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Licenciada em Bioquímica

# Carbon monoxide, autophagy and cytoprotection in response to cerebral ischemia

Dissertação para obtenção do Grau de Mestre em Genética Molecular e Biomedicina

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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**Novembro, 2013**

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.....

# Abstract

There is an increasing need for promoting neuroprotection against cerebral ischemia, which is the main cause of brain damage in adults. Astrocytes are the most abundant cells inboard the central nervous system (CNS), being known as key glial cell for promoting neuronal survival and homeostasis. It is more established in nowdays that astrocytic dysfunction contributes to neurodegenerative processes. Although, carbon monoxide is a well renown as a lethal and toxic gas due to its high affinity to hemoglobin, CO exerts anti-apoptotic, anti-inflammatory and anti-proliferative functions. Recent studies showed likewise that CO induces autophagy, promoting therefore cytoprotective and anti-inflammatory effects. Autophagy is a major catabolic pathway, known as an autodigestive process that degrades cellular organelles and proteins, playing an important role in cellular homeostasis during environmental stress.

Due to the great interest on the signaling and cytoprotective actions of CO, novel strategies have been put forward to exploit the potential therapeutic effects of this gaseous molecule. One of these approaches consist on the development of CO-releasing molecules (CO-RMs), compounds that deliver small quantities of CO to tissues and first identified by the group of Motterlini and co-workers. The aim of this Master thesis was to study the action of CORM-A1, a boron-containing compound that spontaneously releases CO, against cell death in primary culture of astrocytes. In particular, we examined the role of CORM-A1 in autophagy, mitophagy and cell metabolism.

Here, we demonstrated that CORM-A1 promotes the induction of autophagy in primary culture of astrocytes. Furthermore, autophagy is directly involved in the cytoprotective effect of CORM-A1 against cell death. In some preliminary experiments we have shown that CORM-A1 also induced mitophagy, while autophagy and inhibition of cell death promoted by CORM-A1 seem to occur under hypoxia (5% of oxygen). This master thesis has addressed several important questions on the role of CO in astrocyte function but also opened to many other important questions on the mechanism of action of CO. For instance, future work must be undertaken in order to explore whether CO-mediated induction of reactive oxygen species (ROS), which play an important role in cell signaling, which are the factors directly involved in mitophagy and the cross-talk between apoptosis and modulation of autophagy.

**Key words:** Astrocytes, neuroprotection, carbon monoxide, CORM's, autophagy, mitophagy.

# Resumo

Isquemia cerebral é a principal causa de danos cerebrais em adultos, levantado assim uma necessidade crescente para a promoção da neuroprotecção. Os astrócitos são as células mais abundantes no interior do sistema nervoso central (SNC), estando envolvidas na homeostasia e sobrevivência neuronal. Um das causas já estabelecidas para patologias neurodegenerativas está relacionado com a disfunção destas células.

O monóxido de carbono (CO) é geralmente conhecido como um gás tóxico letal devido à sua elevada afinidade com a hemoglobina, no entanto já se sabe que o CO apresenta propriedades benéficas para o organismo, entre elas anti-apoptóticos e anti-inflamatória e anti-proliferativa, para além destas propriedades, estudos recentes mostraram também que CO induz autofagia. Autofagia, é um processo degradação de organelos celulares e proteínas denaficadas, exercendo um papel importante na homeostase celular.

Em linha com o conhecimento já existente, o objetivo deste trabalho foi estudar a acção de CO contra a morte celular em cultura primária de astrócitos, em particular o papel da CORM-A1 na indução de autofagia, mitophagy e metabolismo celular. CORM-A1 é uma nova fonte de CO, designada como "*CO-releasing molecules"*, desenvolvida por *Motterlini* e colegas.

Neste trabalho demonstramos que CORM-A1 induz autofagia em astrócitos. Além disso, autofagia está directamente envolvida no efeito citoprotector de CORM-A1 contra a morte celular, bem como a indução de mitofagia. Para além disto, verificamos que efeito da CORM-A1 em hipoxia continua a ser citoprotector.

Em suma, durante esta dissertação de mestrado muitas "janelas" de investigação foram abertas, deixando caminho para futuras abordagens.

**Palavras chave:** Astrócitos, neuroprotecção, monóxido de carbono, CORM's, autofagia e mitofagia.

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# Abbreviations

- **ATP-** Adenosine triphosphate;
- **AXO –** Alexander disease;
- **$Ca^{+2}$ –** calcium cation;
- **cAMP-** Cyclic adenosine monophosphate;
- **cGMP-** Cyclic guanosine monophosphate;
- **CO –** Carbon monoxide;
- **COHb –** Carboxyhemoglobin;
- **CORM's -** Carbon monoxide releasing molecules;
- **CNS –** Central nervous system;
- **DiOC –** 3,3′-dihexyloxacarbocyanine iodide;
- **DMEM -** Dulbecco's modified eagle medium;
- **dMfn –** Factor mitufusion;
- **DNA -** Deoxyribonucleic acid;
- **EBSS –** Earle's balancel salt solution;
- **EDTA -** Ethylenediamine tetraacetic acid;
- **FBS –** Fetal bovine serum;
- **GABA –** Neurotransmitter glutamate;
- **GC –** Guanilate cyclase;
- **HCQ –** Hydroxycloroquine;
- **HO-** Hemoxygenase;
- **IF –** Imunofluorescense;
- **MMP-** Mitochondrial membrane permeabilization;
- **PBS –** Phosphate buffer saline;
- **PE –** Phosphatidylethanolamine;
- **PI –** Propidium iodide;
- **PI3K-** phosphatidylinositol 3-kinase;
- **PNS-** Peripheral Nervous System;
- **Q-PCR –** Quantitative polymerase chain reaction;
- **RT –** Room temperature;
- **WB –** Western blot;
- **WM-** Wortmannin;
- **ROS –** Reactive species of oxygen;
- **t-BHP -** *tert*-Butylhydroperoxide;
- **T-TBS** – Tris-Buffered Saline with Tween 20;

# I. Introduction

*State of the art*

# 1 Central nervous system

The nervous system coordinates the actions and transmits signals in the body and it consists of two main parts: the central nervous system (CNS) and the peripheral nervous system (PNS). The central nervous system is the processing center and consists of two main organs, spinal cord and brain. It collects, integrates and sends information to the peripheral nervous system. Both organs, brain and spinal cord, are protected by three layers of connective tissue named as meninges (Roberts *et al*. 2004).

At the cellular level, nervous system is defined by the presence of nerve cells, also known by neurons, and special types of supporting cells, known as glial cells (figure 1.1). The neurons are accountable for functions that are unique to the nervous system, while the glial cells are non-neuronal cells that primarily support and protect the neurons (Bordal *et al.* 2010).

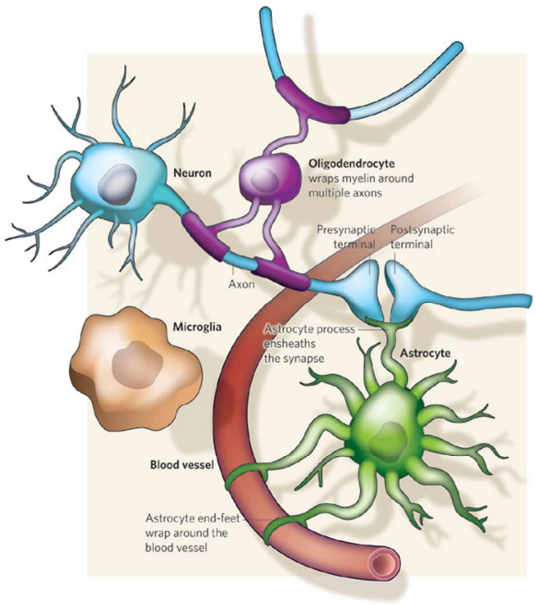


**Figure 1.1 – Schematic representation of several brain cell populations.** Addapted from.(Halassa, Fellin *et al.* 2007; Halassa, Fellin *et al*. 2009)

Neurons are post-mitotic and highly polarized cells, meaning that they develop, in the course of maturation, distinct subcellular domains responsible for different functions. Morphologically three major regions compose neurons: cell body, dendrites and axon. The cell body, or soma, contains the nucleus and the major cytoplasmic organelles. Multiple short dendrites are extensions of the receiving surface and arise from perikaryon, ramifying over a certain volume of gray matter. 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 (Roberts *et al.* 2004).

Neuronal cells are classified into two broad groups, projection neurons and interneurons. Projection neurons are characterized by their capacity to respond to stimuli with an electrical discharge, a nerve impulse, and, further, by their fast conduction of the nerve impulse over long distances. This capacity is allocated to axon that is particularly built to conduct the nerve impulse from cell body to other cells. Therefore a signal can be transmitted in millisecond along the body, the CNS or between them. Interneurons mediate cooperation among neurons that rely grouped together (Roberts *et al.* 2004).

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 does not allow the existence of energy reservoirs. In accordance, brain receives about 15% of the cardiac output at rest (Roberts *et al.* 2004; Bordal *et al.* 2010). Moreover, just few minutes of blood flow interruption may cause neuronal cell death, occurring during cerebral ischemic stroke, which is the third cause of death in west countries.

The CNS is composed by three types of glial cells, oligodendrocytes, astrocytes and microglia. In 1859, Rudolph Virchow, studied this set of cells and coined as neuroglia, or “nerve glue”. This name derives from the notion that glial cells served as a kind of glue, an inactive connective tissue holding neurons together in the CNS. Glial cells are the major cells present in the brain and indispensable for neuronal functioning (Roberts *et al.* 2004; Vilhardt *et al.* 2005).

Oligodendrocytes are the main responsible for the formation of myelin sheath in the CNS and are involved in a short number of processes. The main one is during brain development as responsibles for myelin production. The other cell type that is present in CNS is microglia, which originates from mesoderm and is known as the macrophages of nervous tissue (Vilhardt *et al.* 2005). Some studies indicate that microglia constitute 5 to 20% of all the glial cells and are distributed throughout all parts of the CNS. These cells have a particularly function in CNS that is the role of cleaning the extracellular medium, by “scanning” the environment for foreign material and sick or dead cellular elements (Roberts *et al.* 2004; Bordal *et al*. 2010). Finally the third glial cells are astrocytes, which are discussed in the next section.

## 1.1 The role of astrocytes in central nervous system

Astrocytes are the most abundant cells inboard the CNS, outnumbering neurons in a ratio of 10:1, constituting about 20-50% of the total human brain volume (Sagduyu K. *et al.* 2002; Roberts *et al*. 2004; Izhikevich *et al*. 2007). 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 (Son, Jeong *et al*. 2005; Sofroniew and Vinters *et al.* 2010; Allaman, Bélanger *et al.* 2011).

In the beginning of Neurobiology field, they were the most poorly understood neural cells, thought for simply being the cells that occupy the spaces between neurons. However astrocytes revealed to be

involved in many important functions in the brain: modulation of metabolism, neurotransmitters and homeostatic functions, establishing and maintaining the essential nature of interactions between neurons (Sagduyu K. *et al*. 2002; Son, Jeong *et al*. 2005). Based on *in vivo* and *in vitro* approaches, it is already recognized that astrocytes produce and release growth factors for the modulation of distinct neuronal subpopulations at the level of: morphology, proliferation, differentiation and survival. Additionally, astrocytes also act on the control of development and function of astrocytes and oligodendrocytes. The ability of astrocytes in producing and responding to growth factor and cytokines is the major mechanism underlying the development and regenerative capacity of CNS (Roberts *et al.* 2004).

Since the last decade, astrocytes have been categorized in two subtypes, *protoplasmic* or *fibrous*, based on their unique cytoarchitectural and phenotypic features which allow them to respond to changes in the microenvironment (Sofroniew; Vinters *et al.* 2010). *Protoplasmic* astrocytes are found throughout all gray 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, but both make extensive contacts with intraparenchymal blood vessels *via* specialized processes called endfeets. These endfeets express glucose transporters of the Glut-1 type and are a possible site of glucose uptake (Sagduyu K. *et al.* 2002). In this sense astrocytes have been shown to have 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 (Allaman, Bélanger *et al*. 2011).

At the metabolic level, astrocytes are the only cell types in the brain that comprise the energy storage molecule glycogen, and that 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 (Sagduyu K. *et al.* 2002). Other 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 (Sofroniew; Vinters *et al.* 2010; Allaman, Bélanger *et al*. 2011). Astrocytes are powerfully coupled together by gap junctions, containing aqueous pores, which are permeable to ions and other molecules with low molecular weight (Sagduyu K. *et al.* 2002). 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 (Sofroniew; Vinters *et al.* 2010).

Since the last years the role of astrocytes in the brain has raised a special interest for investigation, these star-shaped cells that lack axons not only supports neuronal activity, but can also modulate the neurotransmission by modulating synapses. This process is defined as the “tripartide 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 (figure 1.2). 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 (Halassa, Fellin *et al*. 2007; Halassa, Fellin *et al.* 2007; Halassa, Fellin *et al.* 2009). Glutamate is the most common amino acid

and neurotransmitter present in brain tissue, which is an excitatory neurotransmitter. Glutamate can be generated from neuronal metabolism of glucose and glutamine is its principal precursor of synaptically released glutamate. For instance, glutamate, which is uptaken by astrocytes, which convert glutamate into glutamine and then release it to the extracellular space, while glutamine is uptaken by neurons, restarting the cycle generating again glutamate and GABA, which are potent excitatory and inhibitory neurotransmitters, respectively (Sagduyu K. *et al*. 2002; Roberts *et al.* 2004).

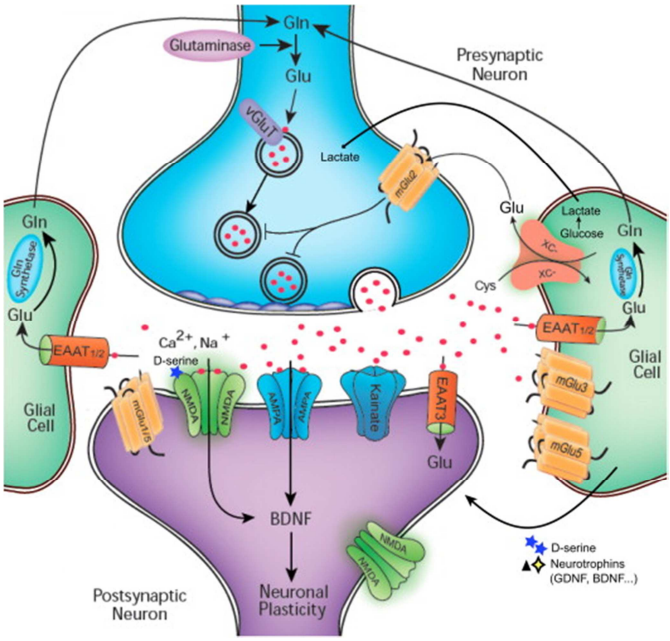


**Figure 1.2 – Representative scheme of communication between neurons and astrocytes- trypartide synaps.** Adapted (Halassa, Fellin *et al.* 2007)

Considering 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. A striking example of one disorder caused by a primary dysfunction of astrocytes is Alexander disease (AXD), the first identified human neurological pathology (Allaman, Bélanger *et al.* 2011). 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 (Sagduyu K. *et al.* 2002; Roberts *et al.* 2004).

