodegeneration. All studies were carried out in neuronal cells including neuroblastoma cell lines, primary cortical neurons, mouse brain cortex and hippocampus, and brain biopsies from idiopathic normal pressure hydrocephalus (iNPH) patients. The specific aims of each study were:

- **PAPER I** Assess contact sites in brain biopsy material from iNPH patients and investigate whether ER-mitochondria contacts are present in the synapse in human brains. Furthermore, we aimed to evaluate how plaques, tangles and cerebrospinal fluid (CSF) markers correlate to MERCS parameters in iNPH brain biopsies.
- **PAPER II** Ascertain the role of MERCS in affecting exocytosis in neuroblastoma cell lines and investigate potential mechanisms linking MERCS function and exocytosis.
- **PAPER III** Study mitochondria, MERCS and amyloid-related pathological pathways *in vitro* in the knock-in *App*$^{NL-F}$ mouse model of AD.
- **PAPER IV** Characterise mitochondria and MERCS dysfunction in hippocampus of tauopathy P301s mice at different stages of tau pathology.
- **PAPER V** Establish a drug discovery platform for mitochondrial drug research to boost mitochondrial bioenergetics. We also aimed to validate the compound found in this study as a mitotherapeutic in models of neurodegeneration.

3 MATERIALS AND METHODS

3.1 Human brain biopsies

Brain biopsies used in **PAPER I**, were taken from patients undergoing iNPH reversal surgery. Surgery and extraction of brain material was performed by our collaborators in Kuopio University Hospital in compliance with the Helsinki declaration and The Kuopio University Hospital Research Ethics Committee (5/2008, 19.3.2008). A small catheter is inserted in the cortical area of the brain to penetrate in the ventricle and alleviate pressure due to build-up of CSF, which leads to improved cognitive function [351]. These patients often present comorbidity with AD, presenting similar pathological hallmarks as AD [352]. 10 minutes after collection, cylinder cortical biopsies (2-5 mm in diameter, 3-7 mm in length) were placed in fixative solution (1 % glutaraldehyde and 3.7 % formaldehyde in sodium phosphate buffer). The samples were fixed for 4–14 days in fixative solution. Sections were Haematoxylin and eosin stained to assess p-tau and Aβ staining. Samples were stratified on dementia diagnosis and according to presence of amyloid plaques and NFTs. Fixed samples were then processed for ultrathin sectioning, see Electron microscopy.

Table 1 **Characteristics of biopsy samples**

	Patient #	*Gender*	*Age*	*Comorbidities*	*MMSE*
Aβ- Tau-	1	F	75	NI	22
	2	F	76	NI	23
	3	F	77	NI	25
	4	M	75	NI	19
A+ Tau-	5	M	86	LBD/VaD	13
	6	F	79	NI	24
	7	M	79	NI	19
	8	F	71	NI	20
	9	F	76	NI	23
AB+ Tau+	10	F	78	NI	23
	11	F	77	NI	28
	12	F	74	NI	24
	13	M	79	AD/VaD	12
	14	F	81	AD	15

3.2 Animal models and primary cortical cultures

Animal models were used to collect brain material or to derive embryonic primary cortical cultures. All experimental procedures were carried out in compliance with the guidelines of the Institutional Animal Care and Use of Committee and the European Community directive (2010/63/EU) and procedures approved by the "Regionala Etikprövningsnämnden" and "Linköpings djurförsöksetiska nämnd" (Regional Ethics Review Board, authorization no. S53–14, ID407 and 12,779/2019), by the Faculty of Medicine, University of Coimbra (authorization no. ORBEA_189_2018/11042018), by the Italian Ministry of Health (authorization no. D2784.N.HEH, 03/07/18) and by the University of Bordeaux (approval number 10137).

In **PAPER IV** we used heterozygous P301s mice, a tauopathy model, which exhibits hippocampal synaptic dysfunction and synaptic loss at 3 months of age and present NFTs pathology at 6 months. By 8 months mice have developed neuronal loss and brain atrophy, primarily in the hippocampus but spreading to other brain regions such as the neocortex [246].

For *in vitro* studies, primary cortical cultures were prepared from WT (**PAPER IV and V**) or *App*$^{NL-F/NL-F}$ (**PAPER V**) mouse embryos. Mothers were sacrificed and pups were collected. Embryonic cortices were then dissociated and plated in poly-D-lysine coated plates. Neurons were kept in neurobasal medium supplemented with B27 and 2 mM L-glutamine. Half of the culture medium was replaced every week with freshly supplemented Neurobasal medium.

3.3 Cell models

SH-SY5Y cells were used in **PAPER II and PAPER V**. The neuroblastoma SH-SY5Y cell line is a widely used cell model of human origin showing neuron-like features. They present a variety of vesicular proteins, and two types of secretory vesicles. Furthermore, these cells are capable of releasing and synthesizing neurotransmitters and neuropeptides such as neuropeptide Y [45]. When SH-SY5Y cells are differentiated, through retinoic acid and Brain-derived neurotrophic factor, as we did and characterized in **PAPER V,** cells obtain mitochondrial characteristics extremely similar to primary cortical neurons. The American Type Culture Collection supplied neuroblastoma SH-SY5Y cells, which were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum. For differentiation protocol see **PAPER V.**

3.4 Synaptosome preparation

Synaptosomes, containing both pre- and post-synaptic sites, are artificial structures with an average diameter of 0.5–1 μm which are frequently used in research for studying synaptic transmission and release mechanisms. They are formed through homogenization of neuronal tissue in isotonic solution, resulting in rupture of nerve terminals from axons forming spherical like structures when its lipid bilayer reseals, holding up all its intracellular components [353]. Adult mouse forebrain was homogenised in Syn-PER reagent (Synaptic proteins extraction reagent, Themofisher, 10 mL per gram of tissue) using a Dounce grinder on ice with 10 strokes before centrifugation at 1200 x g for 10 minutes at 4 °C. The supernatant was separated and centrifuged at 15,000 x g for 20 minutes at 4 °C to produce a pellet containing our crude synaptosomal preparation. Pellets were resuspended in 1 mL of Ca2+-containing Hanks' Balanced Salt Solution (HBSS) and stored on ice before protein quantification.

3.5 Electron microscopy and image analysis

Several methods have been assessed to analyse contacts. While advances have been made in imaging and in microscopy resolution, the golden standard for analysing ER-mitochondria proximity (10-30nm) remains transmission electron microscopy (TEM). While this technique is the best in terms of resolution, it lacks 3D spatial resolution and might underestimate the extent of interacting structures.

