CARBON MONOXIDE AGAINST CEREBRAL ISCHEMIA: ROLE OF MITOPHAGY, MITOCHONDRIA AND CELL METABOLISM

CLÁUDIA FIGUEIREDO PEREIRA

Tese para obtenção do grau de Doutor em Envelhecimento e Doenças Crónicas

Doutoramento em associação entre:

Universidade NOVA de Lisboa (Faculdade de Ciências Médicas | NOVA Medical School - FCM|NMS/UNL)

Universidade de Coimbra (Faculdade de Medicina - FM/UC)

Universidade do Minho (Escola de Medicina - EMed/UM)

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Ao meu avô.

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ABSTRACT

Cerebral ischemic stroke is the third cause of death in west countries and the first in Portugal. An interruption of a few minutes in the blood flow results in severe brain damage. Likewise, it is believed that during the insult there are two phases of damage; i) after hypoxia/ischemia and ii) following reperfusion, as a result of exacerbated oxidative stress. Oxidative stress is a hallmark of cerebral ischemic stroke. There is an increase in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which cause damage in several components of the cell. Moreover, excessive and aberrant ROS generation is one of the major consequences of mitochondrial dysfunction. Therefore, the efficient removal of damaged mitochondria is essential to prevent cellular dysfunction, and this elimination occurs throughout mitophagy. Mitophagy is known to be activated after cerebral ischemia/reperfusion. Several evidences show that carbon monoxide (CO) activates endogenous protective mechanisms and prevents tissue and cellular damage.

The main aim of this PhD thesis was to address the cellular and biochemical pathways, underlying CO promotion of cytoprotection against oxidative stress in astrocytes, with a particular focus on the role of mitophagy and mitochondria function. In **Chapter I**, there is a general introduction concerning oxidative stress and importance of astrocytes in neuroprotection. Particular attention is- given to mitochondria quality control process, namely, mitophagy and mitochondrial biogenesis. Finally, the biological role of CO is addressed.

The capability of CO gas to trigger autophagy and promote cytoprotection dependent on autophagy was already described in several models, but there is no evidence with the use of CO releasing molecule (CORM-A1). Thus, **Chapter II**, evaluates the role of CORM-A1 on the control of autophagic process in yeast models and astrocytes. Here, CORM-A1 partially prevented oxidative stress-induced cell death *via* autophagy. As the main target of CO in the cell is known to be mitochondria, in **Chapter III**, the role of CO in mitochondria turnover was studied. Herein, first insights of CO-mediated cross-talk between mitophagy and mitochondrial biogenesis were observed. Moreover, at early time points CO induced mitophagy mediated by both PINK1/Parkin and also by BNIP3 pathways and activates *novo* synthesis of mitochondria, by stimulating mitochondrial DNA replication and increasing gene expression of peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α), specific mitochondrial transcription factor.

In **Chapter IV is shown,**for the first time, that glutathione appears to regulate CCCP-induced mitophagy via protein glutathionylation of PINK1. However, this is still a work in progress and further studies are needed to fully understand the complete mechanism.

RESUMO

O acidente vascular cerebral (AVC) é a principal causa de morte em Portugal e a terceira nos países ocidentais. Durante um episódio de AVC, a interrupção do fluxo sanguíneo durante alguns minutos pode levar a danos cerebrais graves, resultante de duas fases de resposta por parte do organismo, i) após isquémia e ii) na sequência do restabelecimento da corrente sanguínea, uma vez que durante esta última fase existe um aumento do stress oxidativo, pela produção exacerbada de espécies reativas de oxigénio (ROS). O stress oxidativo é um marcador do AVC. Por sua vez, a produção excessiva de ROS é umas das principais consequências da disfunção mitocondrial. A eficiente e rápida eliminação destas mitocôndrias é fundamental para o prognóstico final. A eliminação das mitocôndrias disfuncionais ocorre por processos mitofágicos. Neste sentido, sabe-se que durante um episódio de AVC, a mitófagia é induzida com o objetivo de reduzir o stress oxidativo. Por outro lado, vários estudos têm mostrado a capacidade anti-apoptótica do monóxido de carbono em diversos tecidos e células.

O principal objectivo deste projecto de doutoramento foi investigar quais as principais vias celulares e bioquímicas subjacentes à proteção do CO contra o stress oxidativo, em astrócitos com foco na função mitocondrial, nomeadamente nos processos mitofágicos e na biogénese mitocondrial. **O Capítulo I** introduz o papel dos astrócitos no contexto de stress oxidativo, com foco no papel na função mitocondrial. Por último, é abordado o papel biológico do CO.

A capacidade do CO promover a citoproteção em vários modelos dependentes de autofagia já é conhecida, contudo não existe nenhuma evidência relativamente ao uso de moléculas dadoras de CO (CORMs). No **Capítulo II** é demostrada, pela primeira vez, a habilidade da CORM-A1 em prevenir a indução de morte celular em contexto de stress oxidativo pela indução de autofagia. Uma vez que, o centro de ação do CO na célula é a mitocondria, no **Capítulo III**, foi estudado o papel do CO na modulação mitocondrial. Foi demonstrado que o CO promove a indução de mitofagia mediada pela via PINK1/Parkin e também pela expressão da proteína BNIP3, ao mesmo tempo que induz a síntese de mitocondria, pelo aumento da replicação do DNA mitocondrial e expressão PGC-1α, factor específico de transcrição mitocondrial.

No **Capítulo IV é demonstrado,** pela primeira vez, o papel regulador do glutatião na mitófagia dependente de PINK1 mediado por CCCP. Este último capítulo mostra resultados preliminares que necessitam de mais estudo para melhor compreensão do processo.

THESIS PUBLICATIONS

1. **Figueiredo-Pereira, C.,** Menezes, R., Ferreira, S., Santos, C., Vieira, H. L. A.. “Carbon monoxide released by CORM-A1 prevents yeast cell death via autophagy stimulation”. FEMS Yeast Research. 2019, Aug 1;19(5): foz051.

LIST OF ABBREVIATIONS

Abbreviation	Full form
AD	Alzheimer disease
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
ANT	ATP/ADP translocase
ATP	Adenosine triphosphate
Bax	Bcl2-like protein 4
BBB	Blood-brain barrier
Bcl2	B-cell lymphoma 2
BNIP3	BCL-2 interacting protein 3
BK	Big conductance K^+ channel
BSO	Buthionine sulfoximine
Ca^{+2}	Calcium cation
cAMP	Cyclic adenosine monophosphate
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
cDNA	Complementar DNA
cGMP	Cyclic guanosine monophosphate

CYTC	Cytochrome *c*
COX	Cytochrome c oxidase
CO	Carbon monoxide
CORM	Carbon monoxide releasing molecule
Cm	Chloramphenicol
CNS	Central nervous system
CsA	Cyclosporin A
DFP	Deferiprone
DNA	Deoxyribonucleic acid
DRP1	Dynamin related protein 1
EDTA	Ethylenediamine tetraacetic acid
EM	Electron microscopy
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FIS1	Mitochondrial fission 1 protein
GABA	γ-Aminobutyric acid
GC	Guanylate cyclase
Glut-1	Glucose transporter-1

GSH	Glutathione
GSSG	Glutathione disulfide (oxidized glutathione)
H_2O_2	Hydrogen peroxide
H_2O	Water
HIF-1α	Hypoxia-inducible factor-1alpha
HO	Heme oxygenase
IP3	Inositol triphosphate
IMM	Mitochondrial inner membrane
IL-1β	Interleukin-1 beta
IL-6	interleukin-6
LPS	Lipopolysaccharide
O_2	Oxygen
O2-	Superoxide
OGD	Oxygen/glucose deprivation
OMM	Mitochondria outer membrane
OXPHOS	Oxidative phosphorylation
PD	Parkinson’s disease
Parkin	E3 ubiquitin ligase

PBS	phosphate-buffered saline
PINK1	PTEN-induced kinase 1
PI	Propidium iodide
PGC-1α	Peroxisome proliferator activator receptor coactivator-1α
PNS	Peripheral nervous system
MCL-1	Myeloid leukemia cell differentiation protein
MDVs	Mitochondria-derived vesicles
MMP	Mitochondrial membrane permeabilization
mRNA	Messenger RNA
MTDR	Mitotracker Deep Red
3-MA	3-methyl adenine
NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide
NIX	BCL2 interacting protein 3-like
NO	Nitric Oxide
NRF-1	Nuclear respiratory factors 1
NRF-2	Nuclear respiratory factors 2
Q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-Q-PCR	Real time quantitative polymerase chain reaction
siRNA	Small interfering RNA
t-BHP	*tert*-butylhydroperoxide
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
TNF-α	Tumor necrosis factor alpha
TSPO	Translocator protein
WB	Western blot

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INTRODUCTION

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Cláudia Figueiredo-Pereira has written the whole chapter based on the referred bibliography

1 CENTRAL NERVOUS SYSTEM

The nervous system is responsible for coordinating the actions and transmit signals in the body and is divided in two main parts; the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is the processing center that collects, integrates and sends information to the peripheral nervous system. It consists of two main organs, spinal cord and brain, due to their extreme importance to the body, they are located in the dorsal body cavity, encased in bone and protected by the blood-brain barrier (BBB) [1–3].

At the cellular level (Figure 1), nervous system is defined by the presence of nerve cells, also known by neurons, and specific types of supporting cells, known as glial cells. Neurons are highly polarized cells, specialized in the transmission of information through the propagation of electrical signals. While glial cells are non-neuronal cells that primarily support and protect neuronal function [4,5].

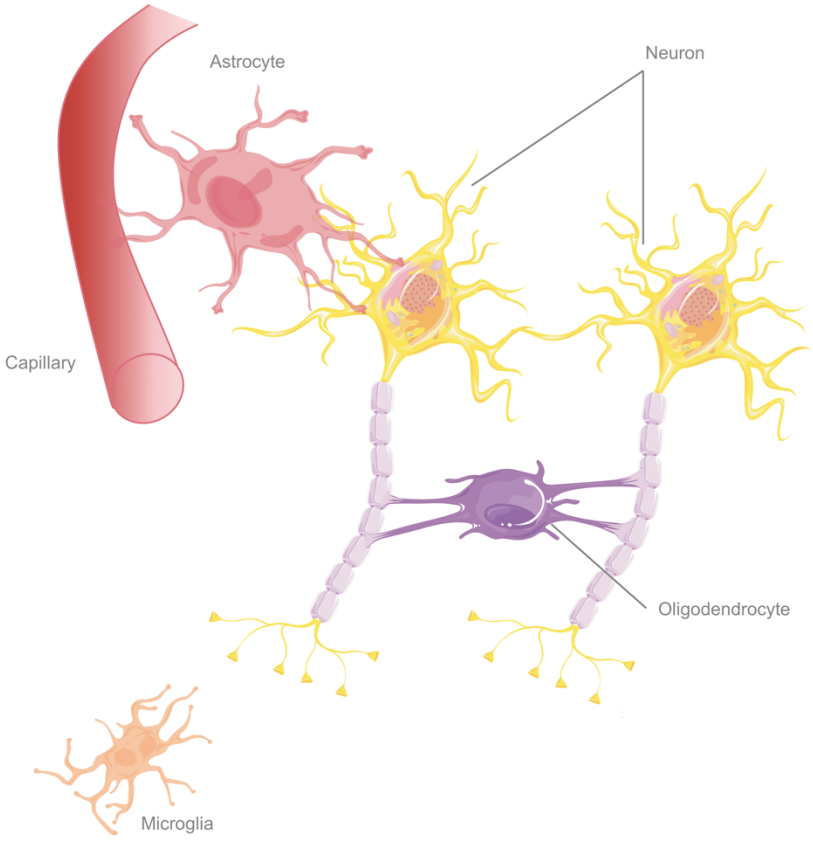

Figure 1- Schematic representation of several brain cell population and there interaction. Graphical elements were adalted from the Servier Medical Art Powerpoint image bank. Servier Medical Art by Servier (http://www.servier.com/Powerpoint-image-bank) is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

1.1 **Neurons**

Neurons have one of the largest and most complex architectures of all cells in the human body. Morphologically they are composed by three major regions: cell body, dendrites and axon. The cell body, or soma, contains the nucleus and the major cytoplasmic organelles. The specialized thin branches, dendrites, are extensions of the receiving surface and arise from perikaryon, ramifying over a certain volume of gray matter [6]. Dendrites can differ in size and shape, depending on the neuronal type. Finally, the axon conducts nerve impulses to other neurons or to muscle cells, which is the most extended part of the neuron. Each neuron might have multiple dendrites, but just one axon [7]. Between all the cell types in the body, neurons are the most dependent on oxygen and nutrient supply, since the specialization on the transmission of information limits the existence of energy reservoirs. In accordance, brain receives about 15% of the cardiac output at rest [1,5]. Moreover, just few minutes of blood flow interruption may cause neuronal cell death [8], occurring during cerebral ischemic stroke, which is the third cause of death in west countries [9] and the first in Portugal [10,11].

In 1859, Rudolph Virchow, studied neuroglia, or “nerve glue”. This name derives from the notion that glial cells served as a glue, and at that time glial cells were considered as an inactive connective tissue holding neurons together in the CNS. Nevertheless, glial cells are the major cells present in the brain and indispensable for neuronal functioning [1,12].

1.2 **Glial Cells**

There are three main glial cells: oligodendrocytes, microglia and astrocytes, with different functions. Oligodendrocytes are responsible for the production of myelin sheath that covers neuronal processes and facilitates electrical signal transmission [13,14]. The other glial cell type that is present in CNS is microglia and is known as the macrophages of nervous tissue [12]. Some studies indicate that microglia constitute 5 to 20% of all the glial cells and are distributed throughout all parts of the CNS [15]. These cells have the particularly function of cleaning the extracellular space in CNS, by “scanning” the environment for foreign material and damaged or dead cellular elements [15]. The third glial cells are astrocytes, which are discussed in deeper detail in the next section.

1.2.1 **Astrocytes**

Astrocytes originate their name from their star-like morphology and represent the majority of the glial cells in CNS. In the recent years, astrocytes have risen a great interest since they play a key role in the CNS, being involved in the regulation of neurodevelopment, neurotransmission, cerebral metabolism and blood flow [16,17]. Astrocytes are the most widely distributed cells inboard the CNS, outnumbering neurons, constituting about 20-50% of the total human brain volume [18]. They are known for being a heterogeneous cell population based on their morphology, function and expression of different sets of receptors, transporters, ions channels and neurotransmitters [19–21].

Since the last decade, astrocytes have been categorized in two subtypes: *protoplasmic* or *fibrous* [21], accordingly to the brain region specificity and to their unique cytoarchitectural and phenotypic features, which allow them to respond to changes in the microenvironment: [21,22]. *Protoplasmic* astrocytes are found throughout all grey matter, whereas the *fibrous* astrocytes are only found in white matter. The *protoplasmic* astrocytes exhibit a morphology of several stem branches, while *fibrous* astrocytes have longer branches and 50 to 60 long fiber-like processes [23], but both make extensive contacts with the neurovascular unit [23,24] *via* specialized processes called endfeets [25]. These endfeets express glucose transporters of the Glut-1 type and are a possible site of glucose uptake [26]. In this sense astrocytes have been shown to play an important role in neurovascular and neurometabolic coupling. Likewise, for enabling the dynamic coupling of cerebral blood flow with energy demand, the astrocytes release vasoactive substance [27].

1.2.1.1 **Astrocytes the crosstalk with neurons: metabolism**

Astrocytes are the only cell type in the brain that comprise the energy storage molecule glycogen. Furthermore, astrocytes also respond to glutamatergic activation by increasing their rate of glucose utilization and releasing lactate in the extracellular space, which might, in turn, be used by neurons to sustain their energy demands, which is called the astrocyte-neuron lactate shuttle [28]. Several astrocytic homeostatic functions have been demonstrated, including: (i) ion and water homeostasis, (ii) defense against oxidative stress, (iii) scar formation and tissue repair, (iv) modulation of synaptic activity *via* the release of gliotransmitters, and (iv) synapse formation and remodeling [14,16,29–31].

Astrocytes are powerfully coupled together by gap junctions, containing aqueous pores, allowing the transfer of ions and other molecules with low molecular weight [26,32]. A set of biological important molecules, comprising nucleotides, small peptides, cAMP, sugars, amino acids, inositol triphosphate (IP3) and Ca^{2+} have accesses by gap junctions [33,34]. Ca^{2+} waves are mediated by the binding of ATP to the P2X7 receptor, which is expelled to the extracellular space by the pannexin hemichannel [35].

Ca^{2+} waves spread through the astrocytic network occurs over hundreds of micrometers, modulating the surrounding neurons activity, by triggering the release of nutrients and regulating blood flow [36]. Recently, it was identified that astrocytes not only regulate the blood flow but supply the building blocks of neurotransmitters, which fuel neuronal metabolism, and are also able to transfer mitochondria into neurons [14,16,29]. Likewise, they are able to phagocyte synapses, modulate neurotrophin secretion, and clear debris [30,31]. This crosstalk between astrocytes and neurons starts early in development namely controlling gliogenesis and synaptogenesis. This occurs during brain development, being the astrocyte maturation the end point of the synaptogenic and neuroplastic periods [37].

These star-shaped cells that lack axons not only supports neuronal activity, but can also regulate the neurotransmission by modulating synapses. This process is defined as the "tripartite synapse" and depending on intracellular levels of Ca^{+2}, astrocytes release gliotransmitters (e.g. glutamate) – in a process termed gliotransmission - that have feedback actions on neurons [38]. The term "tripartite synapse" refers to a concept in synaptic physiology based on the demonstration of the existence of bidirectional communication between astrocytes and neurons [29,38,39]. In fact, astrocytes upregulate and release several neurotransmitters such as glutamate, ATP and D-serine [40], which influence paracrine signaling from astrocytes to neurons, endothelial cells, pericytes, and microglia cells. Glutamate is the most common amino acid and neurotransmitter present in brain tissue, which is an excitatory neurotransmitter. Astrocytes use ATP and Na^+ gradient created by the surrounding neurons to uptake glutamate, which is converted into glutamine and then release it into the extracellular space, where neurons are able to uptake it, and restart the cycle and generate again glutamate [41] and GABA, which are potent excitatory and inhibitory neurotransmitters, respectively [1,26]. This inter-cellular crosstalk plays a key feature in the homeostasis of the brain, by maintaining the neurovascular unit under physiologic conditions and any disruption on this crosstalk results in neurodegeneration conditions [14,42,43].

1.2.1.2 **The role of astrocytes in the protection of neurons against oxidative stress**

Bearing in mind the pivotal role of astrocytes in the brain and the strong cooperation between neurons and astrocytes, direct evidences point to an important role of astrocytes in several pathologies, either through the loss of normal function or the gain of defective functions. CNS injury or disease can be caused by the overproduction of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and the impairment of the cellular antioxidant defense capacity [44], which result in the activation of a coordinated astrocytic response: biochemical and morphological changes named as reactive astrogliosis [41]. Astrocytes have robust antioxidant abilities, which are limited in

neurons [45]. Reactive astrocytes in response to acute stress attempt to limit CNS damage, although chronic astrogliosis can stimulate constant ROS production and release of proinflammatory molecules, which promotes neuronal injury and neurotoxicity [46]. Astrocytes have a key role in protecting neurons against oxidative stress, and, in turn, against neuronal cell death [47,48] occurring in stroke and neurodegenerative disorders, such as Parkinson's disease (PD) or Alzheimer disease (AD). Consequently, in contrast to the classically accepted paradigm that brain function results exclusively from neuronal activity, there is an emerging view, in which brain function actually arises from the coordinated activity of a network comprising both neurons and glia cells.

Ischemic stroke results from the lack of blood supply, limiting oxygen and nutrients availability in the brain, followed by blood reperfusion that can generate oxidative stress. In cerebral ischemia/reperfusion-mediated brain injury, astrocytes can induce two types of reactive astrocytes (A1 and A2), which are related with reversible alterations in gene expression, cell hypertrophy and formation of a glial scar [49]. Astrocytes are much more resilient to ischemia/reperfusion-mediated inflammatory injury than neurons and play a vital role in limiting brain damage [50]. Ischemic cerebral damage is a result of O_2 and nutrient depletion, with subsequent acidosis, inflammation, glutamate excitotoxicity, mitochondria loss, increases in Ca^{+2} levels and oxidative stress [51,52]. In a rodent in vivo model of transient focal cerebral ischemia, it was showed that astrocytes were able to release functional mitochondria that enter in neurons and this entry amplified neuronal survival and metabolic function, improving neuronal outcome [16].

Astrocytes also play a crucial role in neurodegenerative disease, namely, in PD pathogenesis *via* oxidative and nitrosative stress. Samples from brain post-mortem PD patients, as well as experimental animal models, showed that astrocyte activation and elevated levels of oxidative metabolism [53,54], are pathogenic features of PD, it is believed that this is a downstream effect of the disease, although growing evidences suggest that it may contribute to PD [55]. In macaque models, reactive astrocytes were found in early stage PD, associated with persistent proinflammatory signaling [56]. In cases of toxic insult or neuroinflammation, there is an induction of reactive astrogliosis due to an increase on the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) [57], resulting in increased expression of astrocytic glutamate transporters and potassium channels, production of antioxidant glutathione (GSH) and ROS/RNS [58]. The astrocytic release of TNF-α, IL-1β, interleukin-6 (IL-6), and interferon-γ, which initiates neuronal apoptosis through the activation of caspase 3, caspase 8, and cytochrome *c* can be a mechanism by which reactive astrogliosis induces dopaminergic neuronal death [59,60]. Furthermore, reactive astrocytes and oxidative stress result in increased lipid peroxidation, mitochondrial impairment, and DNA strand breaks, which lead to neuronal injury and death [61,62]. Likewise, the levels of GSH and antioxidant enzymes secreted by astrocytes are significantly decreased in PD patients [63]. On the

other hand, there has been also demonstrated in an SH-SY5Y-astrocyte co-culture model and in an α-synuclein transgenic mouse model, that α-synuclein released from neurons might be taken up by astrocytes, leading to reactive astrogliosis and oxidative stress [64–66]. Dysfunctional mitochondria are the main producer of ROS, a byproduct of oxidative phosphorylation, namely produced in complex I [67]. Dysfunction of complex I has been hypothesized to be contributor to PD, leading to impair astrocytic function and toxicity[68]. Thus, mitochondrial defects may contribute to astrocytic oxidative stress[68] .

In conclusion, the role of astrocytes in brain function and dysfunction is of extreme importance. Although many advances have been made in this area, the full understanding of astrocytic cellular mechanism in disease is still unknown. Therefore, targeting astrocytes for promoting neuronal function and viability is an important strategy to protect the brain.

2 CARBON MONOXIDE

Carbon monoxide (CO) is a diatomic colorless small molecule, invisible, chemically inert, nonirritant, and odorless gas [69]. It is commonly known as a lethal gas due to it high affinity to heme-proteins, in particular to hemoglobin, which compromises oxygen delivery to the cells and promotes intoxication and death. High concentrations of CO are cytotoxic by inhibition of mitochondrial respiration *via* binding to cytochrome c oxidase (COX), which leads to excessive ROS generation and mitochondrial uncoupling effect.

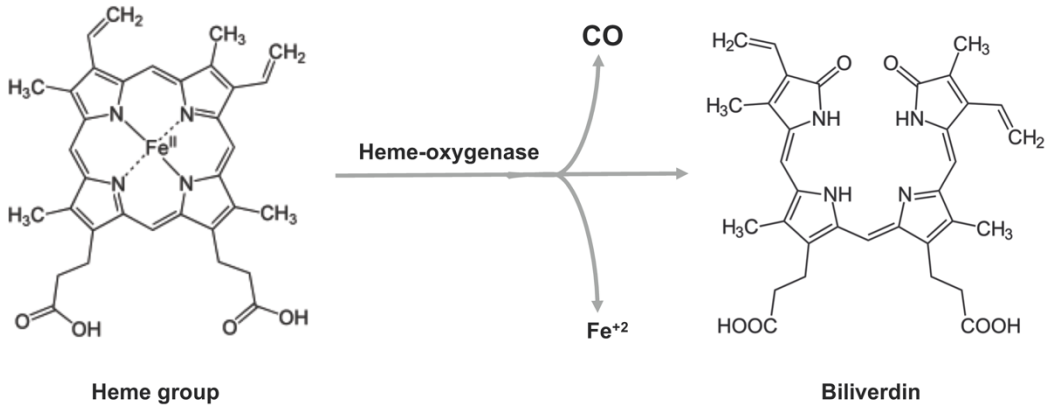

Figure 2 - Endogenous pathway production of carbon monoxides. Heme group is converted in biliverdin, ferric iron and carbon monoxide by the action of heme-oxygenase. Addapted from [70].

Nevertheless, this gas is an endogenous product of heme degradation by heme oxygenase (HO), along with free iron and biliverdin, which is rapidly converted into bilirubin by biliverdin reductase (Figure 2), presenting strong antioxidant properties [68]. HO is a stress induced enzyme comprising of two isoforms of HO: the inducible HO-1 and the constitutive HO-2 [71]. The cytoprotective HO/CO system responds to several stresses, namely: oxidative stress, hypoxia, hyperoxia, hypothermia, inflammation, ER stress and ischemia [72].

2.1 CO releasing molecules (CORM)

Since the last two decades, CO has received great attention as a biological regulator that can have an important role as a therapeutic tool. This led to the development of CO releasing molecules (CORMs) (Figure 3), which can potentially allow CO administration in a clinical context. CORMs are organic and organometallic compounds, with the capacity of delivering CO in a time and tissue-specific manner, allowing a significant reduction in carboxyhemoglobin toxicity [73,74].

It is already described the capacity of CORMs to mimic the effect of the gaseous CO, including vessel relaxation, protection against organ ischemia-reperfusion injury, prevention of organ rejection after transplantation, inhibition of inflammatory response and anti-apoptotic properties, highlighting the efficiency of CO transport by these molecules [75,76].

In 2003, the first water-soluble CORM, called CORM-3 (tricarbonylchloro(glycinato) ruthenium (II)), was described. CORM-3 was obtained by coordinating the amino acid glycine onto the metal center. CORM-3 is fully soluble in water and presents on its structure a metal carbonyl complex with a half-time of about 1 minute in physiological buffers [74]. Another very studied CORMs is CORM-A1 (sodium boranocarbonate), like CORM-3, is fully soluble in water, but it does not possess a transition metal on its structure and is able to release CO with a half-life of 21 minutes being slower releaser under physiological conditions, (37ºC and pH 7,4) [77].
Finally, CORM-2 (trocarbonyldichloro ruthenium (II) dimer) is a first generation CORM containing two ruthenium atoms, which the main disadvantage of being water insoluble [74] .

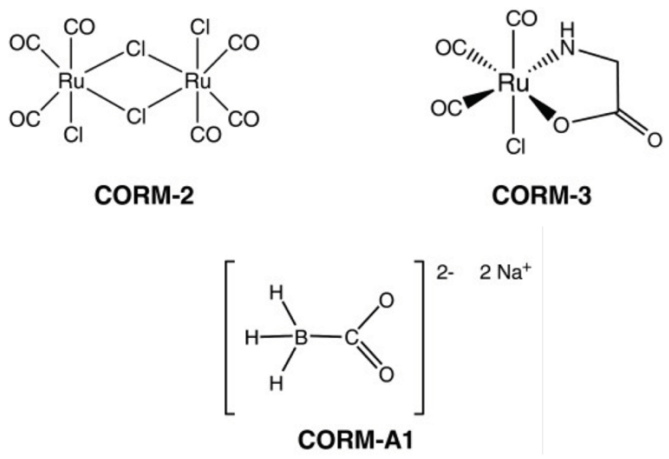

Figure 3 - Chemical structures of CORM'S. CORM's are compounds that contain a heavy metal such as nickel, cobalt, or iron surrounded by carbonyl (CO) groups as coordinated ligands. Addapted from [78].

2.2 Biological functions of carbon monoxide - Cytoprotection

CO was firstly described as a putative neural messenger [79] and is now recognized as a signaling molecule exerting essential regulatory roles in a variety of physiological and pathological process in cardiovascular, nervous and immune systems. The main CO's biological roles are: anti-inflammatory, anti-apoptotic, anti-atherogenic, vasodilator, anti-proliferative and modulator of cell metabolism [75,80,81].

2.2.1 History of CO as gasotransmitter

Some evidence suggests that endogenous CO is a neurotransmitter in CNS. In 1993, Verma and colleagues demonstrated, in primary olfactory neuronal culture, the action of CO as endogenous neuronal transmitter regulating cyclic guanosine monophosphate (cGMP) and guanylate cyclase (GC) [70,79]. Probably the most studied biological function of CO is its anti-inflammatory property [75]. In 2000, it was demonstrated the anti-inflammatory role of low concentrations of CO in vivo and in vitro, *via* a pathway involving mitogen-activated protein kinases [82]. In 2005, Sawle and colleagues have demonstrated for the first time that CORM-2 and CORM-3 prevented LPS-induced inflammation in murine macrophages, reducing the levels of TNF-α and nitrite [77]. Brouard *et al* in 2000 showed for the first time the anti-apoptotic property of the system HO/CO in endothelial cells. Since then, carbon monoxide has been reported as an anti-apoptotic agent in several systems: pulmonary cells, muscle, endothelial cells, neurons and astrocytes [71,83,84]. Likewise, it has been shown that CO confers resistance against cell death following ischemia-reperfusion damage and oxidative stress in many different models, namely lung, heart, kidney, liver and brain [71,83].

2.2.2 **Carbon monoxide and Central Nervous System**

In central nervous system, low concentrations of exogenous CO have been explored as neuroprotective agent. In 2007, Chora *et al.* described that CO reduced neuroinflammation in experimental autoimmune encephalomyelitis, a model of multiple sclerosis [85]. In the same year, in a newborn pig model of brain seizure a systemic administration of CO-releasing molecule (CORM-A1) stimulates the vasodilator and cytoprotective effects of CO in the cerebral circulation, protecting the neonatal brain from cerebrovascular injury [86]. Few years later, stimulation of endogenous CO production, *via* HO activation, promoted vasodilation of pial arterioles of newborn pigs [87]. In 2009, Zeynalov showed that in mouse brain CO reduced injury after transient middle cerebral artery occlusion [88]. In 2011, Wang demonstrated that CO exposure after ischemic stroke provide neurological protection, reducing the infarct size without improving behavioral effects by activating NrF2 pathway [89]. In 2012, Mahan *et al.* showed that CO limited cerebral injury resulting from cardiac bypass procedures using deep hypothermic circulatory arrest [90]. In the same year, Yabluchanskiy and colleagues demonstrated that a CO-releasing molecule (CORM-3) promotes neuroprotection or neurotoxicity after intracerebral hemorrhage in a time of administration dependent manner [91].

In neuronal cultures, our group has showed that the preconditioning of murine primary cerebellar granule cells with exogenous CO prevented neuronal apoptosis induced by excitotoxicity and oxidative stress [92,93]. Likewise, CO limited neuronal death in a perinatal model of cerebral ischemia, by increasing Bcl-2 expression, preventing the release of cytochrome c from the mitochondria and inhibiting caspase-3 activation [94]. Recently, it was demonstrated by the first time that CO (CORM-A1) also positively modulates neuronal differentiation by reducing neuronal cell death and improving neuronal precursor cell proliferation [95]. Furthermore, CO increased neuronal differentiation rate by stimulating the tricarboxylic acid cycle (TCA) activity and improving metabolism in NT2 neuronal model [96]. Likewise, phosphate pentose pathway and the modulation of the GSH system is also involved in CO-induced neuronal differentiation [97].

The role of CO in protecting astrocytes against brain damage will be explored in the next section.

2.2.2.1 **Astrocytes and carbon monoxide**

Regarding the cytoprotective effect of CO in the brain, namely in astrocytes (Figure 4), it is known that the cytoprotective effect of CO can be dependent on the generation of low amounts of ROS, which acts as signaling molecule [98]. In 2010, Queiroga showed for the first time that the antiapoptotic behavior of CO is due to the inhibition of mitochondrial membrane permeabilization (MMP) [98]. In isolated mitochondria from astrocytes, CO partially (i) inhibits loss of mitochondrial potential ($\Delta\Psi m$),

(ii) swelling and (iii) the release of cytochrome c. Furthermore, CO prevents MMP by targeting the inner membrane protein ATP/ADP translocase (ANT) and promoting its glutathionylation and inhibition of its pore activity via ROS signaling [98]. Few years later, it was also shown that low concentration of exogenous CO prevented apoptosis in astrocytes by inducing Bcl-2 expression, which in turn, enhanced ATP generation, cytochrome *c* oxidase enzymatic activity and mitochondrial population [99]. Also, CORM-2 was demonstrated to stimulate peroxisome proliferator activator receptor coactivator-1α (PGC-1α)-mediated mitochondria biogenesis in astrocytes [100]. More recently, *via* RNA sequencing approach, 162 genes were found to be differentially expressed following CO treatment in astrocytes. FosB gene expression was upregulated and validated by RT-Q-PCR and western blot. Nevertheless, knocking down FosB expression with siRNA did not revert the anti-apoptotic effect of CO. In addition, the CO cytoprotective effect was abrogated in the presence of the P2X7 receptor antagonist A-438079 [101]. Finally, the anti-apoptotic effect of CO was also evaluated in astrocyte-neuron communication, CO was able to prevent neuronal cell death in a paracrine manner by targeting astrocytic metabolism via purinergic signaling [102].

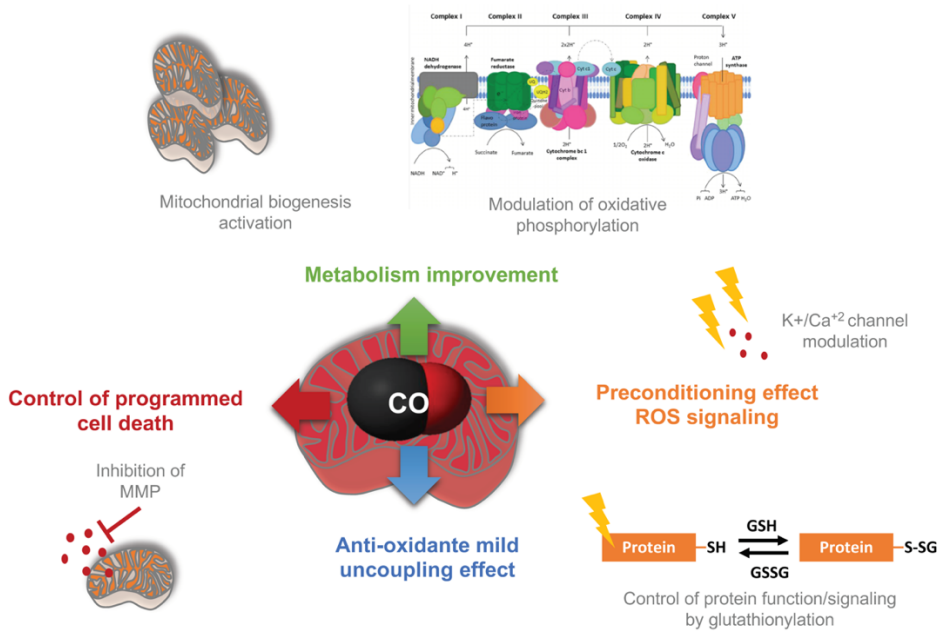

Figure 4 - The main described mechanisms of carbon monoxide on astrocytic mitochondria. Modulation of mitochondrial membrane permeabilization and cell death control; improvement of mitochondrial metabolism, ROS generation and signaling and mild uncoupling effect.

Concerning the role CO in neuroinflammation, in rat astrocytes and hippocampal neurons, CORM-2 protects against Aβ toxicity in a concentration-dependent and HO-1-dependent manner [87,103]. CO suppresses NOX-derived ROS production caused by Aβ [103]. Crosstalk between NF-κB and ROS activates A1 type astrocytes [104]. Therefore, CO may downregulate the ROS/NF-κB pathways in astrocytes, consequently reducing the activation of inflammatory A1 astrocytes during CNS injury.

Carbon monoxide produced in astrocytes has been described to contribute to vasodilation [105], leading to the supply of O_2 and nutrients to surrounding cells. Adenosine diphosphate (ADP) that is a regulator of cerebral blood flow, NO (nitric oxide) and CO are important signaling molecules in the brain [106]. Indeed, astrocyte-derived CO can diffuse into endothelial and smooth muscle cells, activating BK channel and resulting of vasodilation [107]. Thus, CO increased pial arteriolar dilatation in newborn pigs in response to ADP [108]. On other hand, CO and NO are able to activate the big conductance K^+ channel (BK) in endothelial cells [109]. Likewise, CO and NO also activate BK channels in smooth muscle directly [107,109]. In conclusion, CO also emerges as an important player of cerebral vasomodulation.

3 AUTOPHAGY

More than 40 years ago Christian de Duve, described a catabolic process conserved from lower to higher eukaryotes, which is named as autophagy, this word remotes from the Greek and means "selfeating" [110,111]. Autophagy is an intracellular degradation system that carries cytoplasmic constituents into the lysosome. There are three types of autophagy: macroautophagy, microautophagy and chaperone mediated autophagy [112,113]. Hereafter macroautophagy is named as autophagy.

Autophagy was first described as a cell death pathway, although in the last years autophagy reveals to be a protective cellular process that allows cells to survive in response to multiple stressors, such as; starvation, hypoxia, ROS and damaged organelles, strengthening organism defense [112,114,115]. Autophagy is involved in diverse human processes, such as development, longevity, immunity, cancer and neurodegenerative diseases [111,116]. Throughout a possible lethal stress, cells respond with rapid metabolic changes to protect themselves against potential damage. This is orchestrated by a multifaceted cellular program, which involves the action of various stress response pathways. One of the key pathways that arbitrates stress-induced metabolic adaptation and control of cell damage is autophagy [116].