As stated above, the role of astrocytes in brain functioning is of extreme importance. Although many advances have been made in this area, the full understanding of astrocytic intracellular events is still unknown. Consequently in this master thesis we have a great interest in studying astrocytes due to their crucial role on brain homeostasis and neuronal functioning.

# 2 Carbon monoxide

Carbon monoxide (CO) is a diatomic colorless small molecule, invisible, chemically inert, nonirritant, and odorless gas (Idriss, Blann et al. 2008). It is, commonly known as a lethal gas and toxic air pollutant, however, CO is an endogenously produced gaseoustrasmitter. The chemical composition of CO was identified in the 18$^{th}$ century. In 1857, Claude Bernard demonstrated that CO could cause asphyxia, by reversely moving oxygen from hemoglobin and forming carboxyhemoglobin (COHb). During the subsequent years, CO toxicity has been lengthily studied (Ozaki, Kimura *et al.* 2012).

Only after almost one century, in 1949, it was demonstrated that CO is endogenously produced through its identification in exhaled human air (Sjöstrand *et al.* 1949). In 1968 heme oxygenase (HO) was identified as the enzyme that catalyzes heme and endogenously produces CO (Ozaki, Kimura *et al.* 2012). CO is generated by oxidation of the heme group, in particular the α-methene bridge carbon atom of the heme porphyrin ring, being a catabolic byproduct of hemoglobin in the body under abnormal conditions (figure 2.1) (Ozaki, Kimura *et al.* 2012; Ryter ; Choi *et al.* 2013).

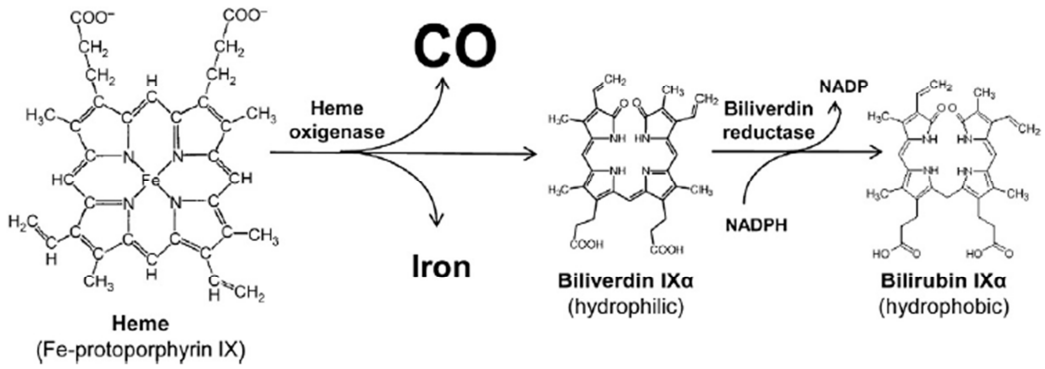


**Figure 2.1- Endogenous pathway production of carbon monoxides (Ozaki, Kimura *et al*. 2012).** Heme group is converted in biliverdin, ferriciron and carbon monoxide by the action of heme oxygenase. Biliverdin is after converted in bilirubin in the presence of biliverdin reductase.

## 2.1 Heme oxygenase (HO)

Heme oxygenase is the rate-limiting enzyme, responsible for metabolizing heme molecules into biliverdin, ferrous iron, and CO (figure 3). HO activity and expression is induced by several different stress stimuli, such as heavy metals or reactive oxygen species (ROS), and is involved in both physiological and pathological processes (Ozaki, Kimura *et al*. 2012). Tenhunen and colleagues were the first to propose the existence of three isozymes, HO-1, HO-2 and HO-3 (Idriss, Blann *et al*. 2008).

HO-1 and HO-2 have a clear and significant biological role, while HO-3 has a substantially lower catalytic activity and is less studied (Ozaki, Kimura *et al.* 2012). Although HO-1 and HO-2 catalyze the same reaction, they differ in expression patterns, HO-1 expression is induced in response to a numerous stimulus, while HO-2 is constitutively expressed (Motterlini, Clark *et al.* 2002). The inducible

HO-1, is a ubiquitous heat shock protein (HSP32), which is particularly protective safeguarding the cell against diverse stress-related conditions (such as redox). HO-1 is upregulated in response to oxidative stress, hyperthermia, hypoxia and proinflammatory stimuli in different tissues and has been shown to exert potent cytoprotective and antiapoptotic properties. The common feature between these inducers is their ability to produce ROS at low levels, and thus with signaling functions, suggesting that HO-1 provides potent cytoprotective effect (Idriss, Blann *et al.* 2008). While, HO-2 is abundant in brain, testis, and liver, and is responsible for particularly high HO activity in these organs during the steady conditions (Ryter; Choi *et al.* 2013).

One of the products originated by heme group degradation is biliverdin, which is rapidly converted in bilirubin by the action of biliverdin reductase. These two molecules are natural antioxidants, usually are present in serum in high levels and are also considered to be responsible for the HO-induced cytoprotection. (Idriss, Blann *et al*. 2008).

It is well known that byproducts generated during the heme catabolism are described as having potential protective role. Nevertheless, in several types of injury the protection has been attributed mainly to CO.

## 2.2 **Biological functions of carbon monoxide**

Firstly, CO was described as a putative neural messenger 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. Some CO's cytoprotective effects are: anti-inflammatory, anti-apoptotic, anti-atherogenic and anti-proliferative (Motterlini *et al.* 2010).

Motterlini and colleagues shown that in isolated aortic tissue CO induced vessel relaxation and prevent coronary vasoconstriction as well as acute hypertension in vivo (Motterlini, Clark *et al.* 2002; Clark, Naughton *et al.* 2003). The CO biological effects concerning the inflammatory processes seem to involve the stimulation of soluble guanylate cyclase (sGC) and cyclic guanosine monophosphate (cGMP) production, but it can also include modulation of MAPK activation and of calcium-dependent potassium channel activity, in smooth muscle models (Ryter, Alam *et al*. 2006).

Some evidence suggests that endogenous CO is a neurotransmitter in CNS. In 1993, Verma and colleagues demonstrated, in primary olfactory neuron culture, the action of CO as endogenous neuronal transmitter regulating cyclic guanosine monophosphate (cGMP) and guanylate cyclase (GC) (Verma, Hirsch *et al.* 1993; Ozaki, Kimura *et al.* 2012). Brouard *et al* in 2000 demonstrated for the first time the anti-apoptotic property of the system HO-1/CO in endothelial cells. Since then, it has been shown that CO confers resistance against cell death following ischemia-reperfusion trauma, in many different models, namely lung, heart, kidney, liver and brain (Ryter, Alam *et al*. 2006; Queiroga, Almeida *et al.* 2012).

### 2.2.1 Carbon monoxide in the CNS

In 2007, Chora *et al.* described that CO reduced neuroinflammation in experimental autoimmune encephalomyelitis, a model of multiple sclerosis. Few years after, in 2009, Zeynalov showed that in mice brain CO reduced injury after transient middle cerebral artery occlusion. In 2012, Mahan *et al.* showed that CO prevented cerebral injury resulting from cardiac bypass procedures using deep hypothermic circulatory arrest. In the same year, Yabluchanskiy and colleagues demostrated that a CO-releasing molecule (CORM-3) promotes neuroprotection or neurotoxicity after intracerebral hemorrhage depending on the time of administration (Smith, Mann *et al.* 2011).

However, in the brain environment, the CO cytoprotective-associated mechanisms have been poorly described. For that reason, along the last years our laboratory has developed a great interest for studying the effect of CO in the prevention of neuronal and astrocytic cell death and its role in mitochondria. Therefore, in 2008, Vieira and colleagues first showed that the preconditioning of murine primary cerebellar granule cells with exogenous CO prevented neuronal apoptosis induced by excitotoxicity and oxidative stress (Vieira, Queiroga *et al*. 2008). Likewise, CO prevents 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 (Queiroga, Tomasi *et al*. 2012).

Because astrocytes are crucial cell for cerebral homeostasis and correct functioning, our group has also focused on CO cytoprotection in this neural cell type. In 2010, Queiroga and colleagues demonstrated that CO prevented apoptosis in primary culture of astrocytes by directly inhibiting mitochondrial membrane permeabilization (MMP), with oxidized glutathione and ROS as signaling factor (Queiroga, Almeida *et al.* 2010). Likewise, CO also prevented astrocytic cell death by improving cell metabolism, namely by: increasing ATP production, increasing cytochrome *c* oxidase enzymatic specific activity and stimulating mitochondrial biogenesis, in a Bcl-2 dependent mode of action (Almeida, Queiroga *et al.* 2012).

Up to the date all generated data in our lab was performed using CO gas or CO-saturated solutions. We are now interested in using CO releasing molecules (CORM) that will be described in the next section.

## 2.3 **CO Releasing molecules**

Since the last decade, CO has received great attention as a biological regulator that can have an important role as a therapeutic tool. The main example is the development of CO-releasing molecules (CORMs) (figure 2.2), which are organic and organometallic compounds, with the capacity of delivering CO in a time and tissue-specific manner, allowing a significant reduction in carboxyhaemglobin toxicity (Queiroga, Almeida *et al.* 2012). It is already known 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 (Motterlini, Clark *et al.* 2002; Motterlini *et al.* 2010).

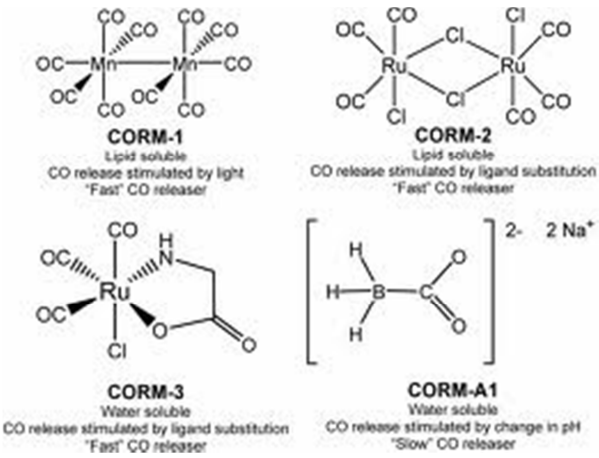


**Figure 2.2 - Chronologic discover of CORM'S.** Schematic diagram showing the types of bioactive CORM's identified until this moment, CORM's are compounds that contain a heavy metal such as nickel, cobalt, or iron surrounded by carbonyl (CO) groups as coordinated ligands. Figure adapted from (Motterlini, Sawle *et al*. 2005).

In 2003 the first water-soluble CORM, CORM-3 (tricarbonylchloro(glycinato) ruthenium (II)), was described, which was obtained by coordinating the amino acid glycine onto the metal center. CORM-3 is fully soluble in water and presents in its structure a metal carbonyl complex with a half-time of about 1minute in physiological buffers. One of the most recently described CORMs is CORM-A1 (sodium boranocarbonate), like CORM-3, it is fully soluble in water, but does not possess a transition metal in 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) (Motterlini, Sawle *et al.* 2005).

The advantage of using this type of molecules is the capability of these small organic and organometallic compounds to deliver CO in a timely and tissue-specific manner, allowing a reduction in the carboxyhaemoglobin formation and toxicity. Opening therefore new windows of opportunity to use CO in clinical applications.

# 3 Autophagy

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 macroautophagy, also known as autophagy (Kroemer, Mariño *et al.* 2010).

More than 40 years ago Christian de Duve, described a catabolic processes conserved from lower to higher eukaryotes, which is named as autophagy, this word remotes from the Greek and means “selfeating” (Gomes; Scorrano ; Rabinowitz; White *et al.* 2010). Autophagy is an intracellular degradation system that carries cytoplasmic constituents to the lysosome. There are three types of autophagy; macroautophagy, microautophagy and chaperone mediated autophagy (Mizushima, Levine *et al.* 2008).

Autophagy is a protective mechanisms that allows cells to survive in response to multiple stressors, such as; starvation, hypoxia, ROS and damaged organelles, strengthening organism defense (Levine; Kroemer *et al.* 2008; Mizushima *et al.* 2008). Autophagy is involved in diverse human processes, such as development, longevity, immunity, cancer and neurodegenerative diseases (Kroemer, Mariño *et al.* 2010; Rabinowitz; White *et al.* 2010).

Furthermore diverse studies suggest that the lifetime of *Drosophila* and *Caenorhadditis elegans* and even mice can be significantly increased by stimulation of autophagy. In the last years autophagy reveals as a protective process (Hansen; Johansen *et al.* 2011).

## 3.1 Autophagic Flux

Autophagic flux refers to all steps of the complete process of autophagy (figure 3.1). Autophagic process initiates with the formation of isolate membrane named phagophore (a and b). This phagophore sequesters and engulfs a portion of cytoplasm to form mature autophagosomes (c) the fusion of this structure with a lysosome forms the autolysosome (d), in which occurs the degradation of the engulfed content by the action of acidic lysosomal hydrolases (e) (Mizushima, Levine *et al.* 2008; Mizushima, Yoshimori *et al.* 2010). Thus, these three main steps (figure 5- steps a,c,d) allow cellular elimination of damaged or harmful components through catabolism and cellular recycling, which maintain nutrient and energy homeostasis (Xie; Klionsky *et al.* 2007; Hansen; Johansen *et al.* 2011). In the absence of stress, autophagy suits a housekeeping function, providing a routine “garbage disposal” to the cells. This function is particularly important to quiescent and terminally differentiated cells, in which the damaged organelles are not disposed by the cell moment of replication (Rabinowitz; White *et al.* 2010).

Autophagy is induced by an extensive range of stimulus (Rabinowitz; White *et al.* 2010). The most usual trigger of autophagy is nutrient deprivation also known as starvation, but other stimuli are involved including damaged organelles, misfolded proteins, DNA damaged, hypoxia, redox stress and mitochondrial damage (Mizushima *et al.* 2007; Kroemer, Mariño *et al.* 2010).