In **PAPER IV** where we aimed to assess MERCS and mitochondria morphology *in vivo*, anesthetised animals were perfused with 2% glutaraldehyde and 1% formaldehyde in 0.1 phosphate buffer solution through intracardial perfusion. Brains were stored in fixing solution until right hemispheres were cut in a brain slicer matrix and coronal slices collected for ultrathin sectioning.

In **PAPER II, III** and **V** cells or synaptosomes were washed twice in PBS (or HBSS for synaptosomes), and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffered saline. Formaldehyde was not used as it is a weaker fixative and it is used in tissue to permeate quickly to the core of the sample, while glutaraldehyde is a fast but impermeable fixative hence is used in monolayer preparations.

Leica Ultracut UCT was used to create ultrathin sections, and uranyl acetate and lead citrate were used as contrasting agents. Sections were examined at 100 kV using a Tecnai 12 BioTWIN transmission electron microscope. Digital images were acquired at 26,500 x magnification in all studies. 5-10 cells per sample were imaged and images acquired were analysed using the freehand tool in ImageJ. Mitochondrial number, perimeter, MERCS number and length were quantified per cell. When the distance between the ER and the mitochondria was less than or equal to 30 nm, contacts were considered as MERCS [154]. The number of MERCS per mitochondria was calculated by dividing the total number of MERCS by the number of mitochondrial profiles. Percent of mitochondrial perimeter in contact with ER was analysed by multiplying MERCS length per 100 times divided by

mitochondrial perimeter. This we believe allows us to normalize MERCS number per the total mitochondrial number, as changes may occur in mitochondria fission/fusion dynamics, while the percentage of mitochondria in contact with ER provides an indication of overall MERCS length.

3.6 Mitochondrial techniques

Seahorse: The oxygen consumption rate (OCR) is the primary indicator of mitochondrial respiratory activity. Complex IV is the ETC's sole oxygen user, and O_2 is the ETC's final electron acceptor. Different respiratory states can be isolated and mitochondrial parameters can be assessed using appropriate pharmacological manipulation [354]. All studies were carried out using a e XF96 Seahorse flux analyser. In **PAPER II**, SH-SY5Y cells OCR analysis was performed in sodium bicarbonate and phenol red free DMEM supplemented with L-glutamine 4 mM, D-glucose 25 mM, pH = 7.4, at 37 °C without CO_2. In neuronal preparations used in **PAPER III and V** OCR was performed in unbuffered DMEM media supplemented with 10 mM glucose, 0.23 mM pyruvate and 2 mM L-glutamine (pH 7.4). Baseline OCR was measured followed by sequential injection of the following drugs: oligomycin 1 μM, FCCP 1.5 μM, and Antimycin A 0.5 μM with rotenone 0.5 μM. OCR values were reported according to the Seahorse XF Cell Mito Stress Test (Agilent) and values obtained in pmolO_2/min were normalized to total amount of protein.

ATP luciferase: Total cellular ATP can be quantified using bioluminescence-based probes such as luciferase systems. The CellTiter-Glo® Luminescent Cell Viability Assay was used to measure total ATP levels according to the manufacturer's instructions. Mitochondrial complex III inhibitor antimycin A was used as a negative control, decreasing total ATP cellular content.

TMRM: TMRM is commonly used as an indirect indicator of $\Delta\psi_m$. TMRM is a membrane-permeant, positively charged fluorescent probe which accumulates in the negatively charged

mitochondrial matrix and is used as an indirect measurement of $\Delta\psi_m$ [354]. In **PAPER V**, SH-SY5Y cells were incubated with TMRM in non-quenching conditions (5 nM), and fluorescence intensity analysed as a measure of $\Delta\psi_m$. In **PAPER III and V** in neurons, TMRM was used in quenching conditions (150 nM). In these experiments, the retention of TMRM by mitochondria was measured in these experiments to predict changes in $\Delta\psi_m$. A microplate reader was used to record basal fluorescence, and FCCP and oligomycin were used to maximise mitochondrial depolarisation leading to mitochondrial probe release. TMRM release was measured as changes in fluorescence after oligomycin/FCCP addition. Protein content was used to normalise the data.

3.7 MERCS functional readouts

Calcium imaging: As ER Ca^{2+} signals to mitochondrial matrix are fundamental for a variety of physiological mechanism, assessing changes in this pathway upon changes in ER-mitochondria proximity is crucial to confirm any morphological changes at MERCS [156]. Neurons were loaded with 2 μM Rhod2-AM probe, which due to its positively charged AM protein is capable of permeating into cellular membranes and particularly into the negative matrix of mitochondria. The Ca^{2+} sensitive form of Rhod2 is liberated in mitochondria by mitochondrial esterase cleaving the AM group. Rhod2 fluorescence was recorded in a confocal microscope through 1s time-lapse recordings. The application of 0.1 mM ATP plus 0.1 mM bradykinin induced IP3 receptor-mediated Ca^{2+} release from ER. Mitochondrial Ca^{2+} fluctuations were then calculated as a ratio of Rhod2 fluorescence after stimulation to baseline fluorescence.

Neutral lipids staining: MERCS are essential in the metabolism lipids, such as cholesterol and triacylglycerol [170]. Triacylglycerol and sterol esters are the primary components of lipid droplets [173], which have been observed to generate nearby ER and mitochondria [174, 175]. Hence, lipid droplet formation has been used to assess MERC function in an indirect

manner [176, 177]. 1X HCS LipidTOX™ Red Neutral Lipid Stain was usued in primary cortical neurons to assess neutral lipid staining according to manufacturer´s instructions and imaged in a Carl Zeiss LSM880 inverted confocal microscope. Single cells per fields were acquired, threshold set and grey intensity measured using ImageJ program

3.8 SynaptopHluorins (SypHy)

SypHy are indicators of presynaptic activity that contain a GFP-based pH sensor (pHluorin) linked to a vesicle protein such as synaptophysin-1. GFP-pHluorin is auto-quenched when exposed to the lumen of synaptic vesicles (pH 5.5). During exocytosis the GFP part of synaptophysin is exposed to the extracellular fluid (pH 7.4) and increases its fluorescence. Hence, this method was used to evaluate release of vesicles and has been validated both in neurons and cell lines[355, 356]. See **PAPER II** for further details on analysis.

