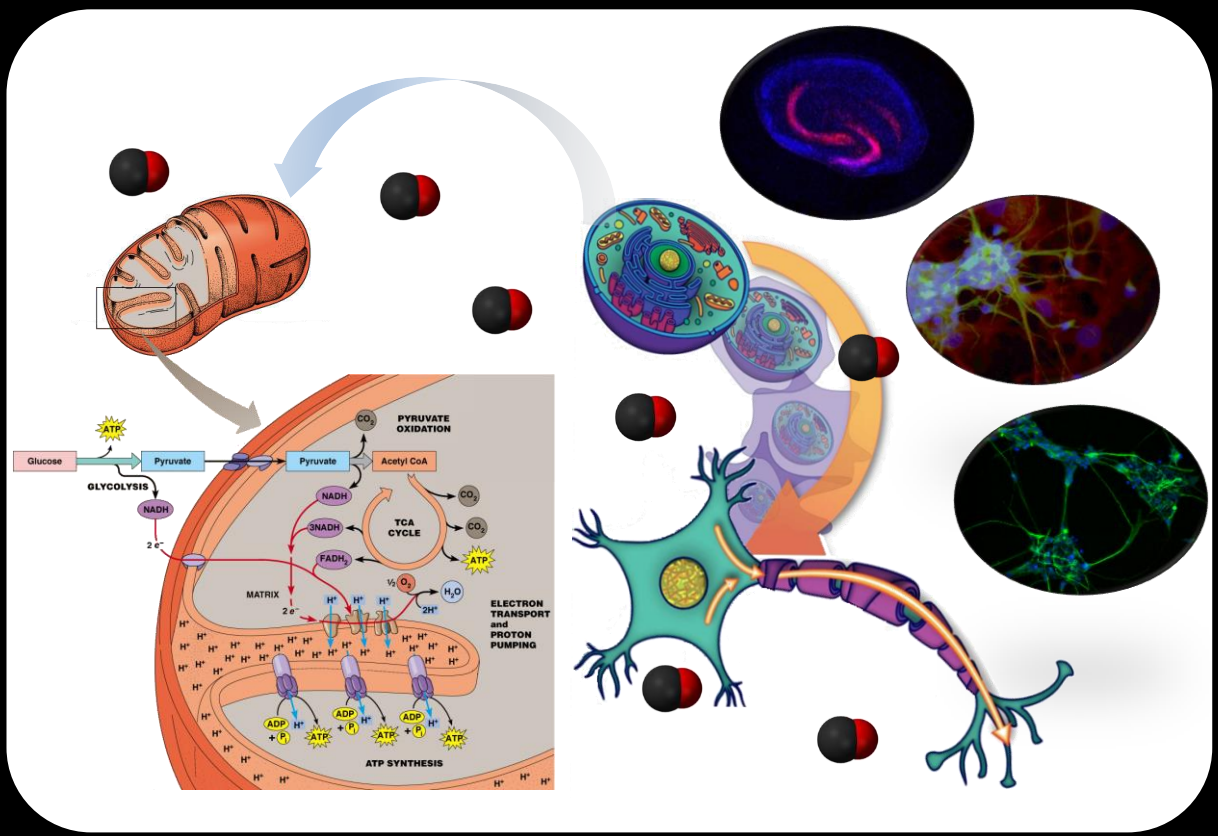
Carbon Monoxide modulation of neuronal differentiation

Disclosing cellular mechanisms

Ana Sofia Cabral e Sousa de Almeida



Dissertation presented to obtain the Ph.D degree in Biology, Neuroscience

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
November, 2016

UNIVERSIDADE NOVA DE LISBOA

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FOREWORD

The present thesis dissertation is the result of four years of research at the Cell Death and Disease Laboratory at Centro de Doenças Crónicas – Faculdade de Ciências Médicas, Universidade Nova de Lisboa (Lisboa, Portugal) and at Animal Cell Technology Unit of Instituto de Tecnologia Química e Biológica – Universidade Nova de Lisboa (Oeiras, Portugal), under the supervision of Dr. Helena L. A. Vieira.

This thesis aims at improving adult neurogenesis yield, using CO as modulator of cellular and biochemical pathways.