4 MITOCHONDRIA

A 1.5 billion years ago, aerobic bacteria colonized eukaryotic cells, and this symbiotic relationship resulted in bacteria evolution into mitochondria, while the host cells acquired the ability to metabolically use oxygen (O_2) [117,118]. Although mitochondria are viewed as a vestigial bacterium living in eukaryotic cells, they still retain several features of their bacterial ancestry; i) Mitochondria are able to replicate autonomously independently of cell division; [117] ii) The mitochondria double membrane is composed by cardiolipin, a specific phospholipid, found only in prokaryotes and in the inner membrane of mitochondria but not in other membranes of eukaryotic cells; [119] iii) they harbor there one genome (circular DNA), lacking histones and containing intronless polycistronic genes that encode 13 mitochondrial proteins, 22 transfer RNAs and 2 ribosomal RNAs in vertebrates [120–122] and iv) mitochondrial protein translation starts with a formylated methionine, an N-terminal modification never found on proteins encoded in the nuclear genome, the same occurs in bacteria [123].

The mammalian mitochondrial proteome consists in approximately 1,200 proteins, but the human mtDNA only encodes approximately 1% of the mitochondrial proteome, being 13 proteins that are mainly involved in oxidative phosphorylation (OXPHOS) [122]. The remaining mitochondrial proteins are encoded by the nuclear genome, translated in the cytosol, and imported into mitochondria [124]. Mitochondria are vitally important, fulfilling crucial functions in the cell, namely: energy production and biomolecules production [125]. They also constitute platforms for the development of protective homeostatic response against oxidative and play a key role in the control of cell fate *via* apoptotic cues [126].

In mammalian cells, energy (ATP) is mostly produced in the inner membrane (IMM) of mitochondria by oxidative phosphorylation OXPHOS, which is constituted by the respiratory chain (Complex I-IV) and ATP synthase complex (ATPase or Complex V) [127,128]. This is a very productive metabolic pathway based on electron transfer along an electron transport chain, from Complex I to Complex VI, through oxidation-reduction reactions [67].

The metabolites produced by glycolysis are used in the TCA cycle that generates compounds to supply the first electronic transporters in the OXPHOS: NADH to complex I (NADH ubiquinone reductase) and succinate to complex II (succinate dehydrogenase). The generated electrons pass to complex III (ubiquinol-cytochrome c oxidoreductase), and lastly to complex IV (cytochrome c oxidase – COX), *via* cytochrome c, in which O_2 is the final electronic receptor, leading to H_2O. During the transport of electrons, H^+ ions are pumped across the IMM leading to the formation of a gradient, maintaining a high $\Delta\Psi m$. ATP is synthesized in the last step of the chain, the enzymatic complex – ATP synthase, by driving back protons to the mitochondrial matrix [129,130]. When an injury occurs, there is a perturbance in the cellular redox homeostasis, due to the leakage of electrons through the

electron transport chain, generating ROS, which, depending on the concentration, constitute valuable secondary messengers or extremely damaging factors [131–134]. Additionally, due to the high importance of mitochondria to the cell, any disruption of mitochondrial homeostasis can lead to a disease. Therefore, mitochondrial quality control related processes: fission/fusion, mitophagy (specific mitochondrial autophagy) and mitochondrial biogenesis must be under tight control and balanced.

4.1 Mitochondrial function and ROS

Brain is rich in mitochondria and uses about 20% of the total consumed oxygen of human body. Moreover, 90% of the brain consumed oxygen is for generating energy, which makes brain cells particularly sensitive to oxidative stress. Oxidative stress is linked to mitochondrial dysfunction because mitochondria generate ROS but are also the target of ROS [135]. Mitochondria are the main cellular structure responsible for managing O_2 levels and ROS production. Thus, a mitochondrial imbalance of ROS production and/or ROS clearance promotes oxidative damage of lipids, nucleic acids and proteins, representing a threat to the cell survival [126,134]. ROS accumulation leads to DNA base modification, DNA strand breaks, inter- and intra-strand crosslinks and DNA-proteins crosslink that eventually affect genomic structure and stability [136]. Similarly, oxidation of cysteine residues, which are very vulnerable to oxidase due to their highly reactive nucleophilic thiol moiety, results in alterations of protein structure and function [137]. One of the most devasting effect of oxidative stress in the cell is lipid peroxidation, that occurs when free radical interact with membrane lipids, forming lipid radicals, which results in alteration of membrane permeability and stability, compromising cellular compartmentalization and overall function [138]. Therefore, the increase on oxidative stress as a result of mitochondrial dysfunction has being associated to several pathologies, namely, atherosclerosis, diabetes, stroke and aging-related physiological disorders (neurodegenerative disease) [139].

ROS have been known as a damaging and pernicious agent to the cell, resulting in oxidative stress, although, ROS play a critical role as a signaling molecules of cellular physiological functions. Under physiological conditions, mitochondrial ROS serve as second messengers to coordinate cellular responses, including adaptation to hypoxia, cellular division, and complex responses to external stimuli. The oxygen anion forms, superoxide (O2-) and hydrogen peroxide (H_2O_2) activate signaling pathways controlled by tyrosine phosphorylation such as NF-kB, PKC, MAPK or JNK [140,141]. ROS reacts with the redox sensitive cysteine residue of tyrosine phosphatase leading to their transient inactivation, which favors unopposed kinase activity [142,143]. Likewise, ROS have been demonstrated to play an adaptive role in many physiological events including, hypoxia, physical activity, regulation

of autophagy, immunity, differentiation and longevity [142]. In this way, mitochondria homeostasis is very important for avoiding cellular accumulation of ROS and a loop of damage, thus the number and quality of this organelle is critical to the good cellular functioning. The elimination of mitochondria leads to the renewing of mitochondria named as mitochondria turnover [135]. Indeed, mitochondria mitigate oxidative stress through quality control, that include balanced of mitochondrial biogenesis, autophagic degradation of dysfunctional mitochondria (mitophagy), mitochondria-specific protein refolding/turnover pathways, and mitochondrial fission/fusion events.

4.2 **Mitophagy**

In 1915, Margaret and Warren Lewis, in the early work of mitochondria dynamics, proposed the idea of mitochondrial degradation or degeneration (Figure 5) [144]. Later in century, with the use of electron microscopy (EM), it was observed in rat tissues for the first time mitochondria within vesicles and lysosomes [145,146]. Christian de Duve postulated the concept of cellular autophagy, the idea of mitochondrial autophagy flourished, and was further support by several studies showing mitochondrial autophagy in the muscle of metamorphosing *Antheraea Polyphemus*, in rabbit hearts under ischemic-reperfusion injury and during rat erythrocyte maturation[147–151].

The term mitophagy was first suggest in 1998, by Sidney Scott and Daniel Klionsky, but just became popularized in 2005 by John Lamesters, describing the selective engulfment of mitochondria into vesicles with autophagosome marker [152,153].

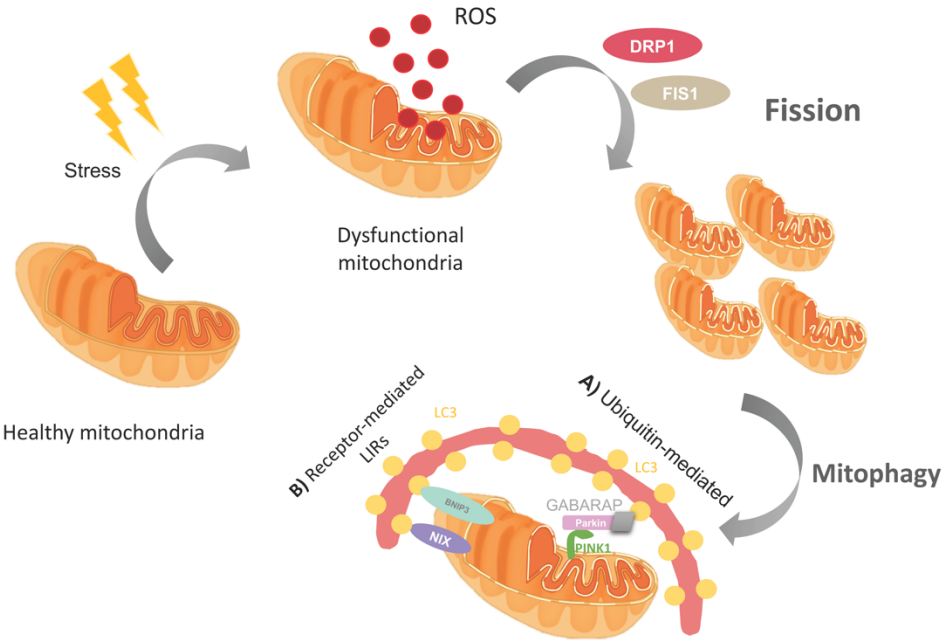

Figure 5 - Mitophagy mechanism; Mitophagy is a specialized autophagic process that eliminate damaged mitochondria in order to maintain the cellular homeostasis. Mitophagy can be triggered by several stress, such as, excessive ROS, DNA damage and aging.

4.2.1 Fission and Mitophagy

Mitophagy depends on mitochondrial morphology and fission processes (Figure 5). Whenever stressful stimulus is high and promotes mitochondrial depolarization and dysfunction, key cellular response involves fission followed by mitophagy. In fact, mitochondrial fission facilitates mitochondrial engulfment by autophagosomes due to a matter of size, fission decreases mitochondrial size. Thus, fission and mitophagy maintain mitochondrial homeostasis and limiting dysfunction mitochondria, increased oxidative stress and metabolic collapse. Mitochondrial fission is mediated by DRP1 and by the outer membrane protein FIS1 [154–156]. Both proteins regulate mitochondrial division, and their knockout or downregulation promotes highly interconnected mitochondrial network. DRP1 is regulated by different mechanisms, including phosphorylation, sumoylation, and ubiquitination [156]. Upon phosphorylation DRP1 translocate to mitochondria and binds to the outer membrane receptor, FIS1 [154,155]. Increased levels of FIS1 expression promote autophagy [157]. Likewise, moderately elevated ROS levels promote mitophagy (but not cell death neither nonspecific autophagy) in a DRP1-dependent manner [158].

4.2.2 **PINK1/Parkin pathways of Mitophagy**

There are several protein receptors described to be related with mitophagy pathway, although the specific mechanisms about how mitochondrial proteins are selectively sorted and regulated within the network for mitophagic elimination are still poorly understood. The most studied and described mitophagy pathway, in mechanistic terms, is mediated by ubiquitin-dependent mitophagy receptor; PTEN-induced kinase 1 (PINK1) and the E3 ligase Parkin genes [159]. Moreover, mutations in these genes are associated with the development of autosomal recessive form of Parkinson Disease [160]. PINK-1 and Parkin have been mostly studied in dopaminergic neurons. PINK1 is a cytosolic and mitochondrial localized serine/threonine kinase, and it is constitutively expressed protein. Under physiological conditions, PINK1 is proteolyzed by mitochondria rhomboid protease PARL, at the mitochondria membrane of healthy mitochondria. This event results in processed forms of PINK-1, which are rapidly degraded by the proteasome. In response to stress, in particular when $\Delta\psi m$ is reduced, there is an accumulation and activation of PINK-1 in the mitochondria surface, phosphorylating Parkin and recruiting it from the cytosol to the mitochondrial outer membrane (OMM) [161]. Mitochondria accumulation of Parkin leads to their degradation via mitophagy (Figure 5A)[162].

Parkin functions are downstream PINK1 activity since overexpression of Parkin partially compensates PINK1 loss [163]. Thus, PINK1 kinase activity is essential to promote translocation of Parkin to depolarize mitochondria. Still, Parkin mediates the formation of two distinct poly-ubiquitin chains, linked through Lys 63 and Lys 27. Parkin-dependent mitophagy requires the ubiquitin-autophagy adaptor protein p62 and the outer membrane protein VDAC1 in response to mitochondrial membrane depolarization [164]. VDAC1 is the mitochondrial target of Parkin-catalyzed Lys 27 ubiquitylation. Under specific conditions, Parkin or PINK1 are dispensable for mitophagy activation. In a Parkin-independent manner, PINK1 recruits NDP52 and optineurin to mitochondria for direct stimulation of mitophagy, being a process upstream of LC3 binding [165]. Accordingly, in the absence of PINK1, Parkin can be recruited to depolarized mitochondria and can promote mitophagy in cardiomyocytes [166]. Finally, Parkin translocation to depolarized mitochondria is antagonized by pro-survival protein of BCL-2 family, induced myeloid leukemia cell differentiation protein (MCL-1) and BCL-XL in a Beclin-independent manner [167].

4.2.3 **BNIP3/NIX pathways of mitophagy**

In the last few years, several proteins have been identified in mitophagy signaling, some of them independent of ubiquitin, namely BNIP3 (the BCL-2/adenovirus E1B 19-kDa-interacting protein 3) and NIX (BNIP3L). BNIP3 and NIX are members of the BH3-only subfamily of the Bcl-2 family proteins [168] such as residence in the outer membrane and the ability to interact with the prosurvival proteins, Bcl-2 and Bcl-X_L, which connects them with cell death modulation [169,170]. Both BNIP3 and BNIP3L have been reported to promote cell death in several models [171,172]. BNIP3 was first found to be a pro-apoptotic protein, since it was upregulated under hypoxia conditions [173]. BNIP3 expression is regulated by the hypoxia-inducible factor-1alpha (HIF-1α) expression [173,174], it becomes activated in response to hypoxia and has been implicated in mitophagy induction in several models, namely in response to stroke [175] and tumor growth [130]. Nevertheless, BNIP3 is also involved in mitophagy modulation and elimination of dysfunctional mitochondria, by directly interacting with LC3 (Figure 5B) [176] and is part of a general mechanism of cell survival that is controlled by HIF-1α [169]. Overexpression of Bcl-2 protects against BNIP3-mediated loss of $\Delta\Psi m$, but does not prevent induction of general autophagy [177]. Likewise, in Bax/Bak knockout cells, BNIP3 does not stimulate mitochondrial membrane depolarization, but does promote mitophagy. Furthermore, BNIP3 reduces levels of both nuclear and mitochondria encoded proteins involved in mitochondrial respiration and activates mitochondrial proteases [177]. NIX is BNIP3 ligand, and was firstly found to be required for mitochondrial clearance during erythrocyte maturation and has a WXXL motif [178,179]. NIX also participates in mitophagy induction under hypoxia conditions, mediated by HIF-1α [180]. Recent studies shown that, although these two receptors are ubiquitin-independent mitophagy mediators and compose a distinct pathway of PINK/Parkin, NIX is also able to interact with Parkin, promoting its recruitment to depolarized mitochondria [181].

4.2.4 **Mitophagy and cell death**

The involvement of mitophagy activation in the promotion of cell death or cytoprotection is a complex subject, with controversial data in the literature. Similar to autophagy, mitophagy was first described to be involved in cell death stimulation, depending on the context and severity of the insult [169].

Oxidative stress stimulates cell death *via* mitophagy. Indeed, in glioma cell line selenite induces mitochondrial damage, mitophagy, and cell death by generation of anion superoxide, and whenever Atg 6 and Atg7 were knocked out, mitophagy and cell death were reverted [182]. Moreover, two anion superoxide generators, diquat and paraquat, are able to trigger mitophagy and cell death [183]. Furthermore, BNIP3, an important mitophagy modulator, has been extensively described to be

involved in cell death promotion. BNIP3 triggers mitochondrial fission in a DRP1-dependent manner and induces cell death via cytochrome *c* (CYTC) release from mitochondria in a Bax/Bak-independent way [184]. Furthermore, whenever autophagy is blocked by treatment with 3-methyl adenine (3-MA) in Bax/Bak knockout cells, BNIP3 induces necrosis[177]. Indeed, BNIP3-induced apoptosis is dependent on CYTC release and loss of $\Delta\Psi m$ [185]. BNIP3 interacts with OPA1, a protein modulating fusion, and promotes OPA1 oligomer disassembly, preventing mitochondrial fusion and inducing their fission. These events are upstream mitochondrial membrane permeabilization and cell death[185]. For instance, BNIP3 expression increases in stressed cardiomyocytes *in vitro*, as well as in cardiac overload *in vivo* and is prevented by 3-MA, an inhibitor of autophagy. Interestingly, 3-MA treatment restores cardiac function [186]. BNIP3 also contributes to cardiac I/R lesion since a TAT-fusion protein encoding the carboxyl terminal transmembrane deletion mutant of BNIP3 blocks BNIP3-induced cell death [187]. Thus, several studies claim that mitophagy, along with autophagy, is another cell death process. Nevertheless, it is still a subject of discussion, whether the cells die by mitophagy (mitophagy triggers cell death) or with mitophagy (mitophagy is a cytoprotective process active in response to stress), since the underlying mechanisms of mitophagy are not yet very clear.

4.2.5 **Mitophagy, cytoprotection and mitochondrial quality control**

Mitophagy is tightly involved in the regulation of mitochondrial dynamics, namely: motility, quality control, and population [110,188]. In the last few years, mitophagy has been described as cytoprotective mechanism by elimination dysfunction mitochondria and limiting oxidative stress, being a key cell process for mitochondrial quality control.

The two key proteins, PINK1 and PARKIN, emerge as important players in mitochondrial dynamics, mitochondrial quality control, and cytoprotection. Accordingly, in HeLa cells, silencing PINK1 increases fission and decreases $\Delta\Psi m$. The same effect is found when human mutated PINK1 gene was overexpressed in the same cell line [189]. In PINK1 knockdown SH-SY5Y cell line, inhibition of autophagy and mitochondrial fission increases cell death, although the expression of Parkin was able to reverse the effect of Pink1 deficiency and promote mitophagy and cytoprotection [190]. Moreover, knocking down of PINK1 gene promoted general autophagy and morphological changes in mitochondria, probably due to accumulation of dysfunctional mitochondria [190].

In lipopolysaccharide (LPS)-induced sepsis, mitogen-activated protein kinase kinase 3 (MKK3) deficiency increases mitochondrial biogenesis and mitophagy through acting on SIRT1, PINK1, and Parkin, and simultaneously reduces lethality in mice with sepsis [191]. Therefore, facilitating mitochondrial turnover and mitochondrial quality control promote cytoprotection. Likewise, PINK1

plays an important protective role in response to hyperoxia, since its deletion increases susceptibility to damage in lung and endothelium, via upregulation in mitophagy, apoptosis, proteasome activation, and oxidant generation [192].

PARKIN directly supports mitochondrial function and protects mitochondrial genomic integrity from oxidative stress [193]. Likewise, Parkin and PINK1 are essential for the formation of mitochondria-derived vesicles (MDVs), which are important for mitochondrial quality control and vesicular trafficking [194]. Mitochondrial ROS generate mitochondrial vesicles in a Parkin-dependent manner. These MDVs are targeted to lysosomes for degradation independently of autophagy and emerge as an important mechanism for elimination of oxidized and damaged mitochondrial proteins [194]. Furthermore, MDV formation and degradation can precede mitophagy [194]. Another important protein controlling mitophagy in response to ROS generation is the translocator protein (TSPO), which is a small protein located in the mitochondrial outer membrane. TSPO prevents mitophagy by inhibiting downstream PINK1/Parkin pathway. In addition, TSPO also binds to VDAC1, which is implicated in the mitophagic process, limiting mitochondrial coupling and increasing ROS generation, which in turn inhibits PARKIN-mediated ubiquitination of proteins and limits mitophagy [195].

In two ischemic stroke models, mouse transient middle carotid artery occlusion and oxygen and glucose deprivation in primary culture of hippocampal neurons, tunicamycin- or thapsigargin-induced endoplasmic reticulum (ER) stress promotes mitophagy in a Parkin-dependent way, which is a cytoprotective response [196]. In addition, in two Parkinson disease patients of a familial form containing Parkin mutation presented upregulation of PGC-1α that modulates mitochondrial biogenesis [197]. Thus, if confirmed in a larger cohort of patients, mitophagy dysfunction would be associated with mitochondrial biogenesis defects. During aging, Parkin overexpression reduces proteotoxicity and extends lifespan [198]. Likewise, Parkin overexpression results in reduced Mitofusin levels in aging flies, with concomitant changes in mitochondrial morphology and an increase in mitochondrial activity [198]. Finally, in neonatal cardiomyocytes, LPS prevents cell death by promoting mitochondrial biogenesis and mitophagy, since LPS increases the expression of mitochondrial transcription factor A (TFAM), PGC-1α and nuclear respiratory factor 1 (NRF-1). Moreover, whenever, autophagy is inhibited by 3-MA, there is an apoptosis increase following LPS treatment [199], indicating that autophagy is key process for maintaining cells alive. Recently, *in vivo* models for hepatic diseases, it was reported that Parkin-mediated mitophagy may decrease apoptosis in hepatocytes, by improving mitochondrial quality control and suppressing steatosis (i.e., lipid accumulation), following acute and chronic ethanol consumption [200,201]. Finally, during atherosclerosis development in human vascular smooth muscle cells, stimulation of mitophagy protects against apoptosis induced by atherogenic stressors. Indeed, overexpression of PINK1 and Parkin rescues VSMC from oxidized low-density lipoproteins (LDL)-triggered apoptosis [202]. In

summary, mitophagy, along with mitochondrial biogenesis, emerges as a cytoprotective process in different tissues and models.

Mitophagy is a key process in the cross talk between cell metabolism and cytoprotection, since metabolic adaptations to the environmental conditions (oxygen and nutrients) prevent cell death. Upon high oxidative phosphorylation activity, the small GTPase Rheb is recruited to the mitochondrial outer membrane, promoting mitophagy through the direct physical interaction with the mitochondrial autophagic receptor NIX and the autophagosomal protein LC3-II [203]. Thus, stimulation of oxidative phosphorylation increases mitochondrial turnover: mitophagy and mitochondrial renewal, which can limit potential oxidative stress due to excessive oxidative metabolism. As a consequence, mitotic arrest is a promising therapy for killing cancer cells. Nevertheless, under prolonged mitotic arrest cells are able to survive due to mitophagy stimulation and metabolic shift to glycolysis in an AMP-activated protein kinase (AMPK)-dependent manner [204]. BNIP3-promoted mitophagy reduces stress-induced ROS generation [205]. Under prolonged hypoxia, increased expression of HIF-1α and BNIP3 stimulates mitophagy, which is then an adaptive process for avoiding excessive ROS production and for limiting cell death induction, since malfunctioning mitochondria are eliminated, [206]. Furthermore, liver of BNIP3-null mice presents higher mitochondrial mass, an increased amount of mitochondria with lower $\Delta\Psi m$, abnormal mitochondrial structure and enhanced ROS levels [207]. Likewise, BNIP3-null liver also presents reduced β-oxidation of fatty acids and defective glucose output under fasting condition due to limitation on mitochondrial integrity and function [207]. Therefore, the pro- or antiapoptotic role of BNIP3 might be dependent on the intensity of the toxic stimulus and the metabolic environment. One can speculate a double role for BNIP3, being a cell metabolism adaptor or an initiating factor for cell death pathways. Taken all together, mitophagy is also implicated in cellular metabolic adaptations to the environment.

4.3 Mitochondrial Biogenesis

Mitochondrial biogenesis is the process of generating mitochondrial population and it is dependent on fission of existing mitochondria followed by a coordinated programme of transcription and translation regulated by the transcription factor PGC-1α [208,209]. This process is essential for the growth and recovery of the existing mitochondrial network following mitochondrial elimination by mitophagy [210], ensuring that the mitochondrial network is capable to efficiently support and adapt to the metabolic needs of the cell, as well as to maintain mitochondrial homeostasis. Mitochondrial biogenesis depends on the coordination of nuclear and mitochondrial-encoded gene expression and import of around 1000 lipids and proteins, as well as on mitochondrial DNA replication [209–211].

PGC-1α is a transcriptional coactivator of several genes, and a known master regulator of mitochondrial biogenesis [208,212]. PGC-1α is unable to bind to DNA directly, therefore, orchestrates the activity of diverse transcription factors, including the nuclear respiratory factors (NRF-1 and NRF-2) [213,214], which in turn control the expression of TFAM [215], as well as nuclear mitochondrial genes, such as mitochondrial respiratory complexes, enzymes of heme biosynthesis, mitochondrial import machinery associated proteins and mitochondrial ribosomal proteins .

Recently, a novel parkin interacting substrate, named PARIS/ZNF746, was identified to link both processes: mitophagy and mitochondrial biogenesis. PARIS/ZNF746 is a member of the family of KRAB zinc-finger proteins transcriptional repressors. PARIS is ubiquitinated by PARKIN for proteasomal degradation [216,217]. As PARIS is physiological transcriptional repressor of the PGC-1α, degradation of this protein upregulates PGC-1α and subsequently PGC-1α -dependent nuclear respiratory factors genes expression, promoting therefore mitochondrial biogenesis [192]. In the literature, many works have demonstrated that mitophagy and mitochondrial biogenesis can occur simultaneously, but their interdependency and the underlying mechanisms controlling both events are not clear yet, being PARIS the first protein connecting both processes.

5 AIMS AND SCOPE OF THE THESIS

The main aim of the PhD thesis is to identify the cellular and biochemical pathways underlying CO promotion of cytoprotection against oxidative stress in astrocytes, with a particular focus on the role of mitophagy and mitochondria function. Thus, two strategies were adopted:

i) **Addressing the role of autophagy in CO-induced cytoprotection in astrocytes and yeast model (chapter I)**; The cytoprotective action of CO in neuronal cells [92] and in astrocytes [98,99,101] have been already described. Furthermore, it has also been demonstrated that the anti-apoptotic effect of CO is mediated by autophagy activation [218,219]. However, at the molecular level little is known. In this thesis yeast model and primary culture of astrocytes were used. The yeast Saccharomyces cerevisiae is an excellent eukaryotic model for studying autophagy associated molecular mechanisms. Several key proteins associated with autophagic process are described in yeast and present homologs in mammals. All the findings discovered in yeast were validated in astrocytes.

ii) **Addressing the cellular pathways of CO-induced mitophagy and its cross-talk with mitochondrial function and biogenesis (chapter II);** The hypothesis is that CO may play a role in mitochondrial turnover and it is based on three main associations. First, ROS are a signaling molecules in several CO-induced pathways [220,221]. Secondly, CO-activated autophagy is ROS dependent [218]. Third, the main cellular target of CO is mitochondria *via* ROS signaling, modulation of membrane integrity and improvement of mitochondria metabolism [98,99], namely, the ability to stimulate mitochondrial biogenesis [100]. Therefore, the role of CO in the cross-talk between mitophagy and mitochondrial biogenesis was assessed. Additionally, the mitochondrial effectors responsible for initiating mitophagy are still a subject of discussion and speculation, since little is known about them. Thus, the factors involved in CO-induced mitophagy were also investigated.

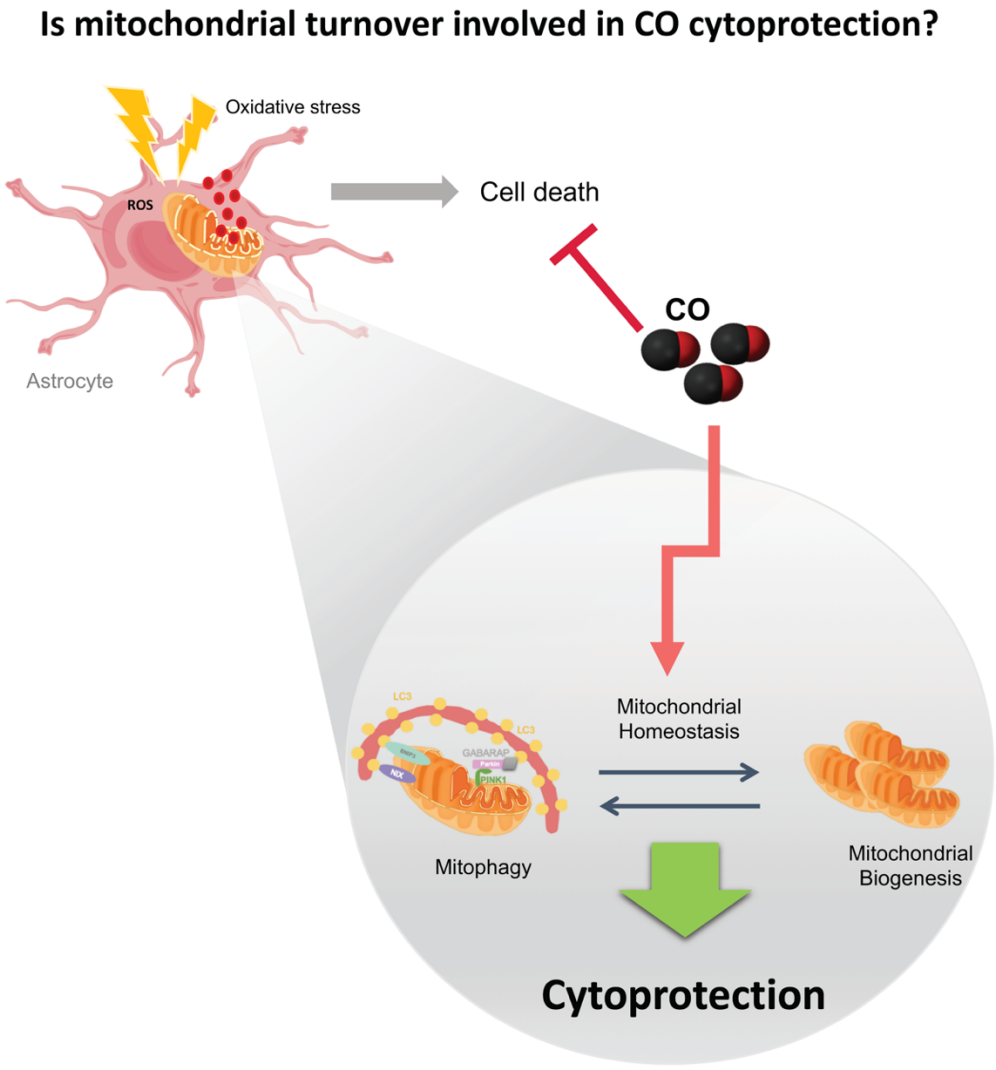

Figure 6 - The main hypothesis of this thesis and model systems. The effect of CO in the cross-talk between mitophagy and mitochondrial biogenesis.

Finally, a third specific aim of this thesis is to disclose how ROS can signal and regulate mitophagy. In fact, dysfunctional mitochondria are clock bombs in the cells, due to their oxidative function. Thus, there is a great need for efficient clearance of damaged mitochondria by mitophagy. Likewise, the last point of the thesis aims to;

iii) **Understanding the role of ROS signaling and pos-translational protein modification in mitophagy process (chapter III).** This part is an in-progress work, and it is mainly focused on mitophagy molecular pathways depend on redox changes, namely ROS signaling and GSH system. In this specific aim, mitophagy was stimulated by the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP).

6 REFERENCES

1. Roberts, J. H. B. J. L. *From Molecules to Nerworks*. (Elsevier, 2004).

2. Obermeier, B., Verma, A. & Ransohoff, R. M. The blood-brain barrier. *Handb. Clin. Neurol.* **133**, 39–59 (2016).

3. Purves, D; Augustine, G. J. Neuroscience. in *Neuroscience* (Sinauer Associates, Inc, 2001).

4. Evans, C. S. & Holzbaur, E. L. F. Quality Control in Neurons: Mitophagy and Other Selective Autophagy Mechanisms. *J. Mol. Biol.* (2019). doi:10.1016/j.jmb.2019.06.031

5. Bordal, P. *The central nervous system; Structure and function*. (Oxford, 2010).

6. Millecamps, S. & Julien, J.-P. Axonal transport deficits and neurodegenerative diseases. *Nat. Rev. Neurosci.* **14**, 161–176 (2013).

7. Pissadaki, E. K. & Bolam, J. P. The energy cost of action potential propagation in dopamine neurons : clues to susceptibility in Parkinson ' s disease. **7**, 1–17 (2013).

8. Lee, J. M., Grabb, M. C., Zipfel, G. J. & Choi, D. W. Brain tissue responses to ischemia. *J. Clin. Invest.* **106**, 723–731 (2000).

9. Chamorro, Á., Dirnagl, U., Urra, X. & Planas, A. M. Neuroprotection in acute stroke: Targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. *Lancet Neurol.* **15**, 869–881 (2016).

10. Ferreira, R. C. *et al.* Doenças Cérebro-Cardiovasculares. (2015).

11. Saúde, direção geral da. Para As Doenças Cérebro-Cardiovasculares. (2017).

12. Vilhardt, F. Microglia: phagocyte and glia cell. *Int. J. Biochem. Cell Biol.* **37**, 17–21 (2005).

13. Bradl, M. & Lassmann, H. Oligodendrocytes : biology and pathology. 37–53 (2010). doi:10.1007/s00401-009-0601-5

14. Abbott, N. J., Ronnback, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* **7**, 41–53 (2006).

15. Nayak, D., Roth, T. L. & Mcgavern, D. B. Microglia Development and function. 367–402 (2014). doi:10.1146/annurev-immunol-032713-120240.Microglia

16. Hayakawa, K. *et al.* Transfer of mitochondria from astrocytes to neurons after stroke. *Nature* **535**, 551–5 (2016).

17. Liddelow, S. A. & Barres, B. A. Reactive Astrocytes: Production, Function, and Therapeutic Potential. *Immunity* **46**, 957–967 (2017).

18. Forno, L. S., DeLanney, L. E., Irwin, I., Di Monte, D. & Langston, J. W. Astrocytes and Parkinson's disease. *Prog. Brain Res.* **94**, 429–436 (1992).

19. Xin, W. & Bonci, A. Functional Astrocyte Heterogeneity and Implications for Their Role in

Shaping Neurotransmission. **12**, 1–7 (2018).

20. Son, E. *et al.* Sequential induction of heme oxygenase-1 and manganese superoxide dismutase protects cultured astrocytes against nitric oxide. *Biochem. Pharmacol.* **70**, 590–597 (2005).

21. Sofroniew, M. V. & Vinters, H. V. Astrocytes: Biology and pathology. *Acta Neuropathol.* **119**, 7–35 (2010).

22. Emsley, J. G. & Macklis, J. D. Astroglial heterogeneity closely reflects the neuronal-defined anatomy of the adult murine CNS. **2**, 175–186 (2007).

23. Watkins, S. *et al.* Disruption of astrocyte–vascular coupling and the blood–brain barrier by invading glioma cells. *Nat. Commun.* **5**, 1–15 (2014).

24. Iadecola, C. The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron* **96**, 17–42 (2017).

25. Takano, T. *et al.* Astrocyte-mediated control of cerebral blood flow. *Nat. Neurosci.* **9**, 260–267 (2006).

26. K, S. *Neuropsychopharmacology: The Fifth Generation of Progress*. (American Journal of Psychiatry 159(7), 2002).

27. Allaman, I., Be, M., Magistretti, P. J., Bélanger, M. & Magistretti, P. J. Astrocyte-neuron metabolic relationships: for better and for worse. *Trends Neurosci.* **34**, 76–87 (2011).

28. Pellerin, L. & Magistretti, P. J. Sweet sixteen for ANLS. *J Cereb Blood Flow Metab* **32**, 1152–1166 (2012).

29. Eroglu, C. & Barres, B. A. Regulation of synaptic connectivity by glia. **468**, 223–231 (2015).

30. Chung, W.-S. *et al.* Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* **504**, 394 (2013).

31. Tasdemir-yilmaz, O. E. & Freeman, M. R. Astrocytes engage unique molecular programs to engulf pruned neuronal debris from distinct subsets of neurons. 20–33 (2014). doi:10.1101/gad.229518.113.20

32. Peters A, Palay SL, W. H. *The fine structure of the nervous system*. (Oxford University Press, 1991).

33. Beckel, J. M. *et al.* Mechanosensitive release of adenosine 5'-triphosphate through pannexin channels and mechanosensitive upregulation of pannexin channels in optic nerve head astrocytes: a mechanism for purinergic involvement in chronic strain. *Glia* **62**, 1486–1501 (2014).

34. Pablo, J., Vaccari, D. R., Dietrich, W. D. & Keane, R. W. Activation and regulation of cellular inflammasomes : gaps in our knowledge for central nervous system injury. **34**, 369–375 (2014).

35. Rubini, P. *et al.* Functional P2X7 receptors at cultured hippocampal astrocytes but not neurons. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **387**, 943–954 (2014).

36. Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S. & Smith, S. J. Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* **247**, 470–473 (1990).

37. Muller, C. M. & Best, J. Ocular dominance plasticity in adult cat visual cortex after transplantation of cultured astrocytes. *Nature* **342**, 427–430 (1989).

38. Halassa, M. M., Fellin, T. & Haydon, P. G. The tripartite synapse: roles for gliotransmission in health and disease. *Trends Mol. Med.* **13**, 54–63 (2007).

39. Karkia Pratap ,Smitha Keisha, Johnson Jra James, Aschnerb Michael, L. E. Genetic dys-regulation of astrocytic glutamate transporter EAAT2 and its implications in neurological disorders and manganese toxicity. **40**, 380–388 (2016).

40. T

41. Joe, E. *et al.* Astrocytes , Microglia , and Parkinson ' s Disease. **27**, 77–87 (2018).

42. Lo, Eng H., Rosenberg, G. A. The Neurovascular Unit in Health and Disease. **40**, 10–13 (2010).

43. Choi, Y. K. *et al.* Dual effects of carbon monoxide on pericytes and neurogenesis in traumatic brain injury. *Nat. Med.* (2016). doi:10.1038/nm.4188

44. Ma, Q. Role of Nrf2 in Oxidative Stress and Toxicity. *Annu. Rev. Pharmacol. Toxicol.* **53**, 401–426 (2013).

45. Yu, X. *et al.* Differences in vulnerability of neurons and astrocytes to heme oxygenase-1 modulation: Implications for mitochondrial ferritin. *Sci. Rep.* **6**, 24200 (2016).

46. Zaichick, S. V, McGrath, K. M. & Caraveo, G. The role of Ca2+ signaling in Parkinson's disease. *Dis. Model. & Mech.* **10**, 519 LP – 535 (2017).

47. Rocha, S. M., Cristovao, A. C., Campos, F. L., Fonseca, C. P. & Baltazar, G. Astrocyte-derived GDNF is a potent inhibitor of microglial activation. *Neurobiol. Dis.* **47**, 407–415 (2012).