3.9 Western blotting

In **PAPER II, III and V** Cells were lysed in equal volumes of RIPA buffer with 1x proteinase, 1x phosphatase inhibitors, and benzonase solution consisting of 50 mM Tris, 4 mM MgSO4 and 1x benzonase. Homogenates were centrifuged for 10 minutes at 10,000 rpm and supernatant stored at -20C.

In **PAPER IV** P301s hippocampi were manually homogenized with a Teflon pestle in lysis buffer (Hepes (1M), NaCl (1M), Glycerol 10%, DDM 0.05% with 1xphosphatase and 1xprotease inhibitors). Homogenates were centrifuged for 10 minutes at 10,000 rpm and supernatant stored at -20 C.

The Pierce^{TM} BCA Protein Assay was used to determine the protein concentration of the samples.15-25 µg of protein were run on 4–12% Bis-Tris gels and transferred to a nitrocellulose membrane. Membranes were blocked with 5% powdered milk in TBS-T, then

probed overnight at 4 °C with appropriately diluted primary antibody in blocking solution. Blots were washed and incubated for 1 hour at room temperature with fluorophore-coupled secondary antibodies. The Odyssey® Infrared Imaging system was used to visualise proteins, and Image Studio Lite 5.2 was used to analyse band intensity, which was normalised to the respective loading control (actin), and data was presented as a percentage of the respective controls.

3.10 Lactate dehydrogenase (LDH) release assay

LDH Assay is commonly used to study cellular cytotoxicity. When the plasma membrane is disrupted, such as during necrosis, LDH a cytosolic enzyme is released into the cell culture medium. LDH assay (#G1780, Cytotox 96®assay, Promega) was used according to the manufacturer's instructions. Maximum LDH release was assessed by incubating cells with 10 µL of Lysis buffer, used as a positive control.

3.11 $A\beta_{40}$ AND $A\beta_{42}$ ELISA

Aβ, one of the thought initiators of AD, has been shown to be abundantly secreted by AD-mutation bearing cells *in vivo* and *in vitro*. Aβ secreted in media can subsequently influence receptors and channels in the plasma membrane of adjacent cells hence propagating Aβ mediated dysfunction [357]. Enzyme-linked immunosorbent assay (ELISA) systems for quantification of $A\beta_{40}$ and $A\beta_{42}$ are the most sensitive methods used in AD research for quantification of these pathological peptides compared to WB. Amyloid 1–40 (IBL, #16340) and Amyloid 1–42 (IBL, #16233) Assay Kits were used according to the manufacturer's instructions.

3.12 β- AND γ- secretase activity

Secretases in the amyloid pathways are involved in the cleavage of APP generating pathologically relevant peptides. The β-site APP Cleaving Enzyme (BACE1) and γ-secretase complex (comprising presenilins (PS), nicastrin, anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2)) have been shown to be overly active in AD brains and in AD models of the disease, indeed PS1 mutations in AD are often thought to result in gain of toxic function, increasing APP cleavage [358]. We thus evaluated the activity of each secretase in **PAPER V**.

β-Secretase Activity Assay Kit (Sigma-Aldrich, # 565785), was used according to the manufacturer´s instructions. Active β-Secretase and β-Secretase inhibitors were used as positive and negative control, respectively.

γ-Secretase activity was measured indirectly by assessing inhibition of its activity by treating neurons with 10 μM γ-secretase inhibitor L-685,458. Through ratiometric quantification of extracellular $A\beta_{40}$ or $A\beta_{42}$ levels with DMSO and L-685,458, we extrapolated changes in γ-secretase activity in our samples.

3.13 Statistics

Data was analysed using GraphPad Prism 8.00 (GraphPad Software). Samples were compared by a non-parametric independent test (Mann–Whitney U-test) when compared as pairs. Multiple sample analysis was performed using Kruskal-Wallis test, followed by Dunn´s multiple comparison test. When existent outliers were evaluated using the ROUT (Q=1%) method and eliminated. All values were expressed as mean ± standard error of the mean (SEM), n = corresponds to number of independent experiments or number of individual measurements, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Values were considered statistically significant when $p \leq 0.05$.

4 RESULTS & DISCUSSION

This section describes the main findings from the five constituent papers contained in this thesis and discusses main results found. Further details on results and further discussion points are included in the respective papers and manuscripts.

4.1 PAPER I: Alterations in mitochondria-endoplasmic reticulum connectivity in human brain biopsies

As previously mentioned, organelles interact with each other to sustain their functions [133]. Several contact sites have been identified in a variety of cells; however, very limited studies have assessed these contact sites in neurons and none, were carried out in human brain material. Here, we have observed several mitochondria-contact sites in human brain biopsies, including OMM interaction with plasma membrane, nucleus, Golgi apparatus, lysosomes and ER, with MERCS being the most widespread contacts detected in this human brain material. Importantly, we found the presence of MERCS in both pre- and post-synaptic terminals for the first time in human brain material, depicted in Figure 8. These observations formally reveal the presence of MERCS in human tissue and confirm their presence and relevance to study these structures in human brain material. Indeed, MERCS have been localised in synapses in mouse brain tissue and modulation of these contacts seems to affect exocytosis [137, 359]. Further studies should clarify the role of these structures in human material using neurons derived from induced pluripotent stem (iPS) cells.

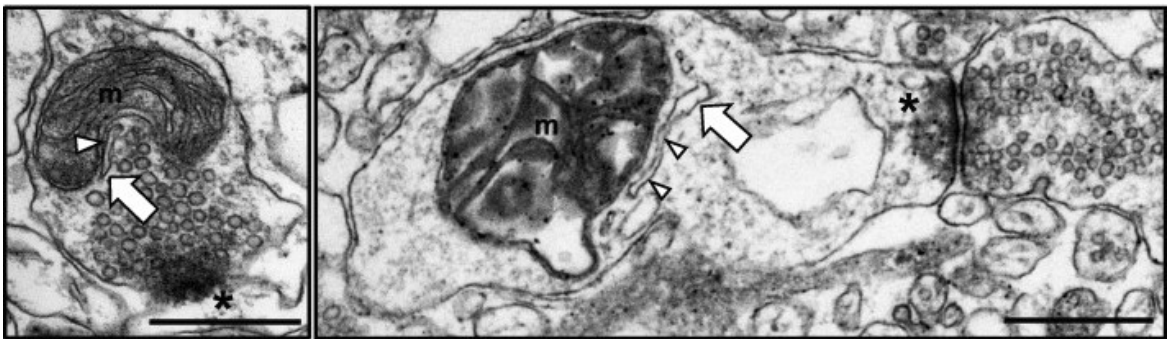

Figure 8 **Electron micrographs of MERCS in presynaptic terminals in human brain biopsies.** Thick arrows show smooth ER and small arrow points shows MERCS. Scale bar 500 nm