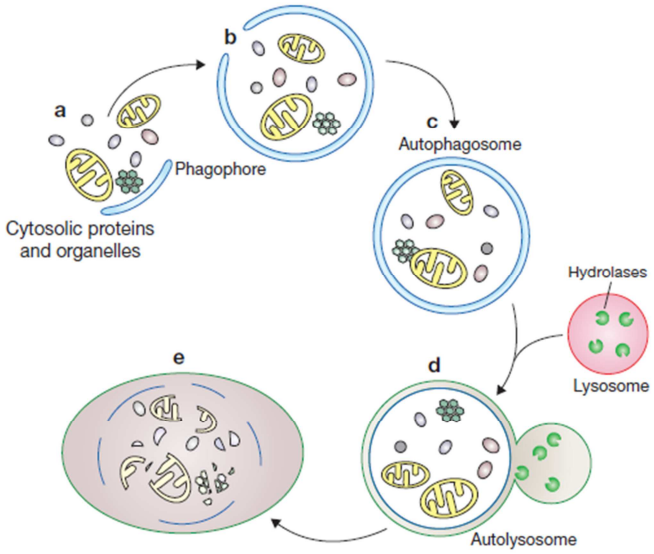


**Figure 3.1- Autophagic flux (Xie and Klionsky 2007).** a,b) Cytosolic material is sequestered by an expanding membrane named as phagophore. c) Resulting in the formation of double-membrane vesicle, autophagosome. d) The outer membrane fuse with a lysosome forming the autolysosome. e) The cargo containing membrane compartment is then lysed and the content is degraded;

Although some of the proteins involved in autophagic process are already known, it is still necessary to disclose many of the cellular factors involved in autophagy. The autophagosome formation requires Beclin-1-class III phosphatidylinositol 3-kinase complex to generate phosphatidylinositol 3-phosphate, as well as generation and insertion of LC3 (Rabinowitz; White *et al*. 2010). This protein specifically associates with autophagosome membranes and usually is present in the cytosol as soluble form of LC3 (LC3-I). When autophagy is activated LC3-I conjugates with phosphatidylethanolamine (PE) and occurs its lipidation, leading to the conversion in autophagic vesicle-associated form, LC3-II (Yang and Klionsky 2010; Yang and Klionsky *et al.* 2010; Yen, Shintani *et al*. 2010; Hansen; Johansen *et al.* 2011). Thus, LC3-II is widely used as a biomarker for autophagy activation.

Many proteins that possess an LC3-interacting region (LIR) are considered as adaptors proteins to target autophagic degradation. The ubiquitinylation on modified proteins or in mitochondria, is recognized and bound by autophagy receptors, such as p62 (also known as sequestosome1, SQSTM1), Nbr1 (Kirkin, McEwan *et al.* 2009), as well as BNIP3L (also known as Nix). These receptors bind to mitochondrial membranes by the interaction with LC3 delivering cargo to autophagosome (Novak, Kirkin *et al.* 2009; Rabinowitz; White *et al.* 2010; Hansen; Johansen *et al.* 2011).

Since alterations in autophagy have a role in pathogeneses of several diseases, there is a great interest in identifying compounds that can be used for therapeutic purposes.

## 3.2 **Mitophagy**

A 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. In this moment mammalian cells rely on mitochondria for vital functions such as; pyruvate oxidation, the Krebs cycle, the metabolism of amino acids, fatty acids, calcium homeostasis, steroids and the generation of energy as ATP. Mitochondria also play a significant role in several mechanisms of cell death because they are sources of pro-apoptotic molecules, such as cytochrome c and are decked with proteins of the Bcl-2 family such as the pro-apoptotic protein Bax (Youle; Narendra *et al.* 2010). On the other hand, mitochondria are an important source of ROS (reactive oxygen species). Mitochondria ROS production contributes to cellular redox signaling, as well as to cellular damage, depending on their concentration (Scherz-Shouval, Shvets *et al.* 2007; Scherz-Shouval; Elazar 2011; Vives-Bauza; Przedborski *et al.* 2011). Recent studies suggest that one major sensor of redox signaling in cellular response, between stress adaptation and cell death, is autophagy (Jisun, Samantha *et al.* 2012).

Brain is rich in mitochondria and uses about 20% of oxygen, while 90% of the consumed oxygen is for generating energy, which makes brain cells particularly sensitive to oxidative stress. Oxidative stress is linked to mitochondrial dysfunction as at the same time mitochondria generate and are the target for ROS. 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. Mitochondria turnover is dependent on autophagy, but the cross-talk between autophagy, redox signaling and mitochondrial dysfunction is not well understood (Jisun, Samantha *et al.* 2012).

Mitophagy was discovered in 2004 in Sacchoromyces cerevisae as well as the first involved protein (Uth1p) (Bhatia-Kiššová; Camougrand *et al.*). The term mitophagy was suggested by Lamesters and colleges in 2005, to describe the engulfment of mitochondria into vesicles with autophagosome marker, therefore, mitophagy concerns to selective process of autophagy (Youle; Narendra *et al.* 2010). Following the loss of mitochondria, cell might maintain the population of mitochondria (lengths and shape) by altering the balance between fission and fusion and by inducing mitochondrial biogenesis (Vives-Bauza; Przedborski *et al.* 2011). In the recent years some studies defend that fission or mitochondrial fragmentation is an event that occurs previously to mitophagy (Poole, Thomas *et al.* 2010). Recent evidences suggests that mitophagy from yeast to mammals is the primary mechanism for the elimination of malfunctioning mitochondria. Thus, mitochondrial remodeling through fission, fusion, mitophagy and mitochondrial biogenesis are important for mitochondrial homoeostasis and quality control (Kim, Rodriguez-Enriquez *et al.* 2007; Jisun, Samantha *et al.* 2012).

### 3.2.1 **BNIP3**

The receptors of mitophagy are still a subject of discussion and speculation, since so far little is known about them. Recent studies suggested the role of a mitochondrial outer membrane protein, named Nix or BNIP3L, that is required for mitochondrial clearance during erythrocyte maturation and has a WXXL motif (Kanki, Wang *et al*. 2009). It may also be a mitochondrial receptor for mitophagy in mammalian cells (Kirkin, McEwan *et al*. 2009; Novak, Kirkin *et al*. 2009).

Similarly, BNIP3 that was initially identified in yeast as Bcl-2 and adenovirus E1B 19-kDa interacting protein-3, is present in the membrane of mitochondria when it is overexpressed (Zhang; Ney *et al.* 2009; Quinsay, Lee *et al.* 2010). Together with Nix, BNIP3 are mitochondrial proteins that have the ability to induce both cell destinies: survival or death.
BNIP3 and Nix share certain features with the BH3-only subgroup of the Bcl-2 family, such as sequence homology in the BH3 domain, residence in the outer membrane and the ability to interact with Bcl-2 and Bcl-$X_L$, which connects them with cell death modulation (Bellot, Garcia-Medina *et al*. 2009).
Furthermore these proteins are also related with autophagy by Beclin-1, an important autophagic protein, that interacts with Bcl2 and Bcl-$X_L$, thus, BH3-only proteins may induce autophagy by competing with Bcl-2 for binding to Beclin-1 (Zhang, Meng *et al.* 2003; Zhang; Ney *et al*. 2009).

### 3.2.2 **PINK1 and Parkin**

The loss of function of the proteins: Parkin (an E3 ubiquitin ligase) or PINK-1 (PTEN-induced kinase 1, a mitochondrial localized serine/threonine kinase) results in a genotype of recessive familial forms of Parkinsonism (Poole, Thomas *et al*. 2010). The malfunction of these two proteins is responsible for male sterility in Drosophila, apoptotic muscle degeneration, defects in mitochondrial morphology and increased sensitivity to multiple stresses including oxidative stress (Clark, Dodson *et al*. 2006). Additionally, Parkin and PINK-1 are also related to mitophagy modulation.

PINK-1 and Parkin are mostly studied in dopaminergic neurons since it was thought that these two proteins are involved in the neurodegenerative Parkinson disease. PINK-1 a constitutively express protein, being proteolysed by mitochondria rhomboid protease PARL, at the mitochondria membrane of healthy mitochondria. This process results in processed forms of PINK-1, which are rapidly degraded by the proteasome. Whenever membrane potential (Δψm) is reduced there is an accumulation and activation of PINK-1, recruiting Parkin from the cytosol to the mitochondria. Mitochondria accumulation of Parkin leads to their degradation via mitophagy (figure 3.2) (Shiba-Fukushima, Imai *et al*. 2012).

In 2011 Cui and colleagues found in dopaminergic MN9D mouse cells that the down-regulation of PINK-1 by RNA interference resulted in abnormal mitochondrial morphology, partial loss of mitochondrial membrane potential and increased production of ROS, leading to mitophagy. In these cells mitophagy was associated with up-regulation of Beclin-1 and the opening of mitochondrial

permeability transition (MPT) pore, suggesting that the mechanisms behind the activation of mitophagy are controlled by opening of MPT pore and by general autophagy regulators (Cui, Fan *et al.* 2011).

All of these processes involving PINK 1/Parkin are thought to be related with the capacity of the mitochondrial machinery regulation through ubiquitination, namely, the mitochondrial fusion-promoting factor mitofusion (dMfn). The steady-state abundance of dMfn is increased by the decrease of PINK1/Parkin complex (Poole, Thomas *et al.* 2010; Youle; Narendra *et al.* 2010; Imai; Lu *et al.* 2011). However, the exact stimuli and mechanism that activate autophagy by the loss of function of PINK-1 and accumulation of Parkin is unclear (Cui, Fan *et al.* 2011).

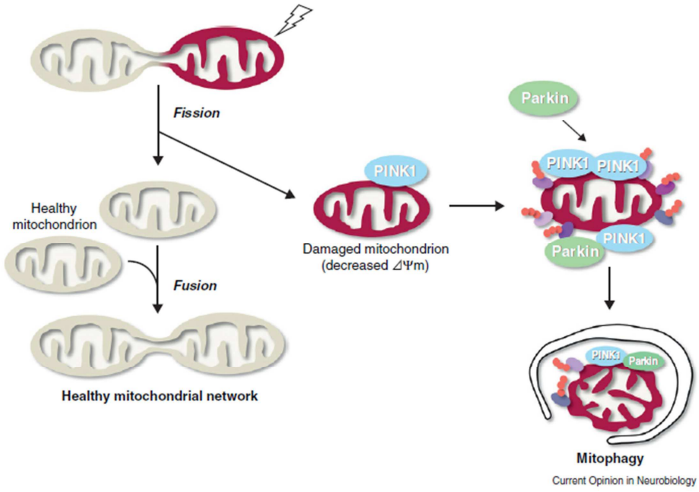


**Figure 3.2 - Mitophagy process by activation of PINK1 and Parkin induction (Imai; Lu *et al.* 2011).** Mitochondrial fusion and fission events are required for the maintenance of healthy mitochondrial population, in the case of mitochondrial fusion it is thought to facilitate the interchange of internal components. On other hand mitochondrial fusion seems to have an important role in the removal of damaged mitochondria and PINK1/Parkin are likely to be the trigger of this all process. In the case of damaged mitochondrias PINK1 accumulates in the mitochondrial membrane and recruits parkin from the cytosol signaling the mitochondria for degradation via a selective process of elimination – mytophagy;

Mitochondrial depolarization, fission and ROS production (malfunctioning mitochondria) are associated with modulation of mitophagy, but how these potential signals are integrated is not clear, being a scientific subject with broad room for development and research.

Therefore this thesis focuses on understanding CO mode of action in primary culture of astrocytes, namely its capability of cytoprotection against cell death *via* modulation of autophagy and/or mitophagy.

# II. Aims

# 4 Aim

The role of astrocytes in the CNS and their involvement with malfunction, such as in neurodegenerative diseases, has raised a great interest along the time, since astrocytic function is crucial for neuronal maintenance. The role of CO in the cytoprotection of astrocytes was already demonstrated in previous studies (Queiroga, Almeida *et al*. 2010; Almeida, Queiroga *et al.* 2012). Nevertheless, the CO-induced cellular pathways are still a matter of debate. In addition, studies carried out in the recent years, in brain and in other models, introduce a new cellular process of cytoprotection: autophagy, previously claimed as a cell death process (Boya, Reggiori *et al.* 2013). In summary, and based in all the existent knowledge, this thesis has as a general aim:

**The study of the cellular and biochemical pathways involved in CO-induced cytoprotection, in particular the role of autophagy, mitophagy and cell metabolism.**

For reaching this aim, three specific objectives will be followed:

1) Understanding whether CO induces autophagy and in particularly mitophagy in astrocytes;
2) The autophagy/mitophagy role in CO-induced cytoprotection in astrocytes;
3) Identification of the main factors involved in CO-induced autophagy/mitophagy;

# III. Material and methods

This section is reserved to describe the material and methods used to evaluate the experimental hypothesis. The description of the experimental work is divided in five main points.

## 4.1 Material, solutions and medium

### 4.1.1 Medium and culture material

All plastic material was provided from Sarstedt (Germany) and is sterilized. Glass material and prepared solutions were sterilized by autoclaving at 121ºC for 20 minutes or by filtration using a pore size of 0.22 µM.

**Table 4.1- Reagents used culture cells, for the extraction of astrocytes**

| Reagent | Reference | Company | Final concentration |
|---|---|---|---|
| **DMEM low glucose** | 31885-0230 | Gibco – Life technologies | |
| **Fetal Bovine Serum (heat inactivated) (FBS)** | 41F3715K | Gibco- Life technologies | 20, 15 and 10% in cell culture medium |
| **Penicillin-streptomycin** | 1514022 | Gibco- Life technologies | 1% (100U/mL) in cell culture medium |
| **Glucose** | | Sigma | 1 g/mL in cell culture medium |

### 4.1.2 Preparation of Buffer solutions

All the solutions presented in the table below were prepared in milli-Q water:

**Table 4.2 - Reagents utilized in the technique of Western Bolt.**

| Buffers | Composition |
|---|---|
| **Phosphate Buffer saline (PBS)** | 1,54M NaCl,34mM $Na_2HPO4$, 20mM $KH_2PO_4$. With a final pH of 9,4. |
| **Cell lysis Buffer** | 2% (w/v) SDS<br>50mM tris-HCL<br>pH= 6.8 |
| **Loading buffer (for samples in WB)** | 10% (v/v) Glycerol<br>10mM DTT<br>0,005% (w/v) Blue Bromofenol |
| **T-TBS (WB)** | 0,25M Tris-HCL; 0,75M NaCL |
| **Blocking Buffer (for blocking WB membranes)** | T-TBS with 5% (w/v) Milk |
| **Running Buffer (protein gel for WB)** | 0,25M Tris base,1,92M Glicine, SDS 20%(v/v) |
| **Transfer Buffer (transferring protein from gel to membranes in WB)** | Runing buffer with 10% (v/v) of Methanol |
| **Permeabilization solution** | 0,1% (w/v) SDS in PBS |
| **Blocking solution** | 10% (v/v) of FBS in PBS |

### 4.1.3 Preparation of CORM-A1

The solution of CORM-A1 was prepared in milli-Q water with a final concentration of 5mM. Then, the solution was filtrated with 0,22 µM filter, aliquoted and stored at -20ºC. For each use, an aliquot was thawed and rapidly added into the culture.