48. Niranjan, R., Nath, C. & Shukla, R. The mechanism of action of MPTP-induced neuroinflammation and its modulation by melatonin in rat astrocytoma cells, C6. *Free Radic. Res.* **44**, 1304–1316 (2010).

49. Anderson, M. A., Ao, Y. & Sofroniew, M. V. Heterogeneity of reactive astrocytes. 23–29 (2015). doi:10.1016/j.neulet.2013.12.030.Heterogeneity

50. Kim, Park & Choi. The Role of Astrocytes in the Central Nervous System Focused on BK Channel and Heme Oxygenase Metabolites: A Review. *Antioxidants* **8**, 121 (2019).

51. O'Donnell, J. C., Jackson, J. G. & Robinson, M. B. Transient oxygen/glucose deprivation causes a delayed loss of mitochondria and increases spontaneous calcium signaling in astrocytic processes. *J. Neurosci.* **36**, 7109–7127 (2016).

52. Dirnagl, U., Becker, K. & Meisel, A. Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use. *Lancet Neurol* **8**, 398–412 (2009).

53. Alam, Z. I. *et al.* Oxidative DNA damage in the parkinsonian brain: an apparent selective

increase in 8-hydroxyguanine levels in substantia nigra. *J. Neurochem.* **69**, 1196–1203 (1997).

54. Giasson, B. I., Ischiropoulos, H., Lee, V. M.-Y. & Trojanowski, J. Q. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. *Free Radic. Biol. Med.* **32**, 1264–1275 (2002).

55. Gelders, G., Baekelandt, V. & Van der Perren, A. Linking neuroinflammation and neurodegeneration in parkinson's disease. *J. Immunol. Res.* **2018**, (2018).

56. Barcia, C. *et al.* Erratum: IFN-γ signaling, with the synergistic contribution of TNF-α, mediates cell specific microglial and astroglial activation in experimental models of Parkinson's disease. *Cell Death Dis.* **3**, e379–e379 (2012).

57. Sofroniew, M. V. Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci.* **32**, 638–647 (2009).

58. Chen, L.-W., Yung, K.-L. & Chan, Y.-S. Reactive astrocytes as potential manipulation targets in novel cell replacement therapy of Parkinson's disease. *Curr. Drug Targets* **6**, 821–833 (2005).

59. Lau, L. T. & Yu, A. C.-H. Astrocytes Produce and Release Interleukin-1, Interleukin-6, Tumor Necrosis Factor Alpha and Interferon-Gamma Following Traumatic and Metabolic Injury. *J. Neurotrauma* **18**, 351–359 (2001).

60. Leal, M., Casabona, J., Puntel, M. & PITOSSI, F. Interleukin-1β and tumor necrosis factor-α: reliable targets for protective therapies in Parkinson's Disease? . *Frontiers in Cellular Neuroscience* **7**, 53 (2013).

61. Teismann, P. & Schulz, J. B. Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. *Cell Tissue Res.* **318**, 149–161 (2004).

62. Umeno, A., Biju, V. & Yoshida, Y. In vivo ROS production and use of oxidative stress-derived biomarkers to detect the onset of diseases such as Alzheimer's disease, Parkinson's disease, and diabetes. *Free Radic. Res.* **51**, 413–427 (2017).

63. Baillet, A. *et al.* The role of oxidative stress in amyotrophic lateral sclerosis and Parkinson's disease. *Neurochem. Res.* **35**, 1530–1537 (2010).

64. Nakabeppu, Y., Tsuchimoto, D., Yamaguchi, H. & Sakumi, K. Oxidative damage in nucleic acids and Parkinson's disease. *J. Neurosci. Res.* **85**, 919–934 (2007).

65. Lee, H. *et al.* Direct Transfer of ⎵ -Synuclein from Neuron to Astroglia Causes Inflammatory Responses in Synucleinopathies * □. **285**, 9262–9272 (2010).

66. Bosco, D. A. *et al.* Elevated levels of oxidized cholesterol metabolites in Lewy body disease brains accelerate α-synuclein fibrilization. *Nat. Chem. Biol.* **2**, 249 (2006).

67. Keeney, P. M., Xie, J., Capaldi, R. A. & Jr, J. P. B. Parkinson ' s Disease Brain Mitochondrial Complex I Has Oxidatively Damaged Subunits and Is Functionally Impaired and Misassembled. **26**, 5256–5264 (2006).

68. Jacobson, J. & Duchen, M. R. Mitochondrial oxidative stress and cell death in astrocytes –

requirement for stored Ca 2 + and sustained opening of the permeability transition pore. (2002).

69. Idriss, N. K., Blann, A. D. & Lip, G. Y. H. Hemoxygenase-1 in cardiovascular disease. *J. Am. Coll. Cardiol.* **52**, 971–978 (2008).

70. Ozaki, K. S., Kimura, S. & Murase, N. Use of carbon monoxide in minimizing ischemia/reperfusion injury in transplantation. *Transplant. Rev. (Orlando).* **26**, 125–139 (2012).

71. Ryter, S. W., Alam, J. & Choi, A. M. K. Heme Oxygenase-1/Carbon Monoxide: From Basic Science to Therapeutic Applications. *Physiol. Rev.* **86**, 583–650 (2006).

72. Gozzelino, R., Jeney, V. & Soares, M. P. Mechanisms of cell protection by heme oxygenase-1. *Annu. Rev. Pharmacol. Toxicol.* **50**, 323–354 (2010).

73. Romão, C. C., Blättler, W. A., Seixas, J. D. & Bernardes, G. J. L. Developing drug molecules for therapy with carbon monoxide. *Chem. Soc. Rev.* **41**, 3571 (2012).

74. Seixas, J. D. *et al.* Characterization of a versatile organometallic pro-drug (CORM) for experimental CO based therapeutics. *Dalt. Trans.* **42**, 5985–98 (2013).

75. Motterlini, R. & Otterbein, L. E. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov* **9**, 728–743 (2010).

76. Motterlini, R. *et al.* Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ. Res.* **90**, e17–e24 (2002).

77. Sawle, P. *et al.* Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264. 7 murine macrophages. *Br. J. Pharmacol.* **145**, 800–810 (2005).

78. Motterlini, R., Haas, B. & Foresti, R. Emerging concepts on the anti-inflammatory actions of carbon monoxide-releasing molecules (CO-RMs). *Med. Gas Res.* **2**, 28 (2012).

79. Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V & Snyder, S. H. Carbon monoxide: a putative neural messenger. *Science (80-.).* **259**, 381 LP – 384 (1993).

80. Queiroga, C. S. F., Vercelli, A. & Vieira, H. L. A. Carbon monoxide and the CNS: challenges and achievements. *Br. J. Pharmacol.* **172**, 1533–1545 (2015).

81. Wegiel, B., Nemeth, Z., Correa-Costa, M., Bulmer, A. C. & Otterbein, L. E. Heme oxygenase-1: a metabolic nike. *Antioxid. Redox Signal.* **20**, 1709–1722 (2014).

82. Otterbein, L. E. *et al.* Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat. Med.* **6**, 422–8 (2000).

83. Queiroga, C. S. F., Almeida, A. S. & Vieira, H. L. A. Carbon Monoxide Targeting Mitochondria. *Biochem. Res. Int.* **2012**, 1–9 (2012).

84. R. Oliveira, S., Queiroga, C. S. F. & Vieira, H. L. A. Mitochondria and carbon monoxide: cytoprotection and control of cell metabolism – a role for Ca 2+ ? *J. Physiol.* **594**, 4131–4138 (2016).

85. Chora, A. A. *et al.* Heme oxygenase-1 and carbon monoxide suppress autoimmune neuroinflammation. *J. Clin. Invest.* **117**, 438–447 (2007).

86. Zimmermann, A., Leffler, C. W., Tcheranova, D., Fedinec, A. L. & Parfenova, H. Cerebroprotective effects of the CO-releasing molecule CORM-A1 against seizure-induced neonatal vascular injury. *Am J Physiol Hear. Circ Physiol* **293**, H2501-7 (2007).

87. Parfenova, H. *et al.* Functional role of astrocyte glutamate receptors and carbon monoxide in cerebral vasodilation response to glutamate. *AJP Hear. Circ. Physiol.* **302**, H2257–H2266 (2012).

88. Zeynalov, E. & Dore, S. Low doses of carbon monoxide protect against experimental focal brain ischemia. *Neurotox. Res.* **15**, 133–137 (2009).

89. Wang, B., Cao, W., Biswal, S. & Doré, S. Carbon monoxide-activated Nrf2 pathway leads to protection against permanent focal cerebral ischemia. *Stroke* **42**, 2605–2610 (2011).

90. Mahan, V. L., Zurakowski, D., Otterbein, L. E. & Pigula, F. A. Inhaled Carbon Monoxide Provides Cerebral Cytoprotection in Pigs. *PLoS One* **7**, e41982 (2012).

91. Yabluchanskiy, A. *et al.* CORM-3, a carbon monoxide-releasing molecule, alters the inflammatory response and reduces brain damage in a rat model of hemorrhagic stroke. *Crit. Care Med.* **40**, 544–552 (2012).

92. Vieira, H. L. A. A. H. L. A., Queiroga, C. S. F. C. S. F. F. & Alves, P. M. P. M. Pre-conditioning induced by carbon monoxide provides neuronal protection against apoptosis. *J. Neurochem.* **107**, 375–384 (2008).

93. Schallner, N. *et al.* Carbon Monoxide Abrogates Ischemic Insult to Neuronal Cells via the Soluble Guanylate Cyclase-cGMP Pathway. *PLoS One* **8**, (2013).

94. Queiroga, C. S. F. *et al.* Preconditioning Triggered by Carbon Monoxide (CO) Provides Neuronal Protection Following Perinatal Hypoxia-Ischemia. *PLoS One* **7**, (2012).

95. Almeida, A. S. A. S., Soares, N. L. N. L., Vieira, M., Gramsbergen, J. B. J. B. & Vieira, H. L. A. A. Carbon Monoxide Releasing Molecule-A1 (CORM-A1) Improves Neurogenesis: Increase of Neuronal Differentiation Yield by Preventing Cell Death. *PLoS One* **11**, e0154781 (2016).

96. Almeida, A. S. A. S., Sonnewald, U., Alves, P. M. P. M. & Vieira, H. L. A. H. L. A. Carbon monoxide improves neuronal differentiation and yield by increasing the functioning and number of mitochondria. *J. Neurochem.* **138**, 423–435 (2016).

97. Almeida, A. S. A. S. *et al.* Improvement of neuronal differentiation by carbon monoxide: Role of pentose phosphate pathway. *Redox Biol.* **17**, 338–347 (2018).

98. Queiroga, C. S. F. *et al.* Glutathionylation of Adenine Nucleotide Translocase Induced by Carbon Monoxide Prevents Mitochondrial Membrane Permeabilization and Apoptosis. *J. Biol. Chem.* **285**, 17077–17088 (2010).

99. Almeida, A. S. A. S., Queiroga, C. S. F. C. S. F. F., Sousa, M. F. Q. M. F. Q. Q., Alves, P. M. P. M. & Vieira, H. L. A. A. H. L. A. H. L. A. Carbon monoxide modulates apoptosis by reinforcing oxidative metabolism in astrocytes: Role of Bcl-2. *J. Biol. Chem.* **287**, 10761–

10770 (2012).

100. Choi, Y. K. *et al.* Carbon monoxide stimulates astrocytic mitochondrial biogenesis via L-type Ca2+ channel-mediated PGC-1α/ERRα activation. *Biochem. Biophys. Res. Commun.* **479**, 297–304 (2016).

101. Oliveira, S. R., Figueiredo-Pereira, C., Duarte, C. B. & Vieira, H. L. A. P2X7 Receptors Mediate CO-Induced Alterations in Gene Expression in Cultured Cortical Astrocytes—Transcriptomic Study. *Mol. Neurobiol.* (2018). doi:10.1007/s12035-018-1302-7

102. Queiroga, C. S. F., Alves, R. M. A., Conde, S. V., Alves, P. M. & Vieira, H. L. A. Paracrine effect of carbon monoxide: astrocytes promote neuroprotection via purinergic signaling. *J. Cell Sci.* **129**, jcs.187260 (2016).

103. Hettiarachchi, N. T. *et al.* Heme oxygenase-1 derived carbon monoxide suppresses Aβ1–42 toxicity in astrocytes. *Cell Death Dis.* **8**, e2884 (2017).

104. Morgan, M. J. & Liu, Z. Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Res.* **21**, 103 (2010).

105. Leffler, C. W., Parfenova, H., Fedinec, A. L., Basuroy, S. & Tcheranova, D. Contributions of astrocytes and CO to pial arteriolar dilation to glutamate in newborn pigs. *Am. J. Physiol. Heart Circ. Physiol.* **291**, H2897-904 (2006).

106. Kanu, A. & Leffler, C. W. Roles of Glia limitans astrocytes and carbon monoxide in adenosine diphosphate-induced pial arteriolar dilation in newborn pigs. *Stroke* **40**, 930–935 (2009).

107. Anlong, L. *et al.* Astrocyte-Derived CO Is a Diffusible Messenger That Mediates Glutamate-Induced Cerebral Arteriolar Dilation by Activating Smooth Muscle Cell KCa Channels. *Circ. Res.* **102**, 234–241 (2008).

108. Kanu, Alie, L. C. W. roles of glia limitans astrocytes and CO in ADP-induced pial arteriolar dilation in newborn pigs. *Stroke* **40**, 930–935 (2009).

109. Kanu, A. & Leffler, C. W. Carbon monoxide and Ca2+-activated K+ channels in cerebral arteriolar responses to glutamate and hypoxia in newborn pigs. *Am. J. Physiol. Circ. Physiol.* **293**, H3193–H3200 (2007).

110. Gomes, L. C. & Scorrano, L. Mitochondrial morphology in mitophagy and macroautophagy. *Biochim Biophys Acta* (2012). doi:S0167-4889(12)00052-3 [pii]10.1016/j.bbamcr.2012.02.012

111. Rabinowitz, J. D. & White, E. Autophagy and metabolism. *Science* **330**, 1344–1348 (2010).

112. Mizushima, N. Autophagy: process and function. *Genes Dev.* **21**, 2861–2873 (2007).

113. Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. *Nature* **451**, 1069–1075 (2008).

114. Levine, B. & Kroemer, G. Autophagy in the pathogenesis of disease. *Cell* **132**, 27–42 (2008).

115. Hansen, T. E. & Johansen, T. Following autophagy step by step. *BMC Biol.* **9**, 39 (2011).

116. Kroemer, G., Mariño, G. & Levine, B. Autophagy and the integrated stress response. *Mol. Cell* **40**, 280–293 (2010).

117. Archibald, J. M. Review Endosymbiosis and Eukaryotic Cell Evolution. *Curr. Biol.* **25**, R911–R921 (2015).

118. Zimorski, V., Ku, C., Martin, W. F. & Gould, S. B. Endosymbiotic theory for organelle origins. *Curr. Opin. Microbiol.* **22**, 38–48 (2014).

119. Osman, C., Voelker, D. R. & Langer, T. Making heads or tails of phospholipids in mitochondria. *J. Cell Biol.* **192**, 7 LP – 16 (2011).

120. Aanen, D. K., Spelbrink, J. N. & Beekman, M. What cost mitochondria? The maintenance of functional mitochondrial DNA within and across generations. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **369**, 20130438 (2014).

121. Shimada, K. *et al.* Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* **36**, 401–414 (2012).

122. Kauppila, T. E. S., Kauppila, J. H. K. & Larsson, N. G. Mammalian Mitochondria and Aging: An Update. *Cell Metab.* **25**, 57–71 (2017).

123. Dahlgren, C., Gabl, M., Holdfeldt, A., Winther, M. & Forsman, H. Basic characteristics of the neutrophil receptors that recognize formylated peptides, a danger-associated molecular pattern generated by bacteria and mitochondria. *Biochem. Pharmacol.* **114**, 22–39 (2016).

124. Annesley, Sarah J., Fisher, P. R. Mitochondria in health and disease. *Indones. Biomed. J.* **11**, 1–15 (2019).

125. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T. & Pfanner, N. Importing mitochondrial proteins: machineries and mechanisms. *Cell* **138**, 628–644 (2009).

126. Valera-alberni, M. & Canto, C. Mitochondrial stress management : a dynamic journey. **2**, 253–274 (2018).

127. Kawamata, H. & Manfredi, G. Proteinopathies and OXPHOS dysfunction in neurodegenerative diseases. *J. Cell Biol.* **216**, 3917 LP – 3929 (2017).

128. Picard, M., Wallace, D. C. & Burelle, Y. The rise of mitochondria in medicine. *Mitochondrion* **30**, 105–116 (2016).

129. Kawamata, H. & Manfredi, G. Proteinopathies and OXP HOS dysfunction in neurodegenerative diseases. *J. Cell Biol.* **216**, 3917–3929 (2017).

130. Nelson, D. L., M. M., C. *Lehninger Principles of Biochemistry*. (W.H. Freeman, 2004).

131. Scherz-Shouval, R. *et al.* Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J.* **26**, 1749–1760 (2007).

132. Scherz-Shouval, R. & Elazar, Z. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem. Sci.* **36**, 30–38 (2011).

133. Vives-Bauza, C. & Przedborski, S. Mitophagy: the latest problem for Parkinson's disease.

Trends Mol. Med. **17**, 158–165 (2011).

134. Kausar, S., Wang, F. & Cui, H. The Role of Mitochondria in Reactive Oxygen Species Generation and Its Implications for Neurodegenerative Diseases. *Cells* **7**, 274 (2018).

135. Lee, J., Giordano, S. & Zhang, J. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *Biochem. J.* **441**, 523–540 (2012).

136. Jena, N. R. Cite as DNA damage by reactive species: Mechanisms, mutation and repair. in 503–517 (Journal of Biosciences, 2012).

137. Araki, K. *et al.* Redox Sensitivities of Global Cellular Cysteine Residues under Reductive and Oxidative Stress. *J. Proteome Res.* **15**, 2548–2559 (2016).

138. Cheeseman, K. H. Mechanisms and effects of lipid peroxidation. *Mol. Aspects Med.* **14**, 191–197 (1993).

139. Migliore, L. & Coppede, F. Environmental-induced oxidative stress in neurodegenerative disorders and aging. *Mutat. Res.* **674**, 73–84 (2009).

140. Finkel, T. Oxygen radicals and signaling. *Curr. Opin. Cell Biol.* **10**, 248–253 (1998).

141. Zhang, J. *et al.* ROS and ROS-Mediated Cellular Signaling. *Oxid. Med. Cell. Longev.* **2016**, 4350965 (2016).

142. Sena, L. A. & Chandel, N. S. Physiological roles of mitochondrial reactive oxygen species. *Mol. Cell* **48**, 158–167 (2012).

143. Rhee, S. G., Bae, Y. S., Lee, S. R. & Kwon, J. Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Sci. STKE* **2000**, pe1 (2000).

144. Lewis, M. R. & Lewis, W. H. Mitochondria (and other cytoplasmic structures) in tissue cultures. *Am. J. Anat.* **17**, 339–401 (1915).

145. CLARK, S. L. Cellular differentiation in the kidneys of newborn mice studies with the electron microscope. *J. Biophys. Biochem. Cytol.* **3**, 349–362 (1957).

146. NOVIKOFF, A. B. & ESSNER, E. Cytolysomes and mitochondrial degeneration. *J. Cell Biol.* **15**, 140–146 (1962).

147. De Duve, C. & Wattiaux, R. Functions of lysosomes. *Annu. Rev. Physiol.* **28**, 435–492 (1966).

148. De Duve, C. General Properties of Lysosomes: The lysosome concept. *Lysosomes* 1–35 (1963).

149. Beaulaton, J. & Lockshin, R. A. Ultrastructural study of the normal degeneration of the intersegmental muscles of Antheraea polyphemus and Manduca sexta (Insecta, lepidoptera) with particular reference to cellular autophagy. *J. Morphol.* **154**, 39–57 (1977).

150. Ultrastructural, I. & Changes, C. Lysosomal Alterations in Hypoxic and Reoxygenated Hearts. *Am J Pathol 98425-444, 1980* (1980).

151. Heynen, M. J. & Verwilghen, R. L. A quantitative ultrastructural study of normal rat

erythroblasts and reticulocytes. *Cell Tissue Res.* **224**, 397–408 (1982).

152. Scott, S. V & Klionsky, D. J. Delivery of proteins and organelles to the vacuole from the cytoplasm. *Curr. Opin. Cell Biol.* **10**, 523–529 (1998).

153. Lemasters, J. J. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. *Rejuvenation Res.* **8**, 3–5 (2005).

154. Yu, R. *et al.* MIEF1/2 function as adaptors to recruit Drp1 to mitochondria and regulate the association of Drp1 with Mff. *Sci. Rep.* **7**, 880 (2017).

155. Loson, O. C., Song, Z., Chen, H. & Chan, D. C. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. *Mol. Biol. Cell* **24**, 659–667 (2013).

156. Smirnova, E., Griparic, L., Shurland, D. L. & van der Bliek, A. M. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol. Biol. Cell* **12**, 2245–2256 (2001).

157. Gomes, L. C. & Scorrano, L. High levels of Fis1, a pro-fission mitochondrial protein, trigger autophagy. *Biochim. Biophys. Acta* **1777**, 860–866 (2008).

158. Frank, M. *et al.* Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner. *Biochim. Biophys. Acta* **1823**, 2297–310 (2012).

159. Thornton, C. *et al.* Mitochondrial dynamics, mitophagy and biogenesis in neonatal hypoxic-ischaemic brain injury. *FEBS Lett.* **592**, 812–830 (2018).

160. Poole, A. C., Thomas, R. E., Yu, S., Vincow, E. S. & Pallanck, L. The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. *PLoS One* **5**, e10054 (2010).

161. Okatsu, K. *et al.* Mitochondrial hexokinase HKI is a novel substrate of the Parkin ubiquitin ligase. *Biochem. Biophys. Res. Commun.* **428**, 197–202 (2012).

162. Shiba-Fukushima, K. *et al.* PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. *Sci. Rep.* **2**, 1002 (2012).

163. Narendra, D. P. *et al.* PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* **8**, e1000298 (2010).

164. Geisler, S. *et al.* PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat. Cell Biol.* **12**, 119–31 (2010).

165. Lazarou, M. *et al.* The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* **524**, 309–314 (2015).

166. Kubli, D. A. *et al.* PINK1 is dispensable for mitochondrial recruitment of parkin and activation of mitophagy in cardiac myocytes. *PLoS One* **10**, 1–16 (2015).

167. Hollville, E., Carroll, R. G., Cullen, S. P. & Martin, S. J. Bcl-2 Family Proteins Participate in Mitochondrial Quality Control by Regulating Parkin/PINK1-Dependent Mitophagy. *Mol. Cell*

55, 451–466 (2014).

168. Willis, S. N. & Adams, J. M. Life in the balance : how BH3-only proteins induce apoptosis. **17**, 617–625 (2010).

169. Bellot, G. *et al.* Hypoxia-Induced Autophagy Is Mediated through Hypoxia-Inducible Factor Induction of BNIP3 and BNIP3L via Their BH3 Domains. *Mol. Cell. Biol.* **29**, 2570–2581 (2009).

170. Boyd, J. M. *et al.* Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. *Cell* **79**, 341–351 (1994).

171. Lee, H. & Paik, S.-G. Regulation of BNIP3 in normal and cancer cells. *Mol. Cells* **21**, 1–6 (2006).

172. Webster, K. A., Graham, R. M. & Bishopric, N. H. BNip3 and signal-specific programmed death in the heart. *J. Mol. Cell. Cardiol.* **38**, 35–45 (2005).

173. Kothari, S. *et al.* BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. *Oncogene* **22**, 4734–4744 (2003).

174. Autophagy, H. *et al.* BNIP3 Is an RB / E2F Target Gene Required for. **27**, 6229–6242 (2007).

175. Shi, R.-Y. *et al.* BNIP3 Interacting with LC3 Triggers Excessive Mitophagy in Delayed Neuronal Death in Stroke. *CNS Neurosci. Ther.* **20**, 1045–1055 (2014).

176. Kubli, DA, Ycaza JE, Gustafsson, A. Bnip3 mediates mitochondrial dysfunction and cell death through Bax and Bak. *Biochem. J.* **415**, 407–415 (2007).

177. Rikka, S. *et al.* Bnip3 impairs mitochondrial bioenergetics and stimulates mitochondrial turnover. *Cell Death Differ.* **18**, 721–31 (2011).

178. Kanki, T., Wang, K., Cao, Y., Baba, M. & Klionsky, D. J. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Dev. Cell* **17**, 98–109 (2009).

179. Sandoval, H. *et al.* Essential role for Nix in autophagic maturation of erythroid cells. *Nature* **454**, 232–235 (2008).

180. Sowter, H. M., Ratcliffe, P. J., Watson, P., Greenberg, A. H. & Harris, A. L. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res.* **61**, 6669–6673 (2001).

181. Ding, W. X. *et al.* Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. *J. Biol. Chem.* **285**, 27879–27890 (2010).

182. Kim, E. H. *et al.* Sodium Selenite Induces Superoxide-Mediated Mitochondrial Damage and Subsequent Autophagic Cell Death in Malignant Glioma Cells. 6314–6325 (2007). doi:10.1158/0008-5472.CAN-06-4217

183. Kim, E. H. & Choi, K. S. A critical role of superoxide anion in selenite-induced mitophagic cell death. *Autophagy* **4**, 76–78 (2008).

184. Shroff, E. H. *et al.* BH3 peptides induce mitochondrial fission and cell death independent of BAX/BAK. *PLoS One* **4**, (2009).

185. Landes, T. *et al.* The BH3-only Bnip3 binds to the dynamin Opa1 to promote mitochondrial fragmentation and apoptosis by distinct mechanisms. *EMBO Rep* **11**, 459–465 (2010).

186. Chaanine, A. H. *et al.* JNK modulates FOXO3a for the expression of the mitochondrial death and mitophagy marker BNIP3 in pathological hypertrophy and in heart failure. *Cell Death Dis* **3**, 265 (2012).

187. Hamacher-Brady, A. *et al.* Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death Differ.* **14**, 146–57 (2007).

188. Winklhofer, K. F. Parkin and mitochondrial quality control: Toward assembling the puzzle. *Trends Cell Biol.* **24**, 332–341 (2014).

189. Exner, N. *et al.* Loss-of-Function of Human PINK1 Results in Mitochondrial Pathology and Can Be Rescued by Parkin. *J. Neurosci.* **27**, 12413–12418 (2007).

190. Dagda, R. K. *et al.* Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J. Biol. Chem.* **284**, 13843–13855 (2009).

191. Mannam, P. *et al.* MKK3 regulates mitochondrial biogenesis and mitophagy in sepsis-induced lung injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **306**, L604-19 (2014).

192. Zhang, Y. *et al.* Endothelial PINK1 mediates the protective effects of NLRP3 deficiency during lethal oxidant injury. *J. Immunol.* **192**, 5296–304 (2014).

193. Rothfuss, O. *et al.* Parkin protects mitochondrial genome integrity and supports mitochondrial DNA repair. *Hum. Mol. Genet.* **18**, 3832–3850 (2009).

194. Mclelland, G., Soubannier, V., Chen, C. X., Mcbride, H. M. & Fon, E. A. Parkin and PINK 1 function in a vesicular trafficking pathway regulating mitochondrial quality control. *EMBO J.* **33**, 282–295 (2014).

195. Gatliff, J. & Campanella, M. The 18 kDa translocator protein (TSPO): a new perspective in mitochondrial biology. *Curr. Mol. Med.* **12**, 356–368 (2012).

196. Zhang, X. *et al.* Endoplasmic reticulum stress induced by tunicamycin and thapsigargin protects against transient ischemic brain injury: Involvement of PARK2-dependent mitophagy. *Autophagy* **10**, 1801–1813 (2014).

197. Pacelli, C. *et al.* Mitochondrial defect and PGC-1alpha dysfunction in parkin-associated familial Parkinson's disease. *Biochim. Biophys. Acta* **1812**, 1041–1053 (2011).

198. Rana, A., Rera, M. & Walker, D. W. Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics, and extends lifespan. *Proc. Natl. Acad. Sci.* **110**, 8638–8643 (2013).

199. Hickson-Bick, D. L. M., Jones, C. & Buja, L. M. Stimulation of mitochondrial biogenesis and autophagy by lipopolysaccharide in the neonatal rat cardiomyocyte protects against programmed cell death. *J. Mol. Cell. Cardiol.* **44**, 411–418 (2008).

200. Williams, J. A., Ni, H.-M., Ding, Y. & Ding, W.-X. Parkin regulates mitophagy and mitochondrial function to protect against alcohol-induced liver injury and steatosis in mice. *Am. J. Physiol. - Gastrointest. Liver Physiol.* **309**, G324–G340 (2015).

201. Zhong, Z. *et al.* Acute ethanol causes hepatic mitochondrial depolarization in mice: Role of ethanol metabolism. *PLoS One* **9**, e91308 (2014).

202. Swiader, A. *et al.* Mitophagy acts as a safeguard mechanism against human vascular smooth muscle cell apoptosis induced by atherogenic lipids. *Oncotarget* **7**, 28821–35 (2016).

203. Melser, S. *et al.* Rheb regulates mitophagy induced by mitochondrial energetic status. *Cell Metab.* **17**, 719–730 (2013).

204. Doménech, E. *et al.* AMPK and PFKFB3 mediate glycolysis and survival in response to mitophagy during mitotic arrest. *Nat. Cell Biol.* **17**, 1304–1316 (2015).

205. Chourasia, A. H. & Macleod, K. F. Tumor suppressor functions of BNIP3 and mitophagy. **8627**, (2015).

206. Zhang, H. *et al.* Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* **283**, 10892–10903 (2008).

207. Glick, D. *et al.* BNip3 Regulates Mitochondrial Function and Lipid Metabolism in the Liver. *Mol. Cell. Biol.* **32**, 2570–2584 (2012).

208. Jornayvaz, François R., Shulman, G. I. Regulation of mitochondrial biogenesis. *Essays Biochem* 1–15 (2010). doi:10.1042/bse0470069.Regulation

209. Leaw, B. *et al.* Mitochondria , Bioenergetics and Excitotoxicity : New Therapeutic Targets in Perinatal Brain Injury. **11**, 1–18 (2017).

210. Anzell, A. R., Maizy, R., Przyklenk, K. & Sanderson, T. H. Mitochondrial Quality Control and Disease : Insights into Ischemia-Reperfusion Injury. 2547–2564 (2018). doi:10.1007/s12035-017-0503-9

211. Hagberg, H., Mallard, C., Rousset, C. I. & Thornton, C. Mitochondria: hub of injury responses in the developing brain. *Lancet. Neurol.* **13**, 217–232 (2014).

212. Gureev, A. P., Shaforostova, E. A. & Popov, V. N. Regulation of Mitochondrial Biogenesis as a Way for Active Longevity : Interaction Between the Nrf2 and PGC-1 α Signaling Pathways. **10**, 1–12 (2019).

213. Evans, M. J. & Scarpulla, R. C. NRF-1: a trans-activator of nuclear-encoded respiratory genes in animal cells. *Genes Dev.* **4**, 1023–1034 (1990).

214. Scarpulla, R. C. Transcriptional Paradigms in Mammalian Mitochondrial Biogenesis and Function. 611–638 (2008). doi:10.1152/physrev.00025.2007.

215. Virbasius, J. V & Scarpulla, R. C. Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors : A potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. **91**, 1309–1313 (1994).

216. Nishida, T. & Yamada, Y. SUMOylation of the KRAB zinc-finger transcription factor

PARIS/ZNF746 regulates its transcriptional activity. *Biochem. Biophys. Res. Commun.* **473**, 1261–1267 (2016).

217. Lupo, A. *et al.* KRAB-Zinc Finger Proteins : A Repressor Family Displaying Multiple Bio- logical Functions. 268–278 (2013).

218. Lee, S. J. *et al.* Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation. *Am J Respir Cell Mol Biol* **45**, 867–873 (2011).

219. Lee, S. *et al.* Carbon Monoxide Confers Protection in Sepsis by Enhancing Beclin 1-Dependent Autophagy and Phagocytosis. *Antioxid. Redox Signal.* **20**, 432–442 (2014).

220. Zuckerbraun, B. S. *et al.* Carbon monoxide signals via inhibition of cytochrome c oxidase and generation of mitochondrial reactive oxygen species. *FASEB J.* **21**, 1099–106 (2007).

221. Almeida, A. S., Figueiredo-pereira, C., Vieira, H. L. A. & Raimundo, N. Carbon monoxide and mitochondria — modulation of cell metabolism , redox response and cell death. **6**, 1–6 (2015).

II

CARBON MONOXIDE RELEASED BY CORM-A1 PREVENTS YEAST CELL DEATH *VIA* AUTOPHAGY STIMULATION

This chapter is based on the following published article:

Carbon monoxide released by CORM-A1 prevents yeast cell death *via* autophagy stimulation

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ABSTRACT

Autophagy is an autodigestive process, promoting cytoprotection by the elimination of dysfunctional organelles, misfolded proteins and toxic aggregates. Carbon monoxide (CO) is an endogenous gasotransmitter that under low concentrations prevents cell death and inflammation. For the first time, the role of autophagy in CO-mediated cytoprotection against oxidative stress was evaluated in the model yeast *Saccharomyces cerevisiae*. The Boron based carbon monoxide releasing molecule, CORM-A1, was used to deliver CO. CORM-A1 partially prevented oxidative stress-induced cell death in yeast. Likewise, CORM-A1 activated autophagy under basal physiological conditions, which were assessed by autophagic flux and the expression of mCherry-Atg8 or GFP-Atg8. Inhibition of autophagy by knocking out key autophagic genes in yeast (*ATG8* or *ATG11*) blocked CORM-A1 cytoprotective effect, indicating the critical role of autophagy on CO-induced cytoprotection. The CO-mediated cytoprotection *via* autophagy induction observed in yeast was validated in primary cultures of astrocytes, a well characterized model for CO's cytoprotective functions. As in yeast, CORM-A1 prevented oxidative stress-induced cell death in an autophagic dependent manner in astrocytes.

Overall, our data support the cytoprotective action of CO against oxidative stress. CO promotes cytoprotection in yeast *via* autophagy, opening new possibilities for the study of molecular mechanisms of CO's biological functions using this powerful eukaryotic model.

CONTENTS

Claudia Figueiredo-Pereira had carried out the majority of the experimental part and was involved on the decisions on how to execute the experiments, as well as on the interpretation and discussion of the results.

INTRODUCTION

Autophagy is a process by which cytoplasmic components (macromolecules and/or organelles) are transported via autophagosomes into lysosome/vacuole to be degraded by hydrolases. Despite being a process known for many decades, only in the 1990's with the discovery of the autophagy-related genes (ATG), the pathophysiological significance of autophagic process started to be disclosed [1]. Although autophagy was firstly recognized as a specific type of cell death, also called type 2, it is now increasingly accepted that autophagy is a cytoprotective process involved in cell adaptation to stress [2]. The autophagic process is associated with several physiological roles, including quality control of intracellular proteins and organelles, anti-aging, adaptive responses to starvation, and suppression of tumour formation [2,3]. Autophagy-related (Atg) proteins play critical roles throughout autophagy, being some responsible for the autophagosome formation. There are more than 30 Atg proteins already described in yeast and higher eukaryotes [4].

The yeast *Saccharomyces cerevisiae* is an excellent eukaryotic model for studying autophagy associated molecular mechanisms [5]. Several key proteins associated with autophagic process are described in yeast and present homologs in mammals. In yeast, Atg8 is an ubiquitin-like protein that plays an essential role in autophagy, being responsible for autophagy initiation, cargo recognition, cargo engulfment and vesicle closure [6]. There are two subfamilies of Atg8 homologs found in mammals: microtubule-associated protein-1 light chain 3 (LC3) and γ-aminobutyric acid (GABA)-receptor-associated proteins (GABARAP) [7]. Furthermore, Atg11 is a key protein for selective autophagy. During mitochondrial specific autophagy (mitophagy), kinase-induced phosphorylation of the core autophagy proteins facilitates their interaction with the scaffold protein Atg11, which is required for autophagosome formation [5].

Carbon monoxide (CO) is a diatomic colorless small molecule and odorless gas. Despite being commonly known as a lethal gas, CO is endogenously produced by the activity of heme-oxygenase (HO), which is considered as a cytoprotective enzyme expressed or activated in response to stress [8,9]. CO has been increasingly recognized as a cytoprotective and homeostatic molecule, with anti-apoptotic and anti-inflammatory activities, and with the ability of modulating cell metabolism [10–12]. Recently, it has been shown that low levels of CO also modulate autophagy in lung epithelial cells [13], in a model of sepsis [14], mesenchymal stromal cells [15] and in islet cells [16]. Data about CO effects on yeast physiology are very scarce. CO was shown to interfere with yeast metabolic cycles [17]. However, no data linking CO and autophagy is available in the literature.

Since the last two decades, CO has received great attention as a potential therapeutic molecule. Therefore, the development of strategies for delivering CO in biological systems are in progress, in

particular CO-releasing molecules (CORMs), which are organic and organometallic compounds, with the capacity of delivering CO in a time and tissue-specific manner [18,19]. CORM-A1 is a boron based water soluble molecule, which releases CO in a pH and temperature dependent manner, being slow releaser under physiological conditions [20]. It is already established the capacity of CORMs to mimic the beneficial effect of CO gas *in vitro* and *in vivo* experimental models, including vasomodulation, anti-inflammatory and anti-apoptotic properties [21–24]. Still, there is no data about the use of CORMs in yeast models for the study of the underlying molecular mechanisms of CO.