iNPH patients, from which the biopsies used in this study originate, often present co-morbidity with AD [352], hence we sought an opportunity to assess MERCS in samples from patients diagnosed with dementia and presenting AD pathogenic hallmarks. Quantitative TEM analysis revealed that number of MERCS per cell were significantly increased in demented patients (Lewy body dementia (LBD), vascular AD (VaD), AD/VaD or only AD; n=3) compared to non-demented patients (n=11). Furthermore, we discovered a strong correlation between the number of MERCS and the ventricular levels of soluble $A\beta_{42}$, which supports data from AD models [190, 205, 318].

However, when we stratified data for presence or absence of amyloid plaques and NFTs, the major hallmarks of AD, we did not see any changes in number of MERCS per cells. Nonetheless, we detected a significant downregulation of MERCS length in biopsies containing both amyloid plaques and neurofibrillary tangles ($A\beta^+/tau^+$) compared to subjects with no AD-related pathology ($A\beta^-/tau^-$), while no changes were seen in subjects with $A\beta^+/tau^-$ samples. Similarly, recent data in *post-mortem* LOAD cortex has shown decreased ER to mitochondria apposition in Braak stages III-IV, while Braak stage VI remained unaffected, using proximity ligation assay [326].

Caution should be taken when interpreting our results as brain material studied had a primary diagnosis of iNPH and not AD; furthermore, we could not differentiate the type of cells imaged in this study. Nevertheless, the study is unique in detecting MERCS in patients diagnosed with dementia and correlating changes in MERCS structure to the presence of pathological hallmarks of AD including amyloid plaques, soluble Aβ in ventricles and NFTs in fixed human brain material. Of note, this study using brain biopsies minimised artefacts induced by *post-mortem* delay as material was quickly fixed post-extraction, giving us a reliable picture of MERCS in the brain. Future studies on post-mortem FAD and LOAD brain tissue should clarify how MERCS progress throughout pathology in patients whose primary diagnosis is AD.

4.2 PAPER II: Increased mitochondria-endoplasmic reticulum contacts result in elevated exocytosis depending on inositol 1,4,5-trisphosphate receptor function in human neuroblastoma cells

MERCS are intracellular hubs regulating several cellular functions including Ca^{2+} shuttling, bioenergetics, phospholipid exchange and autophagy [141]. An underexplored aspect of MERCS physiology is their contribution to exocytosis. Recently, the hypothesis that MERCS structure and function may impact on exocytosis and neuronal communication has been gaining attention, due to their fundamental role in ATP biogenesis and Ca^{2+} buffering, which are crucial mediators of vesicle release [360, 361]. As we mentioned in **PAPER I**, MERCS are present in the presynaptic terminal in human biopsies; however, it is not clear how MERCS influence exocytosis and through which mechanisms.

We firstly tested whether increasing MERCS apposition in neuroblastoma cell line SH-SY5Y could affect vesicle release. As previously reported by us and others [152-154], we saw increased ER to mitochondria apposition upon Mfn2 knockdown (KD), while ATP production, bioenergetics, and cell viability were not affected, suggesting that Mfn2 works as a negative regulator of MERCS in our model. We then tested whether increased ER to mitochondria juxtaposition could affect exocytosis. We found substantial downregulation of vesicular proteins in Mfn2 KD cells, including synaptophysin and synapsin-1, as well as a downregulation of releasable factors such as neuropeptide Y. This data was corroborated by TEM analysis revealing substantial depletion in the vesicular pool. We reported increased vesicle release in Mfn2 KD cells upon KCl mediated depolarization, according to previous findings [207]. Additionally, SNAP25 levels in Mfn2 KD cells, a SNARE complex component, were shown to be significantly upregulated. Hence, these results suggest that increased release mechanism might be responsible for decreased vesicle content, as seen during synaptic depression and exitotoxicity [362], summarised in Figure 9.

As ER to mitochondria communication is upregulated upon Mfn2 KD [153, 154], we sought to pharmacologically block ER to mitochondria Ca^{2+} shuttling by inhibiting IP3Rs Ca^{2+} release, through Xestospongin C (XeC) treatment. XeC is a potent, cell permeable IP3R inhibitor that has been used as a tool to investigate mechanisms depending on Ca^{2+} transfer at MERCS [194, 350]. Abolishing IP3R activity normalized synaptic vesicle protein levels and vesicle release when ER and mitochondria were closely apposed in Mfn2 KD cells. Hence, MERCS Ca^{2+} shuttling seems to be fundamental for exocytosis and this mechanism is dependent on IP3Rs activity.

Further studies should pinpoint the exact mechanism through which MERCS affect exocytosis. We suggest that MERCS might affect propensity of vesicles to be released through either local ATP or Ca^{2+} upregulation around release sites. While this study was carried out in neuroblastoma cells it has great translatable significance for neurons, and future research should confirm that these mechanisms are also relevant in neuronal preparations. Additionally, this study suggests that increased IP3Rs activity and MERCS connectivity could be tackled in diseases where excitotoxicity is abundant, such as in AD.