### 4.1.4 Antibodies

For Western Blot and Immunofluorescence techniques, we used the primary and secondary antibodies described below:

**Table 4.3 - Description of conditions and features of the used antibodies.**

| | | | | Indicated by the company | | Optimized | | |
|---|---|---|---|---|---|---|---|---|
| **Antibody** | **Reference** | **Company** | **Origin** | **[] µg/ml WB** | **[] µg/ml IF** | **Dilution Factor WB** | **Dilution factor IF** | **Molecular Weight (kDa)** |
| **Anti-Glial Fibrillary Acidic Proteins (GFAP)** | | Milipore | Mouse Monoclonal | 1:1000 | | 1:1000 | 1:500 | 50 |
| **Anti SQSTM1 or P62** | 157H000 08878-M01 | Abnova Tebu-bio | Mouse Monoclonal | | | 1:500 | | 62/64 |
| **Anti-LC3B** | ab48394 | abcam | Rabbit Policlonal | 2µg/ml | | 1:500 | 1:400 | 18 LC3 I 17 LC3II |
| **Anti-BNIP3** | ab10433 | abcam | Mouse Monoclonal | 5µg/ml | | 1:500 | | 37 |
| **Amersham ECL Anti-rabbit IgG** | NA934VS | GE Healthcare | Donkey | | | 1:1000 | | - |
| **Amersham ECL Anti-mouse IgG** | NA931V | GE Healthcare | Sheep | | | 1:1000 | | - |
| **Alexa Fluor 488** | A11001 | Life technologies | Goat ant-rabbit | | | | 1:500 | |
| **Alexa Fluor 594** | 1008648 | Life technologies | Goat ant-rabbit | | | | 1:400 | - |
| **Alexa Fluor 647** | | | Goat ant-rabbit | | | | 1:400 | - |

## 4.2 Primary Cultures of Astrocytes: isolation and maintenance

The animal manipulation was realized according to the recommendation of the *Conveção Europeia for Protecção dos animais vertebratos* used for experimental studies and scientific (Directiva do Conselho 86/609/CEE). All efforts were done to minimize the number of used animals and to decrease animal suffering.

Primary culture of astrocytes was obtained from 1-day-old mouse cortex, as described by Schousboe, A., *et al.* 2001. The 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 cm2) in Dulbecco's minimum essential medium supplemented with 20% (v/v) fetal bovine serum (heat-inactivated), 100units/ml penicillin/streptomycin solution. Cells were maintained in a humidified atmosphere of 7%$CO_2$ at 37 °C.

With the aim of achieving a pure culture of primary astrocytes, after 7 days, when cells reach the confluence, the dark phase cells growing on the astrocytic cell layer were eliminated (such as oligodendrocytes) by vigorous shaking and removing the supernatant. The remaining astrocytes were detached by mild trypsinization using trypsin/EDTA (0.25%, w/v) and were subcultured in DMEM supplemented with 15% (v/v) fetal bovine serum, in a new t-flaks of 75$cm_2$ with a final volume of 15mL, for more two weeks , during this period culture medium was renewed twice a week . Finally at the third week, medium was replaced from 15% (v/v) of fetal bovine serum to 10%. Culture medium was renewed twice a week. All the experiments were performed with 3 to 5 week-old cultures. Depending on the used techniques; WB, IF or Q-PCR, cells were cultured in different culture plates with different cell concentration, according with the table 4.4, represented below.

**Table 4.4 - Description of cells cultured in different plates with different concentrations, culture volumes for the different techniques.**

| Plates | Number of plated cells per well | Technique | Volume per well (ml) |
|---|---|---|---|
| **6 well** | $50*10^4$<br>$100*10^4$ | Western Blot<br>Q-PCR | 2,5 |
| **24 well** | $35*10^4$ | Imunofluorence and flow Cytometry | 0,5 |

## 4.3 Role of CORM-A1 in primary culture of astrocytes

### 4.3.1 Inducers and inhibitors of cell death and autophagy

Starvation was used as a positive control for autophagy stimulation. Medium without supplementation of amino acids, Earle´s Balanced Salt Solution (EBSS) (Gibco Invitrogen, Germany) was added to cell culture for promoting starvation, by blocking nutrient supply.

In contrast, hydroxychloroquine (HCQ), which is an inhibitor of autophagy by blocking the autophagic flux, was used for evaluating it (figure 4.1). HCQ is attracted to the inside of organelles, preventing the fusion of lysosome with the autophagosome structure, by neutralizing the lysosomal pH, and at the same time inhibiting the action of lysossomal proteases. Thus, HCQ promotes the accumulation of the autophagy marker LC3I/II.

The levels of LC3-II are tightly correlated with the amount of autophagosomes and for that reason it is considered as the most reliable marker of active autophagosomes and autophagolysosomes (Yorimitsu; Klionsky *et al.* 2005). An increase of cellular LC3-II levels occurs during activation of autophagy, nevertheless accumulation of LC3-II can also be obtained by interrupting the autophagosome-lysosome fusion step or by inhibiting lysosome-mediated proteolysis. Therefore, the use of LC3-II as an autophagic marker needs to be complemented by an assay to estimate the overall autophagic flux, using HCQ. Therefore, measurement of autophagy activity includes (i) the increased synthesis or lipidation of LC3 or an increase in the formation of autophagosomes, and (ii) most importantly, the assessment of autophagic flux through the entire system, including lysosomes or the vacuole, and the subsequent release of the breakdown products (Klionsky, Abdalla *et al*. 2012).

Thus, autophagic substrates need to be monitored dynamically over time to verify that they have reached the lysosome/vacuole, and, when appropriate, are degraded. For measuring the autophagic flux, HCQ is added to the cell culture for blocking the flux and LC3II levels were carried out by Western Blot analysis. In summary, autophagy is stimulated whenever an accumulation of LC3-II is obtained in the presence of HCQ (Klionsky, Abdalla *et al.* 2012).

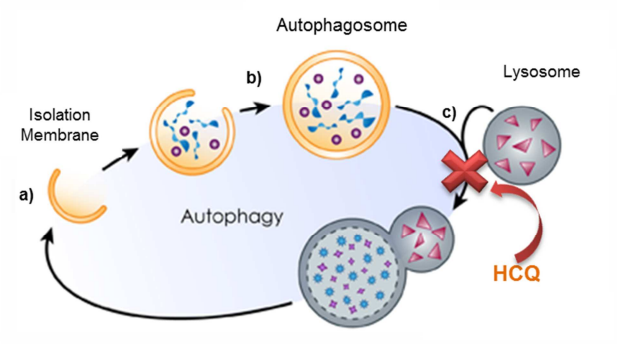


**Figure 4.1 - Autophagic Flux.** a) Phagofore formation, b) Formation of a double-membrane named by autophagosome, with incorporation of damaged components, c) incorporation of lysosome and elimination of cargo. Adapted from (Hansen; Johansen *et al*. 2011)

Another inhibitor of autophagy used along this study was Wortmannin. Wortmannin is a cell-permeable, fungal metabolite that acts as a potent, selective and irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K). PI3K is required for autophagy, thus inhibition of PI3K with wortmannin can prevent autophagic sequestration (at the beginning of the process) (Klionsky, Abdalla *et al.* 2012).

Cell death is induced with *tert*-Butylhydroperoxide (t-BHP), which is an organic peroxide widely used in a variety of oxidation processes. It is a pro-oxidant molecule, causing oxidative stress and resulting in cell or organ injury. Exposure to t-BHP promotes cell death, increasing ROS production and mitochondrial dysfunction. Cell viability was measure by flow cytometry analysis (Queiroga, Almeida *et al*. 2010).

Hence, for achieve this aim we used the following compounds described at table 4.5.

**Table 4.5 - Inducers and Inhibitors of autophagy and cell death inducers;**

| Compounds | Action | Final Concentration |
|---|---|---|
| ***tert*-Butyl hydroperoxide (T-TBS)** | Pro-oxidant agent – cell death inducer | 0, 80, 160, 240, 280, 320µM |
| **EBSS** | Autophagy inducers starvation | |
| **Hydroxychloroquine (HCQ)** | Autophagic flux blocker | 3 mg/ml |
| **Wortmannin (WM)** | Autophagy inhibitor | 100 nm |

### 4.3.2 **Induction of autophagy**

Astrocytes, plated according to the table 4.4, were incubated with CORM-A1 at a final concentration of 12,5 and 25 µM with and without hydroxyclochroquine (HCQ) at 30µg/ml for 30 minutes or 1 hour at 37ºC, as described in figure 4.2 a).

Cells were collected for posterior analysis through Western Blot, Immunochemistry and Q-PCR in order to evaluate autophagy induction, mitophagy and mitochondria population, respectively. These assays were prepared accordingly to figure 4.2.

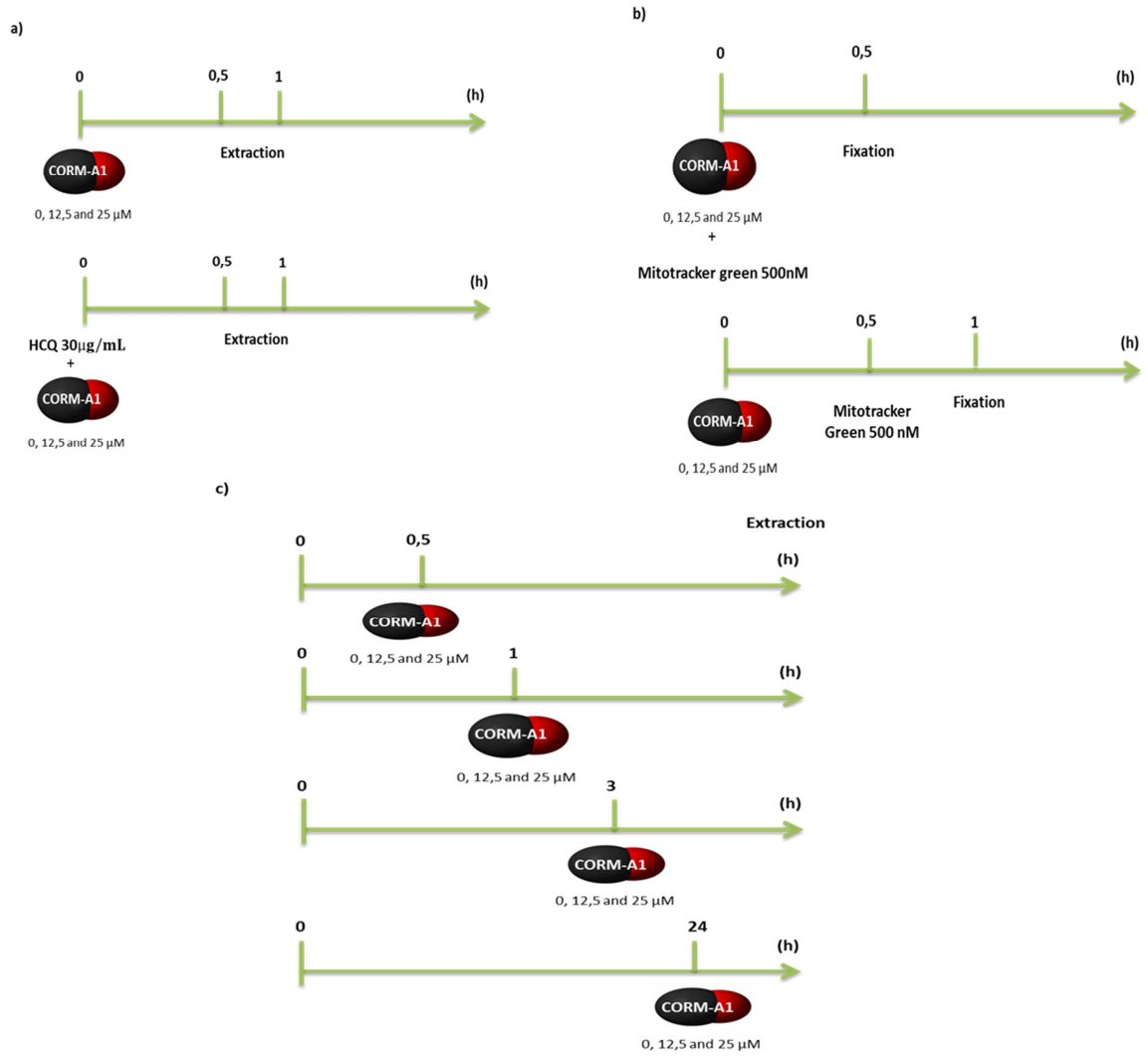


**Figure 4.2- Scheme of autophagy induction in primary culture of astrocytes along time.** Cells were treated with CORM-A1 (a) for assessing autophagic flux HCQ were also added and LC3 analysis by Western Blot, b) for measuring specific mitophagy stimulation, a mitochondrial dye was also added for further analysis by imunofluorescence (IF), c) Mitochondrial population is followed along 24h after CORM-A1 treatment by mitochondrial DNA quantification via Q-PCR analysis.

### 4.3.3 Induction of cell death and assessment of cell viability

Astrocytes were incubated for 18h with *t*-BHP (80-320µM). Then, medium was collected, cells were washed once with PBS and trypsined. Supernatant medium, PBS and cell pellet were pulled together for incubating with the dyes: propidium iodide (PI) and 3,3'-dihexyloxacarbocyanine iodide (DiOC) for 30 minutes at 37ºC, followed by cytofluorometric analysis with FACS scan (BD FACSCalibour). DiOC is used for measuring mitochondrial potential and PI for assessing plasmatic membrane integrity, an indirect way for assessing cell viability.

For evaluating the cytoprotective role of CO, in particular CORM-A1, and the correlation with autophagy, astrocytes were pre-treated with wortmanin 100µM for 1 hour (for preventing autophagic

process), then astrocytes were treated with CORM-A1 at 12,5µM for 30 minutes and 1h, followed by cell death induction as described in figure 4.3:

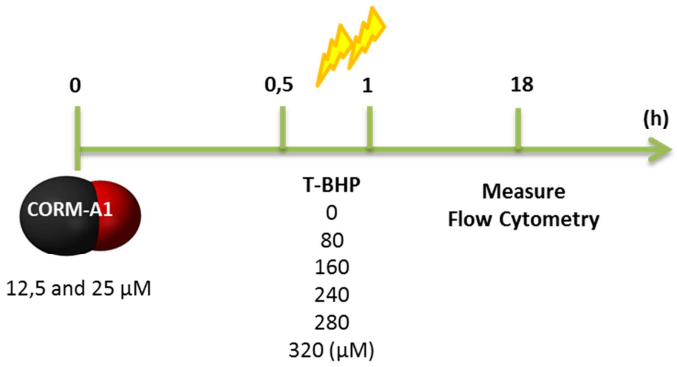


**Figure 4.3 - Scheme of the primary culture of astrocytes treatment for cell viability measurement;**

Results were assessed by FlowJo analysis, version 10.1. For the quantification of cell viability two gates were outlined limiting death cells and alive cells, for the death cells just cells marked with PI positive (PI+) and DiOC negative (DiOC-) were considered and for live cells just cells regarding to PI negative (PI-) and DiOC positive (DiOC +) was showed in figure 4.4.