Herein the role of CO on autophagy modulation and cytoprotection was first assessed in yeast models using CORM-A1 for delivering CO. Yeast cells were challenged with oxidative stress and the role of CO on autophagy induction and the requirement of key autophagy genes (*ATG8* and *ATG11*) were disclosed. Finally, the data were validated in primary cultures of mouse cortex astrocytes. Astrocytes are an optimal model for mammalian validation because our group has previously demonstrated that CO prevents cell death induced by oxidative stress [25–27]. In summary, our data show that CORM-A1-induced cytoprotection is dependent on autophagic process in yeast and primary culture of astrocytes.

MATERIAL AND METHODS

Yeast Strains, Plasmids and Culture Conditions

The following strains were used in this study: BY 4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), *Δatg8* (MATa *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YBL078c::kanMX4*) and *Δatg11* (MATa *ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 YPR049c::kanMX4*) (obtained from EUROSCARF). The plasmids p316-mcherry-*ATG8* [28] and p316-GFP-*ATG8* [29] encoding the respective *ATG8* fusions under the control of *ATG8* promoter, were used to monitor autophagy induction. Yeast transformation procedures were carried out as indicated using lithium acetate standard method [30]. Synthetic complete (SC) medium (0.67% (w/v)) yeast nitrogen base without amino acids (YNB) (Difco), 0.79 g/L complete supplement mixture (CSM) (MP Biomedicals, ref. ICNA114500012), 2% (w/v) glucose (Sigma) used for yeast growth. Synthetic dropout CSM_{-URA} media (0.67% (w/v)) YNB, 0.77 g/L 6-amino acid dropout CSM_{-URA} (MP Biomedicals, ref. ICNA11451121), 2% (w/v) glucose] was used for growth of the yeast cells transformed with p316-mcherry-*ATG8* or p316-GFP-*ATG8*, respectively. A pre-inoculum was prepared in appropriated medium and cultures were incubated overnight at 30°C under orbital shaking. Cultures were diluted in fresh medium and incubated under the same conditions until the optical density at 600 nm (OD_{600}) reached 0.5 ± 0.05 (log growth phase), using the equation OD_i x

$V_i = (OD_f/(2^{(t/gt)}) \times V_f$, where OD_i = initial optical density of the culture, V_i = initial volume of culture, OD_f = final optical density of the culture, t = time (usually 16 h), gt = generation time of the strain and V_f = final volume of culture. Readings were performed in 96-well plates using a Biotek Power Wave XS plate spectrophotometer. Results were expressed as ln of OD_{600} normalized by OD_{600} at time point 0 (designated as OD_{600}) over time.

Mammalian Cell Culture

Primary cultures of astrocytes were prepared from 1-day-old Balb/c mouse cortex, as described [31]. Animals were rapidly decapitated, brain cortex was removed and the meninges were carefully stripped off, then the cortex was washed in ice-cold phosphate-buffered saline (PBS), and mechanically disrupted. Single-cell suspensions were plated in T-flasks (four hemispheres/75 cm^2) in Dulbecco's minimum essential medium supplemented with 20% (v/v) FBS (heat-inactivated), 1% (v/v) of 100 U/mL penicillin/streptomycin solution and glucose (to obtain a final concentration of 10 mM). Astrocytes were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. After 7 days, the dark phase cells growing on the astrocytic cell layer were separated by vigorous shaking and removed. Culture medium was replaced twice a week for 3 weeks with gradual reduction in FBS content (2^{nd} week 15% (v/v); 3^{rd} week 10%(v/v)). At the 3^{rd} week the confluent astrocytes were detached by mild trypsinization using trypsin/EDTA (0.25%, w/v) and sub-cultured, in 24 and 6-well plates at 50 x 10^3 and 100 x10^3 cells/well, respectively, until reaching full confluence.

Preparation of CORM-A1 Solution

The CO releasing molecule, CORM-A1 [20] was used to deliver CO to cell culture. CORM-A1 (SML-0315, Sigma) stock solutions were prepared in water to a final concentration of 500 or 1000 µM and stored at -20°C to avoid loss of released CO. The concentration of CORM-A1 and time points for treatments were selected based on the cytoprotective effects of CO in yeast and astrocytes subjected to oxidative stress.

Cell Viability and Autophagy Assessment in Yeast

Growth assays

Yeast cultures were diluted to OD_{600} 0.12 ± 0.012 in fresh medium supplemented or not with CORM-A1 at 100 µM (twice with 30 min of interval) in 96-well plates. After 1 h incubation with CORM-A1 at 30ºC, *tert*-butylhydroperoxide (*t*-BHP) was added at a final concentration of 1 mM. The cultures were incubated at 30°C with shaking for 24 h and cellular growth was monitored hourly. CO's protection factor against oxidative stress after 15 h incubation with *t*-BHP was estimated according to the equation: % of protection = 100 x (($OD_{control}$ – $OD_{t\text{-}BHP}$) / ($OD_{control}$ – $OD_{CO\ t\text{-}BHP}$)), where:
$OD_{control}$: the OD at 600nm at 15h of growth corresponding to control yeast
$OD_{t\text{-}BHP}$: the OD at 600nm at 15h of growth corresponding to yeast treated with t-BHP (oxidative stress)
$OD_{CO\ t\text{-}BHP}$: the OD at 600nm at 15h of growth corresponding to yeast treated with CO and then with *t*-BHP.

Cell Viability

To analyze cell viability by flow cytometry, cells were grown as indicated and incubated with 5 µg/mL of propidium iodide (PI, Invitrogen) for 30 min protected from light. Cells were gated by forward and side scatter using the flow cytometer (BD FACSCanto™ II), which contains a blue solid state laser (488 nm) FL3 red fluorescence channel for PI detection at 650 nm. The acquisition was done with Diva software and data analysis was performed with FlowJo® software (10.5.3). A minimum of 10,000 events was collected for each experiment.

Induction of autophagy and autophagic flux monitorization

Induction of autophagy was analysed in cells transformed with the mCherry-*ATG8* or GFP-*ATG8* and treated with CORM-A1 at a final concentration of 100 µM (twice with 30 min of interval) using a CyFlow Cube 6 (Sysmex Partec GmbH, Goerlitz, Germany), equipped with a blue solid-state laser (488 nm), green fluorescence channel 530/30 nm, and orange red fluorescence channel 610/20 nm. Cells treated with rapamycin were used as positive control (0.2 µg/mL). Data analysis was performed using FlowJo software. A minimum of 10,000 events was collected for each experiment. To determine the percentage of autophagic cells, cells were grown as described above and GFP and mCherry fluorescence was visualized using a Leica Z2 fluorescence microscope. The percentage of

cells positive for autophagic process was then determined by counting at least 600 cells for each condition using ImageJ software.

For autophagic flux monitorization, protein extraction was performed as previously described [32] Briefly, cells were treated with 10% (v/v) trichloroacetic acid, and washed twice with acetone. The dry cell pellet was resuspended in MURB buffer (50 mM Na_2HPO_4, 25 mM MES, pH 7.0, 1% (v/v) sodium dodecyl sulfate (SDS), 3 M urea, 0.5% (v/v) 2-mercaptoethanol, 1 mM NaN_3 and 0.05% (w/v) bromophenol blue) with proteases and phosphatases inhibitors (Roche, Mannheim, Germany), and disrupted by vortex with an equal volume of acid-washed glass beads, for 5 min. The samples were incubated at 70°C for 10 min and cell debris were removed by centrifugation. Immunoblotting was performed following standard procedures, and GFP (Antibodies Incorporated, Davis, CA, USA) and PGK (Life Technologies, Paisley, UK) antibodies. Autophagic flux was determined using the GFP-Atg8 processing assay [33], by measuring the vacuolar degradation of the Atg8 domain reporter (ratio of free GFP to total GFP signal) [34].

Cell Viability and Autophagy in Mammalian Cells

Administration of chemical compounds to cell culture

The stock solution of Wortmannin (WM) (W-1628, Sigma), which is a selective and irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K) [35], and is required for autophagic sequestration at the beginning of the autophagic process, was prepared in DMSO (Dimethyl sulfoxide) to a final concentration of 100 nM. Hydroxyclochroquine (HCQ) (Plaquinol®, Biosaúde), an inhibitor of autophagic flux, was prepared in H_2O at a final concentration of 3 mg/mL and stored at -20°C. Astrocytic cultures were incubated with 100 nM of WM or 3 µg/mL of HCQ.

Induction of autophagy and autophagic flux monitorization

Astrocytes were treated with CORM-A1 at a final concentration of 12.5 µM with or without HCQ at 30 µg/mL for 1 h at 37ºC. Induction of autophagy in astrocytes was followed by the assessment of LC3 lipidation (LC3II), in particular the ratio between LC3II/I measured by immunoblotting [35]. Furthermore, autophagic flux was measured by using HCQ, an inhibitor of lysosomal function that limits autophagic flux progression.

Apoptosis induction and assessment of cell viability

Astrocytes were treated with CORM-A1 at a final concentration of 12.5 µM for 1 h, followed by apoptosis induction by *t*-BHP at concentrations from 80 to 320 µM for 18 h. In astrocytes, WM (100 µM), was used to modulated CO effect in autophagy process by pre-treating cells 1 h prior to CORM-A1 treatment. To detect apoptosis induced by *t*-BHP, cells were collected by trypsinization and stained with dye propidium iodide (PI, Invitrogen) at 1 g/mL. Cells were gated by forward and side scatter. A flow cytometer (BD FACSCanto™ II) was used to analyse apoptosis-associated parameters. The acquisition was done with Diva software and data analysis was performed with FlowJo® software (10.5.3).

Protein extraction and immunoblotting

After washing the astrocytes twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM K_2HPO_4, 10 mM $NaH_2PO_4 \cdot 2H_20$), total protein was extracted using lysis buffer (50 mM Tris-HCl, 2% SDS, pH 6.8) freshly supplemented with 1X cocktail protease inhibitor (Roche, pH 7.5). Protein concentration was quantified using a Bicinchoninic acid (BCA) protein assay kit Pierce® using the manufacturers' specifications. Absorbance was measured using a TECAN infinite F200PRO microplate reader. Samples from cell extracts were separated under reducing electrophoresis on a 1 mm thick 15% SDS-polyacrylamide (SDS-PAGE) gel. Samples were transferred onto a PVDF membrane (MERK). Membranes were incubated overnight at 4°C with antibodies against LC3I/II protein (Abcam) and actin (Sigma), used as a loading control. Blots were developed using the ECL (enhanced chemiluminescence) detection system after incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), for 1 h at room temperature.

Statistical Analyses

The data concerning yeast and astrocytic culture were carried out at least in three independent preparations (cell isolation). For autophagy evaluation, a representative image of three independent assays is shown. All values are mean S.D. (n =3). Error bars, corresponding to S.D., are shown in the figures.

Statistical comparisons were performed using ANOVA (one-way, two-way) and Student t-test (with Welch's correction) with p <0.05 (n≥3). p <0.05 means that samples are significantly different at a confidence level of 95%.

RESULTS

CORM-A1 promotes cytoprotection against oxidative stress in yeast and primary cultures of astrocytes

CORM-A1 concentrations in yeast were chosen based on the used concentrations in mammal cells (up to 25 μM) and the fact yeast cells present wall. Thus, higher concentrations of CORM-A1 (50 μM or 100 μM) were tested in yeast with one or two additions into culture, with 30 min of interval. The optimal conditions for promoting cytoprotection in yeast were 2 additions of 100 μM of CORM-A1 (data not shown).

In order to assess the potential cytoprotective role of CO in yeast, growth of cells pre-treated or not with CORM-A1 and then subjected to *t*-BHP-induced oxidative stress was followed for 24 h. As shown in Figure 1A, *t*-BHP promotes a decrease on yeast growth as compared to the control condition. CORM-A1 did not affect cell growth under non-stress condition, and it partially recovered the growth of cells exposed to oxidative stress (Figure 1A). Furthermore, yeast cell viability was also assessed by PI dye, an indicator of plasma membrane integrity. At 6, 12 and 15h after *t*-BHP treatment there is an increase on yeast cell death levels, which are partially prevented by CORM-A1 pre-treatment (Figure 1B). These data suggest that CO may protect yeast cells against *t*-BHP-induced oxidative stress.

Likewise, astrocytes were treated with the same pro-oxidant agent *t*-BHP for promoting cell death. Cell viability was assessed by flow cytometry, using PI dye. In support of previous data indicating that CORM-A1 promotes astrocytic survival by preventing caspase-3 cleavage and activation [27], our data also show that CORM-A1 partially inhibited cell death induced by oxidative stress (Figure 1C). In conclusion, CO delivered by CORM-A1 is cytoprotective in yeast and primary cultures of astrocytes.

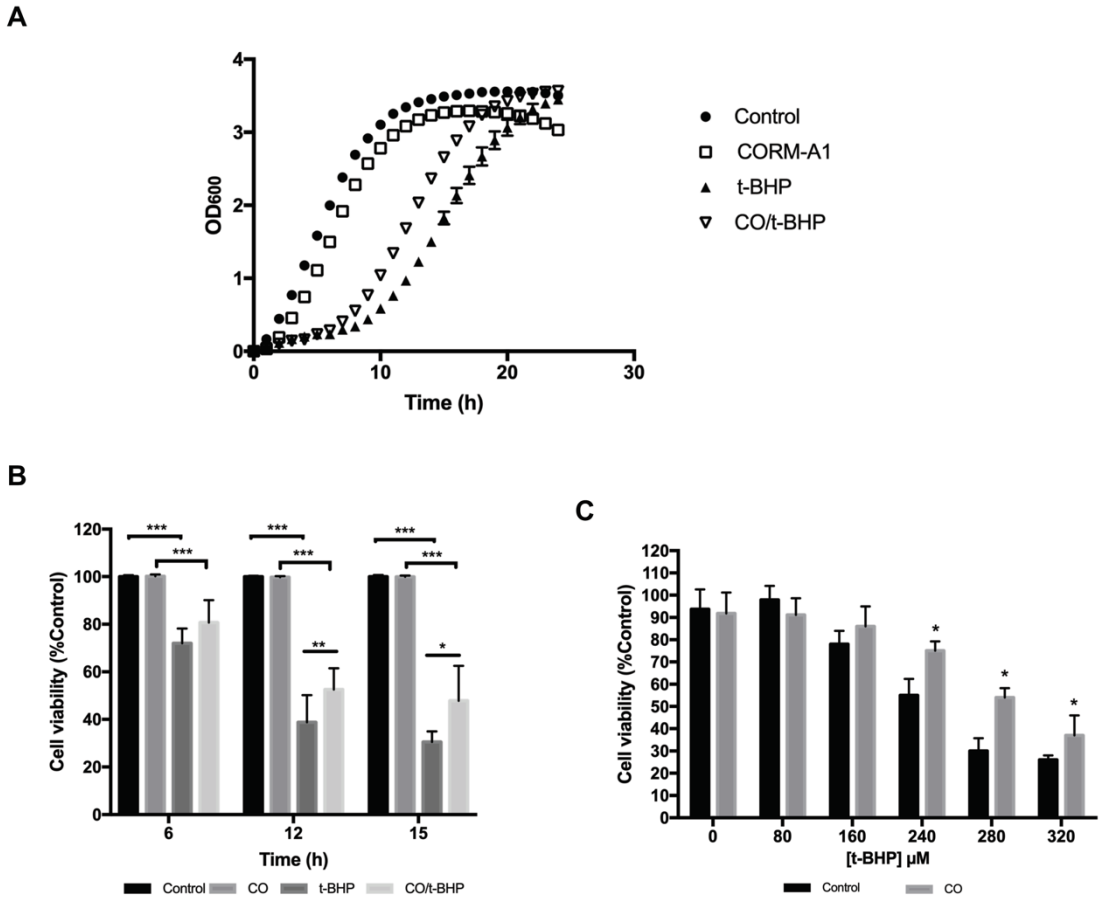


Figure 1- CORM-A1 promotes cytoprotection against oxidative stress. A) BY 4741 cells were pretreated or not with 100 µM CORM-A1 (twice with an interval of 30 min) for a total of 1 h and were incubated for 24 h in the presence or absence of oxidative stress (1 mM t-BHP). The OD600nm was monitored hourly. Results were expressed as ln of OD600 normalized by OD600 at time point 0 (designated as OD600) over time. A representative growth curve is shown. **B)** BY 4741 cells were pretreated or not with 100 µM CORM-A1 (twice with an interval of 30 min) and then were incubated for 6, 12 and 15 h in the presence or absence of oxidative stress (1 mM t-BHP). Cell viability was assessed by PI in flow cytometry. **C)** Primary cultures of astrocytes were pretreated with CORM-A1 at a final concentration of 12.5 µM for 1 h, and then cell death was triggered by t-BHP at 80– 320 µM for 18 h. Cell viability assessment is based on plasmatic membrane integrity using PI dye and measuring its fluorescence by flow cytometry. The results are the mean ± standard error of the mean of at least three independent experiments, performed in different cell preparations. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, as compared to control with two-way ANOVA.

CORM-A1 induces autophagy

CORM-A1 ability to promote autophagy was evaluated by assessing Atg8 expression levels and autophagic flux. Treatment with CORM-A1 increased GFP-Atg8 expression as assessed by flow cytometry (Figure 2A and Supplementary Figure S1). mCherry-Atg8 expression was assessed by fluorescent microscopy (Figure 2B). Both assessments suggest that CORM-A1, by inducing Atg8 expression, may promote autophagy in yeast. Moreover, this effect was not tag-dependent since similar results were obtained by fluorescent microscopy with cells expressing GFP-Atg8 (Supplementary Figure S2). Furthermore, the levels of Atg8 activation are similar whenever induced by CORM-A1 or by rapamycin (Figure 2A and B). To support the hypothesis that CO promotes autophagy, CO-induced autophagic flux was assessed using the GFP-Atg8 processing assay [36]. The data showed that CO promotes autophagy by increasing levels of autophagic flux (Figure 2C). Rapamycin was used as positive control since it is a well characterized autophagy inducer. Taken all together, one can conclude CORM-A1 triggers autophagy in yeast cells.

Primary cultures of astrocytes were used to validate previous data observed in yeast, by the assessment of LC3 lipidation (LC3II). CORM-A1 at 12.5 μM and 25 μM increased LC3II accumulation in astrocytes, indicating that autophagic process is induced (Figure 2D). Cells co-treated with CORM-A1 and HCQ showed an increased accumulation of LC3II, indicating CORM-A1 does promote autophagic flux (Figure 2D). In summary, CORM-A1 stimulates autophagy in yeast and primary culture of cortex astrocytes.

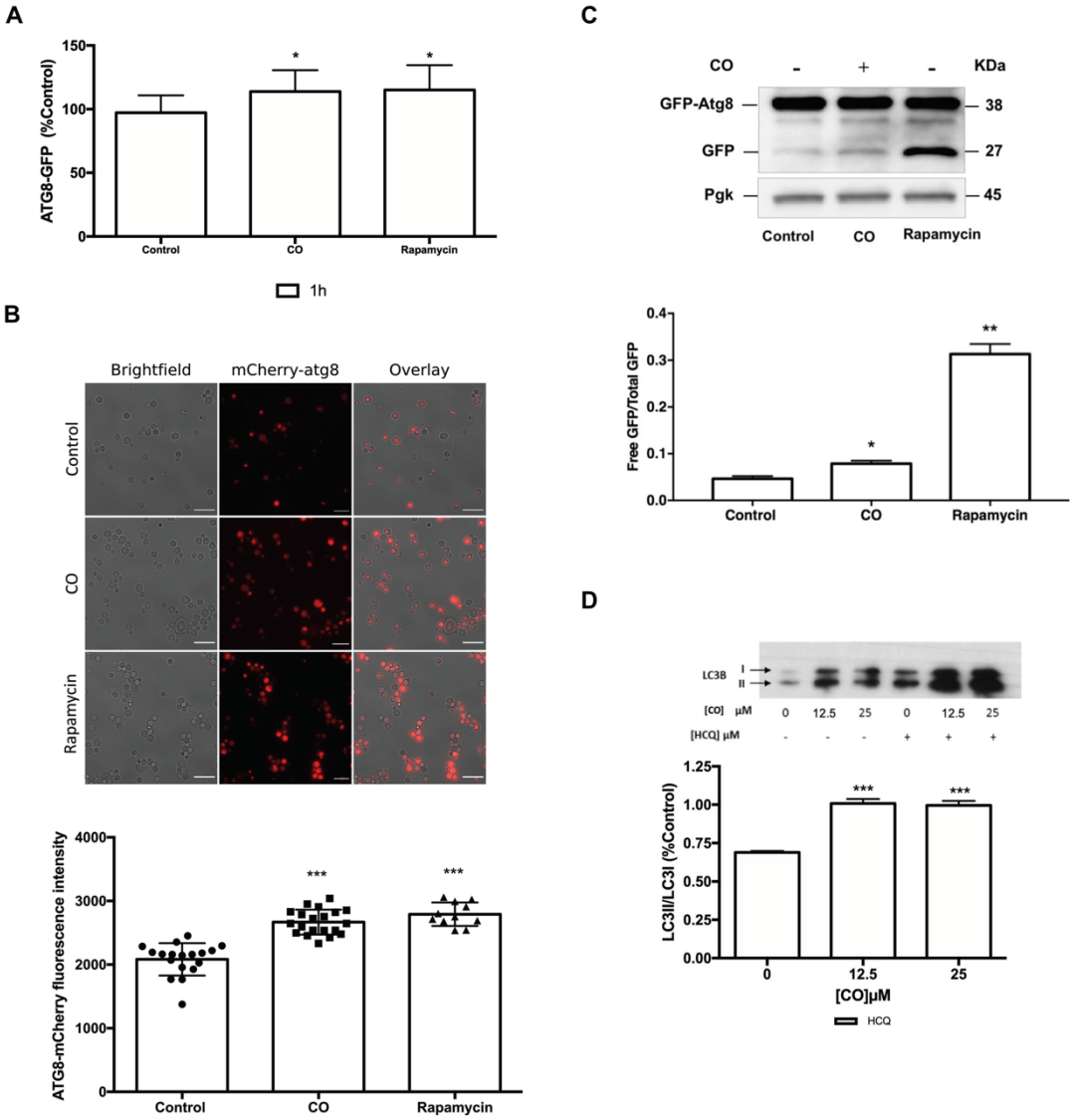


Figure 2 - CORM-A1 induces autophagy. A) BY 4741 cells expressing Atg8-GFP were treated with 100 µM of CORM-A1 or 0.2 µg/mL of rapamycin for 1 h, and fluorescence was quantified by flow cytometry. **B)** BY 4741 cells expressing Atg8- mCherry were treated with 100 µM of CORM-A1 or 0.2 µg/mL of rapamycin for 1 h, and fluorescence was monitored by fluorescent microscopy. A representative image of cells expressing Atg8-mCherry is shown (upper panel). **C)** BY 4741 cells expressing Atg8-GFP were treated with 100 µM of CORM-A1 or 0.2 µg/mL of rapamycin for 1 h and GFP was quantified by measuring the vacuolar degradation of the Atg8 domain reporter (ratio of free GFP to total GFP signal) assessed by immunoblotting. **D)** Primary culture of astrocytes was treated with 12.5 or 25 µM of CORM-A1 with or without 30 µg/mL of HCQ for 1 h, followed by quantification. A representative image of the immunoblot is shown (upper panel). LC3II/LC3I ratio was quantified as a measure of autophagy flux activation (lower panel). The results are the mean ± standard error of the mean of at least three independent experiments, performed in different cell preparations. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001 and ∗∗P < 0.01, as compared to control with one-way ANOVA for **A), B)** and **D)** and t-test with Welch's correction for **C)**.

CO-induced cytoprotection against oxidative stress is dependent on autophagy

It was hypothesized that CO promotes cell survival by autophagy modulation. Thus, we performed genetic analysis to assess the role of key autophagy players on the cytoprotective effect of CORM-A1 against oxidative stress in yeast. The *atg8* and *atg11* knockout mutants were subjected to the same growth curve analysis as the wild type strain (Figure 1A and Supplementary Figure S3) and the protection factor for each strain was calculated at 15h of growth under oxidative stress. As shown in Figure 3A, CO conferred 25% protection against *t*-BHP-induced oxidative stress in the wild type strain. However, protection was completely abolished in the *atg8* and *atg11* mutants, whose protection factors are -33% and -120%, respectively (Figure 3A). In addition, yeast cell viability was also measured by PI dye. In the absence of *ATG8,* the levels of t-BHP-induced cell death were similar with or without CO treatment (Figure 3B upper panel). Likewise, in the absence of *ATG11*, cell death levels were higher in the presence of CORM-A1, suggesting a synergetic toxic effect of CO whenever Atg11 is not expressed (Figure 3B lower panel). Taken all together, these data indicate that Atg8 and Atg11 are essential players in CO-mediated protection. These data indicate that CO-induced cytoprotection in yeast is dependent on Atg8 and Atg11 and consequently on autophagy.

To assess whether autophagy plays a role in the cytoprotective effect of CORM-A1 in primary cultures of astrocytes, the anti-apoptotic effect of CORM-A1 was tested in conditions of autophagy inhibition. Autophagy was pharmacologically inhibited by WM, which reverted CORM-A1-induced cytoprotection at all concentrations of *t*-BHP(Figure 3B). Furthermore, astrocytes treated only with WM did not present any toxic effect when compared to control cells without any treatment, since whenever astrocytes were treated only with WM viability levels were similar to control levels (Figure 3C). Therefore, in astrocytes CO-induced cytoprotection against oxidative stress is partially dependent on autophagy, other cytoprotective pathways might also be involved.

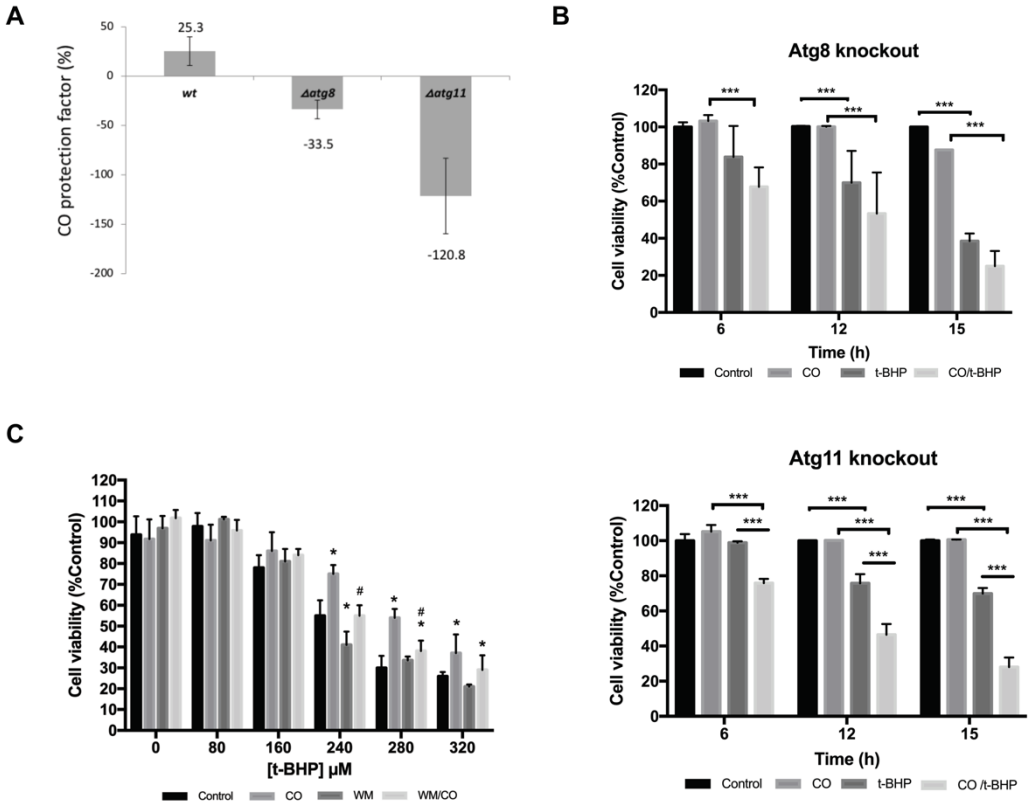


Figure 3 - CO-induced cytoprotection against oxidative stress is dependent on autophagy. A) CO's protection factor is represented for the wild type, and atg8 and atg11 knockout strains pretreated twice with 100 μM CORM-A1 for 30 min (total of CORM-A1 treatment of 1 h) and exposed to oxidative stress (1 mM tBHP). At least three independent replicates were performed to calculate protection factor described in the 'Methods' section. **B)** atg8 and atg11 knockout cells were pretreated or not with 100 μM CORM-A1 (twice with an interval of 30 min) and then were incubated for 6, 12 and 15 h in the presence or absence of oxidative stress (1 mM t-BHP). Cell viability was assessed by PI in flow cytometry. **C)** Primary cultures of astrocytes were pretreated with CORM-A1 at a final concentration of 12.5 μM in the presence or absence of WM at 100 nM for 1 h, and then cell deaths were challenged with t-BHP at 80–320 μM for 18 h. Cell viability assessment is based on plasmatic membrane integrity using PI dye and measuring its fluorescence by flow cytometry. The results are the mean ± standard error of the mean of at least three independent experiments, performed in different cell preparations. ∗P < 0.05 and ∗∗∗P < 0.001 as compared to control and #P < 0.05 as compared to CORM-A1-treated cells both with two-way ANOVA.

DISCUSSION

Herein it was demonstrated that CO promotes autophagy in *S. cerevisiae*. Furthermore, it was also shown that autophagy is necessary for CO to prevent oxidative stress-induced cell death in yeast. These data were validated in a more physiologically relevant and well characterized model of primary culture of astrocytes. This is a pioneer study using CO releasing molecules (CORMs) for assessing CO role on the control of autophagic process. CORM-A1 was chosen because it is water soluble, releases CO under physiological conditions and has been extensively studied as cytoprotective molecule in mammal models. In mammal models (cell culture and *in vivo* approaches), it is not clear whether CORM-A1 is up-taken by the cells and then releases CO, or if CO is released in the extracellular space [19]. Nevertheless, one must take into account that CO is a gas able to diffuse throughout cell membranes and walls. Therefore, despite no evidence about the mechanisms of CORM-A1 to be (or not) up-taken by cells, CO gas can get into intracellular space and act on its biological targets.

The ability of low doses of CO to decrease cell death has been extensively described in mammal models, and some of the underlying molecular mechanisms have been disclosed. For instance, CO's anti-apoptotic effect is dependent on p38 MAPK [37,38], on guanylyl cyclase and nitric oxide synthase [39,40], on caspase-8 activity [41], on Bcl-2 and Bax expression levels [39,42], on mitochondrial membrane permeabilization [25,43] or on modulation of cell metabolism [26,44,45]. Likewise, autophagy also emerges as a cytoprotective process [2,46] and CO might prevent cell death in an autophagy-dependent manner as corroborated by our results. In fact, in 2011, Lee and colleagues showed for the first time that CO regulates autophagy *via* ROS signaling in epithelial cell culture of mice lung [13]. Moreover, CO-induced cytoprotection against hyperoxia is reverted whenever autophagy is blocked [13]. More recently, the CO cytoprotection dependency on autophagic process has been demonstrated in other models, namely in sepsis [14], mesenchymal stromal cells [15] and in islet cells [16].

It has been shown that pulsed administration of CO controls metabolic signaling by promoting respiratory state in yeast [17]. Herein, and for the first time, the link between the beneficial properties of CO and autophagy were assessed in yeast models. Moreover, this work was also pioneer in using CORMs, in particular CORM-A1, in yeast model. CORM-A1 promotes autophagy, reduces cell death and improves cell growth under oxidative stress. Furthermore, the cytoprotective role of CO is lost in yeast strains knocked out for *ATG8* and *ATG11* genes, which present autophagy default. Atg8 is the yeast homolog for mammalian LC3 protein. While, Atg11 is a cytosolic protein, which works as an adaptor protein for selective autophagy and, interestingly this protein is not necessary for non-selective autophagy. Atg11 interacts with the cargo specific receptor-proteins for recruiting the cargo to the pre-autophagosomal structure [47]. Likewise Atg11 is essential for pexophagy and plays a key

role in mitophagy by interacting with Atg32 [47]. Thus, the data indicate that autophagy machinery is important for CO to promote cell survival in yeast under oxidative stress conditions. Still, CO appears to be implicated in selective autophagy since deletion of *ATG11* gene blocks the cytoprotective action of CO. One can also speculate that CO might be involved in the control of mitophagy since Atg11 also modulates selective mitophagy. These data open new possibilities of research on CO underlying mechanisms in mammals, in what concerns specific autophagy, in particular mitophagy, indicating that CO may also promote mitophagy and improve mitochondrial quality control in mammals.

The use of yeast models to decipher the role of CO in the autophagy-dependent cytoprotection open new possibilities for CO Biology Research. Although being the simplest eukaryotic organism, yeast has proven to be a powerful genetic model for mechanistic studies of several human diseases. Yeast cells grown under fermentative conditions are highly proliferative and present a Warburg-like metabolism, which mimics cancer cells behavior and allows mechanistic studies of cell death and autophagy in yeast [48]. Likewise, yeast heterologous expressing key human proteins for the modulation of Parkinson's Disease (PD), namely α-synuclein, β-synuclein or parkin, have been extensively explored for disclosing the underlying molecular mechanisms, in particular mechanisms related to autophagy modulation [49–51]. In addition, yeast cells are simple and cost-effective experimental models for the study of eukaryotic cells. In summary, the use of yeast models with their advantageous genetic tools will facilitate the study of the underlying molecular and cellular mechanisms associated with CO Biology, in particular autophagy and cell death modulation.

ACKNOWLEDGMENTS

This work was supported by Fundação para a Ciência e Tecnologia (FCT) grant FCT-ANR/NEU-NMC/0022/2012, grant PTDC/BIA-MOL/31104/2017 and FCT grant UID/Multi/04462/2013, I&D 2015-2020 iNOVA4Health - Programme in Translacional Medicine. FCT provided individual financial support to CFP (SFRH/BD/106057/2015), HLAV (IF/00185/2012) and CNS (IF/01097/2013).

REFERENCES

1. Tsukada, M. & Ohsumi, Y. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS Lett.* **333**, 169–74 (1993).

2. Kroemer, G. & Levine, B. Autophagic cell death: the story of a misnomer. *Nat Rev Mol Cell Biol* **9**, 1004–1010 (2008).

3. Galluzzi, L., Pedro, J. M. B.-S., Blomgren, K. & Kroemer, G. Autophagy in acute brain injury. *Nat. Rev. Neurosci.* 6–8 (2016). doi:10.1038/nrn.2016.51

4. Ohsumi, Y. Historical landmarks of autophagy research. *Cell Res.* **24**, 9–23 (2014).

5. Farré, J.-C. & Subramani, S. Mechanistic insights into selective autophagy pathways: lessons from yeast. *Nat. Rev. Mol. Cell Biol.* **17**, 537–552 (2016).

6. Mizushima, N., Yoshimori, T. & Ohsumi, Y. The Role of Atg Proteins in Autophagosome Formation. *Annu. Rev. Cell Dev. Biol.* **27**, 107–132 (2011).

7. Schaaf, M. B. E., Keulers, T. G., Vooijs, M. A. & Rouschop, K. M. A. LC3/GABARAP family proteins: autophagy-(un)related functions. *FASEB J.* **30**, 3961–3978 (2016).

8. Motterlini, R. & Otterbein, L. E. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov* **9**, 728–743 (2010).

9. Ryter, S. W., Alam, J. & Choi, A. M. K. Heme Oxygenase-1/Carbon Monoxide: From Basic Science to Therapeutic Applications. *Physiol. Rev.* **86**, 583–650 (2006).

10. Queiroga, C. S. F., Vercelli, A. & Vieira, H. L. A. Carbon monoxide and the CNS: challenges and achievements. *Br. J. Pharmacol.* **172**, 1533–1545 (2015).

11. Motterlini, R. & Foresti, R. Biological signaling by carbon monoxide and carbon monoxide-releasing molecules. *Am. J. Physiol. Physiol.* **312**, C302–C313 (2017).

12. Almeida, A. S. A. S. & Vieira, H. L. A. H. L. A. Role of Cell Metabolism and Mitochondrial Function During Adult Neurogenesis. *Neurochem. Res.* **42**, 1787–1794 (2017).

13. Lee, S. J. *et al.* Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation. *Am J Respir Cell Mol Biol* **45**, 867–873 (2011).

14. Lee, S. *et al.* Carbon Monoxide Confers Protection in Sepsis by Enhancing Beclin 1-Dependent Autophagy and Phagocytosis. *Antioxid. Redox Signal.* **20**, 432–442 (2014).

15. Ghanta, S. *et al.* Mesenchymal Stromal Cells Deficient in Autophagy Proteins Are Susceptible to Oxidative Injury and Mitochondrial Dysfunction. *Am. J. Respir. Cell Mol. Biol.* **56**, 300–309 (2017).

16. Kim, D.-S. *et al.* Carbon Monoxide Inhibits Islet Apoptosis via Induction of Autophagy. *Antioxid. Redox Signal.* **28**, 1309–1322 (2018).

17. Tu, B. P. & McKnight, S. L. Evidence of carbon monoxide-mediated phase advancement of the yeast metabolic cycle. *Proc. Natl. Acad. Sci.* **106**, 14293–14296 (2009).

18. Seixas, J. D. *et al.* Characterization of a versatile organometallic pro-drug (CORM) for experimental CO based therapeutics. *Dalt. Trans.* **42**, 5985–98 (2013).

19. Romão, C. C. & Vieira, H. L. A. *Metal Carbonyl Prodrugs: CO Delivery and Beyond*. *Bioorganometallic Chemistry: Applications in Drug Discovery, Biocatalysis, and Imaging* (Wiley-VCH, 2015). doi:10.1002/9783527673438.ch06

20. Motterlini, R. *et al.* CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. *Faseb J* **19**, 284–286 (2005).

21. Motterlini, R. *et al.* Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ. Res.* **90**, e17–e24 (2002).

22. Sawle, P. *et al.* Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br J Pharmacol* **145**, 800–810 (2005).

23. Marques, A. R. *et al.* Generation of Carbon Monoxide Releasing Molecules (CO-RMs) as Drug Candidates for the Treatment of Acute Liver Injury: Targeting of CO-RMs to the Liver. *Organometallics* **31**, 5810−5822 (2012).