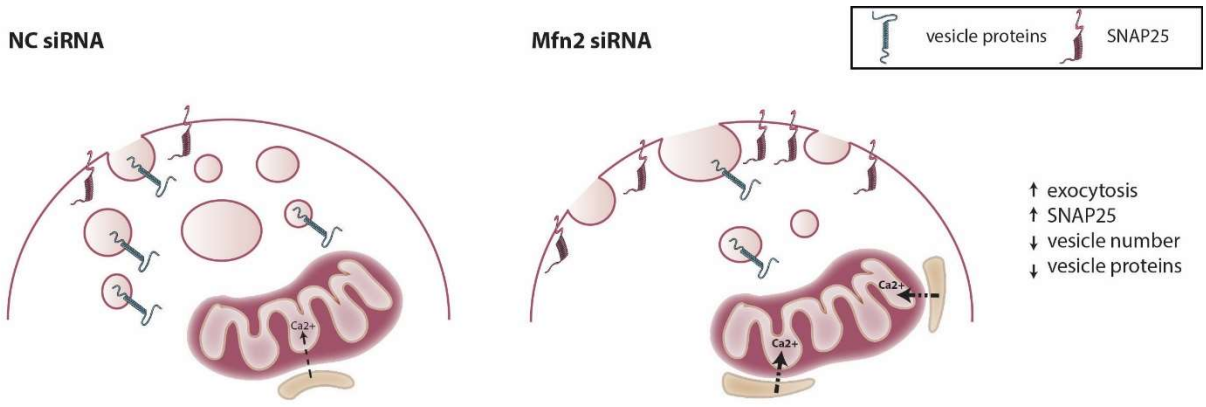

Figure 9 **Graphical abstract of PAPER II**. KD of Mfn2 leads to upregulated ER to mitochondria juxtaposition, decrease number of cytoplasmic vesicles and associated proteins, while increasing SNAP25 levels and exocytosis compared to NC siRNA treated cells.

4.3 PAPER III: In vitro characterization of mitochondrial function and mitochondria-ER contact sites in primary cortical neurons derived from the *App*$^{NL-F}$ mouse model

In **PAPER I** we have identified a correlation between MERCS and soluble Aβ in iNPH patients. While this gave us an indication of MERCS being affected by these pathological hallmarks of AD, we sought to identify the specific contribution of Aβ to mitochondria and MERCS dysfunction prior to plaque deposition, using an *in vitro* model of the disease. Alzheimer´s research has relied on mouse models overexpressing human mutant APP; however, APP overexpression artifacts are quite common in AD models [363]. The *App*$^{NL-F}$ mouse model allows for physiological expression of APP while altering the $A\beta_{42/40}$ ratio expressed in the brain [227]. Thus far no one has thoroughly characterised this animal model *in vitro*. We thus decided to assess in mature primary cortical cultures derived from *App*$^{NL-F}$ mice several pathological, mitochondrial, MERCS and synaptic parameters.

We have shown that mature *App*$^{NL-F}$ primary cortical neurons present increased $A\beta_{42/40}$ ratio and increased secreted $A\beta_{42}$, similarly to adult brains, and can be used as a model to dissect out Aβ-mediated mechanism prior to extracellular amyloid aggregation. Higher mitochondrial respiration capacity was found in *App*$^{NL-F}$ cells compared to WT cells, unlike previously reported in APP overexpressing models [280, 281]. Concomitantly, our data suggests that glycolytic flux is impaired in knock-in neurons, indicative of early damage in bioenergetics as shown in overexpressing models [364]. Furthermore, we found an increased number of MERCS per mitochondria and percentage of mitochondrial perimeter in contact with ER in *App*$^{NL-F}$ cultures, with related functional increase in lipid droplet formation and Ca^{2+} shuttling. Increased MERCS might be an initial cellular compensatory mechanism to sustain cellular activity by increasing ATP production in this model. In fact, upregulation of MERCS was observed to restore locomotor activity in $A\beta_{42}$ overexpressing flies [365]. However, we also observed that *App*$^{NL-F}$ cells seem to be more susceptible to LDH release upon induction of mitochondrial stress, through ETC complex III inhibition, and being uncapable of handling

mild stressors compared to WT cells. Indeed, sustained increase in MERCS can have deleterious effects for the cell eventually leading to apoptosis [143]. As shown by these data, increased MERCS might lead to susceptibility to cell death mediated by mitochondria, likely through increased vulnerability to mPTP activation. Overall, these studies support the hypothesis that mitochondria and MERCS are affected early in AD; however, whether these changes are beneficial or harmful remains unanswered.

Furthermore, we detected an impairment in mitochondrial transport along neurites. In fact, *App*$^{NL-F}$ neurons displayed a substantial upregulation of stationary mitochondria and associated decrease in moving mitochondria, particularly evident in anterograde transport. Decreased anterograde transport indicates that mitochondria might not reach synaptic terminals, leading to synaptic dysfunction. Accordingly, we saw decreased levels of vesicle protein synaptophysin, suggestive of alterations in vesicle release. Hence, this data supports the idea that impaired mitochondrial function and increased MERCS might impact on mitochondrial movement and consequently synaptic health, as recently seen in Drosophila's neuromuscular junction [366].

Overall, these studies suggest that early mitochondrial and MERCS dysfunction is present before plaque formation and is an important hallmark in the development of the disease. Future studies should address how modulation of MERCS through genetic or pharmacological interventions can improve overall AD phenotype.

4.4 PAPER IV: Early mitochondrial and mitochondria-endoplasmic reticulum contact sites dysfunction in the P301s tauopathy model

Limited studies exist on the role of MERCS in tau pathology. The research to date has focused mainly on the effect of aberrant tau on MERCS in peripheral tissue [321, 322]. In **PAPER I** we have shown decreased MERCS length in subjects with AD-related pathology compared to AD-hallmarks negative biopsies. Here, we specifically investigated the effects of tau on MERCS in the hippocampus using the P301 pure tauopathy model, overexpressing human mutant tau [246]. Thus far, to our knowledge, no study has assessed in brain tissue and particularly in hippocampus, how tau pathology progression time-dependently affects MERCS and mitochondria. The hippocampus is an important structure in AD and it is affected by NFTs deposition early in disease course, hence our interest in this region.

Firstly, we assessed hippocampal tau phosphorylation burden using AT8, a phosphorylated tau antibody. Interestingly, even if human tau is expressed at 22 days post-natal (P22) in P301S mice, hyperphosphorylation of tau only appears at 6 months and progresses between 6 and 10 months. We thus divided samples as pre-AT8 staining (early pathology) P22, mid-AT8 staining (mid pathology) at 6 months of age and advanced-AT8 staining (late pathology) at 10 months of age. As previously reported in advanced NFTs pathology in transgenic tau models [286], we report an early impairment in oxygen consumption rate, both in coupled and uncoupled mitochondrial respiration, starting at P22 being sustained until 10 months of age. Complex IV activity was decreased in the hippocampus of transgenic animals only at later stages of the disease, suggesting that progressive damage to ETC might lead to impairment of complex activity. Additionally, as shown in peripheral tissue, we have observed an early upregulation of juxtaposition between ER and mitochondria, along with a process of mitochondrial fragmentation which progressively worsened as disease progressed in the CA1 area of the hippocampus. Later pathological changes were also associated with reduced TOM70 levels in P301s mice, a protein mediating protein import at mitochondria and working as a IP3Rs

modulator at MERCS [178]. Nevertheless, most mitochondrial and MERCS protein levels were unaffected in this model.