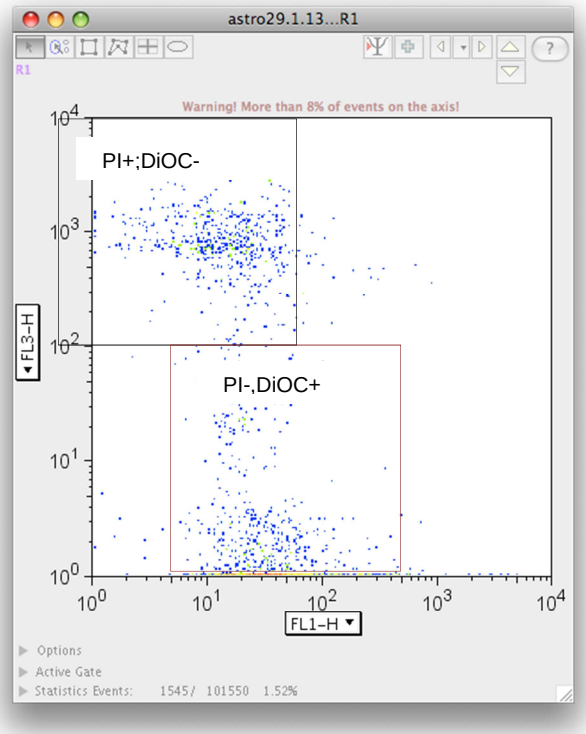


**Figure 4.4 – Example of the gates used to evaluate the cell viability;** Primary culture of astrocytes treated with and without CORM-A1 and pro-oxidante agent, *t*-BHP.

## 4.4 Western Blot

For measuring autophagy induction, astrocytes, previously treated with CORM-A1 in the presence or absence of HCQ, were washed in cold PBS at 4ºC and lysed in a lysis buffer, according to Patricia Boya (González-Polo, Boya *et al.* 2005).

Protein concentration was quantified by Pierce BCA Protein Assay kit (ref). Standard curve (figure 4.5) was done in the range of concentration: 0.0625, 0.125, 0.25, 0.5, 1 and 2 mg/mL of BSA prepared in mill-Q water. Each sample was diluted for 1/5 to a final volume of 100µL and the final concentration was measure in spectrophotometer. Cellular extracts were prepared in Loading buffer (table 4.2).

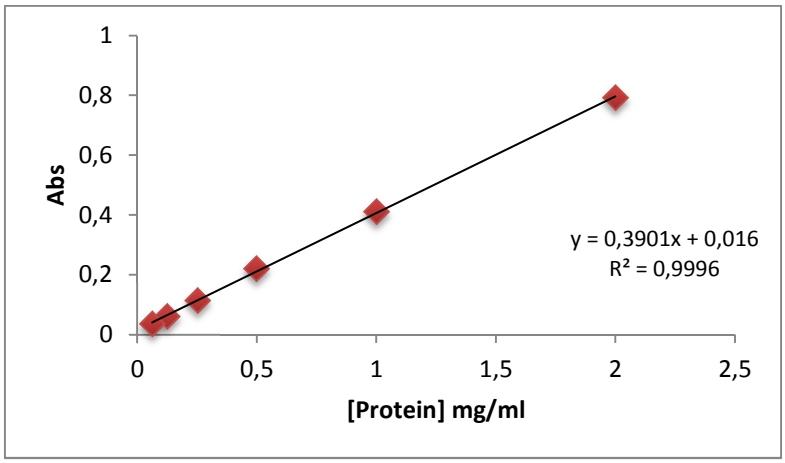


**Figure 4.5 - Example of Standard curve,** used for protein quantification, was prepared with BSA solution in a range of concentrations; 0.0625, 0.125, 0.25, 0.5, 1 and 2 mg/mL.

Protein gel was prepared according to table 4.6 and the settings of the run and transference:

**Table 4.6 - Protocol for gel preparation at 12 and 15% of acrylamide.**

| | Volume (mL) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Reagents** | **H2O mill-Q** | **30% Acrylamide (Bio-RAD, EUA)** | **Tris-HCl (0,5M, pH 8,8) (Bio-RAD, EUA)** | **Tris-HCl (0,5M, pH 6,8) (Bio-RAD, EUA)** | **SDS 10% (Sigma, Japon)** | **APS 10% (Sigma, Japon)** | **TEMED (Sigma, chine)** | **Total Volume** | **Settings** |
| **Resolving 15%** | 2,3 | 5 | 2,5 | - | 0,1 | 0,1 | 0,004 | 10 | 135 - 150V (fixed), 30 min for 12% gels and 45min for 15% gels; |
| **Resolving 12%** | 3,3 | 4 | 2,5 | - | 0,1 | 0,1 | 0,004 | 10 | |
| **Staking** | 2,77 | 0,83 | - | 1,26 | 0,05 | 0,05 | 0,005 | 5 | 500 mA (fixed) for 1h; |

30µg of protein from cellular extracts were loaded on a 15% or 12% SDS-PAGE (table 4.6) and transferred to a nitrocellulose membrane. These two types of gel concentrations were used since the molecular weight of proteins were very different. LC3 I/II is a low weight protein with approximately 16-17kDA, using a 15% SDS-PAGE gel, while p62, GFAP, BNIP3, are higher weight proteins with, 64, 50, 37kDA, respectively and 12% SDS-PAGE gel is sued. The membrane was incubated for 1h in milk solution for protein blocking. Primary antibodies (anti-SQSTM1, anti-LC3, anti-BNIP3) (table 4.3) were incubated for 1h30 at RT or overnight at 4ºC and detected with the appropriated peroxidase-labeled secondary antibodies (GE), which were incubated for 2h at RT. Rouge Ponceau was used as internal control for checking total protein loading. Blots were developed using ECL (enhanced chemiluminscence, GE-Healthcare, RPN 2232) detection system after incubation with HRP-labeled anti-mouse IgG antibody and anti-rabbit IgG antibody for 1h at 25ºC, followed by consecutive washes. Protein detection was performed by film (Amersham hyperfilm ECL 28-9068-36, GE Healthcare life science) development. The results obtained were analyzed by Fiji software, enabling the quantification of the intensity of the bands that correlate with the amount of proteins present in each sample.

## 4.5 **Imunofluorescente Microscopy**

In order to evaluate the autophagy, particularly the autophagy of mitochondria, mitophagy, astrocytes cultured in 24-well plate (table 4.4) with coverslips were exposed to CORM-A1 at 12,5 or 25µM during 30 minutes or 1h. Then, astrocytes were rinsed with PBS, fixed with a solution of 4% PFA and 4% saccharose during 15 min at RT, incubated with methanol for 2 min at -20ºC and washed again with PBS twice. The cells were permeabilized with 0.1% SDS in PBS during 30 minutes at RT. Afterwards, cells were incubated with the rabbit polyclonal antibody anti-LC3B (table 4.3) and with the monoclonal antibody anti-Cyto-c, diluted in PBS containing 10% FBS during 2 hours at RT. Cells were then incubated with the secondary antibody Alexa Fluor 594/647 goat anti-rabbit (table 4.3), during 1h at RT. Finally, cultures were mounted in ProLong mounting media (with DAPI – Invitrogen) and images were captured using a Leica DM 5500B 710 fluorescent microscope (Leica, France) with a camara Andor Luca DL-604M#VP.

## 4.6 **Quantitative PCR - Measure of mitochondrial DNA**

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, with the following conditions (table 4.7).

**Table 4.7 - Representation of the experimental run protocol for DNA amplification.**

| Steps | Cycles | Temperature (ºC) | Time (s) |
|---|---|---|---|
| **Pré-incubation** | 1 | 95 | 600 |
| **Amplification** | 45 | 95 | 10 |
| | | 60 | 10 |
| | | 72 | 10 |
| **Melting** | 1 | 95 | 10 |
| | | 65 | 60 |
| | | 97 | 10 |
| **Cooling** | 1 | 37 | 30 |

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.

## 4.7 **Statistical Analyses**

The data concerning astrocytic culture were carried out at least in three independent preparations (cell isolation). For Western blotting, autophagy avaliation, 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: single factor with replication, with $p$ <0.05 ($n$ ≥3). $p$ >0.05 means that samples are significantly different at a confidence level of 95%.

# IV. Results

# 5 Results

In the beginning of this Master thesis work many questions were without answers, since autophagy, mitophagy and the role of CO in these two cellular processes was unclear. Therefore, this section will be divided in three main parts, in order to uncover some of these issues; all parts were performed in primary culture of astrocytes. The first part (5.1.) concerns the role of CORM-A1 in cytoprotection and autophagy activation, as well as the cross-talk of these two processes. The second part (5.2.) focuses on specific process, namely the role of mitophagy and the potential involved proteins. Finally, the third part (5.3.) will be reserved to the role of CO in low levels of oxygen, describing some preliminary results that open new windows for future research development.

## 5.1. CORM-A1 modulation cytoprotection and autophagy

### 5.1.1 CORM-A1 prevents cell death in primary culture of astrocytes

The role of CO as anti-apoptotic factor in astrocytes was already demonstrated by Queiroga, Almeida and colleagues (Queiroga, Almeida *et al.* 2010; Almeida, Queiroga *et al.* 2012). Herein, we were interested in evaluating the potential cytoprotective effect of a CO releasing molecule (CORM's) developed by *Motterlini et al*. (Motterlini, Sawle *et al.* 2005), called CORM-A1, as described in the section 2.3, page 9. CORM-based CO delivery approaches present more potential for applications and mimic the endogenously produced CO molecule.

We used primary cultured of astrocytes isolated from cortex to mimic better the physiologic conditions in the organism, since these cells are obtained by direct extraction from the organism.

The evaluation of cell culture purity was obtained by fluorescent microscopy. Cells were incubated with GFAP antibody (developed with a secondary Alexa488 antibody - green) and DAPI (blue) that marks for astrocytic protein and nucleus, respectively (figure 5.1). The correlation of the number of positive cells for DAPI and GFAP, allows the evaluation of astrocytic purity. Therefore the cell cultures used in all the assays had about 85% of purity.

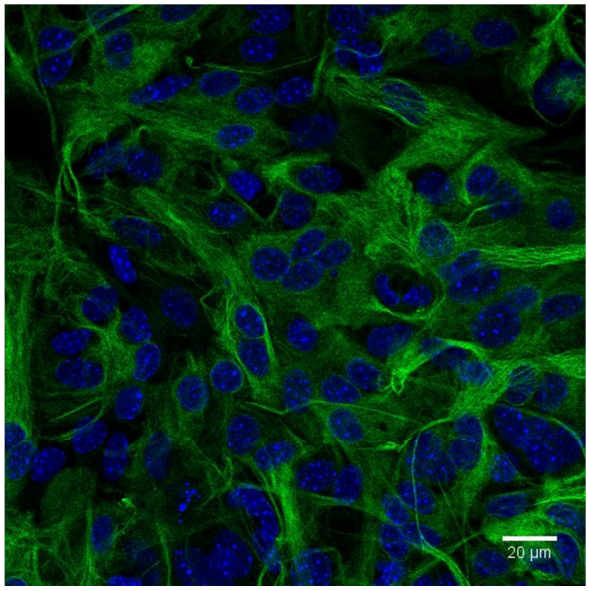


**Figure 5.1 – Purity of primary culture of astrocytes.** In blue, DAPI-stained nuclei; in green, GFAP positive astrocytes cells. Fluorescent microscopic analysis of primary culture if astrocytes, with an amplification of 40x. Quantification of astrocytes per nuclei was obtained by image j.

For evaluating the cytoprotective effect of CORM-A1 cells were cultured at a concentration of $35*10^4$cells/well in 24 well plates, with different conditions: control (no CORM-A1 treatment) and CORM-A1-treated at final concentration of 12,5µM for 1 hour. Cell death was induced with *t*-BHP, an oxidant agent, for 18 hours and measured by flow cytometry, using two different dyes, DiOC and PI. DiOC measures mitochondrial potential and PI quantifies plasmatic membrane integrity, which is an indirect way for assessing cell viability.

As observed in figure 5.2, cells treated only with t-BHP (control group), particularly with high concentrations, 240 until 320 µM, have a decrease in cell viability of approximately 50-80%. Whereas cells pre-treated with CORM-A1 until the concentration of 240 µM of *t*-BHP seem to maintain the cell viability close to the one observed in control cells (without any treatment), between 240-320 µM, there is a slight reduction of cell viability, approximately 40%. Nonetheless, at all concentrations of the pro-oxidant, cell viability is higher in cells pre-treated with CORM-A1 whenever compared to control.
In summary, CORM-A1 partially prevents astrocytic cell death, in particular at higher concentrations of the pro-oxidant t-BHP. This is in accordance with previously observed with CO gas (Queiroga, Almeida *et al*. 2010; Almeida, Queiroga *et al.* 2012).

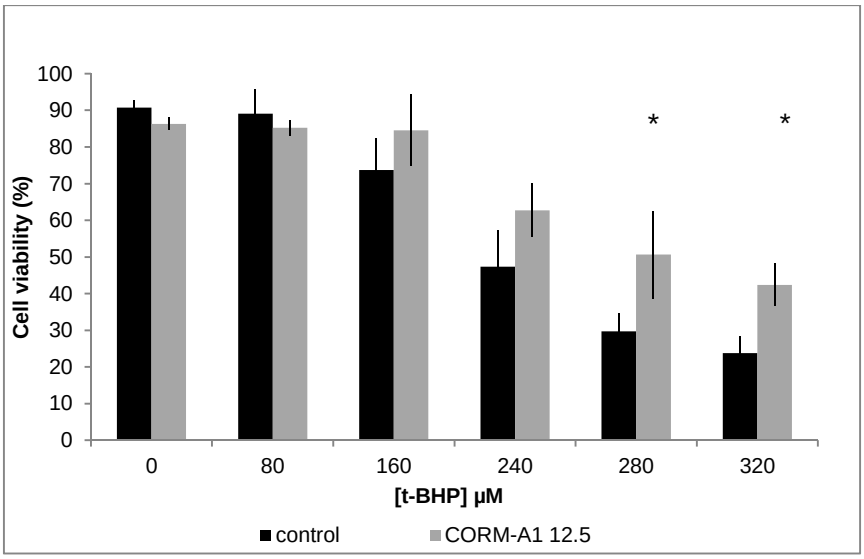


**Figure 5.2 – CORM-A1 confers protection against cell death.** Primary culture of astrocytes was pretreated with 12,5µM of CORM-A1 along 1h, following cell death induction with the pro-oxidant, t-BHP (from 0 to 320µM) for 18h. The measurement of cell viability was assessed by flow cytometry. Black bars- control; gray bars- cells treated with CORM-A1. Cells presenting high mitochondrial potential (detected by DiOC) and containing intact plasma membrane (assessed by propidium iodide) are considered viable cells. All values are mean±SD, n= 5 and *p<0,05 compared with control and CORM-A1-treated cells for each concentration of t-BHP.