24. Schallner, N. *et al.* Carbon Monoxide Abrogates Ischemic Insult to Neuronal Cells via the Soluble Guanylate Cyclase-cGMP Pathway. *PLoS One* **8**, (2013).

25. Queiroga, C. S. F. *et al.* Glutathionylation of Adenine Nucleotide Translocase Induced by Carbon Monoxide Prevents Mitochondrial Membrane Permeabilization and Apoptosis. *J. Biol. Chem.* **285**, 17077–17088 (2010).

26. Almeida, A. S. A. S., Queiroga, C. S. F. C. S. F. F., Sousa, M. F. Q. M. F. Q. Q., Alves, P. M. P. M. & Vieira, H. L. A. A. H. L. A. H. L. A. Carbon monoxide modulates apoptosis by reinforcing oxidative metabolism in astrocytes: Role of Bcl-2. *J. Biol. Chem.* **287**, 10761–10770 (2012).

27. Oliveira, S. R., Figueiredo-Pereira, C., Duarte, C. B. & Vieira, H. L. A. P2X7 Receptors Mediate CO-Induced Alterations in Gene Expression in Cultured Cortical Astrocytes—Transcriptomic Study. *Mol. Neurobiol.* (2018). doi:10.1007/s12035-018-1302-7

28. Suzuki, K., Kondo, C., Morimoto, M. & Ohsumi, Y. Selective Transport of α-Mannosidase by Autophagic Pathways. *J. Biol. Chem.* **285**, 30019–30025 (2010).

29. Suzuki, K. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J.* **20**, 5971–5981 (2001).

30. Gietz, R. D. & Schiestl, R. H. Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast* **7**, 253–263 (1991).

31. Sá Santos, S. *et al.* Culturing primary brain astrocytes under a fully controlled environment in a novel bioreactor. *J. Neurosci. Res.* **79**, 26–32 (2005).

32. Cheong, H. *et al.* Atg17 Regulates the Magnitude of the Autophagic Response. *Mol. Biol. Cell* **16**, 3438–3453 (2005).

33. Cheong, H. & Klionsky, D. J. Chapter 1 Biochemical Methods to Monitor Autophagy-Related Processes in Yeast. in 1–26 (2008). doi:10.1016/S0076-6879(08)03201-1

34. Graef, M. & Nunnari, J. Mitochondria regulate autophagy by conserved signalling pathways. *EMBO J.* **30**, 2101–2114 (2011).

35. Klionsky, D. J. et. al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **12**, 1–222 (2016).

36. Macedo, D. *et al.* (Poly)phenols protect from α-synuclein toxicity by reducing oxidative stress and promoting autophagy. *Hum. Mol. Genet.* **24**, 1717–1732 (2015).

37. Brouard, S. *et al.* Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med* **192**, 1015–1026 (2000).

38. Zhang, X. *et al.* Carbon monoxide modulates Fas/Fas ligand, caspases, and Bcl-2 family proteins via the p38?? mitogen-activated protein kinase pathway during ischemia-reperfusion lung injury. *J. Biol. Chem.* **278**, 22061–22070 (2003).

39. Nakao, A. *et al.* Carbon monoxide inhalation protects rat intestinal grafts from ischemia/reperfusion injury. *Am. J. Pathol.* **163**, 1587–1598 (2003).

40. Vieira, H. L. A. A. H. L. A., Queiroga, C. S. F. C. S. F. F. & Alves, P. M. P. M. Pre-conditioning induced by carbon monoxide provides neuronal protection against apoptosis. *J. Neurochem.* **107**, 375–384 (2008).

41. Kim, H. S., Loughran, P. A., Kim, P. K., Billiar, T. R. & Zuckerbraun, B. S. Carbon monoxide protects hepatocytes from TNF-α/Actinomycin D by inhibition of the caspase-8-mediated apoptotic pathway. *Biochem. Biophys. Res. Commun.* **344**, 1172–1178 (2006).

42. Queiroga, C. S. F. *et al.* Preconditioning Triggered by Carbon Monoxide (CO) Provides Neuronal Protection Following Perinatal Hypoxia-Ischemia. *PLoS One* **7**, (2012).

43. Queiroga, C. S., Almeida, A. S., Alves, P. M., Brenner, C. & Vieira, H. LA. Carbon monoxide prevents hepatic mitochondrial membrane permeabilization. *BMC Cell Biol.* **12**, 10 (2011).

44. Almeida, A. S. A. S., Sonnewald, U., Alves, P. M. P. M. & Vieira, H. L. A. H. L. A. Carbon monoxide improves neuronal differentiation and yield by increasing the functioning and number of mitochondria. *J. Neurochem.* **138**, 423–435 (2016).

45. Almeida, A. S. A. S. *et al.* Improvement of neuronal differentiation by carbon monoxide: Role of pentose phosphate pathway. *Redox Biol.* **17**, 338–347 (2018).

46. Boya, P., Reggiori, F. & Codogno, P. Emerging regulation and functions of autophagy. *Nat. Cell Biol.* **15**, 713–720 (2013).

47. Kanki, T., Furukawa, K. & Yamashita, S. Mitophagy in yeast: Molecular mechanisms and physiological role. *Biochim. Biophys. Acta - Mol. Cell Res.* **1853**, 2756–2765 (2015).

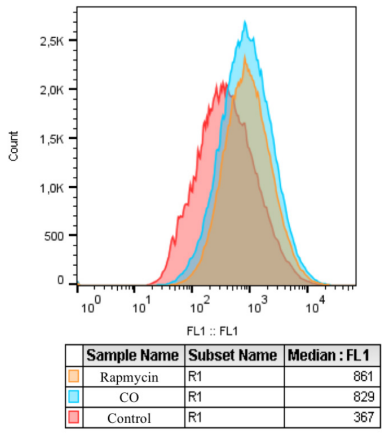
48. Carmona-Gutierrez, D. *et al.* Ceramide triggers metacaspase-independent mitochondrial cell death in yeast. *Cell Cycle* **10**, 3973–3978 (2011).

49. Tenreiro, S. *et al.* Yeast reveals similar molecular mechanisms underlying alpha- and beta-synuclein toxicity. *Hum. Mol. Genet.* **25**, 275–290 (2016).

50. Tenreiro, S. *et al.* Phosphorylation Modulates Clearance of Alpha-Synuclein Inclusions in a Yeast Model of Parkinson's Disease. *PLoS Genet.* **10**, e1004302 (2014).

51. Pereira, C., Costa, V., Martins, L. M. & Saraiva, L. A yeast model of the Parkinson's disease-associated protein Parkin. *Exp. Cell Res.* **333**, 73–79 (2015).

SUPPLMENTARY DATA



	Sample Name	Subset Name	Median : FL1
	Rapmycin	R1	861
	CO	R1	829
	Control	R1	367

Figure S1: CORM-A1 induces autophagy A) Yeast cells expressing Atg8-GFP were treated with 100µM of CORM-A1 and 0.2 µg/mL of rapamycin for 1h, fluorescent cells were quantified by flow cytometry.

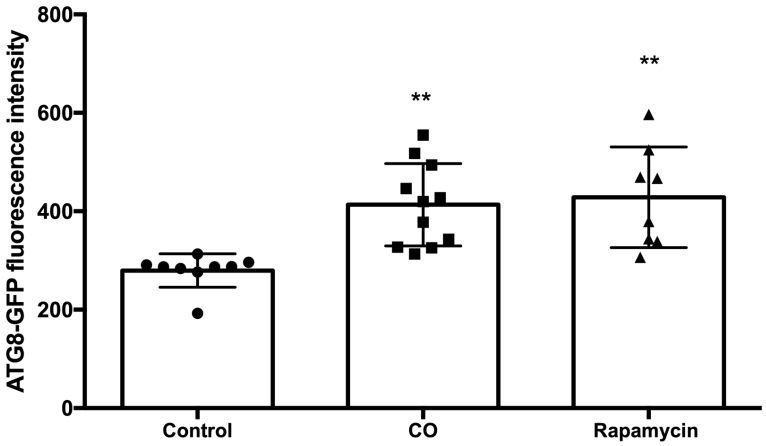


Figure S2 - CORM-A1 induces autophagy A) Yeast cells expressing Atg8-GFP were treated with 100µM of CORM-A1 and 0.2 µg/mL of rapamycin for 1h, fluorescent cells were quantified by fluorescent microscopy.

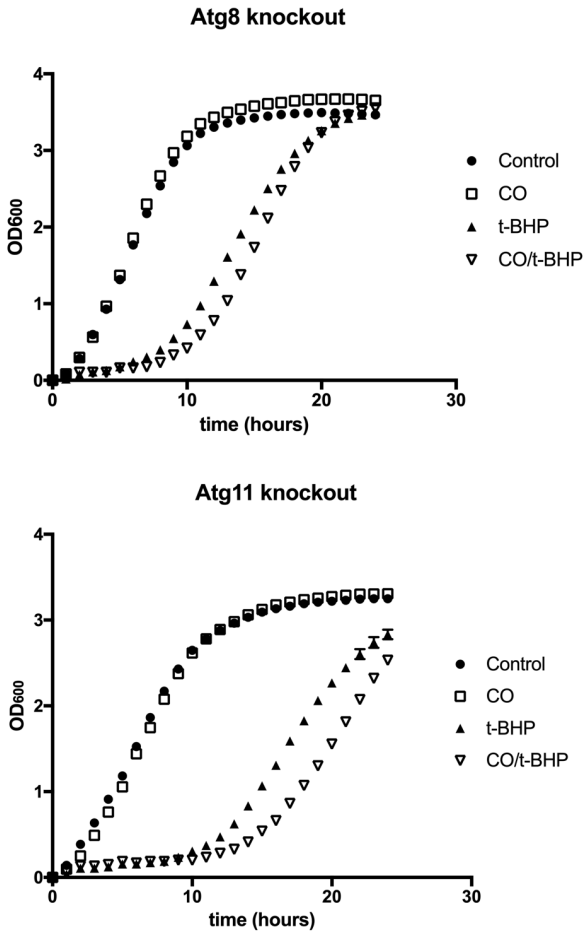


Figure S3 - CO-induced cytoprotection against oxidative stress is dependent on autophagy A) Representative yeast growth curves for *atg8* and *atg11* knockout strains in the presence of oxidative stress (t-BHP treatment at 1mM), with or without CORM-A1 pre-treatment for 90 minutes at final concentration of 100μM.

III

CARBON MONOXIDE STIMULATION OF MITOCHONDRIAL TURNOVER AS KEY FACTOR FOR CYTOPROTECTION AGAINST OXIDATIVE STRESS

This chapter is based on data to be published as:

Carbon monoxide stimulation of mitochondrial turnover as key factor for cytoprotection against oxidative stress

Cláudia Figueiredo-Pereira and Helena L.A. Vieira.

Manuscript under preparation

ABSTRACT

Mitochondria quality control system is essential for maintaining the mitochondrial homeostasis and cell health. Mitochondria can become dysfunction in response to stress, resulting in ROS production. Therefore, the efficient removal of these mitochondria is essential to prevent cellular dysfunction, and occurs through mitophagy. Autophagy turnover of mitochondria, named mitophagy, is an essential mitochondrial quality-control (MQC) mechanism to avoid cell death in mammals. Although as mitochondria are cleared from the cell, new ones need to be produced, this occurs by mitochondrial biogenesis. Carbon monoxide (CO) is known to confer protection against oxidative stress, whereas the potential involvement of MQC in CO anti-apoptotic role remains partly understood. Here, we demonstrate that CO quickly induced mitophagy (within 1h), mediated by both PINK1/Parkin and BNIP3 pathways. Likewise, CO promotes mitochondrial biogenesis (within 24h), by stimulating mitochondrial DNA replication and increasing gene expression of peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1α), specific mitochondrial transcription factor, at 1h. Furthermore, preliminary data suggest that CO promotes a cross-talk between mitophagy and mitochondrial biogenesis. In summary, CO appears as a novel agent modulating mitochondria, promoting clearance of harmful cell components and cytoprotection.

CONTENTS

Claudia Figueiredo-Pereira had carried out the majority of the experimental part and was involved on the decisions on how to execute the experiments, as well as on the interpretation and discussion of the results.

INTRODUCTION

Carbon monoxide (CO) is a diatomic colourless small molecule, invisible, chemically inert, non-irritant, and odourless gas [1]. It is, commonly known as a lethal gas and toxic air pollutant; however, CO is produced endogenously during the degradation of heme by the enzyme heme oxygenase (HO-1 and HO-2) and has been recognized as a ubiquitous signalling molecule necessary for cells to counteract oxidative stress and injury [2]. In the brain environment, CO has firstly been recognized to act as a neurotransmitter [3,4]. In the last years, the study of CO effect in the prevention of neuronal and astrocytic cell death and its role in mitochondrial function have raise a great interest [5]. In primary culture of astrocytes, CO stimulates mitochondrial biogenesis and improves oxidative metabolism in a Bcl-2 dependent manner, which provides protection against oxidative stress [6]. CO also prevents apoptosis by modulation of P2X7 receptor [7] and by targeting mitochondria, generating signalling ROS and preventing mitochondrial membrane permeabilization [8].

Mitochondria are key cellular organelles in maintaining cellular homeostasis by producing biomolecules, generating bioenergy and for controlling cell death [9]. Mitochondria can become damaged in response to their dysfunction, aging or disease, resulting in an increase on mitochondrial ROS production and ultimately apoptosis [10–12]. In fact, excessive and aberrant ROS generation is one of the major consequences of mitochondrial dysfunction. Therefore, the efficient removal of damaged mitochondria is essential to prevent cellular dysfunction, and this elimination occurs through autophagy. Autophagy is an evolutionarily conserved process in which intracellular components are engulfed in a double membrane structure (autophagosome) and further degraded in the lysosomes [13]. Increasing evidences point to autophagy as a protective mechanism in the brain, by maintaining the optimal balance and recycling of cellular components, in particular in response to stress. Specific mitochondrial autophagy is named mitophagy, and is essential for mitochondrial turnover and mitochondrial quality-control (MQC) to avoid cell death in mammals [14].

Mitophagy, involves sensing, sequestration, and trafficking of damaged mitochondria into lysosomes for final degradation. In healthy mammalian cells, mitochondria repair normally occurs trough fusion. Nevertheless, when mitochondrial stress is too high, damaged mitochondria must be eliminated by mitophagy, the only mechanism capable of eliminating whole mitochondria [15]. There are two main pathways for mitophagy process: the ubiquitin-mediated (molecules containing LC3-interacting regions) and receptor-mediated (LC3-interacting regions - LIRs) [11,15]. Ubiquitin-dependent mitophagy, is mediated by the coordinated activities of the mitochondrial serine-threonine PTEN-induced putative kinase 1 (PINK1) and Parkin, an E3 ligase, this are called PINK1/Parkin pathway [16]. Mutations in PINK1 or Parkin genes are mainly associated with the development of autosomal recessive form of Parkinson disease (PD) [14,17]. In response to stress, in particular when $\Delta\psi m$ is

reduced, PINK1, which is usually rapidly degraded by the proteasome, does not degrade anymore and accumulates on the outer membrane of the mitochondria (OMM). PINK1then recruits Parkin to the damaged mitochondria and phosphorylates Parkin, recruiting it from the cytosol into the mitochondria [18]. PINK1 also phosphorylates ubiquitin, which would activate Parkin upon binding. The ubiquitination of OMM proteins by Parkin initiate the mitophagy process by the recruitment of the autophagy-associated adaptors, LC3/GABARAP [19]. Mitochondria accumulation of Parkin leads to their degradation via mitophagy, being the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) the most studied inducer of this mitophagic pathway [20]. The receptor-mediated pathway, or also called mitophagy receptors, are BNIP3/NIX (OMM LIR-containing proteins) and trigger mitophagy under different conditions [21]. BNIP3/Nix were first described to mediate mitophagy during erythrocytes maturation [11] and their expression is upregulated under hypoxia conditions [11,22]. Likewise, Nix interacts directly with LC3 mediating the sequestration of mitochondria to the phagophore (double membrane of autophagosome). As damaged mitochondria are cleared from cell, new mitochondria are generated in a process termed mitochondrial biogenesis [23]. Biogenesis is a tightly controlled process that is regulated by nuclear transcription factors; such as nuclear respiratory factor 1 (NRF1) and its coactivator peroxisome proliferator-activated receptor γ coactivator 1 (PGC1α), which upregulates expression of the mitochondrial transcription factor A (TFAM), thus allowing replication of the mitochondrial DNA (mtDNA) [23–28].

We and others have already demonstrated that CO triggers autophagy in several models [29–31], including astrocytes [32], and CO-induced cytoprotection is dependent on autophagy [30,32,33]. Furthermore, it also known that the main cellular target of CO is mitochondria, and CO is anti-apoptotic via ROS signalling [6,8,34]. Nevertheless, the role of mitophagy in CO-mediating cytoprotection in glial cells has never been explored. Herein, we investigate whether the CO protective role is mediated by mitophagy and which are the underlying mitophagic pathways. We also evaluate the capability of CO in maintaining mitochondria homeostasis and turnover by assessing mitochondrial biogenesis. Our data indicate that at short time points (1h) CO induces mitophagy and activates the machinery for mitochondrial new production (mitochondrial DNA synthesis), while at 24h CO promotes mitochondrial biogenesis for the reestablishment of mitochondria population. Likewise, CO-induced mitophagy is not exclusive to one single pathway; being involved PINK1/Parkin pathway and also the mitophagy receptor, BNIP3. Likewise, preliminary data suggest a cross-talk control between mitophagy and mitochondrial biogenesis promoted by CO. Finally, CO's prevention of astrocytic cell death is dependent on mitophagy.

MATERIAL AND METHODS

Primary culture of astrocytes

Primary cultures of astrocytes were prepared from 1-day-old Balb/c mouse cortex, as described [35]. Animals were rapidly decapitated, brain cortex was removed and the meninges were carefully stripped off, then the cortex was washed in ice-cold phosphate-buffered saline (PBS), and mechanically disrupted. Single-cell suspensions were plated in T-flasks (four hemispheres/75 cm^2) in Dulbecco's minimum essential medium supplemented with 20% (v/v) FBS (heat-inactivated), 1% (v/v) of 100 U/mL penicillin/streptomycin solution and glucose (to obtain a final concentration of 10 mM). Astrocytes were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. After 7 days, the dark phase cells growing on the astrocytic cell layer were separated by vigorous shaking and removed. Culture medium was replaced twice a week for 3 weeks with gradual reduction in FBS content (2^{nd} week 15% (v/v); 3^{rd} week 10%(v/v)). At the 3^{rd} week the confluent astrocytes were detached by mild trypsinization using trypsin/EDTA (0.25%, w/v) and sub-cultured, in 24 and 6-well plates at 50 x 10^3 and 100 x10^3 cells/well, respectively, until reaching full confluence.

Carbon monoxide releasing molecule A1 (CORM-A1)

The CO releasing molecule, CORM-A1 [36] was used to deliver CO to cell culture. CORM-A1 (SML-0315, Sigma) stock solutions were prepared in water to a final concentration of 5mM. Then, the solution was filtrated with 0,2µM filter and stored at -20°C to avoid loss of released CO. For each use, an aliquot was thawed and immediately used. The concentration of CORM-A1 and time points for treatments were selected based on the cytoprotective effects of CO in astrocytes subjected to oxidative stress [32].

Mitochondrial population assessment (Mitophagy and mitochondrial biogenesis)

Astrocytes were plated in a concentration of 50*10^4 cells/well into the 24-well plate for flow cytometer analysis and 100*10^4 cells/well into the 12-well plates for Q-PCR analysis. For mitophagy assessment cells were incubated for 1h with and without the mitophagy inhibitor Cyclosporin A (CsA) at 5µM, the mitophagy inducer (uncoupling agent) carbonyl cyanide chlorophenylhydrazone (CCCP) at 25µM and for 24h with deferiprone (DFP) at 1mM and CORM-A at 12,5µM. Likewise, for mitochondrial biogenesis assessment cells were incubated for 24h with and without the CsA at 5µM, CCCP at 25µM, the protein synthesis inhibitor, chloramphenicol (Cm) at 10µM and CORM-A at 12,5µM.

Cells were collected for analysis by flow cytometry, western blot and Q-PCR in order to assess mitophagy and mitochondrial biogenesis.

Determination of mitochondrial population by flow cytometry

After astrocyte treatment, as previously described, cells were trypsinized for 5 min at 37 ºC and resuspended in complete medium with 10 nM Mitotracker Deep Red (MTDR, M22426, Invitrogen) incubated for 15 min at 37 ºC followed by cytofluorometric analysis with FACS scan (BD FACSCalibour). MTDR is used for mitochondria labeling. Fluorescence intensity for each incubation time was analyzed in 10,000 cells and fluorescence value per cell was determined and plotted against cell number. Median fluorescence in the FL4 channel in the viable cell (PI-negative) population was plotted and normalized against that of untreated cells.

Inmunoflourescent microscopy

After astrocyte treatment, cells were fixed for 30 min with 4% paraformaldehyde in PBS, washed and permeabilized with 0.1% SDS for 30 min and incubated for 16 h with the primary antibodies; anti-LC3B (abcam) 1:800 and TOM20 (Santa-Cruz) 1:500. Cells were then incubated for 1h with the secondary antibodies (GE Healthcare) 1:200, followed by 1h with horseradish peroxidase-conjugated streptavidin (GE Healthcare) 1:1000. Confocal analysis was performed with a Zeiss LSM 710 Confocal microscope. Images were taken every 0.2 mm.

Quantitative-Polymerase chain reaction (Q-PCR)

Q-PCR was used for quantification of mitochondrial DNA (mtDNA), which is an indirect measurement of mitochondrial population. Total cell DNA (containing mitochondrial DNA) was extracted from astrocytes using QIAGEN kit (ref. 69504). Polymerase chain reaction (PCR) was performed using specific forward and reverse primers designed for the mitochondrial cytochrome b gene, (5'TTCATGTCGGACGAGGCTT-3'), (3'- TCCTCATGGAAGGACGTAGC-5') and for the nuclear GAPDH gene (5'- CCTTCATTGACCTCAACTACAT-3'), (3'-CCAAAGTTGTCATGGATGACC-5') to be used for controlling the cellular amount in each sample. "Fast Strand DNA Master Plus SYBR Green I" (Roche) was used in the experimental run protocol, denaturation program was 95ºC for 10min, followed by 45 cycles of 95ºC for 10'', 60ºC for 10'' and 72ºC for 10'.

The results were analyzed by relative quantification, with aid of the software light cycle 96 of ROCHE, reference 00 000000 0010225, software version 1.01.00.0045. Mitochondrial population was quantified by the amount of mitochondrial DNA relatively to nuclear DNA (internal control). All results were normalized relatively to the control cell sample without any treatment.

Evaluation of gene expression (mRNA quantification by reverse transcriptase quantitative PCR)

For evaluation of gene expression, mRNA was extracted from astrocytes using High Pure RNA isolation kit (Roche Diagnostics), and cDNA synthesis was performed using the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics). PCR was performed using specific forward and reverse primers designed for PGC-1alpha, (CACAGATTCAGGCCAGTGCT) (TTCTGGTGCTGCAAGGAGAG).

Fast Strand DNA Master Plus SYBR Green I" (Roche) was used in the experimental run protocol, denaturation program was 95ºC for 10min, followed by 45 cycles of 95ºC for 10'', 60ºC for 10'' and 72ºC for 10'.

Cell viability assessment

Astrocytes were treated with CORM-A1 at a final concentration of 12.5 µM for 1 h, followed by apoptosis induction by (*tert*-butylhydroperoxide) *t*-BHP at concentrations from 80 to 320 µM for 18 h. In astrocytes, CsA (5µM), was used to modulated CO effect in mitophagy process by pre-treating cells 1 h prior to CORM-A1 treatment. To detect apoptosis induced by *t*-BHP, cells were collected by trypsinization and stained with dye propidium iodide (PI, Invitrogen) at 1 g/mL. Cells were gated by forward and side scatter. A flow cytometer (BD FACSCanto™ II) was used to analyse apoptosis-associated parameters. The acquisition was done with Diva software and data analysis was performed with FlowJo® software (10.5.3).

Isolated mitochondria from primary culture of astrocytes

Primary culture of astrocytes were grown in 175 cm^2 t-flask up to confluence, then cells were washed with ice-cold PBS and collected by trypsinisation. Samples were centrifugated at 200 g for 10 min, and cells (pellet) were washed in PBS by centrifugation at 200 g for 10 min. The supernatant was

discarded and the pellet (cells) incubated in 3.5mL of hypotonic buffer (0.15mM MgCl2, 10mM KCl, 10mM Tris-HCL, pH 7.6) at 4ºC for 15min, After the addition of an equal volume of homogenization buffer (0.15mM MgCl2, 10mM KCl, 10mM Tris-HCL, 0.4mM PMSF, 250mM saccharose, pH7.6) twice concentrated, samples were manually homogenized with Douncer potter. Cell extracts were centrifugated at 900 g for 10 min, followed by supernatant centrifugation at 10000 g for 10 min. The mitochondrial pellet was resuspended in 110µL of the homogenization buffer and the total amount of protein was quantified using BCA assay (Pierce, Illinois). All the steps were carried out at 4ºC.

Protein extraction and immunoblotting

Isolated mitochondria were separated under reducing electrophoresis on a 1 mm thick 15% SDS-polyacrylamide (SDS-PAGE) gel. Samples were transferred onto a PVDF membrane (MERK). Membranes were incubated overnight at 4°C with antibodies against Pink1 protein (GeneTex) and Parkin protein (Abcam) and actin (Sigma), used as a loading control. Blots were developed using the ECL (enhanced chemiluminescence) detection system after incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare), for 1 h at room temperature.

siRNA Transfection

Pink1 expression was silenced by Pink1 coding siRNA transfection according to the manufacturer's instructions (Invitrogen). Astrocytes at 40% of confluence were transfected using LipofectamineTM RNAiMAX and Opti-MEM medium (Invitrogen); for 3.9 cm^2 of astrocytic culture area 10pmol of siRNA were used. At room temperature, siRNA and culture medium were mixed gently with Lipofectamine for forming liposomes, and then astrocytes were transfected in the absence of antibiotics. 24, 48 and 72h after transfection Pink1 expression was assessed by Western blot assay, silencing was more efficient between 24h, and then Pink1 silenced astrocytes were used in this time frame.

Statistical Analyses

The data concerning yeast and astrocytic culture were carried out at least in three independent preparations (cell isolation). For autophagy evaluation, a representative image of three independent assays is shown. All values are mean S.D. (n =3). Error bars, corresponding to S.D., are shown in the figures.

Statistical comparisons were performed using ANOVA (one-way, two-way) and Student t-test (with Welch's correction) with $p < 0.05$ ($n \geq 3$). $p < 0.05$ means that samples are significantly different at a confidence level of 95%.

RESULTS

CORM-A1 induces mitophagy

The ability of CO to trigger mitophagy in primary culture of astrocytes was evaluated at 1h of CO treatment by two distinct approaches. First, it was used immunocytochemistry, the classical technique for mitophagy assessment, by the detection of co-localization of the mitochondrial marker (TOM20) with autophagosomal marker (anti-LC3II labeling) (Figure 1A). Following 1h of CORM-A1 treatment at a final concentration of 12,5 and 25 μM, there is co-localization of mitochondria with autophagosome, indicating mitophagy.

In a second strategy, mitophagy was determined by flow cytometry using MitoTracker Deep Red (MTDR) to assess mitochondrial population [37]. Along with CO treatment, selective inhibitors and inducers of mitophagic flux were used to confirm that alterations in mitochondrial population are due to mitophagy activation [37]. The uncoupler CCCP (carbonyl cyanide chlorophenylhydrazone) is used to trigger mitophagy by reducing mitochondrial membrane potential ($\Delta\Psi m$) [38]. While, cyclosporine A (CsA) limits mitophagy by preventing $\Delta\Psi m$ reduction [39]. Astrocytes were treated with CORM-A1 at a final concentration of 12,5 μM for 1 hour in the presence or absence of CCCP or of CsA; followed by mitochondrial population assessment by flow cytometry MTDR dye (Figure 1B). CORM-A1 decreases fluorescence intensity of MTDR, indicating that at 1h, CORM-A1 reduces mitochondrial population by inducing mitophagy. Accordingly, CCCP treatment reduced MTDR levels, while CsA treatment increased MTDR fluorescence, indicating an activation and prevention of mitophagy, respectively. Moreover, whenever astrocytes were co-treated with CsA and CORM-A1, there is a partial reversion of mitophagy activation, assessed by a partial increase on mitochondrial population (Figure 1B). Of note, the used CCCP concentration is low, is not toxic and does not promote cell death; thus, it only decreases mitochondrial potential and promotes mitophagy, as described in [11].

In conclusion, CORM-A1 at short time period (1h) promotes mitophagy, which was assessed by the reduction of mitochondrial population and co-localization of mitochondria and autophagosome.

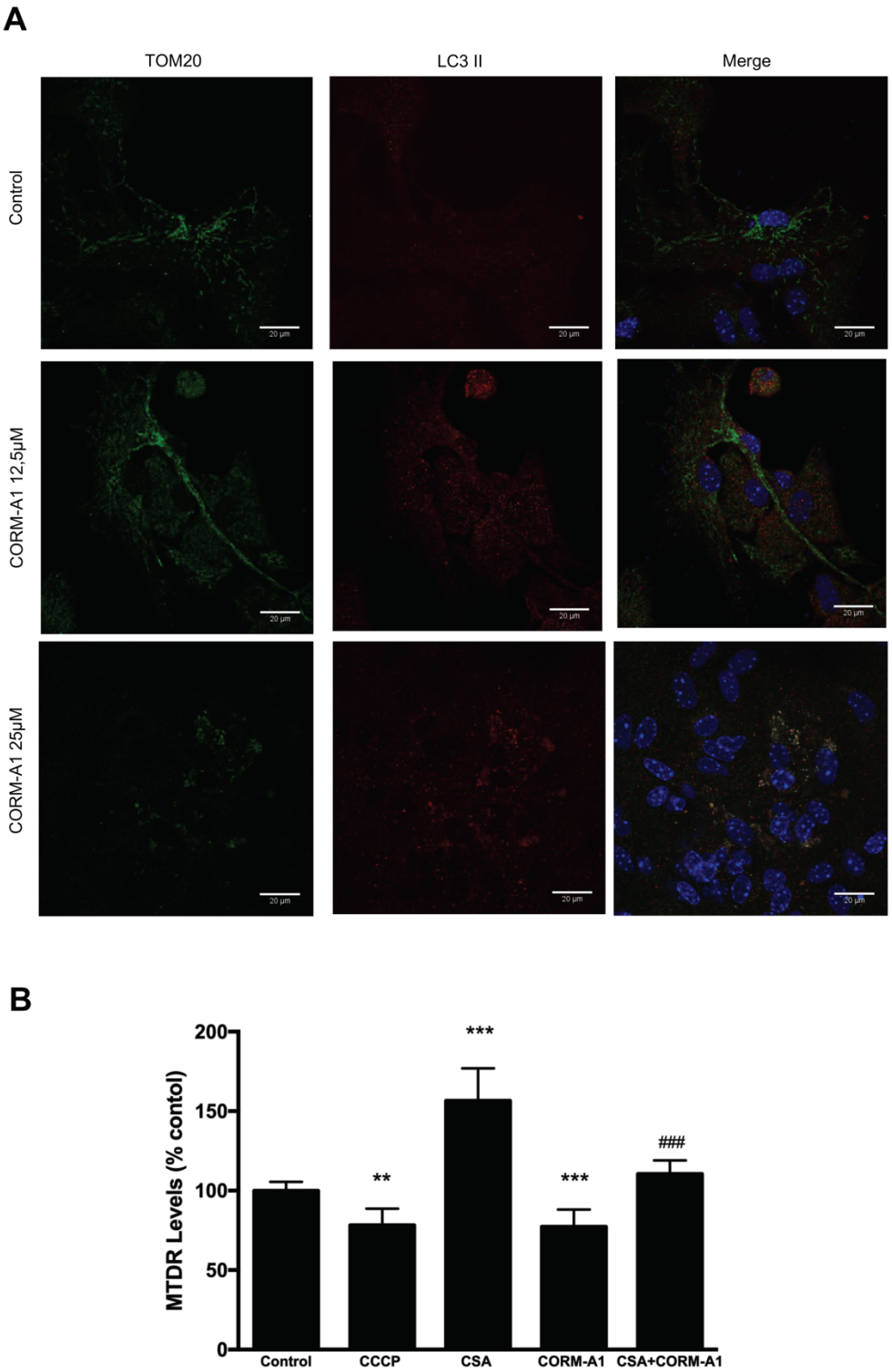


Figure 1- CORM-A1 induces mitophagy in astrocytes at short time points (1h); A) Primary culture of astrocytes were pretreated with 12,5 or 25µM of CORM-A1 for 1h. Astrocytes were immunostained with LC3-B (red) and TOM20 (green). Magnification 63x (preliminary figure). **B)** Primary cultures of astrocytes cultured in 24-well plate were treated with 25µM CCCP or 12,5µM of CORM-A1 along 1h with and withou 5µM CsA added at the same time to the cells. MTDR was used to determine mitochondrial mass by flow cytometry. The intensity in the FL4 channel was normalized to untreated control cells. Graphs represent the median ± SD of seven experiments performed in triplicate. ** $p<0,1$, *** $p<0,001$ compared with control treatment and ### $p<0,001$ compared to CORM-A1 without CsA treatment.

CORM-A1-induced mitophagy is associated with PINK1/Parkin pathway

The receptors mediating mitophagy are still a subject of discussion and speculation, since little is known about them [22,40]. PINK1/Parkin-mediated mitophagy is the mostly studied pathway, since it is relevant for the autosomal form of Parkinson disease [41,42]. Therefore, in order to study the involved receptors of CO-induced mitophagy, we evaluated by Western blot the presence of PINK1 and Parkin, in mitochondria enriched fraction following CORM-A1 treatment in astrocytes. CORM-A1 treatment increased the level of PINK1 and Parkin at mitochondria. Indeed, during mitophagy there is an increase of PINK1 in the outer mitochondrial membrane and translocation of Parkin from the cytosol into mitochondria [43]. Accordingly, and as positive control, it was also observed a higher level of these proteins in the mitochondria following CCCP treatment in astrocytes (Figure 2). These findings suggest that PINK1/Parkin pathway is involved in CORM-A1 activation of mitophagy.

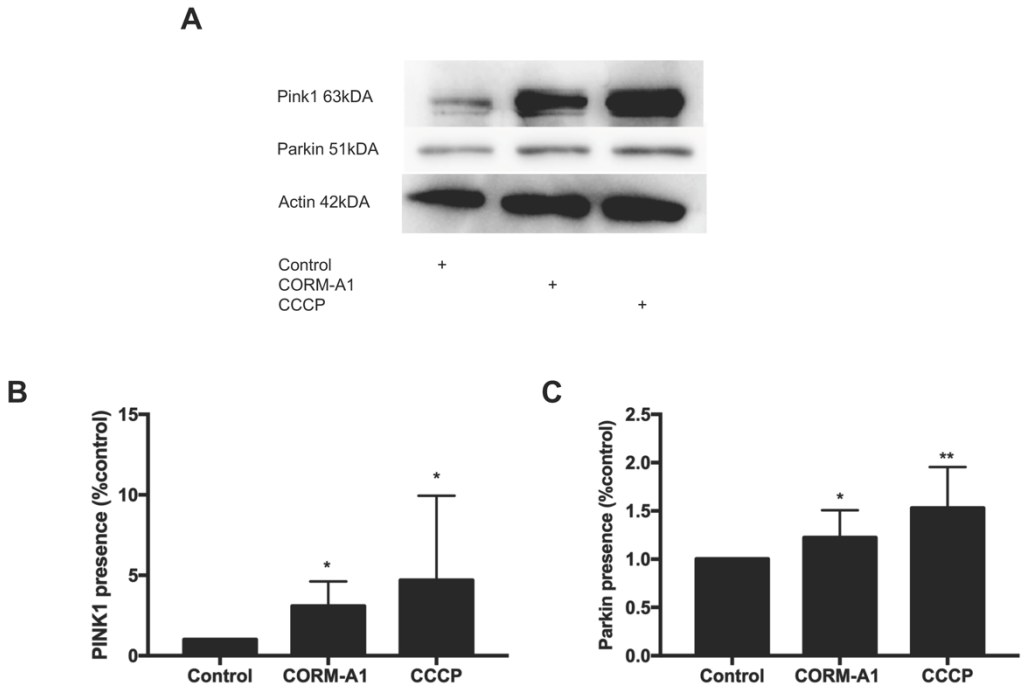


Figure 2- CORM-A1-induced mitophagy in astrocytes is associated with PINK1/Parkin pathway; A) Mitochondrial fractions isolated from primary culture of astrocytes treated with CORM-A1 at a final concentration of 12,5 µM or with CCCP at 25µM during 1h were analyzed by immunoblotting using antibodies against PINK1 and Parkin. **B)** Quantification of PINK1 presence in mitochondrial enriched fraction. **C)** Quantification of Parkin presence in mitochondrial enriched fraction. Graphs represent the median ± SD of four experiments. *p<0,5, ** p<0,1 compared to control treatment.