Here, we have identified impairment of mitochondrial respiration and an upregulation of MERCS in the hippocampus as early events in tauopathy pathology development; however, further work is required to establish whether these two mechanisms are connected in this model. It is possible that early deficiency in OCR might result in MERCS-mediated compensatory mechanisms to boost ATP supply, which as pathology progresses might result in sustained mitochondrial Ca^{2+} influx leading to cell death. Indeed, progressive decrease of OCR and MERCS Ca^{2+}-mediated damage could underlie the alterations of hippocampal synaptic function and activity in late pathology. Hence, pharmacological rescue of mitochondrial function and decrease in ER to mitochondria apposition may be a potential therapeutic strategy to implement in neurodegenerative tauopathies.

4.5 PAPER V: Neuronal cell-based high-throughput screen for enhancers of mitochondrial function reveals luteolin as a modulator of mitochondria-endoplasmic reticulum coupling

As AD and neurodegenerative disorders are often characterised by brain hypometabolism and reduced mitochondrial activity [367], finding a drug to boost ATP production could be used as therapeutic strategy to tackle neurodegeneration. Hence, we established a cell-based assay for high throughput screening (HTS) using differentiated neuroblastoma SH-SY5Y cells to search for mitochondrial activity modulators. A pilot screen using the Prestwick library with 1200 FDA approved compounds was performed. Significantly increased ATP level with no increase in cell death was reported with 61 compounds. After further validation 9 compounds were subjected to dose response curve-dependent selection and a single molecule, the flavonoid luteolin, was identified as capable of significantly boosting cellular

ATP up to 20% compared to DMSO. We further validated this drug in neuronal tissue, using primary mouse cortical neurons. We showed that 2.5 μM luteolin increased both basal and maximal respiration and oligomycin-sensitive ATP production, and total cellular ATP from 6-16 h of treatment, while not affecting ROS production, mitochondrial area, structure, nor nuclear-dependent mitochondria biogenesis. By preincubating cells with XeC, an IP3R inhibitor, luteolin ATP-boosting effect was abolished through decreased Ca^{2+} shuttling from ER to mitochondria. Indeed, luteolin-treated neurons presented increased Ca^{2+} ER to mitochondria transfer, leading to increased Krebs cycle activity measured through increased NADH levels and increased PDH dephosphorylation, depicted in Figure 10. Similarly, in *ex vivo* tissue, mouse cortex synaptosomes acutely incubated with luteolin presented increased ATP production, which was abrogated by XeC treatment. Furthermore, luteolin can act on isolated mitochondria by enhancing complex I and complex II ETC activity. Considering these positive mitochondrial boosting effects, we sought to assess luteolin´s therapeutic potential in HD models, which show decreased bioenergetics and mitochondrial dysfunction [368]. Luteolin appeared to have a neuroprotective effect in HD models by enhancing respiration in mouse primary cortical neurons and increasing movement in *C.elegans.*

These findings support luteolin's function as a mitochondrial activity enhancer in neuronal models and show that we have established a robust drug screening platform in differentiated neuroblastoma cell lines that can be used for mitochondrial drug development in the central nervous system. Luteolin has been shown to have antioxidant properties in a variety of studies [369] though we could not reproduce these effects in our models, our results feature luteolin as a MERCS modulator and ER to mitochondria Ca^{2+} transfer enhancer. We suggest that luteolin could be used as one of the few drugs discovered thus far showing effects on this subcellular hub. Furthermore, the ATP boosting effect of luteolin could be used to target disorders where dysfunctional MERCS and bioenergetics mechanisms occur. Indeed, diseases such as PD, HD and ALS which present hypometabolism and reduced ER to

mitochondria apposition, may benefit from luteolin´s MERCS boosting effect. It would be interesting to show in disorders such as AD, where decreased mitochondrial function but increased MERCS are observed, if luteolin may boost ATP production and stabilize ER to mitochondria interaction. It is worth noting that increasing ER to mitochondria apposition has been shown to be neuroprotective in an AD model of *Drosophila melanogaster* [365]. While our data suggests a neuroprotective effect of luteolin on HD models, future studies should thoroughly address the neuroprotective activity *in vitro* and *in vivo* of this compound in various models of neurodegeneration.