### 5.1.2 **CORM-A1 promotes autophagy induction.**

Autophagy is an autodigestive process promoting cell survival through degradation and recycling of long-lived proteins, misfolded proteins, ubiquitinated proteins, and injured organelles, plays an important role in maintaining cellular homeostasis during environmental stress (Kroemer, Mariño *et al.* 2010; Lee, Ryter *et al.* 2011). In contrast to what was primarily thought about CO, a toxic molecule, over the last decade studies have revealed cyto- and tissue-protective effects against apoptosis when applied at low concentrations in several models, including our findings (Lee, Ryter *et al.* 2011; Lancel, Montaigne *et al.* 2012; Mahan *et al.* 2012). However, little is known about the effect of CO on the autophagy pathway (Lee, Ryter *et al.* 2011). Herein we were interested in studying the effect of CO in autophagy, using CORM's as CO-source. Our experimental hypothesis is that CORM-A1 prevents cell death by autophagy induction. For the first time, at our knowledge, it is used CORM's in brain and for autophagy modulation. For the assessment of autophagy in primary culture of astrocytes, western blot analysis was performed for the assessment of LC3 lipidation (LC3II), which is a key marker of autophagy, as well as the ratio between LC3II/I. The levels of LC3 II are tightly correlated with the amount of autophagosomes and for that reason it is considered the most reliable marker of active autophagosomes and autophagolysosomes (Yorimitsu; Klionsky *et al*. 2005; Klionsky, Abdalla *et al.* 2012). Furthermore, LC3II levels must be quantified in the presence of HCQ for assessing autophagic flux, as described in Material and Methods (section 4.3.2, page 22).

Cells were treated with CORM-A1 at a final concentration of 12.5 and 25 µM with and without hydroxyclochroquine (HCQ) at 30µg/ml for 1 hour. Rouge Ponceau was used for controlling the amount of charged protein.

Figure 5.3A is a representative example of western blot films obtained for the autophagy assessment, while (B) corresponds to the respective quantification of LC3I and II, CORM-A1 at 12.5 and 25 µM increased LC3II accumulation, in the presence or absence of HCQ, indicating that autophagy is stimulated. To confirm this result, we block autophagosome formation by the addition of HCQ, an inhibitor of autophagic flux. As observed in figure 5.3A and 5.3B, cells treated with CORM-A1 and HCQ have an increase of LC3 accumulation, when compared with cells just treated with HCQ, which represent the basal levels of autophagy. Another approach to evaluate autophagic induction is the quantification of the LC3II/LC3I ratio (figure 5.3C), which quantifies the conversion of LC3I into LC3II. The ratio LC3II/LC3I was calculated in HCQ-treated cells for confirming CO-induced autophagy in primary culture of astrocytes. Nevertheless, it is necessary to take into account that the quantification of this process is not straightforward and plenty of conditions need to be considered. Figure 5.3D, is the compilation of all the blots obtained for autophagy induction by measuring LC3II accumulation. As it was already observed in figure 5.3B, CORM-A1 at 12.5 or 25 µM promotes autophagy induction, and the result is confirmed whenever autophagic flux is blocked with HCQ.

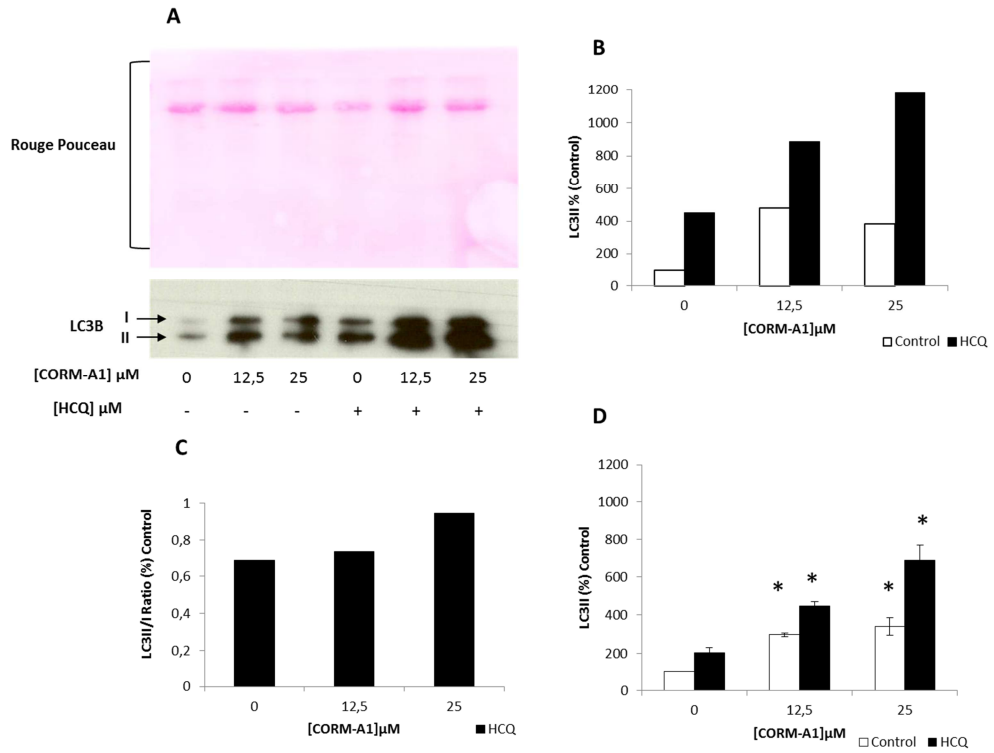


**Figure 5.3 - CORM-A1 induces autophagy in astrocytes.** Primary culture of astrocytes were pretreated with 12,5 or 25µM of CORM-A1 along 1h, with and without HCQ at 30µg/ml. The measurement of autophagy induction was assessed by western blot analysis. **A)** representative figure of immunodection of LC3I and II, Rouge Ponceau was used as internal control for checking total protein loading. **B)** Quantification of LC3II amount in CORM-A!-treated cells for the immunoblot film represented in A; **C)** Ratio of LC3I/LC3II in the immunoblot film represented in A for cells treated with CORM-A1 with HCQ. **D)** Quantification of LC3II for control and CORM-A1-treated cells in the presence or absence of HCQ. All the values are mean±SD, n=3, * p<0,05 compared with control for each.

Therefore treatment of CORM-A1 at 12,5 or 25µM for 1h increases autophagy above the basal level in astrocytes.

### 5.1.3 **Autophagy involvement in the cytoprotective role of CORM-A1**

In order to verify whether autophagy plays a role in the cytoprotective effect of CORM-A1, we used another experimental approach by testing the anti-apoptotic effect of CORM-A1 in the presence of an inhibitor of autophagy, Wortmannin (WM). Wortmannin is a selective and irreversible inhibitor of phosphatidylinositol 3-kinase (PI3K). PI3K is required for autophagy, therefore inhibition can inhibit autophagic sequestration (at the beginning of the process) (Klionsky, Abdalla *et al*. 2012), section 4.3.1. in material and methods, page 23. Cell viability was assessed by flow cytometry by measuring membrane potential and plasmatic membrane integrity using two dyes, PI and DiOC, as previously described. The assay consisted in culturing astrocytes in the presence of WM at final concentration of 100nM for 1h, followed by CORM-A1 treatment at 12,5µM for 1 hour, then challenging astrocytes to cell death through pro-oxidant stress, induced with t-BHP, for i18 hours.

The figure 5.4, shows that WM reverts CORM-A1-induced cell death prevention at high concentration of the pro-oxidant (black stripes) when compared to CORM-A1-treated cells (gray bars). While astrocytes treated only with WM do not present any toxic effect when compared to control cells without any treatment.

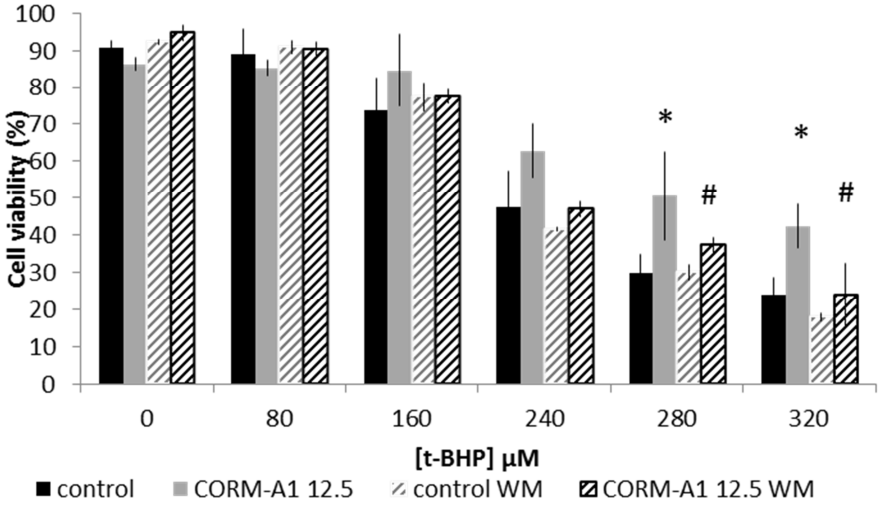


**Figure 5.4 - CORM-A1 confers protection against apoptosis by inducing autophagy.** Primary cultures of astrocytes were pretreated with 12,5µM of CORM-A1 along 1h, with or without wortmannin 100nM, following cell death induction by 18h of exposure to the pro-oxidant, t-BHP (from 0 to 320µM). The measurement of cell viability was assessed by flow cytometry. Cells presenting high mitochondrial potential (detected by DiOC) and containing intact plasma membrane (assessed by propidium iodide) are considered viable cells. All the values are mean±SD, n=3, * $p<0,05$ compared with control for each concentration of t-BHP and $^{\#}$ $p<0,05$ compared with CORM-A1 treated cells without WM for each t-BHP concentration.

Furthermore, when we correlate the results obtained so far it is possible to suggest that the protective effect of CORM-A1 observed against cell death in primary culture of astrocytes is related to autophagy induction, a cell process already known for being a protective process (Lee, Ryter *et al.* 2011). Nevertheless, future work must be done using genetic inhibition of autophagy.

## 5.2 The action of CORM-A1 in mitophagy

Autophagy - general mechanism – is known as protective process under various stress conditions, including nutrient starvation and hypoxia (Levine; Kroemer *et al.* 2008; Kroemer, Mariño *et al.* 2010; Lee, Ryter *et al.* 2011; Lancel, Montaigne *et al.* 2012). The protective effects of CO on apoptotic cell death is already characterized in several cell types, including astrocytes. Nevertheless, in the results described in the previous section we showed that CORM-A1 mimic CO gas and promote autophagy activation. Based on our results and on the literature there is evidence showing CO gas involved in (i) the control of mitochondrial function, (ii) modulating oxidative metabolism and substrate utilization (Mahan *et al.* 2012), (iii) increasing mitochondrial population and (v) enhancing the anti-apoptotic protein Bcl-2 expression (iv) stimulating mitochondrial biogenesis and (v) enhanced Bcl-2 expression (Almeida, Queiroga *et al.* 2012), we hypothesize that CO is involved in the activation of the specific mitochondrial autophagy, namely mitophagy. Mitophagy is a mechanism of quality control of mitochondria, where damaged mitochondria are eliminated avoiding an increase of ROS, which can be very harmful due to their uncontrolled reactive oxygen species (ROS) generation (Klionsky, Abdalla *et al.* 2012). Mitochondrial network dynamics is a balance between biogenesis of new mitochondrial and autophagy of damaged organelles; this balance controls the quality of mitochondrial population. Hence, in this part of the work we have evaluated (in progress) the role of CORM-A1 in the activation of mitophagy, although these results are still preliminary results, requiring further development.

### 5.2.1 CORM-A1 apparently promotes mitophagy activation by BNIP3 expression

Mytophagy was assessed by western blot analysis of the total extract of cells, following the expression of BNIP3, in presence of CORM-A1 and HCQ. Moreover, BNIP3 (also known as BNIP3L) is a BH3-only pro-apoptotic protein, known for being associated with mitophagy, as well as with hypoxia-induced autophagy, by HIF-1 activation (Bellot, Garcia-Medina *et al.* 2009; Lee, Ryter *et al.* 2011). Nevertheless, the activation of mitophagy and its biochemical pathways are not yet well described, in particular in mammals.

Therefore, for the evaluation of mitophagy in primary culture of astrocytes, cells were incubated with CORM-A1 at final concentration of 12,5 and 25µM along 30 minutes and 1hour, as described in the section of material and methods, for autophagy induction.

According to the figure 5.5, CORM-A1 promotes the expression of BNIP3 in both concentrations and times. Although, the expression varies according to time, for 30 minutes the increase of expression is higher in cells treated with CORM-A1 at 25 µM, increasing with higher concentrations of CORM-A1, whereas for 1 hour treatment the expression increases is higher at 12,5 µM.

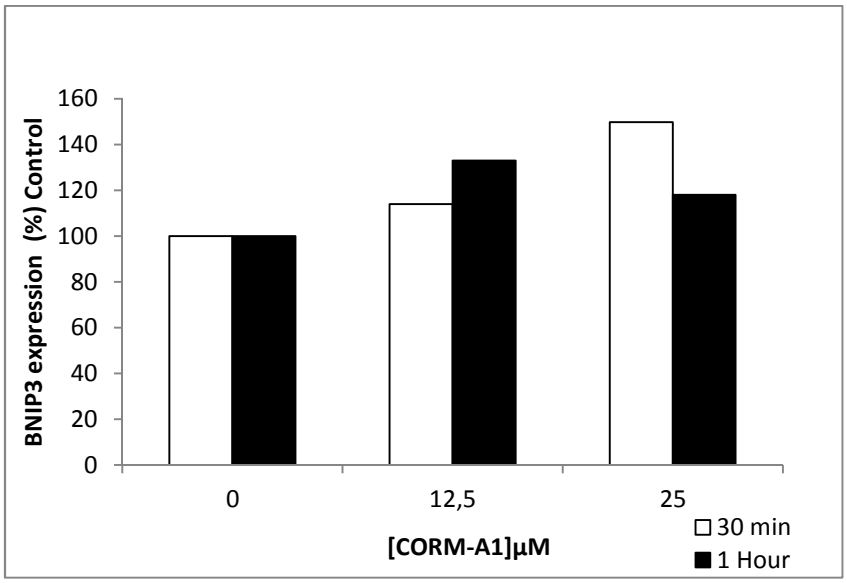


**Figure 5.5 - CORM-A1 seem to be involved in mitophagy induction in astrocytes.** Primary culture of astrocytes were pretreated with 12,5 and 25µM of CORM-A1 along 1h. The measurement of mitophagy induction was assessed western blot, n=2.

In summary, CORM-A1 increased BNIP3 expression in astrocytes, which indicates that there might be involved mitophagy stimulation. Nevertheless, different approaches must be performed for confirming CORM-A1-induced mitophagy in astrocytes.

### 5.2.2 The role of CORM-A1 in mitophagy

The main experimental approach used for assessment of mitophagy is immunocytochemistry, by the detection of co-localization between mitochondrial markers (such anti-cytochrome c or MitoTracker dye) with autophagosomal markers (such as anti-LC3II labeling). In Figure 5.6 some preliminary pictures of astrocytes marked for mitochondria are represented, as well as autophagosomes.