CORM-A1 enhances mitophagy independently of PINK1/Parkin and *via* BNIP3 pathways

To examine whether CO-activated mitophagy requires PINK1/Parkin pathway, we used siRNA to knock down PINK1 expression (Figure 3A). Unexpectedly, PINK1-targeted siRNA did not abolish CO-induced mitophagy, since CORM-A1 treatment still decreases fluorescence intensity of MTDR, indicating lower mitochondrial population via mitophagy promotion (Figure 3B). In contrast, whenever astrocytes were treated with CCCP, PINK1-targeted siRNA significantly abolished the capability of CCCP to promote mitophagy (Figure 3B). These results suggest that CO-promoted mitophagy might be mediated by several different pathways. According to the literature, there are two different classes of mitophagy receptors, the ubiquitin-dependents (PINK1 and Parkin) and ubiquitin-independents (BNIP3 and NIX) [44]. CO has been shown to induce autophagy through ROS [29,30]. BNIP3 and NIX are associated with mitophagy induction under hypoxia conditions [45–47]. Therefore, we investigated BNIP3 implication in CORM-A1-induced mitophagy. In fact, BNIP3 expression was increased in a dose-response manner following CORM-A1 treatment (Figure 3C). Additionally, mitochondria population was measure in the presence of deferiprone (DFP), an iron chelator that induces mitophagy in PINK1/Parkin independent manner. DFP treatment reduced MTDR levels (Figure 3D) and the co-treatment of DFP with CORM-A1 impaired mitochondrial population in a synergetic manner (Preliminary data).

Thus, CO promotes mitophagy by different pathways that can work in a compensatory manner. Although PINK1/Parkin pathway is associated with CO promotion of mitophagy, when this pathway is knock down, CO appears to sustain mitophagy induction via the alternative pathway BNIP3/NIX.

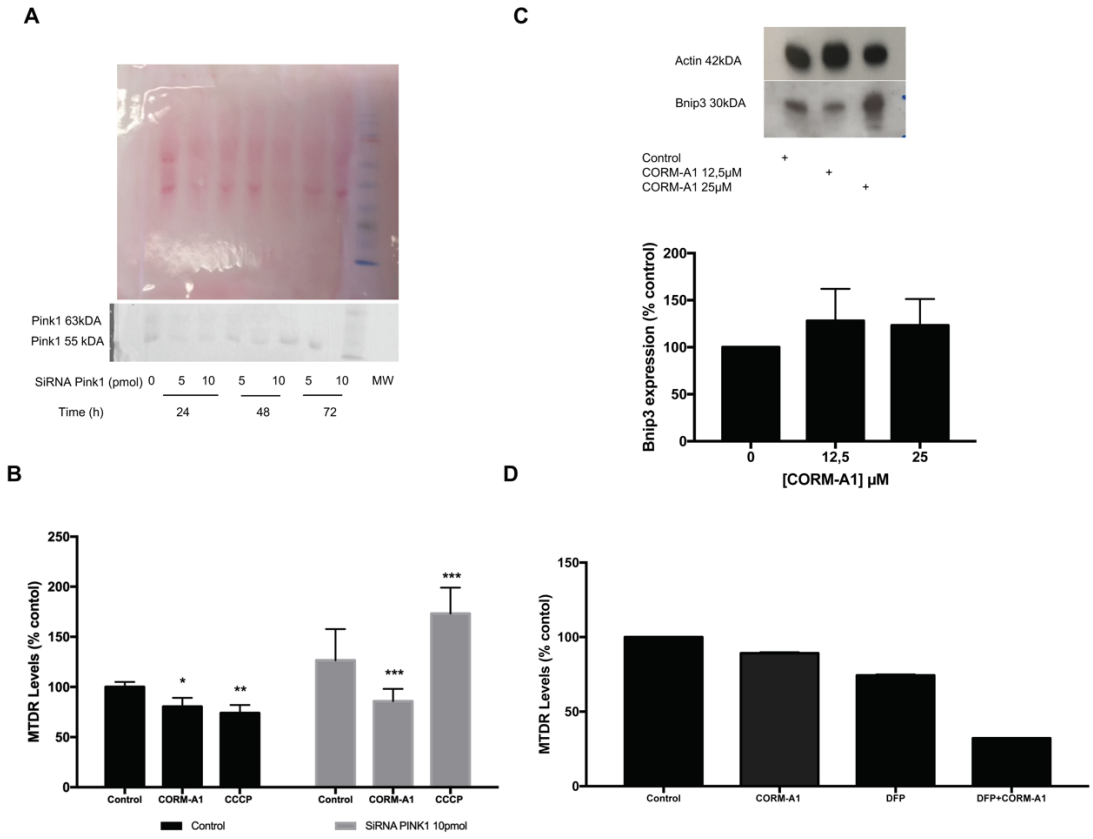


Figure 3- CORM-A1-induced mitophagy in astrocytes is associated with PINK1/Parkin pathway; A) Primary culture of astrocytes were transfected with SiRNA PINK1 for 24h, 48h and 72h and proteins level were determined by immunoblotting. **B)** Primary cultures of astrocytes cultured in 24-well plate were transfected with SiRNA PINK1 for 24h and treated with 25μM CCCP or 12,5μM of CORM-A1 along 1h. MTDR was used to determine mitochondrial mass by flow cytometry. The intensity in the FL4 channel was normalized to untreated control cells. Graphs represent the median ± SD of five experiments performed in triplicate. *p<0,5, ** p<0,1, *** p<0,001 compared with control treatment. **C)** Primary cultures of astrocytes treated with 12,5, and 25μM of CORM-A1 along 1hour. The levels of BNIP3 were determined by immunoblotting, n=2. **D)** Primary cultures of astrocytes were treated with 1mM DFP for 24h followed by treatment with or CORM-A1 at 12,5μM for 1h. MTDR was used to determine mitochondrial mass by flow cytometry. The intensity in the FL4 channel was normalized to untreated control cells n=2.

CORM-A1 promotes mitochondrial biogenesis

CO-induced mitochondrial biogenesis in primary culture of astrocytes was evaluated by three distinct approaches. First, mitochondrial population was assessed by flow cytometry using MTDR dye. Astrocytes were treated with CORM-A1 at a final concentration of 12,5 μM, or 25 μM of CCCP, and CsA at a final concentration of 5μM for 24h. CORM-A1 treatment enhanced about 50% the MTDR fluorescence intensity compared to CORM-A1 treatment at 1h, indicating that CO promotes a return to mitochondrial basal levels after 24h (Figure 4A). As negative control CCCP treatment reduced mitochondrial population, indicating that mitophagy still occurs after 24h of treatment. Likewise, chloramphenicol (Cm) is used as control since it prevents protein synthesis and inhibits

mitochondrial biogenesis. Astrocytes treated with Cm presented mitochondrial population levels similar to control (Figure 4A). While, astrocytic co-treatment of CORM-A1 with Cm abolished the capability of CO to enhance mitochondrial biogenesis at 24h (Figure 4A). These data reinforce the existence of a two-step response of CO: at 1h CO promotes mitophagy (Figure 1B) and at 24h there is a compensatory effect and CO increases mitochondrial biogenesis (Figure 4A), promoting mitochondrial turnover.

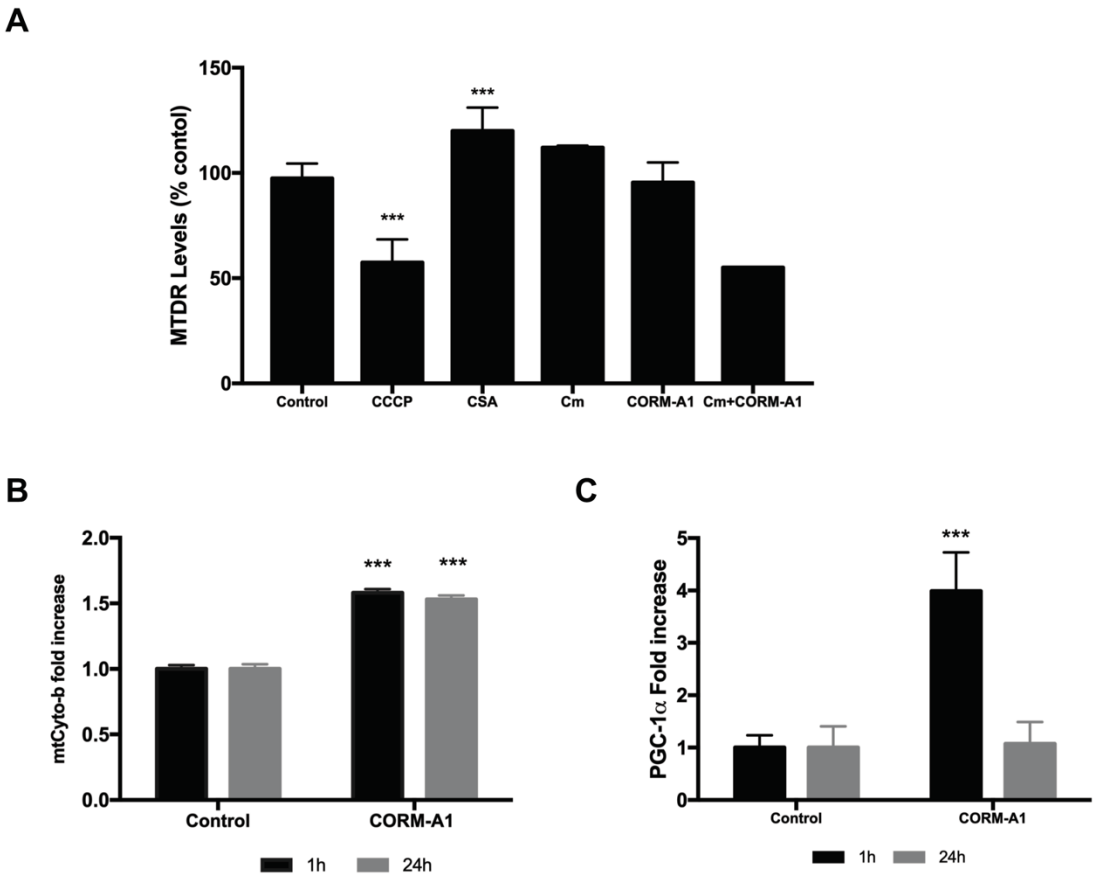


Figure 4- CORM-A1 stimulates mitochondrial biogenesis in astrocytes at long periods of treatment; A) Primary cultures of astrocytes cultured in 24-well plate were treated with 25μM CCCP or 12,5μM of CORM-A1 along 24h with and without 10μM chloramphenicol (Cm) added at the same time to the cells. MTDR was used to determine mitochondrial mass by flow cytometry. The intensity in the FL4 channel was normalized to untreated control cells. Graphs represent the median ± SD of seven experiments performed in triplicate. ***p<0,001 compared with control treatment. Cm data are preliminary with n=2 **B)** Primary cultures of astrocytes were treated with 12,5μM of CORM-A1 for 1h and 24h followed by DNA extraction for measuring mitochondrial cytochrome b (mtCyt b) gene to assess mitochondrial DNA amount, which is represented by fold increase when compared with control without CO treatment. All values are mean±SD of four experiments performed in triplicate. ***p<0,001 compared with control for 1 and 24 h treatment, respectively. **C)** Primary cultures of astrocytes were treated with 12,5μM of CORM-A1 for 1h and 24h followed by mRNA extraction for measuring mitochondrial PGC-1α gene to assess mitochondrial biogenesis, which is represented by fold increase when compared with control without CORM-A1 treatment. All values are mean ± SD of four experiments performed in triplicate. ****p<0,001 compared with control.

In addition, the role of CORM-A1 treatment on cellular mitochondrial population was also assessed by quantification of mitochondrial DNA. Quantitative real-time PCR was used to quantify mitochondrial coding gene for cytochrome b. Indeed, 1h of CORM-A1 treatment induced a 50% increase on the amount of mitochondrial cytochrome b DNA in astrocytes (Figure 4B), demonstrating that CO stimulates mitochondrial DNA replication and, consequently, mitochondrial biogenesis. At 24 h, the amount of cytochrome b DNA is maintained (Figure 4B).

Finally, the role of CO on mitochondrial biogenesis control was assessed in a more accurate manner by the direct quantification of PGC-1α gene expression, which is a transcriptional coactivator, known to be a master regulator of mitochondrial biogenesis. PGC-1α mRNA was quantified by reverse transcriptase quantitative real-time PCR. Indeed, 1h of CORM-A1 treatment upregulated the expression of PGC-1α, increasing mRNA approximately 150% (Figure 4C). Nevertheless, at 24h the expression of PGC-1α decreased, returning to basal levels. In conclusion, based on the assessment of mitochondrial population by MTDR, mtDNA quantification and the expression of a key transcription factor involved on mitochondrial biogenesis, PGC-1α; CORM-A1 emerges as a molecule able to modulate mitochondrial biogenesis. Indeed at 1h, despite the decrease on mitochondrial population (stimulation of mitophagy) assessed by MTDR, CORM-A1 appears to activate mtDNA replication and expression of PGC-1α. Thus, one may speculate that CO triggers mitophagy and mitochondrial biogenesis simultaneously. Likewise, at 24h following CORM-A1 treatment, mitochondrial population and PGC-1α expression return to basal levels. These data suggest that CO is an important player in the turnover of mitochondria and in mitochondrial quality control. Likewise, mitochondrial quality control is not limited to the elimination of dysfunctional mitochondria, but it must also involve generation of new mitochondria. Thus, those two processes might be interconnected and interdependent.

CORM-A1-induced mitochondrial biogenesis is associated with of mitophagy

Mitochondrial quality control involves both events: mitochondrial biogenesis and autophagic degradation of dysfunctional mitochondria (mitophagy). CO has been previously shown to induce mitophagy [31] and mitochondrial biogenesis [48] in other models, although the interdependence of these two mechanisms remains unclear. Thus, to address this question we inhibited mitophagy with CsA 1h prior to CORM-A1 treatment for 24h. In fact, this co-treatment partially reverted the CO-induced mitochondrial population maintenance, which might indicate that CO did not promote the compensatory mitochondrial biogenesis following mitophagy (Figure 5). Although additional experiments with genetic approaches must be done, these results suggest that when mitophagy is

inhibited by CsA, CO is no longer able to activate the machinery to induce compensatory mitochondria biogenesis following mitophagy.

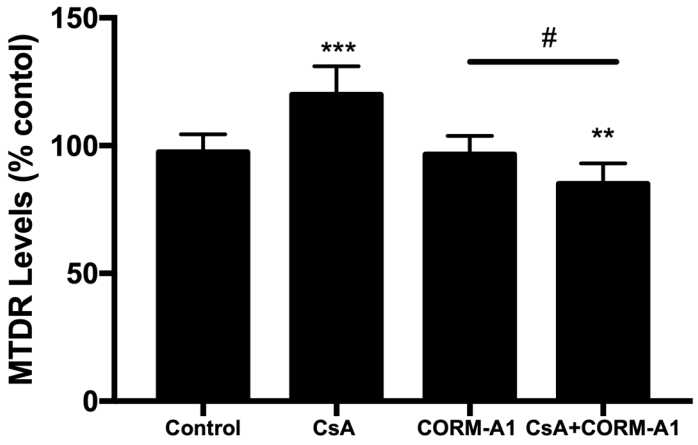


Figure 5- CORM-A1-induced mitochondrial biogenesis in astrocytes is associated with mitophagy inducation; Primary cultures of astrocytes cultured in 24-well plate were treated with 12,5μM of CORM-A1 along 24h with and without 5μM CsA added 1h prior to the cells. MTDR was used to determine mitochondrial mass by flow cytometry. The intensity in the FL4 channel was normalized to untreated control cells. Graphs represent the median ± SD of four experiments performed in triplicate. **p<0,1, ***p<0,001 compared with control treatment, # p<0,5 compared with CORM-A1 without CsA treatment.

CORM-A1-induced cytoprotection is dependent on mitophagy

In order to assess whether CO modulation of mitophagy and mitochondrial biogenesis is important for the established role of CO in preventing astrocytic cell death [6–8,32], pharmacological inhibition of mitophagy was used and CO's cytoprotective role was assessed. Indeed, pretreated astrocytes with CORM-A1 presented higher viability following oxidative cell death promotion with the pro-oxidant *tert*-butylhydroperoxide (tBHP), indicating CO's ability to limit cell death (Figure 6). Nevertheless, whenever astrocytes were pre-treated with CsA and CORM-A1, there is a partial reversion on CO-induced cytoprotection (Figure 6). In conclusion, mitophagy is an important cellular process for CO to be cytoprotective in astrocytes.

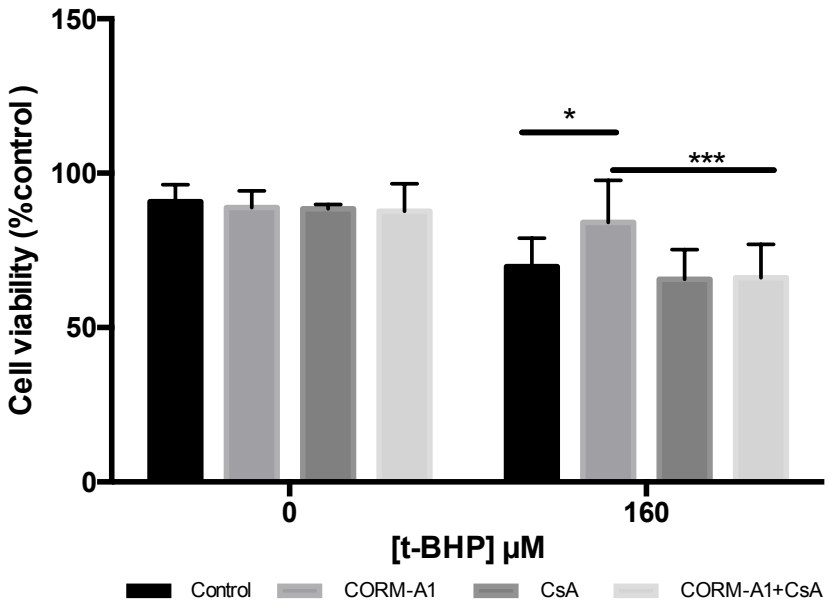


Figure 6- CORM-A1 confers protection against apoptosis by inducing mitophagy; Primary culture of astrocytes were pretreated with 12,5 of CORM-A1 along 1h, with and without CsA 25µM (mitophagy inhibitor), following cell death induction by 18h of exposure to the pro-oxidant, t-bhp (from 0 to 320µm). the measurement of cell viability was assessed by flow cytometry. cells presenting intact plasma membrane (assessed by propidium iodide) are considered viable cells. all the values are mean±sd, of three experiments performed in triplicate. * p<0,5 compared with control and ***p<0,01 compared with CORM-A1 treated cells without CsA for t-BHP treatment.

DISCUSSION

In the present study, we demonstrate that CO confers protection against oxidative stress-induced apoptosis in primary culture of astrocytes in a mitophagy dependent manner and also that CO promotes mitochondrial biogenesis. For the first time, it has been shown that CO-mediated mitophagy is associated with both PINK1/Parkin and BNIP3 pathways. Moreover, preliminary data suggest a cross-talk between mitophagy and mitochondrial biogenesis promoted by CO, process associated with mitochondria quality control.

Several studies have already demonstrated that the cytoprotective role of CO is dependent on autophagy activation. In sepsis CO confers protection in an autophagy dependent manner by increasing Beclin-1 expression [30]. In the same model, knocking out LC3 and Beclin-1 abolished CO's cytoprotective effect [49]. Likewise, CO inhibits hypoxia-induced apoptosis in islets by upregulation of Atg16L1-mediated autophagy [50]. Recently, we have showed that autophagy is important for CORM-A1 to prevent cell death in a model of yeast and primary culture astrocytes by activating the autophagic flux [51]. Herein, the role of mitophagy in CO-mediated cell survival was explored. Indeed, the removal of harmful mitochondria mediated by mitophagy [15] is essential to maintain the mitochondrial homeostasis [23] and cell survival. In the present study, oxidative stress-induced

astrocytic cell death was partially inhibited by CORM-A1 treatment, which in turn promotes mitophagy. Furthermore, whenever mitophagy is prevented by CsA, which maintain mitochondrial potential, CO failed to inhibit cell death. Further studies based on genetic prevention of mitophagy must be performed to validate the role of mitophagy in CO-mediated cytoprotection.

Although PINK1/Parkin cooperation is the most described pathway for mitophagic process, growing number of PINK1 and/or Parkin-independent pathways of selective mitophagy have been reported and they are not exclusive pathways. Therefore, more than one mechanism can be activated at the same time. Indeed, cross-regulation, potential redundancy, and multiplication of mitophagy mechanisms, demonstrate the importance of mitophagy in the cell. In the present study, we demonstrated that CO-mediated mitophagy is associated with an enrichment of PINK1 and Parkin in mitochondrial fraction isolated from CO-treated astrocytes. Nevertheless, knocking down PINK1 did not revert CO's activation of mitophagy, while CCCP-induced mitophagy decreased. Thus, these data indicate that PINK1/Parkin pathway is not exclusive for CO to activate mitophagy. Furthermore, CO-treated astrocytes demonstrated an upregulation of BNIP3 expression. Thus, one can speculate that CO promotes at least two parallel pathways for mitophagy in astrocytes. Other studies shown that while PINK/Parkin are essential for CCCP-triggered mitophagy, they may not be essential for mitophagy triggered by different stimulus, such as ROS or iron deficiency [52]. In fact, in many different biological processes CO acts *via* ROS signaling [53,54], likewise CO triggers autophagy by generation of mitochondrial ROS [29]. Thus, the increased expression of BNIP3 following CO treatment in astrocytes can be associated with BNIP3's regulation of mitophagy in response to hypoxia and redox signaling. Further work must be done to disclose whether BNIP3 is essential CO-regulated pathways of mitophagy, namely by silencing its expression and assessing mitophagy activation.

Mitochondrial biogenesis is important for the maintenance of mitochondrial homeostasis, in particular following mitophagic elimination of dysfunctional mitochondria. We and others have demonstrated that CO stimulates mitochondrial biogenesis in cardiomyocytes [55] and in astrocytes [6,48]. Herein the same effect was observed, CORM-A1 enhanced mitochondrial DNA levels, PGC-1α expression and mitochondrial population. Interestingly, at 1h following CO treatment, mitophagy occurs along with increased mitochondrial DNA content and expression of PGC-1α, suggesting that both events must be activated in parallel. Moreover, one can speculate CO-mediating mitophagy and mitochondrial biogenesis are interdependent processes, although further experiments need to be done to evaluate this question. To reinforce this hypothesis, inhibition of mitophagy with CsA also prevented CO-induced mitochondrial population back to basal levels at 24h, suggesting that CsA-inhibited mitophagy also prevents mitochondrial biogenesis. In 2018, Kim and colleagues showed that CORM2-induced TFEB nuclear translocation enhances mitophagy and mitochondrial biogenesis in hepatocytes and protects the liver against inflammation [31]. These data support the hypothesis that

mitophagy and mitochondrial biogenesis present a common regulation. A potential candidate to regulate both processes is the protein PARIS since it seems to inter-connect Parkin and PGC-1α. In fact, PARIS is a transcriptional repressor (member of the family of KRAB zinc-finger proteins), which is ubiquitinated by Parkin targeting it for proteasomal degradation [56,57]. Moreover, PARIS is physiological transcriptional repressor of the PGC-1α, thus its degradation upregulates PGC-1α and subsequently PGC-1-dependent nuclear respiratory factors genes expression, promoting therefore mitochondrial biogenesis [58]. Further work must be performed to disclose the potential implication of CO in PARIS's regulation of mitophagy and mitochondrial biogenesis.

In summary, our data indicate that CO regulates the autophagic turnover of damaged mitochondria and their subsequent replacement through mitochondrial biogenesis. CO has a protective role against oxidative stress in astrocyte via mitophagy induction. These results suggest that CO may represent a potentially strategy to prevent astrocytic cell death in a context of oxidative stress.

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REFERENCES

1. Idriss, N. K., Blann, A. D. & Lip, G. Y. H. Hemoxygenase-1 in cardiovascular disease. *J. Am. Coll. Cardiol.* **52**, 971–978 (2008).

2. Motterlini, R. & Otterbein, L. E. The therapeutic potential of carbon monoxide. *Nat. Rev. Drug Discov.* **9**, 728 (2010).

3. Riego, G., Redondo, A., Leánez, S. & Pol, O. Mechanism implicated in the anti-allodynic and anti-hyperalgesic effects induced by the activation of heme oxygenase 1/carbon monoxide signaling pathway in the central nervous system of mice with neuropathic pain. *Biochem. Pharmacol.* **148**, 52–63 (2018).

4. Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V & Snyder, S. H. Carbon monoxide: a putative neural messenger. *Science (80-.).* **259**, 381 LP – 384 (1993).

5. Queiroga, C. S. F., Vercelli, A. & Vieira, H. L. A. Carbon monoxide and the CNS: Challenges and achievements. *Br. J. Pharmacol.* **172**, 1533–1545 (2015).

6. Almeida, A. S., Queiroga, C. S. F., Sousa, M. F. Q., Alves, P. M. & Vieira, H. L. a. Carbon monoxide modulates apoptosis by reinforcing oxidative metabolism in astrocytes: Role of Bcl-2. *J. Biol. Chem.* **287**, 10761–10770 (2012).

7. Oliveira, S. R., Figueiredo-Pereira, C., Duarte, C. B. & Vieira, H. L. A. P2X7 Receptors Mediate CO-Induced Alterations in Gene Expression in Cultured Cortical Astrocytes—Transcriptomic Study. *Mol. Neurobiol.* (2018). doi:10.1007/s12035-018-1302-7

8. Queiroga, C. S. F. *et al.* Glutathionylation of adenine nucleotide translocase induced by carbon monoxide prevents mitochondrial membrane permeabilization and apoptosis. *J. Biol. Chem.* **285**, 17077–17088 (2010).

9. Wang, Z., Figueiredo-Pereira, C., Oudot, C., Vieira, H. L. A. & Brenner, C. Mitochondrion: A Common Organelle for Distinct Cell Deaths? *Int. Rev. Cell Mol. Biol.* **331**, 245–287 (2017).

10. Lane, R. K., Hilsabeck, T. & Rea, S. L. The role of mitochondrial dysfunction in age-related diseases. *Biochim. Biophys. Acta - Bioenerg.* **1847**, 1387–1400 (2015).

11. Chu, C. T. Multiple pathways for mitophagy: A neurodegenerative conundrum for Parkinson's disease. *Neurosci. Lett.* 0–1 (2018). doi:10.1016/j.neulet.2018.04.004

12. Chistiakov, D. A., Sobenin, I. A., Revin, V. V., Orekhov, A. N. & Bobryshev, Y. V. Mitochondrial aging and age-related dysfunction of mitochondria. *Biomed Res. Int.* **2014**, (2014).

13. Choi, A. M. K., Ryter, S. W. & Levine, B. Autophagy in human health and disease. *N. Engl. J. Med.* **368**, 651–662 (2013).

14. Rodger, C. E., McWilliams, T. G. & Ganley, I. G. Mammalian mitophagy – from in vitro molecules to in vivo models. *FEBS J.* **285**, 1185–1202 (2018).

15. Martinez-Arroyo, O. *et al.* Mitophagy in human astrocytes treated with the antiretroviral drug Efavirenz: Lack of evidence or evidence of the lack. *Antiviral Res.* **168**, 36–50 (2019).

16. Bayne, A. N. & Trempe, J. F. Mechanisms of PINK1, ubiquitin and Parkin interactions in mitochondrial quality control and beyond. *Cell. Mol. Life Sci.* (2019). doi:10.1007/s00018-019-03203-4

17. McWilliams, T. G. *et al.* Basal Mitophagy Occurs Independently of PINK1 in Mouse Tissues of High Metabolic Demand. *Cell Metab.* **27**, 439-449.e5 (2018).

18. Okatsu, K. *et al.* Mitochondrial hexokinase HKI is a novel substrate of the Parkin ubiquitin ligase. *Biochem. Biophys. Res. Commun.* **428**, 197–202 (2012).

19. Fiesel, F., Hudec, R. & Springer, W. Non-radioactive in vitro PINK1 Kinase Assays Using Ubiquitin or Parkin as Substrate. *Bio-Protocol* **6**, 1–11 (2016).

20. Shiba-Fukushima, K. *et al.* PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. *Sci. Rep.* **2**, 1002 (2012).

21. Novak, I. *et al.* Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep.* **11**, 45–51 (2010).

22. Liu, L., Sakakibara, K., Chen, Q. & Okamoto, K. Receptor-mediated mitophagy in yeast and mammalian systems. *Cell Res.* **24**, 787–795 (2014).

23. Hickson-Bick, D. L. M., Jones, C. & Buja, L. M. Stimulation of mitochondrial biogenesis and autophagy by lipopolysaccharide in the neonatal rat cardiomyocyte protects against programmed cell death. *J. Mol. Cell. Cardiol.* **44**, 411–418 (2008).

24. Andersson, U. & Scarpulla, R. C. PGC-1-Related Coactivator, a Novel, Serum-Inducible Coactivator of Nuclear Respiratory Factor 1-Dependent Transcription in Mammalian Cells. *Mol. Cell. Biol.* **21**, 3738–3749 (2001).

25. Dairaghi, D. J., Shadel, G. S. & Clayton, D. A. Human mitochondrial transcription factor A and promoter spacing integrity are required for transcription initiation. *BBA - Mol. Basis Dis.* **1271**, 127–134 (1995).

26. Falkenberg, M. *et al.* Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat. Genet.* **31**, 289–294 (2002).

27. Gleyzer, N., Vercauteren, K. & Scarpulla, R. C. Control of Mitochondrial Transcription Specificity Factors (TFB1M and TFB2M) by Nuclear Respiratory Factors (NRF-1 and NRF-2) and PGC-1 Family Coactivators. *Mol. Cell. Biol.* **25**, 1354–1366 (2005).

28. Kelly, D. P. & Scarpulla, R. C. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev.* **18**, 357–368 (2004).

29. Lee, S. J. *et al.* Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation. *Am J Respir Cell Mol Biol* **45**, 867–873 (2011).

30. Lee, S. *et al.* Carbon monoxide confers protection in sepsis by enhancing Beclin 1-dependent autophagy and phagocytosis. *Antioxidants Redox Signal.* **20**, 432–442 (2014).

31. Kim, H. J. *et al.* Carbon monoxide-induced TFEB nuclear translocation enhances mitophagy/mitochondrial biogenesis in hepatocytes and ameliorates inflammatory liver injury. *Cell Death Dis.* **9**, (2018).

32. Figueiredo-Pereira, C., Menezes, R., Ferreira, S., Santos, C. N. & Vieira, H. L. A. Carbon monoxide released by CORM-A1 prevents yeast cell death via autophagy stimulation. *FEMS Yeast Res.* **19**, (2019).

33. Lee, S.-J. *et al.* Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation. *Am. J. Respir. Cell Mol. Biol.* **45**, 867–873 (2011).

34. R. Oliveira, S., Queiroga, C. S. F. & Vieira, H. L. A. Mitochondria and carbon monoxide: cytoprotection and control of cell metabolism – a role for Ca^{2+}? *J. Physiol.* **594**, 4131–4138 (2016).

35. Sá Santos, S. *et al.* Culturing primary brain astrocytes under a fully controlled environment in a novel bioreactor. *J. Neurosci. Res.* **79**, 26–32 (2005).

36. Motterlini, R. *et al.* CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **19**, 284–286 (2005).

37. Mauro-Lizcano, M. *et al.* New method to assess mitophagy flux by flow cytometry. *Autophagy* **11**, 833–843 (2015).

38. Narendra, D., Tanaka, A., Suen, D.-F. & Youle, R. J. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J. Cell Biol.* **183**, 795–803 (2008).

39. Crompton, M., Virji, S., Doyle, V., Johnson, N. & Ward, J. M. The mitochondrial permeability transition pore. in *Biochem Soc Symp* **66**, 167–179 (1999).

40. Zhu, Y. *et al.* Monitoring mitophagy in Mammalian cells. *Methods Enzymol.* **547**, 39–55 (2013).

41. McLelland, G.-L., Soubannier, V., Chen, C. X., McBride, H. M. & Fon, E. A. Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control. *EMBO J.* **33**, 282–295 (2014).

42. Valente, E. M. *et al.* Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* **304**, 1158–1160 (2004).

43. Narendra, D. P. *et al.* PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* **8**, e1000298 (2010).

44. Gomes, L. C. & Scorrano, L. Mitochondrial morphology in mitophagy and macroautophagy. *Biochim Biophys Acta* (2012). doi:S0167-4889(12)00052-3 [pii]10.1016/j.bbamcr.2012.02.012

45. Shi, R.-Y. *et al.* BNIP3 interacting with LC3 triggers excessive mitophagy in delayed neuronal death in stroke. *CNS Neurosci. Ther.* **20**, 1045–1055 (2014).

46. Ding, W. X. *et al.* Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. *J. Biol. Chem.* **285**, 27879–27890 (2010).

47. Kothari, S. *et al.* BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. *Oncogene* **22**, 4734–4744 (2003).

48. Choi, Y. K. *et al.* Carbon monoxide stimulates astrocytic mitochondrial biogenesis via L-type Ca2+ channel-mediated PGC-1α/ERRα activation. *Biochem. Biophys. Res. Commun.* **479**, 297–304 (2016).

49. Ghanta, S. *et al.* Mesenchymal Stromal Cells Deficient in Autophagy Proteins Are Susceptible to Oxidative Injury and Mitochondrial Dysfunction. *Am. J. Respir. Cell Mol. Biol.* **56**, 300–309 (2017).

50. Kim, D.-S. *et al.* Carbon Monoxide Inhibits Islet Apoptosis via Induction of Autophagy. *Antioxid. Redox Signal.* **28**, 1309–1322 (2018).

51. Almeida, A. S., Figueiredo-pereira, C., Vieira, H. L. A. & Raimundo, N. Carbon monoxide and mitochondria — modulation of cell metabolism , redox response and cell death. **6**, 1–6 (2015).

52. Han, J. Y. *et al.* Nitric oxide induction of parkin translocation in PTEN-induced putative kinase 1 (PINK1) deficiency: Functional role of neuronal nitric oxide synthase during mitophagy. *J. Biol. Chem.* **290**, 10325–10335 (2015).

53. Zuckerbraun, B. S. *et al.* Carbon monoxide signals via inhibition of cytochrome c oxidase and generation of mitochondrial reactive oxygen species. *FASEB J.* **21**, 1099–106 (2007).

54. Vieira, H., Almeida, A., Figueiredo-Pereira, C. & Queiroga, C. Carbon monoxide targeting mitochondria in astrocytes: modulation of cell metabolism, redox response and cell death. *Springerplus* **4**, L42 (2015).

55. Suliman, H. B., Carraway, M. S., Tatro, L. G. & Piantadosi, C. A. A new activating role for CO in cardiac mitochondrial biogenesis. *J. Cell Sci.* **120**, 299–308 (2007).

56. Nishida, T. & Yamada, Y. SUMOylation of the KRAB zinc-finger transcription factor PARIS/ZNF746 regulates its transcriptional activity. *Biochem. Biophys. Res. Commun.* **473**, 1261–1267 (2016).

57. Lupo, A. *et al.* KRAB-Zinc Finger Proteins : A Repressor Family Displaying Multiple Biological Functions. 268–278 (2013).

58. Zhang, Y. *et al.* Endothelial PINK1 mediates the protective effects of NLRP3 deficiency during lethal oxidant injury. *J. Immunol.* **192**, 5296–304 (2014).

IV

PINK1 GLUTATHIONYLATION THE CRUCIAL STEP IN THE MODULATION OF CCCP-INDUCED MITOPHAGY

This chapter is based on data to be published as:

PINK1 glutathionylation the crucial step in the modulation of CCCP-induced mitophagy
Cláudia Figueiredo-Pereira and Helena L.A. Vieira.

Unpublished data

ABSTRACT

Mitochondria are key cellular organelles for producing bioenergy and for controlling cell death. Once, dysfunctional mitochondria are the main responsible for reactive oxygen species production (ROS), therefore, it is crucial for the cell to sequester and remove them before damage. This process, is named mitophagy, it comprises mitochondria engulfment by autophagosome and delivery to the lysosomes. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), is the classical inducer of mitophagy by promoting mitochondrial uncoupling and decrease of mitochondrial potential. Some recent studies have also revealed ROS as signaling molecules promoting mitophagy in response to mitochondrial disorder. Glutathione provides protection from oxidative stress-induced damage through the reduction of reactive oxygen species for the maintenance of oxidant homeostasis. Herein, the preliminary data generated suggest that dysfunctional mitochondria induced by low concentrations of CCCP stimulate mitophagy via GSH signaling. CCCP-damaged mitochondria generate ROS that, in turn, activate GSH synthesis by increasing GCLC expression. Additionally, pharmacological modulation of GSH generation (inhibitor BSO or addition of GSH substrate NAC) regulates mitophagy. Finally, GSH appears to regulate mitophagy *via* S-glutathionylation of PINK1.

CONTENTS

Claudia Figueiredo-Pereira had carried out the majority of the experimental part and was involved on the decisions on how to execute the experiments, as well as on the interpretation and discussion of the results.

INTRODUCTION

Mitochondria are essential cellular organelles responsible for energy generation, production of cellular building blocks and controlling cell death [1]. Therefore, mitochondrial function must be tightly regulated, along with elimination of dysfunctional mitochondria. Indeed, dysfunctional oxidative phosphorylation can generate excessive amounts of reactive oxygen species (ROS) and promote oxidative stress, leading eventually to cell death. Mitophagy, specific mitochondrial autophagy, is a key cellular process for mitochondrial quality control and for dysfunctional mitochondria elimination, being its dysregulation associated with several diseases [2,3]. The most studied mitophagy inducer is carbonyl cyanide m-chlorophenylhydrazone (CCCP), which promotes mitochondrial uncoupling and decrease of mitochondrial potential [4,5]. Some recent studies have also revealed ROS as signaling molecules promoting mitophagy in response to mitochondrial disorder. Moderate exogenous levels of hydrogen peroxide do not induce cell death, but trigger mitophagy in a fission dependent manner, since knocking down Drp1 reverted mild oxidative stress induced-mitophagy [6]. In a more elegant model, the genetically encoded photosensitizer protein, KillerRed, which is targeted to mitochondria (mtKR) was applied to assess the effect of a short burst of ROS in the mitochondria. mtKR-induced an increase on ROX levels in mitochondrial matrix, leading to Parkin dependent mitophagy, while overexpression of mitochondrial superoxide dismutase-2 reverted mitophagy activation [7]. Likewise, irradiation of mtKR promoted fragmentation of mitochondria and recruitment of Parkin in neuronal axons [8]. Thus, ROS can be signaling molecules, triggering mitophagy.