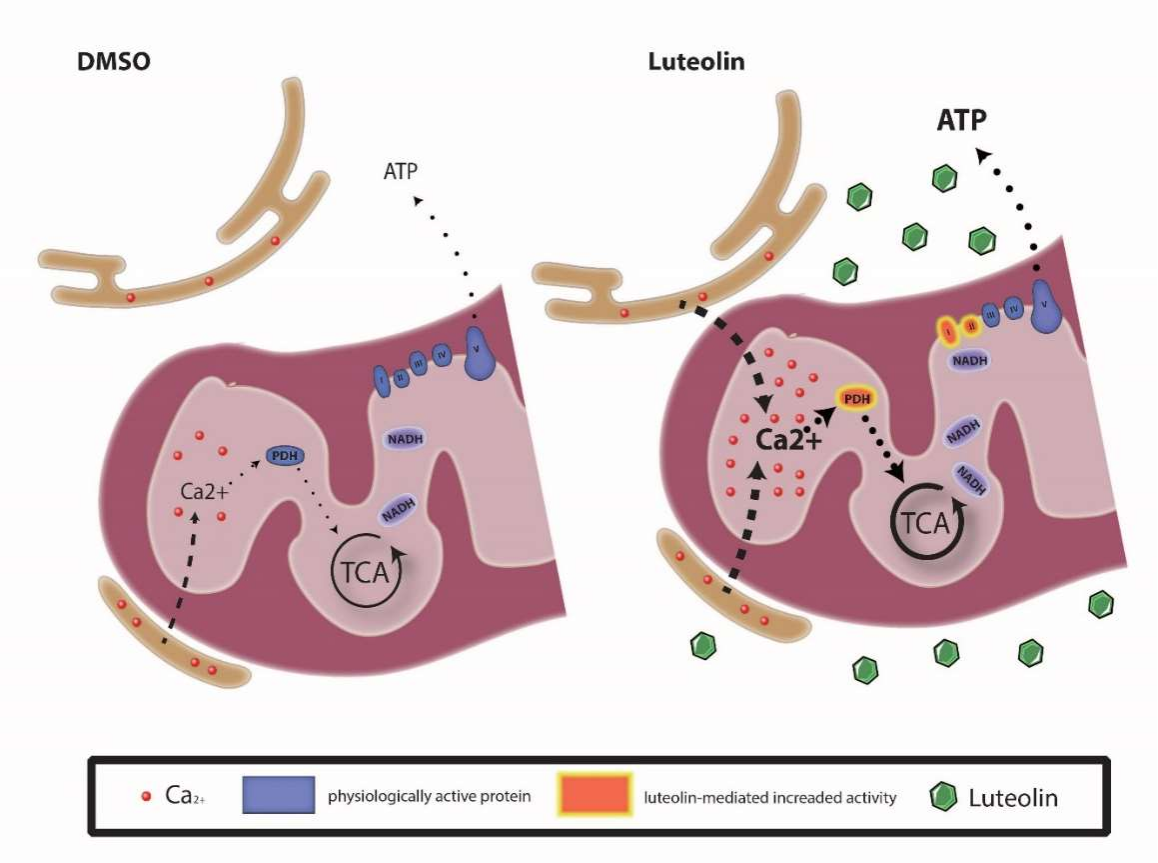

Figure 10 **Graphical abstract of PAPER V**. Luteolin leads to upregulated ER to mitochondria juxtaposition, increase in Ca^{2+} shuttling leading to increase in PDH activity through its dephosphorylation, increasing TCA cycle activity and resulting in increased NADH levels. This consequently results in increase complex I and complex II activity resulting in upregulated overall ATP production.

5 CONCLUSIONS & FUTURE PROSPECTIVES

Overall, this thesis has increased the knowledge in the field of mitochondria and specifically MERCS in both physiology and AD pathology, and we have provided an HTS platform for drug studies in which one compound with promising neuroprotective potential has been identified.

The physiological role of mitochondria at the synapse has been extensively studied, including these organelles´ role in buffering Ca^{2+} and ATP production in axonal boutons [360]. However, conflicting results have been reported on the role of mitochondria in the distal processes of neurons. Up to 50% of synapses contain mitochondria in axonal terminals [370], hence suggesting that mitochondria-free synapses might present different release characteristics. In mitochondria-free synapses exocytosis seems to be carried out through glycolysis [370], further complicating our understanding of the role of these organelles in synapses. Mitochondria-generated ATP has been shown to diffuse from axons to the synapses to sustain synaptic transmission [371]; while on the other hand synapses containing mitochondria, once ETC was blocked behaved similarly to those without mitochondria, sparing presynaptic Ca^{2+} homeostasis [121]. The role of mitochondria in synaptic dynamics is further complicated by their buffering Ca^{2+} properties. Mitochondrial Ca^{2+} clearance was firstly attributed to their ATP provision activating the plasma membrane Ca^{2+} ATPase (PMCA) [372]; however, recent evidence points to mitochondrial Ca^{2+} handling being fundamental in synaptic health [360]. Up to 40% of Ca^{2+} is taken up by these organelles in rat sensory synapses modulating SV releae, as shown by MCU inhibition upon synaptic stimulation [373]. Furthermore, as previously mentioned, Ca^{2+} influx in mitochondria increases ETC activity, identifying this ion as a master regulator of bioenergetics in the presynaptic terminal [131]. An overarching problem of these studies is that data is extrapolated from mammalian and non-mammalian cortical neurons and neuromuscular junction, thus different presynaptic pathways may occur in these distinct

models. In **PAPER I and II** we have revealed an extra layer of complexity to synaptic transmission by showing that mitochondria interact with ER in the presynaptic terminals in human brain biopsies and MERCS mediated Ca^{2+} shuttling are important contributors for vesicle release in neuroblastoma cells. As MERCS are starting to be considered important players in synapse physiology [360, 361], it would be fundamental to establish the percentage of presynaptic terminals containing MERCS, whether axonal MERCS can exert their effects to nearby synaptic boutons, and if MERCS are formed at the synapse for basal transmission or exclusively during sustained synaptic activity to couple Ca^{2+} buffering and influx, to ATP provision. Furthermore, while our and others' studies have addressed MERCS exocytosis overall role in the cell, it would be fundamental to assess selectivity the role MERCS at the synapse. Optogenetic tethering complexes have been engineered [374], which could allow to locally and time dependently increase ER to mitochondria juxtaposition and assess vesicle release, ATP and Ca^{2+} dynamics in presynaptic terminals. While in our hands this plasmid was incompatible with neuronal transfection, next generation optogenetic tethering regulators combined with super-resolution imaging could address this question.

Regarding the pathological aspects of AD in the studies presented in this thesis, one problem facing AD research is that despite over 30 years of studies investigating basic mechanisms and over 100 trials tackling amyloid, no substantial advances have been made on therapeutic progress of the disorder. Furthermore, the sequence of events leading to AD is virtually still unknown. Are the classical pathological species of Aβ and tau the initiators or simply a result of other underlying pathological mechanism in AD? While in FAD the mechanism leading to dementia appear more understandable, in LOAD questions remain whether the same mechanism as FAD apply. More studies should concentrate on FAD vs LOAD differences and assess which mechanisms initiate the disease in LOAD patients-derived cells and brain materials. While the role of both Aβ and phosphorylated tau on neurotoxicity is undeniable, it might be a good time to consider alternative theories explaining AD pathogenesis. AD research

is slowly shifting towards considering the disorder as a multifactorial disease, characterised by different types of microdamage and different factors that lead towards the last decades of one's life to cognitive decline [375]. The Ca^{2+} and mitochondria theories of AD, previously described in the introduction, have emerged as plausible alternative hypothesis to the amyloid one. It is worth noting that one of the few symptomatic treatment used, memantine, an NMDA receptor antagonist, tackles Ca^{2+}-mediated pathways such as excitotoxicity [376]. While this does not give automatic plausibility to these hypotheses, it nevertheless gives some food for thought. Another problem worth addressing in AD research is the animal models used. While overexpression models have provided new mechanistic understanding of Aβ and tau mediated dysfunction, they do not mimic the whole neuropathology of disease, lacking the typical neurodegenerative process observed in AD brains. In fact, humans do not overexpress APP, PS1 or MAPT genes, therefore newer approaches need to be sought to avoid neurotoxicity due to protein overexpression. While in **PAPER III** we have addressed the positive aspects of knock-in mouse models, some limitations using these animals also arise including: the long progression of pathology might be problematic for past-paced research, introducing different mutations such as the Swedish and Beyreuther/Iberian mutations, which do not present together in humans might introduce artifacts in the AD phenotype observed in these animals, and the Swedish mutation by increasing CTF-β might lead to downstream artifacts [377]. Regardless of these limitations, knock-in murine models provide the best alternative that resembles human pathology developed thus far.