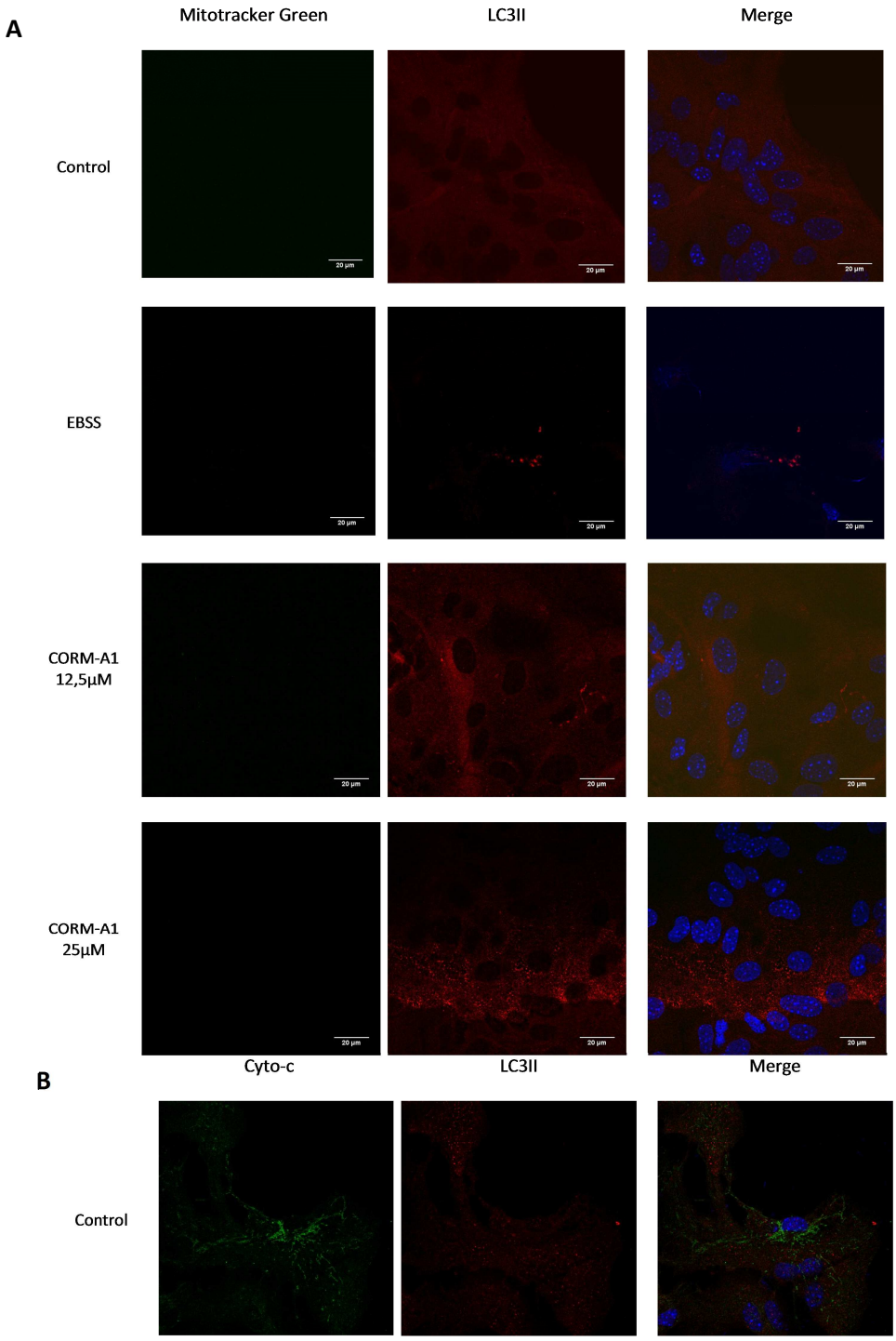


**Figure 5.6 – Optimization of the assay to measure *co-localization of autophagy/mitophagy in* astrocyte primary cultures**. A) Double immunostaining with LC3 and mitochondria (mitotracker) in astrocyte primary cultures. Astrocytes were immunostained with LC3-B (red) and mitotracker (green). B) Double immunostaining with LC3 and mitochondria (Cyto-c) in astrocyte primary cultures. Astrocytes were immunostained with LC3-B (red) and Cyto-c (green). Magnification 63x.

Mitotacker for marking mitochondria was too weak, thus no conclusions were taken for co-localization between mitochodnria and autophagosomes. While the use of anti-cytochrome c (anti-COX) immunocytochemistry is under progress (Figure 5.6B). Further experiments are needed for confirming mitophagy involvement in CO-stimulated autophagy using anti-COX.

### 5.2.3 The role of CORM-A1 in mitochondrial biogenesis

The main experimental approach used for assessment of mitochondrial population is Q-PCR. In Figure 5.7 some preliminary of quantification of mitochondria population are represented.

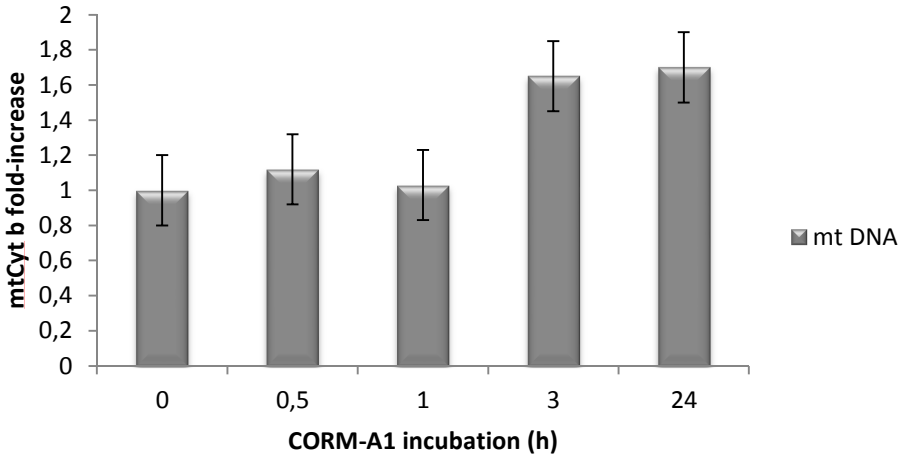


**Figure 5.7 – Effect of CORM-A1 on cytochrome c oxidase activity and mitochondria biogenesis.** Preliminary assay. Primary culture of astrocytes were treated with CORM-A1 at 12,5µM along 0; 0,5; 1; 3; 24 hours.

Accordingly to previous data published by the lab (Almeida, Queiroga et al. 2012), showing that CO increases mitochondrial population after 3 and 24h of treatment, CORM-A1 seems to respond in the same way. However, at 30 min and 1h no signs of decrease on mitochondrial population was observed, which would be expected in the case an increase on mitophagy occurs. Likewise, more experiments are need for confirming this result, as well as other kinetics must be performed.

## 5.3 The role of oxygen in CORM-A1 action

This work was developed at Dr Motterlin's lab (Univesité Paris-Est, France) under the scope of COST (COST-STSMECOST-STSM-BM1005-010413-024687) for 2 months, where we add as aim study the protective effect of CORM-A1, under hypoxia.

Once CO competes with molecular oxygen in regulating mitochondrial bioenergetics and functioning, we were interested in studying the cytoprotective role of CO under hypoxia. In these conditions we were able to mimic the physiological oxygen concentration conditions, since the level of oxygen in the organism is between 3-8%, allowing working closely to the *in vivo* environment.

In the previously points of study we verified that CORM-A1 promotes a protective effect against cell death by inducing autophagy. On the other hand, we have now some starting evidences that CORM-A1 induces also mitophagy. Indeed, some studies postulate that BNIP3 expression is activated in response to hypoxia by the expression of HIF-1 (Boya, Reggiori *et al.* 2013). Also cells can maintain minimal levels of mitochondrial respiration to produce ATP under hypoxia, displaying an increase of

autophagy, mitophagy and intermediates resulting from protein and lipid catabolism (Boya, Reggiori *et al*. 2013). Therefore this point of work is a preliminary assay for future studies, where is very important to understand the interaction of CO and oxygen for heme group proteins and the pathway that is triggered in astrocytes, for promoting the protective effect. In summary, hypoxia might also play a role in the axis CO-autophagy-cytoprotection in astrocytes.

### 5.3.1 Cytoprotective effect of CORM-A1 in astrocytes is maintained at low concentration of oxygen, 5%$O_2$

Our previous data showed that at 21% of oxygen, the CO's cytoprotective role was related to autophagic pathways. Herein, we were interested in evaluating the potential cytoprotective effect of CORM-A1 at 5% of oxygen, and its potential dependence on autophagy. Therefore, we used primary culture of astrocytes, which was maintained at 5% of oxygen for one week before starting the assay. The assay consisted in culturing cells in 24 well plates at different conditions; control, CORM-A1-treated cells and Wortmannin-treated cells for blocking autophagy (inhibitor of autophagy). After the treatment with CORM-A1 and Wortmannin, cell death was induced with *t*-BHP and the cell viability was measure after 18 hours in Flow Cytometry, with two different dyes, DiOC and PI, using the same protocol as for 21% of oxygen.

The result observed at 5% of oxygen haves showed a partial protection conferred by CORM-A1. As it is possible to observe in the figure 5.8, for higher concentration for *t*-BHP there are less cell death when compared to normoxia values (section 5.1.1), meaning that astrocytes seem to be more resistant.

What concerns to autophagy induction is observed lower levels of reversion for CO-induced prevention of cell death in the presence of WM, indicating that cytoprotection under hypoxia probably is less dependent on autophagy process.

It is pure speculation since more experiments must be done for taking further conclusions and confirming these speculative observations.

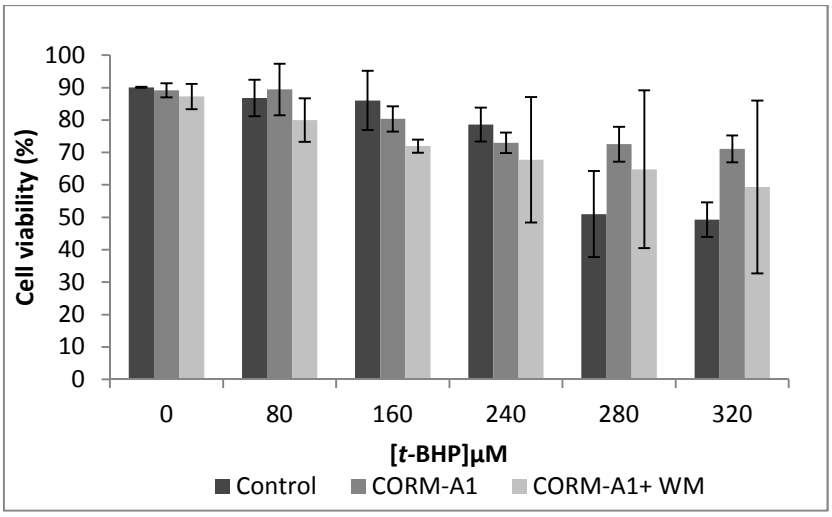


**Figure 5.8 – CORM-A1 confers protection against apoptosis by inducing autophagy at low levels of 0xygen.** Preliminary data of primary culture of astrocytes were pretreated with 12,5µM of CORM-A1 along 1h, following cell death induction by 18h of exposure to the pro-oxidant, t-BHP (from 0 to 320µM). The measure of cell viability was assessed by flow cytometry. Percentage of cells presenting high mitochondrial potential (detected by DiOC6(3)) and containing intact plasma membrane (assessed by propidium iodide). All the values are mean±SD, n=2.

Finally, taking all results together until this moment, i) cytoprotective effect of CORM-A1; ii) Reduce of cell viability with WM under both conditions of oxygen; iii) inducing of autophagy by CORM-A1; iii) inducing of mitophagy, we hypothesized that the signaling mechanism behind the protective role of CORM-A1 is ROS.

# V.Discussion

*The window for the future, where the spirit raises the body…*

Brain is a dynamic and interconnected network of cells and information. The most abundant cells inboard the CNS are astrocytes (Roberts *et al.* 2004; Halassa, Fellin *et al.* 2009). Currently, it is known that astrocytic dysfunction might cause neuronal irreversible loss contributing to neurodegenerative process. There are direct evidence that show that dysfunction of astrocytes triggers neurobiological disorders, as Alzheimer's and Parkinson's disease and epilepsy (Allaman, Bélanger *et al.* 2011). Therefore, there is a need of focusing the therapeutic strategies also into the astrocytic field, complementing the current therapies and research lines, which target mainly neuronal cell type.

Carbon monoxide (CO) is emerging as a possible therapeutic molecule, based on the protective effects largely described, having mitochondria as one of the main targets. Beyond that it is an endogenously produced gaseoustrasmitter. One of the novelties of the present work is the use of a CO-release molecule, CORM-A1. This study is the first to demonstrate that CORM-A1 confers protection against cell death in primary culture of astrocytes, mimicking CO gas (Queiroga, Almeida *et al.* 2010; Almeida, Queiroga *et al.* 2012).

Therefore, *in vitro* approaches –primary culture of astrocytes - were performed to address the role of CORM-A1 in autophagy and mitophagy induction.

Autophagy is an autodigestive process, degrading cellular organelles and proteins, plays an important role in maintenance of cellular homeostasis during environmental stress. It is involved in many physiological processes, promoting the adaptation of cell to starvation, cell differentiation and development, tumor suppression, innate and adaptative immunity, lifespan extension and cell death (Boya, Reggiori *et al*. 2013). In 2011, Lee and colleagues, showed by the first time in epithelial cell culture of mice lung, that autophagy can be regulated by CO exposure (Lee, Ryter *et al.* 2011). After this, in 2013, the same group demonstrated in sepsis that CO is anti-inflammatory and this effect was dependent on Beclin 1, a protein involved in autophagy control and its cross-talk with apoptosis (Bcl-2-Benclin 1 complex). Taking these in consideration, we decide that autophagy is a point of interest in our model of study.

The current study showed that CORM-A1 induces autophagy **(figure 5.3, section 5.1.2)** and autophagy is involved in the anti-apoptotic effect of CORM-A1, **(figure 5.4, section 5.1.3).**

These results bring relevant points for the utilization of CO-releasing molecules as a novel source of CO, namely, concerning the efficiency and safety of their utilization. Proving, that CORM's are safe and efficient source of CO and producing similar effects to the ones already obtained with CO gas. Indeed, the cross-talk between autophagy and apoptosis is linked with the formation of the complex Bcl-2–Beclin1. Correlating this with the previous studies carried out by the group, where it was demonstrated that CO increasing Bcl-2 expression (Bellot, Garcia-Medina *et al.* 2009; Queiroga, Almeida *et al*. 2010; Almeida, Queiroga *et al.* 2012). We postulate that CORM-A1 increases the expression of Bcl-2, increases the formation of the complex Bcl-2–Beclin1 and thus increases mitophagy. So, for the future prospects it will be

important to study Bcl-2 in CO-induced cytoprotection by autophagy stimulation. Furthermore, it is also crucial as future further experiment, the use of genetic inhibition of autophagy, such as knockout models for Atg5 protein, for confirming the role of autophagy in CO's cytoprotection.