Glutathione (GSH) or γ-L-glutamyl-cisteinyl-glycine is a ubiquitous tri-peptide, formed by glutamate, cysteine, and glycine and is the most abundant non-protein thiol [9]. GSH is present in two forms: the thiol-reduced (GSH), the most abundant (>98%) [10] and disulfide-oxidized (GSSG) [11], being the system GSH/GSSG the main cellular antioxidant defense [10,12]. GSH homeostasis is controlled by *de novo* synthesis and by its utilization, recycling and cellular export [13]. The rate-limiting enzyme involved in GSH production is γ-glutamyl-cysteine synthetase (GCL), which is formed by two subunits, the glutamate-cysteine ligase modifier subunit (GCLM) and the glutamate-cysteine ligase catalytic subunit (GCLC). This last subunit is responsible for the catalysis and conversion of glutamate and cysteine into γ-glutamyl cysteine in an ATP-dependent manner, the glutathione precursor [9,14,15]. Moreover, GSH is also a key element of redox signaling, by modulation of ROS levels and by maintaining the thiol status of proteins [12,16]. Likewise, S-glutathionylation is a reversible post-translational protein modification occurring in cysteine residues whose main functions are: (i) modulation of protein function, (ii) reduction of cellular GSSG levels and/or (iii) protection of critical thiols from irreversible oxidation [13,17]. Protein S-glutathionylation can occur spontaneously by a dramatic decrease of GSH/GSSG ratio, which is less likely to occur *in vivo*; or in a physiologically

relevant manner, by the activation of thiol derivatives (such as S-nitrosyl, sulfenic acid, thiyl radical, etc) along with GSH as substrate [17].

A deep decrease in the concentration of GSH leads to enhance in ROS production, oxidative stress with subsequent cell death [12]. Nevertheless, regulation of GSH homeostasis is also critical for redox cell signaling and for the activation of cellular mechanisms of defense, namely via autophagy and mitophagy [13,17,18]. Accordingly, there is a cross-talk between GSH levels and autophagic regulation. Downregulation of p62/SQTM1 (which is an adaptor protein and autophagy substrate) decreases intracellular GSH levels along with an accumulation of dysfunctional mitochondria [19]. Likewise, GSH system also control mitochondrial morphology and function. Glutathione-S-transferase (GST) binds to GSH to react with lipids and proteins and detoxify cellular oxidative stress. In fly models, lack of GST increased mitochondrial fusion levels in neuronal axons *via* an enhancement of oxidized GSSG cellular levels [20]. Still, in primary culture of rodent neurons, pharmacological inhibition of GSH synthesis or promotion of its oxidation into GSSG also leaded to mitochondrial fusion [20].

The present study is a in progress work aiming to disclose whether and how GSH regulates mitophagy and in turn promotes cell survival. In astrocytes, CCCP induces PINK1-dependent mitophagy along with ROS generation. Moreover, mitophagy activation is regulated by GSH levels (pharmacological modulation of its synthesis) and affects the expression of GCLC, the rate limiting enzyme in GSH *de novo* synthesis. Finally, during mitophagic process, Pink1 is modified by S-glutathionylation. These preliminary data indicate that GSH regulates mitophagy via protein post-translational modification.

MATERIAL AND METHODS

Primary culture of astrocytes

Primary cultures of astrocytes were prepared from 1-day-old Balb/c mouse cortex, as described [21]. Animals were rapidly decapitated, brain cortex was removed and the meninges were carefully stripped off, then the cortex was washed in ice-cold phosphate-buffered saline (PBS), and mechanically disrupted. Single-cell suspensions were plated in T-flasks (four hemispheres/75 cm^2) in Dulbecco's minimum essential medium supplemented with 20% (v/v) FBS (heat-inactivated), 1% (v/v) of 100 U/mL penicillin/streptomycin solution and glucose (to obtain a final concentration of 10 mM). Astrocytes were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. After 7 days, the dark phase cells growing on the astrocytic cell layer were separated by vigorous shaking and removed. Culture medium was replaced twice a week for 3 weeks with gradual reduction in FBS

content (2^{nd} week 15% (v/v); 3^{rd} week 10%(v/v)). At the 3^{rd} week the confluent astrocytes were detached by mild trypsinization using trypsin/EDTA (0.25%, w/v) and sub-cultured, in 24 and 6-well plates at 50 x 10^3 and 100 x10^3 cells/well, respectively, until reaching full confluence.

Mitochondria isolation;

Isolation of Non-synaptic Mitochondria from Cortex

Enriched fractions of non-synaptic mitochondria were isolated from male BALB/c mice according to [22]. Briefly, the cortex was removed and washed in an ice-cold isolation buffer containing 225 mM manitol, 75 mM sucrose, 1mM EGTA, and 5mM HEPES, pH 7.4. The tissue was minced with scissors and homogenized manually with Potter Elvehjem in isolation buffer. The homogenate was centrifuged at 1300 *g* for 3 min, and the resuspended pellet was recentrifuged at 1300 *g* for 3 min. Both supernatants were pooled together and centrifuged at 21200 *g* for 10 min. The remaining pellet was resuspended in 3.5 ml of 15% Percoll solution and layered into centrifuge tubes containing a preformed two-step discontinuous density gradient consisting of 3.7 ml of 24% Percoll on top of 1.7 ml of 40% Percoll. The gradient was centrifuged at 31700 *g* for 9 min. The mitochondrial fraction, located between the layers of 24 and 40% Percoll, was removed, diluted 1:8 in isolation buffer, and centrifuged at 16,700 *g* for 10 min. The pellet was resuspended in 10 ml of isolation buffer containing 5 mg/ml bovine serum albumin (to remove lipids) and centrifuged at 6,800 *g* for 10 min. The mitochondrial pellet was resuspended in 100µl of isolation buffer, and the total amount of protein was quantified using a BCA assay (Pierce). All of the steps were carried out at 4 °C. The analysis of enriched mitochondrial fractions was performed in modified brain buffer containing 125mMKCl, 2mMK2HPO4,1mMMgCl2, 15 M EGTA, 20mM Tris, 5mM glutamate, and 5mM malate, pH 7.3, unless stated otherwise. CCCP (carbonyl cyanide m-chlorophenylhydrazone) treatment in isolated mitochondria was performed with a final concentration of 25µM for 1 hour at 37 °C.

Isolated mitochondria from primary culture of astrocytes

Primary cultures of astrocytes were grown in 175 cm^2 t-flask up to reaching confluence, then cells were washed with ice-cold PBS and collected by trypsinisation. Samples were centrifugated at 200 g for 10 min, and cells (pellet) were washed in PBS by centrifugation at 200 g for 10 min. The supernatant was discarded and the pellet (cells) incubated in 3.5mL of hypotonic buffer (0.15mM MgCl2, 10mM KCl, 10mM Tris-HCL, pH 7.6) at 4ºC for 15min, After the addition of an equal volume of homogenization buffer (0.15mM MgCl2, 10mM KCl, 10mM Tris-HCL, 0.4mM PMSF, 250mM

saccharose, pH7.6) twice concentrated, samples were manually homogenized with Douncer potter. Cell extracts were centrifugated at 900 g for 10 min, followed by supernatant centrifugation at 10000 g for 10 min. The mitochondrial pellet was resuspended in 110µL of the homogenization buffer and the total amount of protein was quantified using BCA assay (Pierce, Illinois). All the steps were carried out at 4ºC. CCCP treatment in isolated mitochondria was performed with a final concentration of 25µM for 1 hour at 37 °C.

Mitochondrial population assessment (Mitophagy)

Astrocytes were plated in a concentration of $50*10^4$ cells/well into the 24-well plate for flow cytometer analysis. Cells were pre-treated with NAC (N-acetylcysteine) at 1µM or with BSO (L-buthionine-(S,R)-sulfoximine) at 100µM for 24h, following by incubation for 1h with and without CCCP at 25µM for triggering mitophagy. Cells were collected for further analysis by flow cytometry to assess mitophagy.

Determination of mitochondrial population by flow cytometry

After astrocyte treatment, as previously described [23], cells were trypsinized for 5 min at 37 ºC and resuspended in complete medium with 10 nM Mitotracker Deep Red (MTDR, M22426, Invitrogen) incubated for 15 min at 37 ºC followed by cytofluorometric analysis with FACS scan (BD FACSCalibour). MTDR is used for mitochondria labeling. Fluorescence intensity for each incubation time was analyzed in 10,000 cells and fluorescence value per cell was determined and plotted against cell number. Median fluorescence in the FL4 channel in the viable cell (PI-negative) population was plotted and normalized against that of untreated cells.

Measurement of ROS Generation

ROS generation was followed by assessing hydrogen peroxide (H_2O_2) that converts 2',7'-dichlorofluorescein diacetate (H2DCFDA at 5µM) (Invitrogen) into fluorescent 2',7'-dichlorofluorescein (DCF). While superoxide anion formation was quantified using 5µM MitoSOX Red mitochondrial superoxide indicator (Life Technologies, Scotland). Astrocytes were treated for 1h with CCCP, supernatant was removed, and cells were incubated for 20 min with 10 MitoSOX prepared in PBS. Cells were washed twice, and fluorescence was measured (λex 510 nm/λem 580 nm) using a TECAN infinite F200 PRO spectrofluorimeter. ROS generation was calculated as an

increase over base-line levels, determined for untreated cells (100%) and normalized by total cell count for each condition.

Immunoprecipitation

100 µg of mitochondrial protein (isolated from astrocytes or from mice cortex) was incubated in 100 µl of homogenization buffer containing 0.5% of Triton X-100 in the presence of 20µl of the antibody α-GSH (1 mg/ml; ViroGen) for 90 min at 37 °C, followed by immunoprecipitation with 15 µl of protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) for 30 min at 37 °C. After 10 min of 10,000 *g* centrifugation, supernatant was discarded, and pellet was washed four times with PBS. Proteins attached to the beads were solubilized by Laemmli buffer for further Western blot analysis.

Immunoblotting

Several samples from cell extracts or isolated mitochondria were separated under reducing electrophoresis on a 1 mm thick 15% SDS-polyacrylamide (SDS-PAGE) gel. Samples were transferred onto a PVDF membrane (MERK). Membranes were incubated overnight at 4°C with antibodies against Pink1 protein (GeneTex), Parkin protein (Abcam), GCLC (NOVUS bio) and actin (Sigma), used as a loading control (CONCENTRAÇÕES). Blots were developed using the ECL (enhanced chemiluminescence) detection system after incubation with horseradish peroxidase-labeled anti-mouse IgG antibody (Amersham Biosciences) at 1:5000 dilution and 1 h of room temperature incubation. The area and intensity of bands were quantified by densitometry analysis and are presented as a percentage relative to the positive control (100%). These experiments have been repeated three times with similar results.

siRNA Transfection

Pink1 expression was silenced by Pink1 coding siRNA transfection according to the manufacturer's instructions (Invitrogen). Astrocytes at 40% of confluence were transfected using LipofectamineTM RNAiMAX and Opti-MEM medium (Invitrogen); for 3.9 cm^2 of astrocytic culture area 10pmol of siRNA were used. At room temperature, siRNA and culture medium were mixed gently with Lipofectamine for forming liposomes, and then astrocytes were transfected in the absence of antibiotics. 24, 48 and 72h after transfection Pink1 expression was assessed by Western blot assay, silencing was more efficient between 24h, and then Pink1 silenced astrocytes were used in this time frame.

Statistical Analyses

The data concerning yeast and astrocytic culture were carried out at least in three independent preparations (cell isolation). For autophagy evaluation, a representative image of three independent assays is shown. All values are mean S.D. (n =3). Error bars, corresponding to S.D., are shown in the figures.

Statistical comparisons were performed using ANOVA (one-way, two-way) and Student t-test (with Welch's correction) with p <0.05 (n≥3). p <0.05 means that samples are significantly different at a confidence level of 95%.

RESULTS

CCCP-induced mitophagy is dependent on PINK1/Parkin pathway in astrocytes

In primary culture of astrocytes, the ability of the uncoupler CCCP was assessed for mitophagy activation. Of note, low CCCP concentrations were used in order not to induced cell death, but to promote mitochondrial uncoupling. In astrocyte, 1h of CCCP treatment decreases mitochondrial population (assessed by MTDR) and is reverted by cyclosporin A (CsA), indicating that this uncoupler promotes mitophagy in primary culture of astrocytes (Figure 1A – similar to Figure XX at Chapter III). CCCP is usually described to trigger mitophagy *via* PINK1/Parkin-mediated pathway [24,25] (with PINK1 accumulation in mitochondrial membrane and Parkin translocation into mitochondria). In astrocytes the presence of PINK1 and Parkin in mitochondria following CCCP treatment was evaluated. In fact, CCCP treatment increased the level of PINK1 and PARKIN in mitochondrial enriched fractions (Figure 1B and C).

To examine whether CCCP-activated mitophagy requires PINK1/Parkin pathway, we used siRNA to knock down PINK1 expression (Figure 1D). As expected, whenever astrocytes were treated with CCCP, PINK1-targeted siRNA significantly abolished the capability of CCCP to promote mitophagy. These findings are in accordance with the literature, where it is shown that in other models, namely neurons, CCCP promotes mitophagy *via* PINK1/Parkin [8,24].

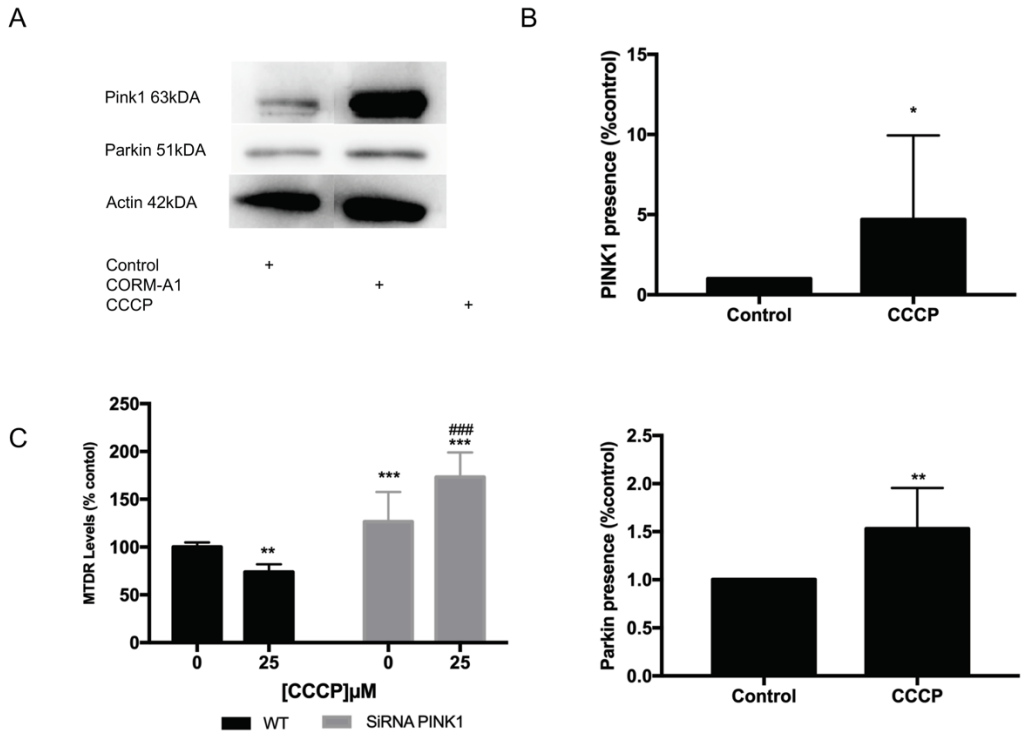

Figure 1- CCCP-induced mitophagy in astrocytes is associated with PINK1/Parkin pathway; A) Mitochondrial fractions isolated from primary culture of astrocytes treated with CCCP at 25µM during 1h were analyzed by immunoblotting using antibodies against PINK1 and Parkin. **(also published in Chapter III). B)** Quantification of PINK1 and Parkin presence in mitochondrial enriched fraction. Graphs represent the median ± SD of four experiments. *p<0,5, ** p<0,1 compared to control treatment. **C)** Primary cultures of astrocytes cultured in 24-well plate were transfected with SiRNA PINK1 for 24h and treated with 25µM CCCP along 1h. MTDR was used to determine mitochondrial mass by flow cytometry. The intensity in the FL4 channel was normalized to untreated control cells. Graphs represent the median ± SD of five experiments performed in triplicate. *p<0,5, *** p<0,001 compared to control treatment and ### p<0,001 compared to wt cells treated with CCCP.

CCCP-mediated mitophagy promote ROS production

Mitophagy induction *via* CCCP treatment involves mitochondrial depolarization and ROS generation [26,27]. Thus, to examine if CCCP induces ROS generation in astrocytes, MitoSOX Red mitochondrial superoxide indicator fluorescence dye was used and anion superoxide production was determined after 1h of CCCP treatment. CCCP significantly increases intracellular ROS of about 50% compared to the control condition. Thus, these data in astrocytes are in accordance with previous published studies in other models, where CCCP induces ROS generation to promote mitophagy activation [26,27].

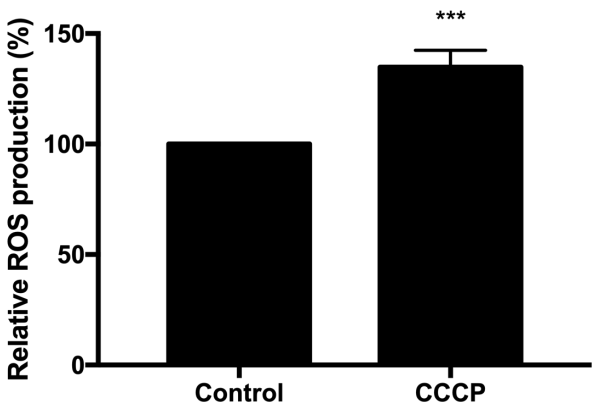

Figure 2- CCCP-induced ROS generation; Quantification of intracellular ROS in primary culture of astrocytes after 1h treatment with CCCP, ROS quantification was performed using MitoSOX Red mitochondrial superoxide indicator fluorescence dye. Graphs represent the median ± SD of three experiments performed in triplicate. *** p<0,001 compared to control treatment.

CCCP-induced mitophagy increases the expression of the rate-limiting enzyme (GCLC) of GSH synthesis

Because CCCP promotes ROS generation and GSH is the major cellular anti-oxidant system [16], one can hypothesized that GSH can be an intermediary factor in PINK1/Parkin-dependent mitophagy. Thus, an indirect way to measure GSH role in CCCP-induced mitophagy is by quantification of the rate limiting enzyme for GSH synthesis: γ-glutamyl-cysteine synthetase (GCL), in particular its catalytic subunit the glutamate-cysteine ligase (GCLC). We used total cell extracts of astrocytes from control or 1h CCCP-treated astrocytes to immunodetect GCLC protein. CCCP treatment increased the expression level of GCLC in astrocytes (Figure 3). Therefore, CCCP generation of ROS might promote the synthesis of GSH. Moreover, further experiments must be performed to: (i) quantify cellular GSH content following CCCP and (II) validate the role of GCLC using siRNA to knock down its expression. In conclusion, CCCP-induced mitophagy also modulates GSH synthesis by increasing GCLC enzyme levels.

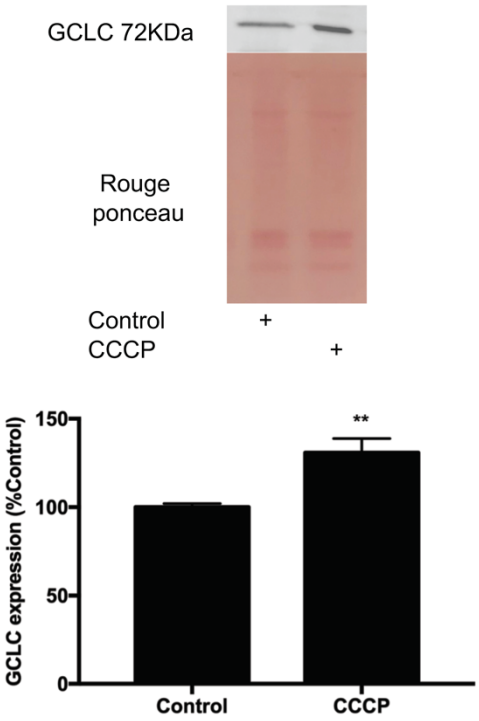

Figure 3- CCCP promotes the expression GCLC enzyme; A) Total cell extract of astrocytes treated with or without CCCP ate 25µM final concentration for 1h were analyzed by immunoblotting using antibody against GCLC. Graphs represent the median ± SD of three experiments. ** p<0,1 compared with the control (PINK1-GSH).

Modulation of glutathione synthesis modulates mitophagy

In order to further understand the role of GSH in mitophagy regulation, two strategies were followed: pharmacological enhancement and inhibition of GSH synthesis. The anti-oxidant N-acetyl-cysteine (NAC) is a derivate of L-cysteine and a precursor of glutathione, increasing its synthesis [28,29]. Mitochondrial population was evaluated by MTDR assay in astrocytes treated with CCCP in the presence and absence of NAC, for quantification of mitophagy (Figure 4A). Whenever astrocytes were treated with CCCP and NAC, there is a higher decrease on mitochondrial population, indicating a synergetic effect of both molecules. This might suggest that glutathione facilitates CCCP-induced mitophagy.

Likewise to confirm the involvement and importance of glutathione synthesis and the role of increased expression of GCLC during mitophagy mediated by CCCP, we treated astrocytes with and without L-buthionine-(S,R)-sulfoximine (BSO). BSO is a specific pharmacological tool known to deplete GSH by decreasing its synthesis since it is a highly selective inhibitor of GCL [10,12]. Astrocytes treated for 1h with CCCP promoted mitochondrial clearance, which is in accordance with the previous data (Figure 1, Chapter III). Nevertheless, co-treatment with BSO rescues the effect of

CCCP, preventing mitochondrial elimination and reestablishing mitochondrial population levels close to the control levels (Figure 4B). Additionally, astrocytes treated with BSO alone lead to an increase of MTDR levels of about 150%. This great accumulation of mitochondrial population in the presence of BSO could be explained by BSO-induced inhibition of basal mitophagy, since mitochondrial turnover is a constant event always occurring in the cell. Nevertheless, further experiments must be done to deeper disclose this result, namely by assessing mitophagy via co-localization of autophagossomes and mitochondria.

Finally, CCCP seems to feed GSH synthesis system, by inducing ROS production and acting at the level of GCLC (Figure 3). This hypothesis, is reinforced by the promotion of mitophagy in the presence of NAC (Figure 4A) and inhibition with BSO (Figure 4B), that selectively inhibits GCL function. In conclusion, glutathione appears to be an important factor for the regulation of mitophagy.

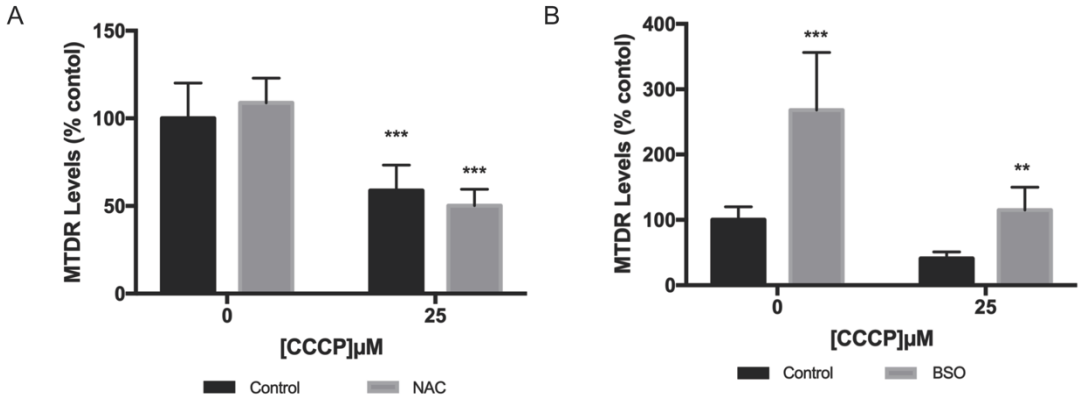

Figure 4- Glutathione synthesis modulated CCCP-mediated mitophagy; A) Primary culture of astrocytes were pretreated with NAC 1µM for 24h, followed by CCCP treatment at the final concentration of 25µM for 1h. MTDR was used to determine mitochondrial mass by flow cytometry. The intensity in the FL4 channel was normalized to untreated control cells. Graphs represent the median ± SD of five experiments performed in triplicate. *** p<0,001 compared with the control. **B)** Astrocytes were pretreated with BSO 100µM for 24h, followed by CCCP treatment at the final concentration of 25µM for 1h. MTDR was used to determine mitochondrial mass by flow cytometry. The intensity in the FL4 channel was normalized to untreated control cells. Graphs represent the median ± SD of four experiments performed in triplicate. **p<0,1, ***p<0,001 compared with the respective control.

Protein S-glutathionylation is involved in the modulation CCCP-induced mitophagy targeting PINK1

Reversible protein S-glutathionylation is a post-translational process involved in cellular response to redox changes, which can modulate protein function or protect cysteine residues against irreversible damage due to oxidative stress.

In order to evaluate protein S-glutathionylation pattern, two different approaches were performed. Purified mitochondria from brain extracts were treated with CCCP followed by immunoprecipitation; thus it is targeted the changes occurring only at mitochondrial level. In the second approach, astrocytes were treated with CCCP, followed by mitochondrial fraction enrichment and immunoprecipitation, therefore intact cells are targeted. Mitochondrial fractions were incubated with α-GSH to immunoprecipitated glutathionylated proteins, followed by immunodetection of PINK1 by a western blot assay (Figure 5A-B). The presence of CCCP augments the levels of glutathionylated PINK1 in both approaches. Thus, these data provide evidence that CCCP treatment modifies PINK1 protein by S-glutathionylation. In conclusion, this data indicates that S-glutathionylation modulates mitophagy. Nevertheless, other targeted proteins for S-glutathionylation must still be explored.

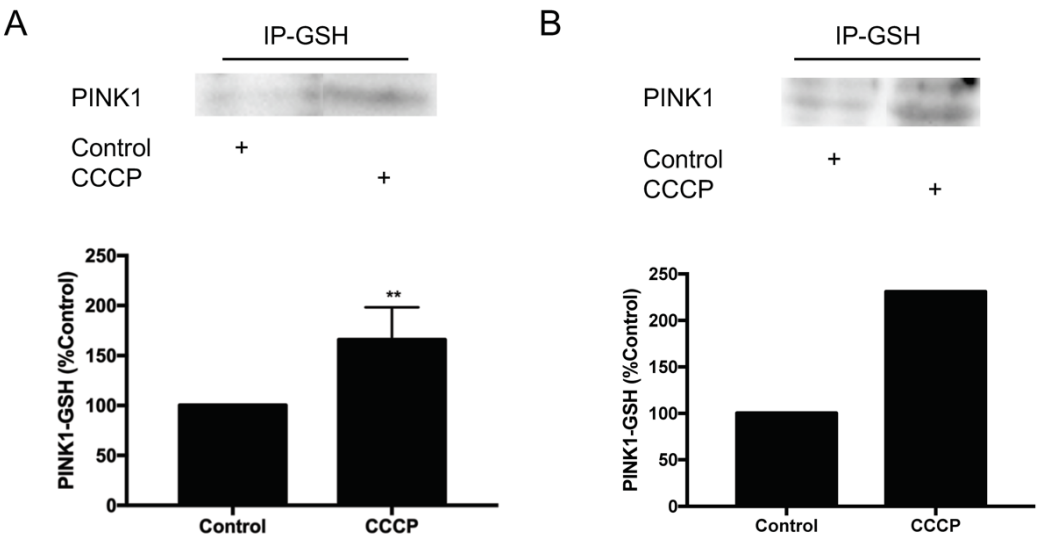

Figure 5- CCCP promotes PINK1 and gluthionylation; A) Purified mitochondria from adult mouse brains were treated with or without CCCP ate 25μM final concentration for 1h. GSH was immunoprecipitated and PINK1 and Parkin were immunodetected by Western blot from the immunoprecipitated proteins. Graphs represent the median ± SD of three experiments. ** p<0,1, compared with the control (PINK1-GSH). **B)** Primary cultures of astrocytes were treated with or without CCCP ate 25μM final concentration for 1h, followed by mitochondria isolation; GSH was immunoprecipitated and PINK1 and Parkin were immunodetected by Western blot. This experiment has been repeated two times, with similar results.

DISCUSSION

The preliminary data generated herein suggest that dysfunctional mitochondria, which was experimentally induced by low concentrations of CCCP and mild uncoupling effect, stimulate mitophagy via GSH signaling. CCCP-damaged mitochondria generate ROS that, in turn, activate GSH synthesis by increasing GCLC expression. Accordingly, in adenocarcinoma cell line HT-29, mitochondrial uncouplers increase mitochondrial ROS generation and GCLC expression (mRNA and protein) [30]. In addition, we have also demonstrated that pharmacological modulation of GSH generation (inhibitor BSO or addition of GSH substrate NAC) also regulates mitophagy. Finally, GSH appears to regulate mitophagy *via* S-glutathionylation of PINK1.

This is an ongoing project that needs more data, namely: (i) quantification of intracellular and mitochondrial GSH; (ii) assessment of mitophagy a S-glutathionylation of PINK1 after knocking down GCLC expression; (iii) disclosing the PINK1 critical cysteine involved in S-glutathionylation and/or (iv) characterization of the enzyme implicated in PINK1 S-glutathionylation post-translational modification. Furthermore, the identification of other potential target proteins for S-glutathionylation is an important research line to be follow. For instance, Cys431 is a key cysteine for Parkin's ubiquitin ligase activity [31].

Literature data suggest that protein S-glutathionylation regulates autophagy and cytoprotection. In murine macrophages aflatoxin B1 induces apoptosis that is inhibited by autophagy activation [32]. In this model, knocking down glutathione-S-transferase omega 1 (GSTO1-1) expression, which promoted a decrease on protein S-glutathionylation levels, decreased autophagy, increasing aflatoxin B1-induced apoptosis [32]. Thus, lower levels of protein S-glutathionylation limits autophagy and sensitizes cells to die. Likewise, restoration of GSTO1 in *Drosophila* Parkin or PINK1 mutants (which present lower levels of GSTO) restores F_1F_0 ATP synthase activity by stimulating S-glutathionylation of ATP synthase β subunit and reverts dopaminergic neuron loss [33,34]. Protein glutathionylation also emerged as a protective process in a Parkinson disease model based on paraquat-induced toxicity and dopaminergic neuronal cell death [35]. In fact, overexpression o glutaredoxin (GRX1) reverted protein deglutathionylation occurring during dopaminergic neuronal cell death, in particular FLI-I and REPS2 proteins [35]. Thus. Protein S-glutathionylation emerges as a cytoprotective cellular process by activating autophagy and preventing cell death [32,35].

In contrast, in three different carcinoma cell lines, starvation-induced autophagy promotes a depletion in cellular GSH due to GSH extrusion, GLC inhibition and formation of protein glutathionylation, indicating that changes in cellular redox state activates autophagy [36]. Likewise, GSH deficiency also trigger autophagy in germ cells as an adaptative stress response [37]. This apparent contradictory data can be explained by the subcellular localization of GSH and the levels

of ROS occurring in the mitochondria. In fact, at mitochondria ROS generation promotes *de novo* GSH production, which in turn modulates mitophagy signaling proteins for the elimination of dysfunction mitochondria.

Finally, in a recent work done, Oh and colleagues have demonstrated the importance of S-nitrosylation in PINK1's kinase activity [38]. In fact, nitrosative stress promotes S-nitrosylation of PINK1, which in turn blocks its kinase activity and activation of Parkin. Furthermore, this post-translational modification of PINK1 mimics mutations occurring in Parkinson disease's patients and models, which lead to a default of mitophagy and neuronal cell death [38]. Therefore, post-translational modifications of PINK1 emerge as new pathways for regulating mitophagy that must be further explored. This work may build a bridge of knowledge between ROS signaling in response to mitochondrial damage and mitochondrial quality control and the underlying molecular mechanisms based on GSH and protein S-glutathionylation.

ACKNOWLEDGMENTS

This work was supported by Fundação para a Ciência e Tecnologia (FCT) grant FCT-ANR/NEU-NMC/0022/2012, grant PTDC/MEC-NEU/28750/2017 and FCT provided individual financial support to CFP (SFRH/BD/106057/2015) and HLAV (IF/00185/2012).

REFERENCES

1. Galluzzi, L., Kepp, O. & Kroemer, G. Mitochondria: Master regulators of danger signalling. *Nature Reviews Molecular Cell Biology* **13**, 780–788 (2012).

2. Esteban-Martínez, L., Sierra-Filardi, E. & Boya, P. Mitophagy, metabolism, and cell fate. *Mol. Cell. Oncol.* **4**, e1353854 (2017).

3. Wang, Z., Figueiredo-Pereira, C., Oudot, C., Vieira, H. L. A. & Brenner, C. Mitochondrion: A Common Organelle for Distinct Cell Deaths? *Int. Rev. Cell Mol. Biol.* **331**, 245–287 (2017).

4. Youle, R. J. & Narendra, D. P. Mechanisms of mitophagy. *Nat. Rev. Mol. Cell Biol.* **12**, 9–14 (2011).

5. Narendra, D., Tanaka, A., Suen, D.-F. & Youle, R. J. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J. Cell Biol.* **183**, 795–803 (2008).

6. Frank, M. *et al.* Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner. *Biochim. Biophys. Acta* **1823**, 2297–2310 (2012).

7. Wang, Y., Nartiss, Y., Steipe, B., McQuibban, G. A. & Kim, P. K. ROS-induced mitochondrial depolarization initiates PARK2/PARKIN-dependent mitochondrial degradation by autophagy. *Autophagy* **8**, 1462–1476 (2012).

8. Ashrafi, G., Schlehe, J. S., LaVoie, M. J. & Schwarz, T. L. Mitophagy of damaged mitochondria occurs locally in distal neuronal axons and requires PINK1 and Parkin. *J. Cell Biol.* **206**, 655–670 (2014).

9. Orellana, C. A., Marcellin, E., Gray, P. P. & Nielsen, L. K. Overexpression of the regulatory subunit of glutamate-cysteine ligase enhances monoclonal antibody production in CHO cells. *Biotechnol. Bioeng.* **114**, 1825–1836 (2017).

10. Lu, S. C. GLUTATHIONE SYNTHESIS. *Biochim Biophys Acta* **1830**, 3143–3153 (2013).

11. Kaplowitz, N., Aw, T. Y. & Ookhtens, M. The regulation of hepatic glutathione. *Annu. Rev. Pharmacol. Toxicol.* **25**, 715–744 (1985).

12. González-Fraguela, M. E. *et al.* Glutathione depletion: Starting point of brain metabolic stress, neuroinflammation and cognitive impairment in rats. *Brain Res. Bull.* **137**, 120–131 (2018).

13. Espinosa-Diez, C. *et al.* Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biol.* **6**, 183–197 (2015).

14. Nava, G. M., Lee, D. Y., Ospina, J. H., Cai, S.-Y. & Gaskins, H. R. Genomic analyses reveal a conserved glutathione homeostasis pathway in the invertebrate chordate Ciona intestinalis. *Physiol. Genomics* **39**, 183–194 (2009).

15. Sun, J. *et al.* High GCLC level in tumor tissues is associated with poor prognosis of hepatocellular carcinoma after curative resection. *J. Cancer* **10**, 3333–3343 (2019).

16. Forman, Henry Jay, Zhang, Hongqiao, Rinna, A. Glutathione: Overview of its protective roles, measurement, and biosynthesis. *Mol Asp. Med* **30**, 1–12 (2009).

17. Gallogly, M. M. & Mieyal, J. J. Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr. Opin. Pharmacol.* **7**, 381–391 (2007).

18. Mailloux, R. J. & Treberg, J. R. Protein S-glutathionlyation links energy metabolism to redox signaling in mitochondria. *Redox Biol.* **8**, 110–118 (2016).

19. Lam, H. C. *et al.* p62/SQSTM1 Cooperates with Hyperactive mTORC1 to Regulate Glutathione Production, Maintain Mitochondrial Integrity, and Promote Tumorigenesis. *Cancer Res.* **77**, 3255–3267 (2017).

20. Smith, G. A. *et al.* Glutathione S-Transferase Regulates Mitochondrial Populations in Axons through Increased Glutathione Oxidation. *Neuron* **103**, 52-65.e6 (2019).

21. Sá Santos, S. *et al.* Culturing primary brain astrocytes under a fully controlled environment in a novel bioreactor. *J. Neurosci. Res.* **79**, 26–32 (2005).

22. Queiroga, C. S. F. *et al.* Glutathionylation of adenine nucleotide translocase induced by carbon monoxide prevents mitochondrial membrane permeabilization and apoptosis. *J. Biol. Chem.* **285**, 17077–17088 (2010).

23. Mauro-Lizcano, M. *et al.* New method to assess mitophagy flux by flow cytometry. *Autophagy* **11**, 833–843 (2015).

24. Narendra, D. P. *et al.* PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* **8**, e1000298 (2010).

25. Chen, Daohong, Chen, Xi, Li, Min, Ding, Wen-Xing, Ying, X.-M. CCCP-Induced LC3 Lipidation Depends on Atg9 Whereas FIP200/Atg13 and Beclin 1/Atg14 are Dispensable. *Biochem Biophys Res Commun* **432**, 226–230 (2013).

26. Xiao, B. *et al.* Superoxide drives progression of Parkin/PINK1-dependent mitophagy following translocation of Parkin to mitochondria. *Cell Death Dis.* **8**, 1–12 (2017).

27. Ding, W. X. *et al.* Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. *J. Biol. Chem.* **285**, 27879–27890 (2010).