MERCS are slowly emerging as central subcellular hubs in the pathogenesis of AD. MERCS play a vital role in a variety of mechanism that seem to be affected in AD including bioenergetics, Ca^{2+} handling, Aβ metabolism, mitochondrial dynamics, mitochondrial transport, exocytosis, inflammation, autophagy and apoptosis. Furthermore, we have shown in **PAPER III and PAPER IV** that in both tau and amyloid models increased apposition of MERCS presents early in pathology progression, suggesting that it might be a fundamental and

pathologically relevant mechanism in disease. Some scientists have ventured to hypothesise that increased MERCS and increased MAM function are pivotal and might be the initiator of AD, known as the MAM hypothesis of AD [378]. An interesting experiment to confirm these speculations would be to selectively increase MERCS in areas of the brain mostly affected by AD, such as the hippocampus, with an inducible synthetic linker and assess how this might recapitulate AD related phenotype, including pathological hallmarks, cell death and cognitive impairment. MERCS in AD is a relatively recent field and while extensive research has been carried out in the last decade to understand the basic mechanism and nature of MERCS, several questions remain withstanding. Is increased ER to mitochondria apposition observed in AD models a compensatory mechanism in the cell to avoid bioenergetic crisis or a damaging event slowly leading to apoptosis? Could increased MERCS be an early compensatory mechanism gone wrong? While Aβ and tau seem to have a role in increasing juxtaposition in most AD models, another aspect that would be pressing to understand is how Aβ and phosphorylated tau interact with MERCS. Can these pathological hallmarks interact with tethering proteins at MERCS or indirectly through protein expression or MERCS-directed signalling pathways? Target validation studies could shed light on such questions. Another issue arising in this field is whether different tethering proteins may be selectively affected in neurodegeneration, generating a diverse phenotypical presentation of diseases. While it has been suggested that different types of MERCS could exist in the cells depending on MERCS distance [348], it is not yet known if specific tethers are involved in specialised MERCS functions or whether MERCS are homogeneous within a cell and mitochondria. Furthermore, studies thus far have considered MERCS as a static entity; however, these structures have been shown to be quite dynamic disassembling and changing depending on the bioenergetics requirements of the cells [195]. Lack of studies on the subject might be partially due to high resolution needed to assess these structures and lack of suitable and reversible constructs to visualize ER to mitochondria proximity. In future

investigations, MERCS dynamics in neurodegeneration should be focusing on time dependent alterations of these subcellular hubs and introducing MERCS dynamics as another aspect in MERCS physiology and pathology, additionally to number and length of MERCS.

Presently only symptomatic therapies are available to AD patients. These drugs do not alter the progression of the disease, meaning the condition ultimately results in worsening of life quality for patients and their families. This limitation is due to our lack of knowledge of this multifactorial disorder. As argued in the previous sections and in the constituent papers of this thesis, both impairment in mitochondrial function and ER to mitochondria apposition could be tackled pharmacologically to improve neurodegenerative disorders. Mitotherapeutics are underrepresented in drug research, with less than 25% of clinical trials targeting mitochondrial dysfunction and very limited number of compounds reaching phase III [379]. Hence, establishing robust screening methods targeting mitochondria is fundamental in drug discovery to boost mitotherapeutics reaching clinical trials. Drug screening in primary neurons [380] and differentiated neuroblastoma cells such as ours in **PAPER V** are of fundamental importance for drug discovery targeting mitochondria. This combined with *in vivo* validation, provides a relevant platform for mitotherapeutics in brain disease research. Flavonoids, such as luteolin identified in **PAPER V**, have promising therapeutic potential in neurodegeneration and in AD. Several *in vitro* and *in vivo* studies have reported positive effects of luteolin on reducing ROS production [381], restoring ATP levels [381], increasing synaptic markers expression [382], reducing pathological hallmarks of AD [383, 384] and restoring cognitive functions [385]. A recent clinical trial assessing the effect of luteolin on memory in healthy subjects was started in January 2020 but halted due to COVID-19 pandemic (https://clinicaltrials.gov/, NCT04468854), hence this compound is currently being considered for treatment of neurodegenerative disorders in humans. However, flavonoids are present in low amounts in dietary sources, some of them, such as luteolin, are greatly unstable depending on temperatures and light condition and show low intestinal absorption and bioavailability in the brain.

Furthermore, the effect required for neuroprotection in the brain would require supplementation of extremely high doses of luteolin which in the periphery, where it is better absorbed, might lead to cytotoxic effects, making this compound particularly tricky for human testing [386]. Improving drug delivery to the brain, better understanding of mitochondria and MERCS physiology and substantial increased investment in mitotherapeutics might provide us with neurodegeneration modifying drugs in the future.

According to World Health Organization (WHO) figures for 2020, AD and other dementias are the seventh leading cause of death worldwide and the second leading cause of death in high-income countries, preceded only by ischemic heart disease. However, AD and dementias research is vastly underfunded compared to other research fields. According to Alzheimer´s Foundation UK, 2% of total medical research charity spending in 2016/17 went towards dementia research. Thus, it is not surprising that with such limited budget, no drug has been identified to rescue AD. Nearly 35.6 million people are estimated to live with dementia globally according to WHO. Worryingly, this figure is projected to double (65.7 million) by 2030 and more than triple by 2050 (115.4 million). This is due to increased life expectancy and health advances, which have resulted in a significant increase in the elderly population globally, especially in low- and middle-income countries. It is expected that more than 70% of patients diagnosed with dementia will be living in low- and middle-income countries by 2050 (WHO). Hence, a strategic rehaul of research and spending priorities together with increased dementia awareness campaigns should be carried out globally to invest on these diseases. Programs supporting healthy ageing and improving support and care of elderly people affected by dementia and their families should be given a priority in health spending in the next decade.

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