Mitophagy is a specific process of autophagy, known as a quality control of mitochondria, which is important for removing damaged mitochondrias to prevent the accumulation of ROS (Levine; Kroemer *et al.* 2008; Kroemer, Mariño *et al*. 2010). As described in the section 3.2 of the introduction, the most known receptors of mitophagy are, BNIP3, BNI3L (NIX), PINK 1 and PARKIN. What concerns to this point, we showed the results corresponding to BNIP3 expression under normoxia. The other proteins are still under optimization process. The preliminary results show that CORM-A1 seems to induce mitophagy by increasing the expression of BNIP3 **(figure 5.5, section 5.2.1)**. Thus, for the future we have a great interest in assessing the other molecules, and in studying mitophagy under hypoxia conditions, for BNIP3 and the others. Still, co-localization of mitochondrial and autophagosomes is another approach that must be optimized and finished for confirming mitophagy induction of CO (preliminary data - **figure 5.6, section 5.2.2).**

Because i) CO competes with oxygen to bind to heme group (Motterlini *et al.* 2010); ii) The physiological levels of oxygen are lower that 21%, being approximately between 0,3 and 5%, known as hypoxia; the action of oxygen on CO effect is extremely important to study. Thus, we decide to test our hypothesis under physiological conditions, meaning under hypoxia, using a 5% oxygen chamber. We have shown that CORM-A1 under hypoxia protects astrocytes against cell death in the same levels of at normoxia, **figure 5.8, section 5.3.1**. In addition, hypoxia promotes the expression of HIF-1 (Bellot, Garcia-Medina *et al*. 2009; Kroemer, Mariño *et al*. 2010) which in turn induce the expression of BNIP3 (Bellot, Garcia-Medina *et al.* 2009) and induce autophagy (Boya, Reggiori *et al.* 2013). Moreover, it is known that hypoxia promotes the generation of ROS at low levels. Likewise, the inhibition of ROS generation decreases anti-apoptotic effect of CO, working as signaling molecule (involved in protective pathways) (Vieira, Queiroga *et al.* 2008; Queiroga, Almeida *et al.* 2010) and low levels of ROS also induce autophagy (Lee, Ryter *et al.* 2011). Therefore, we intend to test whether under hypoxia CORM-A1 induces autophagy and in particular mitophagy One of the possible explanation, is that the signaling mechanism of CORM-A1-induced autophagy and cytoprotection in astrocytes is ROS-signaling dependent; there are other models where this effect was demonstrated (Lee, Ryter *et al*. 2011). Thus as future work ROS generation will be measured in this model, as well as the use of anti-oxidants will permit understanding the importance of ROS signaling.

It is known that the hypoxia-induced autophagy depends on hypoxia-inducible factor, HIF-1 α, which activates transcription of BNIP3 and BNIP3L (NIX), proteins that are responsible for disrupting Beclin-1-Bcl-2 interaction. Under normoxia BNIP3 seems to reduce the half-life of BNIP3L; however, co-expression of these two proteins triggers autophagy under normoxia. While, under hypoxia it is essential the expression of both proteins to activate autophagy.

Anyway, the process of autophagy regulation of these proteins: BNIP3 and BNIP3L is still a matter of debate (Bellot, Garcia-Medina *et al.* 2009). Therefore, as future work, it will be interesting to evaluate ROS generation and signaling in CORM-A1-induced autophagy/mitophagy under normoxia and hypoxia, and the role of BNIP3 and BNIP3L.

This thesis presents several indications that CORM-A1 can be a potential therapeutic agent against astrocytic injury and cell death. In conclusion, here we proved that CORM-A1 is a cytoprotective agent against cell death in primary culture of astrocytes, under normoxia and hypoxia. Autophagy is involved in the anti-apoptotic effect of CORM-A1 under normoxia and under hypoxia. And last, this work has pointed out some clues about mitophagy induction in the anti-apoptotic role of CORM-A1.
This master thesis has opened many research lines and questions for future work.

# VI. References

Allaman, I., M. Bélanger, et al. (2011). "Astrocyte–neuron metabolic relationships: for better and for worse." Trends in neurosciences **34**(2): 76-87.

Almeida, S. S., C. S. Queiroga, et al. (2012). "Carbon monoxide modulates apoptosis by reinforcing oxidative metabolism in astrocytes: role of Bcl-2." Journal of Biological Chemistry.

Bellot, G., R. Garcia-Medina, et al. (2009). "Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains." Molecular and cellular biology **29**(10): 2570-2581.

Bhatia-Kiššová, I. and N. Camougrand "Mitophagy: A process that adapts to the cell physiology." The International Journal of Biochemistry & Cell Biology(0).

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

Boya, P., F. Reggiori, et al. (2013). "Emerging regulation and functions of autophagy." Nature cell biology **15**(7): 713-720.

Clark, I. E., M. W. Dodson, et al. (2006). "Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin." Nature **441**(7097): 1162-1166.

Clark, J. E., P. Naughton, et al. (2003). "Cardioprotective actions by a water-soluble carbon monoxide–releasing molecule." Circulation research **93**(2): e2-e8.

Cui, T., C. Fan, et al. (2011). "Silencing of PINK1 induces mitophagy via mitochondrial permeability transition in dopaminergic MN9D cells." Brain Research **1394**(0): 1-13.

Gomes, L. C. and L. Scorrano "Mitochondrial morphology in mitophagy and macroautophagy." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research(0).

González-Polo, R.-A., P. Boya, et al. (2005). "The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death." Journal of cell science **118**(14): 3091-3102.

Halassa, M. M., T. Fellin, et al. (2007). "The tripartite synapse: roles for gliotransmission in health and disease." Trends in Molecular Medicine **13**(2): 54-63.

Halassa, M. M., T. Fellin, et al. (2009). "Tripartite synapses: roles for astrocytic purines in the control of synaptic physiology and behavior." Neuropharmacology **57**(4): 343-346.

Halassa, M. M., T. Fellin, et al. (2007). "Synaptic islands defined by the territory of a single astrocyte." The Journal of Neuroscience **27**(24): 6473-6477.

Hansen, T. E. and T. Johansen (2011). "Following autophagy step by step." BMC biology **9**(1): 39.

Idriss, N. K., A. D. Blann, et al. (2008). "Hemoxygenase-1 in cardiovascular disease." Journal of the American College of Cardiology **52**(12): 971-978.

Imai, Y. and B. Lu (2011). "Mitochondrial dynamics and mitophagy in Parkinson's disease: disordered cellular power plant becomes a big deal in a major movement disorder." Current Opinion in Neurobiology **21**(6): 935-941.

Izhikevich, E. M. (2007). Dynamical Systems in Neuroscience: The Geometry of Excitability and Bursting. Cambrige, London.

Jisun, L., G. Samantha, et al. (2012). "Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling." Biochemical Journal **441**(2): 523-540.

Kanki, T., K. Wang, et al. (2009). "Atg32 Is a Mitochondrial Protein that Confers Selectivity during Mitophagy." Developmental Cell **17**(1): 98-109.

Kim, I., S. Rodriguez-Enriquez, et al. (2007). "Selective degradation of mitochondria by mitophagy." Archives of biochemistry and biophysics **462**(2): 245-253.

Kirkin, V., D. G. McEwan, et al. (2009). "A role for ubiquitin in selective autophagy." Molecular cell **34**(3): 259-269.

Klionsky, D. J., F. C. Abdalla, et al. (2012). "Guidelines for the use and interpretation of assays for monitoring autophagy." Autophagy **8**(4): 445-544.

Kroemer, G., G. Mariño, et al. (2010). "Autophagy and the integrated stress response." Molecular cell **40**(2): 280-293.

Lancel, S., D. Montaigne, et al. (2012). "Carbon monoxide improves cardiac function and mitochondrial population quality in a mouse model of metabolic syndrome." PLoS ONE **7**(8): e41836.

Lee, S.-J., S. W. Ryter, et al. (2011). "Carbon monoxide activates autophagy via mitochondrial reactive oxygen species formation." American Journal of Respiratory Cell and Molecular Biology **45**(4): 867.

Levine, B. and G. Kroemer (2008). "Autophagy in the pathogenesis of disease." Cell **132**(1): 27-42.

Mahan, V. L. (2012). "Neurprotective, neurotherapeutic, and neurometabolic effects of carbon monoxide." Medical gas research **2**(1): 32.

Mizushima, N. (2007). "Autophagy: process and function." Genes & Development **21**(22): 2861-2873.

Mizushima, N., B. Levine, et al. (2008). "Autophagy fights disease through cellular self-digestion." Nature **451**(7182): 1069-1075.

Mizushima, N., T. Yoshimori, et al. (2010). "Methods in mammalian autophagy research." Cell **140**(3): 313-326.

Motterlini, R., J. E. Clark, et al. (2002). "Carbon monoxide-releasing molecules characterization of biochemical and vascular activities." Circulation research **90**(2): e17-e24.

Motterlini, R., P. Sawle, et al. (2005). "CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule." The FASEB journal **19**(2): 284-286.

Motterlini, R. a. L. E. O. (2010). "The therapeutic potential of carbon monoxide." Nat Rev Drug Discov **9(9)**: 728-743. .

Novak, I., V. Kirkin, et al. (2009). "Nix is a selective autophagy receptor for mitochondrial clearance." EMBO reports **11**(1): 45-51.

Ozaki, K. S., S. Kimura, et al. (2012). "Use of carbon monoxide in minimizing ischemia/reperfusion injury in transplantation." Transplantation Reviews **26**(2): 125-139.

Ozaki, K. S., S. Kimura, et al. (2012). "Use of carbon monoxide in minimizing ischemia/reperfusion injury in transplantation." Transplantation reviews (Orlando, Fla.) **26**(2): 125-139.

Poole, A. C., R. E. Thomas, et al. (2010). "The Mitochondrial Fusion-Promoting Factor Mitofusin Is a Substrate of the PINK1/Parkin Pathway." PLoS ONE **5**(4): e10054.

Queiroga, C. S., A. S. Almeida, et al. (2010). "Glutathionylation of adenine nucleotide translocase induced by carbon monoxide prevents mitochondrial membrane permeabilization and apoptosis." Journal of Biological Chemistry **285**(22): 17077-17088.

Queiroga, C. S., A. S. Almeida, et al. (2012). "Carbon monoxide targeting mitochondria." Biochemistry Research International **2012**.

Queiroga, C. S., S. Tomasi, et al. (2012). "Preconditioning Triggered by Carbon Monoxide (CO) Provides Neuronal Protection Following Perinatal Hypoxia-Ischemia." PLoS ONE **7**(8): e42632.

Quinsay, M. N., Y. Lee, et al. (2010). "Bnip3 mediates permeabilization of mitochondria and release of cytochrome c via a novel mechanism." Journal of Molecular and Cellular Cardiology **48**(6): 1146-1156.

Rabinowitz, J. D. and E. White (2010). "Autophagy and metabolism." Science Signaling **330**(6009): 1344.

Roberts, J. H. B. J. L. (2004). From Molecules to Nerworks. San Diego, California, Elsevier.

Ryter, S. W., J. Alam, et al. (2006). "Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications." Physiological reviews **86**(2): 583-650.

Ryter, S. W. and A. M. Choi (2013). "Carbon monoxide in exhaled breath testing and therapeutics." Journal of breath research **7**(1): 017111.

Sagduyu K. (2002). "Neuropsychopharmacology: The Fifth Generation of Progress." American Journal of Psychiatry **159**(7): 1251-a-1252.

Scherz-Shouval, R. and Z. Elazar (2011). "Regulation of autophagy by ROS: physiology and pathology." Trends in biochemical sciences **36**(1): 30-38.

Scherz-Shouval, R., E. Shvets, et al. (2007). "Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4." The EMBO journal **26**(7): 1749-1760.

Shiba-Fukushima, K., Y. Imai, et al. (2012). "PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy." Scientific reports **2**.

Sjöstrand, T. (1949). "Endogenous formation of carbon monoxide in man under normal and pathological conditions." Scandinavian Journal of Clinical & Laboratory Investigation **1**(3): 201-214.

Smith, H., B. E. Mann, et al. (2011). "The carbon monoxide-releasing molecule, corm-3 (ru (co) 3cl (glycinate)), targets respiration and oxidases in campylobacter jejuni, generating hydrogen peroxide." IUBMB life **63**(5): 363-371.

Sofroniew, M. V. and H. V. Vinters (2010). "Astrocytes: biology and pathology." Acta neuropathologica **119**(1): 7-35.

Son, E., J. Jeong, et al. (2005). "Sequential induction of heme oxygenase-1 and manganese superoxide dismutase protects cultured astrocytes against nitric oxide." Biochemical Pharmacology **70**(4): 590-597.

Verma, A., D. J. Hirsch, et al. (1993). "Carbon monoxide: a putative neural messenger." science-new york then washington- **259**: 381-381.

Vieira, H. L., C. S. Queiroga, et al. (2008). "Pre-conditioning induced by carbon monoxide provides neuronal protection against apoptosis." Journal of neurochemistry **107**(2): 375-384.

Vilhardt, F. (2005). "Microglia: phagocyte and glia cell." The International Journal of Biochemistry & Cell Biology **37**(1): 17-21.

Vives-Bauza, C. and S. Przedborski (2011). "Mitophagy: the latest problem for Parkinson's disease." Trends in Molecular Medicine **17**(3): 158-165.

Xie, Z. and D. J. Klionsky (2007). "Autophagosome formation: core machinery and adaptations." Nature cell biology **9**(10): 1102-1109.

Yang, Z. and D. J. Klionsky (2010). "Eaten alive: a history of macroautophagy." Nature cell biology **12**(9): 814-822.

Yang, Z. and D. J. Klionsky (2010). "Mammalian autophagy: core molecular machinery and signaling regulation." Current Opinion in Cell Biology **22**(2): 124-131.

Yen, W.-L., T. Shintani, et al. (2010). "The conserved oligomeric Golgi complex is involved in double-membrane vesicle formation during autophagy." The Journal of cell biology **188**(1): 101-114.

Yorimitsu, T. and D. J. Klionsky (2005). "Autophagy: molecular machinery for self-eating." Cell Death & Differentiation **12**: 1542-1552.

Youle, R. J. and D. P. Narendra (2010). "Mechanisms of mitophagy." Nature reviews Molecular cell biology **12**(1): 9-14.

Zhang, J. and P. A. Ney (2009). "Role of BNIP3 and NIX in cell death, autophagy, and mitophagy." Cell Death & Differentiation **16**(7): 939-946.

Zhang, Y. Y., X. M. Meng, et al. (2003). "Studies on the synthesis and spectra characteristics of stilbenylcoumarin organic materials." Dyes and Pigments **56**(3): 189-194.