28. Gil-Martínez, A. L. *et al.* Effect of NAC treatment and physical activity on neuroinflammation in subchronic Parkinsonism; is physical activity essential? *J. Neuroinflammation* **15**, 1–13 (2018).

29. Aldini, G. *et al.* N-Acetylcysteine as an antioxidant and disulphide breaking agent: the reasons why. *Free Radic. Res.* **52**, 751–762 (2018).

30. Ravuri, C., Svineng, G. & Huseby, N.-E. Differential regulation of γ-glutamyltransferase and glutamate cysteine ligase expression after mitochondrial uncoupling: γ-glutamyltransferase is regulated in an Nrf2- and NFκB-independent manner. *Free Radic. Res.* **47**, 394–403 (2013).

31. Trempe, J.-F. & Fon, E. A. Structure and Function of Parkin, PINK1, and DJ-1, the Three Musketeers of Neuroprotection. *Front. Neurol.* **4**, (2013).

32. Paul, S., Jakhar, R., Bhardwaj, M. & Kang, S. C. Glutathione-S-transferase omega 1 (GSTO1-1) acts as mediator of signaling pathways involved in aflatoxin B1-induced apoptosis–autophagy crosstalk in macrophages. *Free Radic. Biol. Med.* **89**, 1218–1230 (2015).

33. Kim, K. & Yim, J. Glutathione S-transferase omega suppresses the defective phenotypes caused by PINK1 loss-of-function in Drosophila. *Biochem. Biophys. Res. Commun.* **437**, 615–619 (2013).

34. Kim, K., Kim, S.-H., Kim, J., Kim, H. & Yim, J. Glutathione S -Transferase Omega 1 Activity Is Sufficient to Suppress Neurodegeneration in a Drosophila Model of Parkinson Disease. *J. Biol. Chem.* **287**, 6628–6641 (2012).

35. Rodriguez-Rocha, H. *et al.* Glutaredoxin 1 Protects Dopaminergic Cells by Increased Protein Glutathionylation in Experimental Parkinson's Disease. *Antioxid. Redox Signal.* **17**, 1676–1693 (2012).

36. Desideri, E., Filomeni, G. & Ciriolo, M. R. Glutathione participates in the modulation of starvation-induced autophagy in carcinoma cells. *Autophagy* **8**, 1769–1781 (2012).

37. Mancilla, H. *et al.* Glutathione Depletion Induces Spermatogonial Cell Autophagy. *J. Cell. Biochem.* **116**, 2283–2292 (2015).

38. Oh, C.-K. *et al.* S-Nitrosylation of PINK1 Attenuates PINK1/Parkin-Dependent Mitophagy in hiPSC-Based Parkinson's Disease Models. *Cell Rep.* **21**, 2171–2182 (2017).

V

DISCUSSION AND CONCLUSION

Cláudia Figueiredo-Pereira has written the whole chapter based on the referred bibliography and her own results in chapters II to IV.

CONTENTS

1 DISCUSSION

Brain is a dynamic and complex network of cells and information. The most abundant cells inboard the CNS are astrocytes. Over the past decades, the focus on physiology of astrocytes, as well as their interaction with neurons in normal brain conditions, have suffered a great revolution. The dogma that these star-shaped cells only serve to support neuronal activity has accepted for decades [1], however nowadays several function have been attributed to astrocytes, including; i) Regulation of neurotransmission *via* synapsis modulation, ii) scar formation and tissue repair and iii) defense against biological stress, [2–6]. Currently, it is known that astrocytic dysfunction might cause neuronal irreversible loss, since astrocytes have a key role in protecting neurons against oxidative stress [7,8], contributing to the bad prognosis, our occurrence of several disease, such us, neurodegenerative disorders and stroke (cerebral ischemia). Therefore, this thesis has focused on studying astrocytic function and dysfunction, presenting as main objective the development of strategies to protect astrocytes.

Cerebral ischemic stroke is the third cause of death in west countries [9] and the first in Portugal [10,11]. The interruption of blood flow for a short time period can result in severe brain damage [12], and it is considered that during reperfusion phase it occurs a delayed secondary brain damage, due to the huge increase in oxidative stress [13,14]. Astrocytes are much more resilient to oxidative stress than neurons and perform a crucial role in limiting brain damage [15]. This resilience is due to the fact that astrocytes play a primary role in the maintenance of the redox balance in the brain, since, they are the main responsible for antioxidant production and detoxification of reactive oxygen species (ROS) [16]. The levels of astrocytic antioxidant are much higher than in neurons, neurons have low levels of endogenous antioxidants. Astrocytes are responsible for the production of the powerful antioxidant, glutathione (GSH). Glutathione is responsible for the reduction of reactive oxygen species, maintaining the levels of ROS at normal levels. Herein, astrocytes were challenged with oxidative stress to mimic damaging consequences of ischemic stroke.

Carbon monoxide (CO) is an endogenous molecule that is emerging as a possible therapeutic molecule, due to its protective actions. Likewise, in ischemia-reperfusion injury, CO confers resistance against cell death and oxidative stress in many different models, namely lung, heart, kidney, liver and brain [17,18]. The anti-apoptotic effect of CO in astrocytes is due to its action at mitochondrial level, namely, i) inhibition of mitochondrial membrane permeabilization; ii) improvement of mitochondrial metabolism, iii) generation of low amounts of ROS for signaling and iv) promoting mitochondrial mild uncoupling effect [19,20]. In the present thesis CO was explored to prevent cell death in astrocytes against oxidative stress. In fact, this thesis has explored the action of CO on autophagy modulation, with a great focus on mitochondrial level, in particular its role in mitochondrial quality control, namely mitophagy and mitochondrial biogenesis, as presented in

Figure 6 (**Chapter I**). The main achievements concerning CO modulation of mitochondrial quality control are summarized in Figure 1 and are discussed throughout this chapter. The findings contribute to clarify and spread knowledge about the role of CO at mitochondrial level, opening a new window of molecular mechanisms about CO role against oxidative stress.

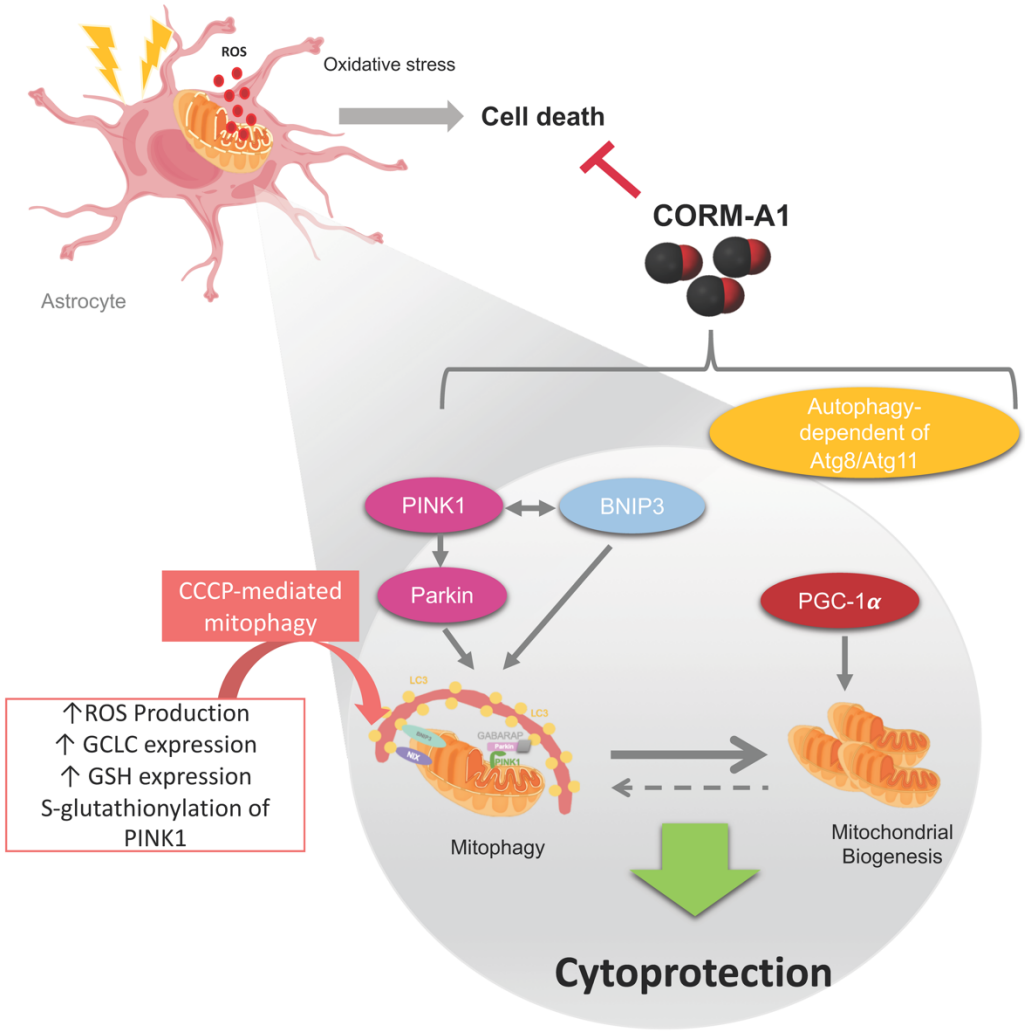

Figure 1 - Main achievements of this PhD thesis. CO prevents cell death against oxidative stress via autophagy, mediated by Atg8 and Atg11 (**Chapter II**). At mitochondrial level CO induces mitophagy dependent of PINK1/Parkin and BNIP3 pathway, increases mtDNA replication and PGC-1α expression at 1h and mitochondrial biogenesis ate 24h. Mitophagy inhibition block induction of mitochondrial biogenesis by CO (**Chapter III**). Concerning to mitophagy pathway, CCCP treatment induces ROS production, promoting glutathione (GSH synthesis), by increasing GCLC expression and GSH appears to regulate mytophagy by S-glutathionylation of PINK1 (**Chapter IV**).

1.1 Mitochondrial quality control; mitophagy and mitochondrial biogenesis as cellular processes for protection against oxidative stress

Mitochondria are key cellular organelles for producing bioenergy and for controlling cell death [21]. Dysfunctional mitochondria are related with the propagation of acute and chronic neurodegenerative disorders, stroke and Alzheimer/Parkinson disease, respectively [22,23]. Mitochondrial appear as the main target of CO at the cellular level for preventing cell death against oxidative stress [19,20]. Recent studies have shown that in reperfusion phase after ischemia there is a enhance in mitochondrial damage, such as mitochondrial permeability transition pore opening, mitochondrial morphological damage, Ca^{2+}-induced mitochondrial swelling, and the release of mitochondrial cytochrome c [24–26]. Thus, elimination of damaged mitochondria is a key process to maintain cell survival.

Autophagy, as a highly regulated process, degrades cellular organelles and proteins, plays an important role in maintenance of cellular homeostasis during environmental stress. Autophagy is involved in many pathophysiological processes, promoting the adaptation of cell to starvation, cell differentiation and development, tumor suppression, innate and adaptive immunity, neurodegenerative development, lifespan extension and cell death [27].

1.1.1 CO and Autophagy

In **Chapter II,** it was demonstrated that CO prevents oxidative stress-induced cell death in yeast *via* autophagy activation. This was a pioneer study using the CO releasing molecule-A1 (CORM-A1) for assessing CO role on the control of autophagic process. Autophagy activation by CO was dependent on Atg8 and Atg11 proteins, since knocking down of these proteins reverted the capability of CO-mediated protection. These data were validated in primary culture of astrocytes, which is a more complex model and the main focused model in this thesis. Likewise, these results are in accordance with the literature, where several studies demonstrated the cytoprotective effect of CO is dependent on autophagy activation. In 2011 was demonstrated for the first time that in sepsis CO confers protection in an autophagy dependent manner by increasing Beclin-1 expression and ROS production [28]. In the same model, CO's cytoprotective effect was abolish by knocking out LC3 and Beclin-1 [29]. Likewise, CO inhibits hypoxia-induced apoptosis in islets by upregulation of Atg16L1-mediated autophagy [30]. It is also well stablished that CO mediates autophagy activation in ROS-dependent manner [28]. Despite all these published data, the role of CO-mediated mitophagy is still poorly explored.

1.1.2 **Mitophagy against oxidative stress**

From previous work from our group and others it is known that the CO cellular target are mitochondria [31,32]. Mitochondria are the main producer of ROS in the cell. During brain damage, aging and other insults, mitochondria produce ROS that become harmful to the cell. Indeed, the removal of dysfunctional mitochondria is mediated by mitophagy [33] and is essential to maintain mitochondrial homeostasis [34] and cell survival. Mitophagy is a selective process of autophagy for removing damaged mitochondria and is an important mechanism of mitochondrial quality control for maintaining normal function of this organelle. Mitophagy was first described to be involved in cell death stimulation, depending on the context and severity of the insult [35]. However, in the last few years, mitophagy has been described as cytoprotective mechanism by elimination dysfunction mitochondria and limiting oxidative stress, being a key cell process for mitochondrial quality control. Mitophagy often occurs following damage or stress of mitochondria, with a number of studies indicating mitophagy is activated after cerebral ischemia/reperfusion [36,37]. In fact, CORM-A1 treatment activates mitophagy under physiological conditions (without stress challenge) and partially inhibited oxidative stress-induced astrocytic cell death in a mitophagy dependent manner (**Chapter III**).

Mitophagy is a very dynamic and complex process, mediated by several proteins. As described in the **Chapter I**, the most described pathway for mitophagic process is PINK1/Parkin, however along the years there have been an increase on the number of PINK1 and/or Parkin-independent pathways of selective mitophagy reported [21,38,39]. In **Chapter III**, CO-mediated mitophagy was observed to be associated with PINK1/PARKIN-dependent mitophagy. Nevertheless, knocking down PINK1 did not revert CO's activation of mitophagy, while CCCP-induced mitophagy decreased. These data indicate that CO-induced mitophagy is not totally dependent on PINK1 while CCCP-induced mitophagy is dependent on PINK1. Furthermore, CO-treated astrocytes demonstrated an upregulation of BNIP3 expression. Consequently, these results suggest that PINK1/Parkin pathway is not exclusive for CO to activate mitophagy. Likewise, due to the dynamism of mitophagy process, one cannot discard that these pathways function in a cooperating manner, being more than one pathway activated at the same time. The PINK1/Parkin-dependent mitophagy is stimulated by the loss of mitochondrial membrane potential and mediated by ubiquitin for LC3 interaction[40,41]. In contrast, BNIP3/NIX are receptor-mediators of mitophagy, therefore are able to interact directly with LC3, they are described to induce mitophagy under hypoxia conditions, where there is excessive ROS generation [41–43]. Although BNIP3 may also regulate mitophagy through other mechanisms. BNIP3 was shown to play a vital role in regulating PINK1 mitochondrial outer membrane localization [41]. Still, recent data showed that NIX, which is a BNIP3 ligand, interacts with Parkin and promote its recruitment to depolarized mitochondria [43]. This can be explained by the fact that these pathways act at different steps of mitophagic process, being BNIP3/NIX responsible for the mobilization of autophagy

machinery, since it involves ROS signaling and PINK1/Parkin for the priming of the mitochondria for the recognition by the autophagy machinery, once Parkin promote mitochondrial ubiquitination [43]. Thus, CO-induced mitophagy may act in the same pattern promoting protection against oxidative, by eliminating in a very efficient way dysfunctional mitochondria, and promoting their biogenesis.

1.2 Carbon monoxide the cross-talk between mitophagy and mitochondrial biogenesis.

As addressed in the previous section, the maintenance of mitochondrial homeostasis, is a crucial process for cell viability. Mitochondrial quality control is compromised by several processes, responsible to maintain mitochondrial homeostasis, by balancing mitochondrial elimination and mitochondrial biogenesis. Moreover, in case of injury or stress, it can result in mitochondria dysfunction, thus it is vital for the cell to rapidly promote mitochondrial clearance and at the same time to produce new ones, by a process termed mitochondrial biogenesis [34,44]. From previous works, we and others have demonstrated that CO stimulates mitochondrial biogenesis in cardiomyocytes [45] and in astrocytes [19,46]. Accordingly, in astrocytes it was observed that CORM-A1 enhanced mitochondrial DNA replication, upregulated PGC-1α expression (transcription factor involved in mitochondrial biogenesis control in cellular nucleus) and increased mitochondrial population (**Chapter III**). Also, it was observed that CO-induced mitophagy simultaneously increased mitochondrial DNA content (replication) and expression of PGC-1α, suggesting that both events must be activated in parallel. In contrast, inhibition of mitophagy also prevented CO-induced mitochondrial biogenesis. Although further research is necessary to completely assess this question, these data support the hypothesis that CO mediates an interdependency of mitophagy and mitochondrial biogenesis.

Along the past recent years, the dogma that mitophagy and mitochondrial biogenesis are related and inter-dependent has been greatly discussed and accepted as proved. Nevertheless, the first experimental evidence of this correlation was just showed in 2018, where CORM-2-induced TFEB nuclear translocation enhance mitophagy and mitochondrial biogenesis in hepatocytes and protects the liver against inflammation [47]. CO-induced mitophagy was mediated by PINK1 and Parkin recruitment to mitochondria through TFEB, and nuclear translocation of TFEB also induced mitochondrial biogenesis [47]. These data support the hypothesis that mitophagy and mitochondrial biogenesis present a shared regulation, and suggest TFEB as a regulator of mitochondria turnover. Another potential regulator is the protein PARIS once it seems to inter-connect Parkin and PGC-1α. In fact, PARIS is a transcriptional repressor (member of the family of KRAB zinc-finger proteins), which is ubiquitinated by Parkin targeting it for proteasomal degradation [48,49]. Furthermore, PARIS

is physiological transcriptional repressor of the PGC-1α, thus its degradation upregulates PGC-1α, promoting mitochondrial biogenesis [50]. There is no evidence in the literature that CO regulates PARIS function. Nevertheless, concerning the findings in **Chapter III**, indicating that CO mediated mitophagy *via* Parkin and at the same time promoted of PGC-1α expression; we can speculate that CO-mediated mitochondrial turnover in astrocytes mat be regulated by PARIS. Therefore, PARIS is a promising candidate to be a target of CO in mitochondrial turnover modulation.

1.3 Glutathionylation of proteins and mitophagy

The last point of this thesis is still an ongoing project and is the only part where CO role was not assessed (**Chapter IV**). Because it was found a very interesting and promising result concerning GSH regulation of CCCP-induced mitophagy, this chapter deeper explores a novel molecular mechanism underlying mitophagy activation. This molecular mechanism is dependent on Parkin/PINK1 pathway and modulated by GHS levels and the post-translational modification glutathionylation.

Along the past years, the importance of ROS signaling in transduction have gained recognition. Reversible post-translational modifications of protein cysteine (Cyst) thiols, play a significant role in redox signaling and regulation of a variety of cellular functions and processes [51] . One of the most common forms of post-translational modifications is the formation of disulfides bridges between protein thiols and glutathione (GSH), known as protein S-glutathionylation (SSG) [53]. Thus, this process ensures protection of protein thiols against over-oxidation, working as redox switch where cells respond in an immediate and reversible way to oxidative stress, acting as signaling regulator at organelle level [52]. In the same way, mitophagy is a cytoprotective mechanism that promotes cell survival and adaptation to oxidative stress conditions [35]. The most studied receptors mediating mitophagy are PINK1/Parkin. Recently it was demonstrated that PINK1/Parkin-mediated mitophagy improves cell survival against oxidative stress [54]. Thus, further studies are needed concerning the role of S-glutathionylation of PINK1 in mitophagy modulation; but it is already known that glutathionylation is prone to occur in modulation of metabolism and apoptosis. Concerning our study, further validations are needed, such as; (i) ROS scavenger prior to CCCP treatment in order to verify that CCCP-induced ROS is promoting GSH synthesis, (ii) quantification of intracellular and mitochondrial GSH; (iii) assessment of mitophagy a S-glutathionylation of PINK1 after knocking down GCLC expression; (iv) disclosing the PINK1 critical cysteine involved in S-glutathionylation and/or (v) characterization of the enzyme implicated in PINK1 S-glutathionylation post-translational modification.

2 FUTURE PROSPECTS

In this thesis it was demonstrated that CO has the ability to modulate mitochondrial population and that has a crucial role in the CO protection against cell death. Several questions were disclosed at the subcellular level. Nonetheless, other questions were raised that must be addressed to further understand the role of mitochondrial quality control in CO-induced protection against oxidative. Thus, the further work will be presented in the follow sections;

◊ **In Vitro approaches;**

a) Once CO appears to modulation two pathways of mitophagy: Parikin/PINK1 and BNIP3/NIX (**Chapter III**), there is the need of better explore these pathways with a functional validation. Particularly, it is important to understand how CO promotes the activation of these two pathways, since in the literature, just few works correlate PINK1/Parkin-dependent mitophagy with BNIP3/NIX-mitophagy [41,43], and there is no evidence regarding CO action in these subject. For instance, downregulation of BNIP3/NIX proteins would bring vital insights about the role of this pathway in CO-induced mitophagy, by assessing whenever PINK1 is downregulated CO is still able to promote mitophagy (**Chapter III**). Moreover, this strategy would disclose which are the steps/phases and the players involved in CO-induced mitophagy.

b) Generated data also suggest CO-mediated mitophagy and mitochondrial biogenesis may be interdependent processes. Thus, it is important to confirm their interaction, by downregulating their main controlling proteins in both pathways and assessing the activation of each pathway independently. For instance, to assess whether knocking down BNIP3 would affect mitochondrial biogenesis activation, or whether knocking down PGC-1α would affect mitophagy.

c) In the **Chapter IV**, to better understand the generated results, it is necessary to promote additional experimental approaches. For instance, it must be confirmed the ROS signaling role in mitophagy promoted by CCCP by inhibiting ROS generation. Also, it was observed that CCCP-induced mitophagy promote glutathione synthesis acting at the rate limiting step, GCLC enzyme. Therefore, to confirm these results, it is important: (i) to measure cellular GSH levels during mitophagy stimulation and (ii) to downregulate this enzyme and understand the impact in the all pathway and in the proteins glutathionylation (receptors mediating mitophagy).

d) It is known that cerebral O_2 physiological concentrations are approximately between 2%-9%, since by the time that inspired air reaches organs and tissues, O_2 levels have

drastically decreased. This is nowadays accepted to constitute physiologic normoxia [55]. One cannot discard the fact that *in vivo* the direct competitor of CO to hemo-proteins binding is O_2, therefore the oxygen levels might also modulate CO biological action. Thus, it is the extreme importance to mimic oxygen levels existing *in vivo* (in the brain) when studying the cytoprotective effect of CO in astrocytes. Moreover, we already have some preliminary results regarding this point, demonstrating that under lower oxygen levels, CO promotes astrocytic cell protection against oxidative stress (data not shown). Likewise, the results obtained in Chapter III show that CO induced BNIP3 expression, which is a protein known to be induced under hypoxic conditions [23]. Thus, one can be speculated that in hypoxic conditions, mitophagy is also related with the CO cytoprotective role in astrocytes.

e) CO-releasing molecules (CORMs) are safe and efficient source of CO and produce similar effects to the ones already obtained with CO gas [56]. These molecules are emerging as promising molecules that increase the potential application of CO in therapeutics, once they can have the ability to release CO under specific conditions in a controlled-directed manner and can be tissue specific [57–59]. Nevertheless, their ability to cross the blood-brain barrier can be an obstacle. Moreover, metabolization of foreigner chemical structures in the organism can also be a limitation. Thus, the endogenous stimulation of CO by modulation of HO-1 activity must be explored for promoting neuroprotection and astrocytic cytoprotection. Indeed, there are some evidences in literature about the pro-survival function of HO-1 activity involving mitochondrial quality control modulation in astrocytes[60] and in other models [61].

◊ **<u>In Vivo approches;</u>**

a) A great limitation of working in the field of mitophagy is the lack of tractable tools and models, since mitophagy is a very dynamic process, that can be induced by different players. Measuring mitophagy until recently was done using the classic technique of co-localization of autophagosome and mitochondria by assessing overexpressed proteins or dyes. Nevertheless, there is the need of more precise and accurate techniques to assess mitophagy and its balance with mitochondrial biogenesis. The major studies on Parkin/PINK1 dependent mitophagy have induced mitophagy in a manner tahat does not represent physiological conditions by using of overexpressed proteins or treating mitochondrial with uncoupling agents, creating asymmetry between *in vitro* and *in vivo* studies [62]. In 2016, Thomas McWilliams developed “mito-QC,” a transgenic mouse with a pH-sensitive fluorescent mitochondrial signal, which enables the subcellular

visualization of mitochondrial architecture and mitophagy in tissues [62]. Therefore, using the in vivo models (mito-QC, mito-QC Pink1-/- and mito-QC NIX/BNIP3$^{-/-}$) it will possible to understand the phycological action of CO in the cross-talk between mitophagy and mitochondrial biogenesis and how these affects cell fate in oxidative stress context. This approach will be used to better evaluate the biochemical pathways of mitophagy, in particular by addressing the role of CO in mitophagy activation in vivo and the molecular mechanisms of PINK1-glutathionylation pathways.

3 CONCLUSION

This thesis added new insights in the role the of CO as a potential therapeutic molecule against brain damage, resulting from oxidative stress. Likewise, there is not an unique pathway for the CO cytoprotective action in neural cells, due to the extreme importance of brain and complexity, CO can act in several levels in response to injury.

In conclusion, during oxidative stress CO, i) activates the autophagy process promoting cell survival, ii) prevents cell death by mitophagy and mitochondrial biogenesis activation and iii) promotes mitochondrial turnover. Additionally, concerning the importance of mitochondria in oxidative stress injury, this thesis gives new insights in the regulation and signal of mitophagy by ROS, based on GSH and glutathionylation signalling.

Moreover, disclosing the molecular mechanism underlying mitophagy has the potential to be used in the development of novel therapeutics in the field of neuroprotection, namely, concerning ischemic brain damage.

4 REFERENCES

1. Allaman, I., Belanger, M. & Magistretti, P. J. Astrocyte-neuron metabolic relationships: for better and for worse. *Trends Neurosci.* **34**, 76–87 (2011).

2. Eroglu, C. & Barres, B. A. Regulation of synaptic connectivity by glia. **468**, 223–231 (2015).

3. Chung, W.-S. *et al.* Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* **504**, 394 (2013).

4. Tasdemir-yilmaz, O. E. & Freeman, M. R. Astrocytes engage unique molecular programs to engulf pruned neuronal debris from distinct subsets of neurons. 20–33 (2014). doi:10.1101/gad.229518.113.20

5. Abbott, N. J., Ronnback, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* **7**, 41–53 (2006).

6. Hayakawa, K. *et al.* Transfer of mitochondria from astrocytes to neurons after stroke. *Nature* **535**, 551–555 (2016).

7. Rocha, S. M., Cristovao, A. C., Campos, F. L., Fonseca, C. P. & Baltazar, G. Astrocyte-derived GDNF is a potent inhibitor of microglial activation. *Neurobiol. Dis.* **47**, 407–415 (2012).

8. Niranjan, R., Nath, C. & Shukla, R. The mechanism of action of MPTP-induced neuroinflammation and its modulation by melatonin in rat astrocytoma cells, C6. *Free Radic. Res.* **44**, 1304–1316 (2010).

9. Chamorro, Á., Dirnagl, U., Urra, X. & Planas, A. M. Neuroprotection in acute stroke: Targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. *Lancet Neurol.* **15**, 869–881 (2016).

10. Ferreira, R. C. *et al.* Doenças Cérebro-Cardiovasculares. (2015).

11. Saúde, direção geral da. Para As Doenças Cérebro-Cardiovasculares. (2017).

12. Lee, J. M., Grabb, M. C., Zipfel, G. J. & Choi, D. W. Brain tissue responses to ischemia. *J. Clin. Invest.* **106**, 723–731 (2000).

13. Lan, R. *et al.* Mitophagy is activated in brain damage induced by cerebral ischemia and reperfusion via the PINK1/Parkin/p62 signalling pathway. *Brain Res. Bull.* **142**, 63–77 (2018).

14. Riaz, N., Wolden, S. L., Gelblum, D. Y. & Eric, J. Oxidative stress and DNA damage after cerebral ischemia: Potential therapeutic targets to preserve the genome and improve stroke recovery. **118**, 6072–6078 (2016).

15. Kim, Park & Choi. The Role of Astrocytes in the Central Nervous System Focused on BK Channel and Heme Oxygenase Metabolites: A Review. *Antioxidants* **8**, 121 (2019).

16. McBean, G. J. Cysteine, glutathione, and thiol redox balance in astrocytes. *Antioxidants* **6**, 1–13 (2017).

17. Queiroga, C. S. F., Almeida, A. S. & Vieira, H. L. A. Carbon monoxide targeting mitochondria. *Biochem. Res. Int.* **2012**, (2012).

18. Ryter, S. W., Alam, J. & Choi, A. M. K. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol. Rev.* **86**, 583–650 (2006).

19. Almeida, A. S., Queiroga, C. S. F., Sousa, M. F. Q., Alves, P. M. & Vieira, H. L. a. Carbon monoxide modulates apoptosis by reinforcing oxidative metabolism in astrocytes: Role of Bcl-2. *J. Biol. Chem.* **287**, 10761–10770 (2012).

20. Queiroga, C. S. F. *et al.* Glutathionylation of adenine nucleotide translocase induced by carbon monoxide prevents mitochondrial membrane permeabilization and apoptosis. *J. Biol. Chem.* **285**, 17077–17088 (2010).

21. Wang, Z., Figueiredo-Pereira, C., Oudot, C., Vieira, H. L. A. & Brenner, C. Mitochondrion: A Common Organelle for Distinct Cell Deaths? *Int. Rev. Cell Mol. Biol.* **331**, 245–287 (2017).

22. Cai, Q. & Tammineni, P. Alterations in Mitochondrial Quality Control in Alzheimer's Disease. *Front. Cell. Neurosci.* **10**, 24 (2016).

23. Chu, C. T. Multiple pathways for mitophagy: A neurodegenerative conundrum for Parkinson's disease. *Neurosci. Lett.* 0–1 (2018). doi:10.1016/j.neulet.2018.04.004

24. Dave, K. R. *et al.* Activation of protein kinase C delta following cerebral ischemia leads to release of cytochrome C from the mitochondria via bad pathway. *PLoS One* **6**, e22057 (2011).

25. Li, J. *et al.* Reperfusion promotes mitochondrial dysfunction following focal cerebral ischemia in rats. *PLoS One* **7**, e46498 (2012).

26. Sun, J. *et al.* Inhibition of mitochondrial permeability transition pore opening contributes to the neuroprotective effects of ischemic postconditioning in rats. *Brain Res.* **1436**, 101–110 (2012).

27. Levine, B. & Kroemer, G. Autophagy in the pathogenesis of disease. *Cell* **132**, 27–42 (2008).

28. Lee, S. J. *et al.* Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation. *Am J Respir Cell Mol Biol* **45**, 867–873 (2011).

29. Ghanta, S. *et al.* Mesenchymal Stromal Cells Deficient in Autophagy Proteins Are Susceptible to Oxidative Injury and Mitochondrial Dysfunction. *Am. J. Respir. Cell Mol. Biol.* **56**, 300–309 (2017).

30. Kim, D.-S. *et al.* Carbon Monoxide Inhibits Islet Apoptosis via Induction of Autophagy. *Antioxid. Redox Signal.* **28**, 1309–1322 (2018).

31. Vieira, H., Almeida, A., Figueiredo-Pereira, C. & Queiroga, C. Carbon monoxide targeting mitochondria in astrocytes: modulation of cell metabolism, redox response and cell death. *Springerplus* **4**, L42 (2015).

32. Queiroga, C. S. F., Almeida, A. S. & Vieira, H. L. a. Carbon monoxide targeting mitochondria. *Biochem. Res. Int.* **2012**, (2012).

33. Martinez-Arroyo, O. *et al.* Mitophagy in human astrocytes treated with the antiretroviral drug Efavirenz: Lack of evidence or evidence of the lack. *Antiviral Res.* **168**, 36–50 (2019).

34. Hickson-Bick, D. L. M., Jones, C. & Buja, L. M. Stimulation of mitochondrial biogenesis and autophagy by lipopolysaccharide in the neonatal rat cardiomyocyte protects against programmed cell death. *J. Mol. Cell. Cardiol.* **44**, 411–418 (2008).

35. Bellot, G. *et al.* Hypoxia-Induced Autophagy Is Mediated through Hypoxia-Inducible Factor Induction of BNIP3 and BNIP3L via Their BH3 Domains. *Mol. Cell. Biol.* **29**, 2570–2581 (2009).

36. Li, Q. *et al.* Rapamycin attenuates mitochondrial dysfunction via activation of mitophagy in experimental ischemic stroke. *Biochem. Biophys. Res. Commun.* **444**, 182–188 (2014).

37. Yuan, Y., Zhang, X., Zheng, Y. & Chen, Z. Regulation of mitophagy in ischemic brain injury. *Neurosci. Bull.* **31**, 395–406 (2015).

38. Novak, I. *et al.* Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep.* **11**, 45–51 (2010).

39. Glick, D. *et al.* BNip3 regulates mitochondrial function and lipid metabolism in the liver. *Mol. Cell. Biol.* **32**, 2570–2584 (2012).

40. Martinez-Arroyo, O. *et al.* Mitophagy in human astrocytes treated with the antiretroviral drug Efavirenz: Lack of evidence or evidence of the lack. *Antiviral Res.* **168**, 36–50 (2019).

41. Zhang, T. *et al.* BNIP3 protein suppresses PINK1 kinase proteolytic cleavage to promote mitophagy. *J. Biol. Chem.* **291**, 21616–21629 (2016).

42. Sowter, H. M., Ratcliffe, P. J., Watson, P., Greenberg, A. H. & Harris, A. L. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res.* **61**, 6669–6673 (2001).

43. Ding, W. X. *et al.* Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. *J. Biol. Chem.* **285**, 27879–27890 (2010).

44. Kauppila, T. E. S., Kauppila, J. H. K. & Larsson, N. G. Mammalian Mitochondria and Aging:

An Update. *Cell Metab.* **25**, 57–71 (2017).

45. Suliman, H. B., Carraway, M. S., Tatro, L. G. & Piantadosi, C. A. A new activating role for CO in cardiac mitochondrial biogenesis. *J. Cell Sci.* **120**, 299–308 (2007).

46. Choi, Y. K. *et al.* Carbon monoxide stimulates astrocytic mitochondrial biogenesis via L-type Ca2+ channel-mediated PGC-1α/ERRα activation. *Biochem. Biophys. Res. Commun.* **479**, 297–304 (2016).

47. Kim, H. J. *et al.* Carbon monoxide-induced TFEB nuclear translocation enhances mitophagy/mitochondrial biogenesis in hepatocytes and ameliorates inflammatory liver injury. *Cell Death Dis.* **9**, (2018).

48. Nishida, T. & Yamada, Y. SUMOylation of the KRAB zinc-finger transcription factor PARIS/ZNF746 regulates its transcriptional activity. *Biochem. Biophys. Res. Commun.* **473**, 1261–1267 (2016).

49. Lupo, A. *et al.* KRAB-Zinc Finger Proteins : A Repressor Family Displaying Multiple Bio- logical Functions. 268–278 (2013).

50. Zhang, Y. *et al.* Endothelial PINK1 mediates the protective effects of NLRP3 deficiency during lethal oxidant injury. *J. Immunol.* **192**, 5296–304 (2014).

51. Mailloux, R. J. & Treberg, J. R. Protein S-glutathionlyation links energy metabolism to redox signaling in mitochondria. *Redox Biol.* **8**, 110–118 (2016).

52. González-Fraguela, M. E. *et al.* Glutathione depletion: Starting point of brain metabolic stress, neuroinflammation and cognitive impairment in rats. *Brain Res. Bull.* **137**, 120–131 (2018).

53. Su, D. *et al.* Proteomic Identification and Quantification of Sglutathionylation in Mouse Macrophages Using Resin-Assisted Enrichment and Isobaric Labeling. *Free Radic. Biol. Med.* 460–470 (2014). doi:10.1016/j.freeradbiomed.2013.12.004.Proteomic

54. Zhao, C. *et al.* PINK1/Parkin-Mediated Mitophagy Regulation by Reactive Oxygen Species Alleviates Rocaglamide A-Induced Apoptosis in Pancreatic Cancer Cells. *Front. Pharmacol.* **10**, 1–13 (2019).

55. Brahimi-Horn, M. C. & Pouyssegur, J. Oxygen, a source of life and stress. *FEBS Lett.* **581**, 3582–3591 (2007).

56. Figueiredo-Pereira, C., Menezes, R., Ferreira, S., Santos, C. N. & Vieira, H. L. A. Carbon

monoxide released by CORM-A1 prevents yeast cell death via autophagy stimulation. *FEMS Yeast Res.* **19**, (2019).

57. Marques, A. R. *et al.* Generation of Carbon Monoxide Releasing Molecules (CO-RMs) as Drug Candidates for the Treatment of Acute Liver Injury: Targeting of CO-RMs to the Liver. *Organometallics* **31**, 5810–5822 (2012).

58. Motterlini, R. *et al.* Carbon monoxide-releasing molecules characterization of biochemical and vascular activities. *Circ. Res.* **90**, e17–e24 (2002).

59. Sawle, P. *et al.* Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264. 7 murine macrophages. *Br. J. Pharmacol.* **145**, 800–810 (2005).

60. Gorojod, R. M. *et al.* Heme Oxygenase-1 protects astroglia against manganese-induced oxidative injury by regulating mitochondrial quality control. *Toxicol. Lett.* **295**, 357–368 (2018).

61. Piantadosi, C. A., Carraway, M. S., Babiker, A. & Suliman, H. B. Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via nrf2-mediated transcriptional control of nuclear respiratory factor-1. *Circ. Res.* **103**, 1232–1240 (2008).

62. McWilliams, T. G. *et al.* Mito-QC illuminates mitophagy and mitochondrial architecture in vivo. *J. Cell Biol.* **214**, 333–345 (2016).