Ao Ricardo

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ABSTRACT

Several evidences support carbon monoxide (CO) for modulating cellular differentiation, in particular for neuronal cells. First, there are some reported studies documenting the biogenesis of mitochondria during spontaneous cell differentiation and it was demonstrated that CO promotes mitochondrial biogenesis. Secondly, ROS are signalling molecules in CO-induced pathways and are also key-players in neuronal differentiation. Third, CO has been described as anti-proliferative molecule in different cell types, namely smooth muscle, cancer and T cells, which can be involved in the balance between differentiation and proliferation that occurs during neurogenesis. Therefore, CO presents a strong potential for modulating neuronal differentiation, opening windows for the development of novel cell therapy strategies for neurological disorders. Thus, the main goal of this PhD thesis was to improve adult neurogenesis yield, using CO as modulator of cellular and biochemical pathways. In **Chapter I** one can find a general introduction about adult neurogenesis and cellular processes that directly modulate this process.

During regeneration process and adult neurogenesis, all precursor cells must maintain the capacity to self-renew and a positive balance of proliferation versus differentiation or death. Thus, in **Chapter II**, CO effect in proliferation, differentiation and cell death during neuronal differentiation was studied using three different

models with increasing complexity: human neuroblastoma SH-S5Y5 cell line, human teratocarcinoma NT2 cell line and hippocampal organotypic slice cultures (HOSC). Here it was proved that CO improves neurogenesis by preventing cell death. In **Chapter III**, hMVbcl-X_L cell line was used to assess other mechanisms of CO for modulating neuronal differentiation besides prevention of cell death. The cell model was generated from human fetal ventral mesencephalic region, presenting genuine characteristics of their regional identity, which discharged the need of complex patterning procedures, however these cells over-express the anti-apoptotic protein BclX_L. Thus, this model allowed to understand that, besides cell death protection, CO was promoting a metabolic modulation during the process of neuronal differentiation. Therefore, in **Chapter IV,** NT2 cell line was used to deeper understand the effect of CO in metabolism modulation, namely the shift from glycolysis to oxidative metabolism. In fact, it was observed that CO stimulated neuronal production by promoting oxidative metabolism over glycolysis. Finally, one cannot disregard that during neurogenesis there is the need of building blocks such as nucleotides, electron donors like NADPH and antioxidant defenses, obtained by glutathione recycling. Thus, in **Chapter V**, CO modulation of pentose phosphate pathway and glutathione metabolism was assessed using SH-SY5Y cell line.

In **Chapter VI** is presented a general discussion that integrates this thesis' results in the current scientific context. In conclusion, this

thesis highlights biochemical modulation of neurogenesis and reveals CO as a promising therapeutic molecule to improve neuronal production for cell therapy strategies.

RESUMO

Várias evidências suportam que exista modulação da diferenciação celular pelo monóxido de carbono (CO), em particular para as células neuronais. Em primeiro lugar, alguns estudos documentam a biogénese de mitocôndrias durante a diferenciação de células e demonstrou-se que o CO promove a biogénese mitocondrial. Em segundo lugar, as espécies reactivas de oxigénio são moléculas de sinalização nas vias induzidas pelo CO e também são fulcrais na diferenciação neuronal. Em terceiro lugar, o CO foi descrito como molécula anti-proliferativa em diferentes tipos de células, nomeadamente músculo liso, células cancerígenas e células T, pelo que pode estar envolvido no equilíbrio entre a proliferação e diferenciação que ocorre durante a neurogénese. Portanto, o CO apresenta um forte potencial para modular a diferenciação neuronal, surgindo oportunidades para o desenvolvimento de novas estratégias de terapia celular para doenças neurológicas. Assim, o principal objetivo desta tese de doutoramento foi para melhorar o rendimento da neurogénese adulta, usando CO como modulador de vias celulares e bioquímicas. No **Capítulo I** encontra-se uma introdução geral sobre a neurogénese em adultos e os processos celulares que modulam directamente este processo.

Durante a regeneração e neurogénese em adultos, todas as células precursoras mantêm a capacidade de auto-renovação e um

saldo positivo de proliferação relativamente a diferenciação ou morte. Assim, no **Capítulo II**, o efeito do CO na proliferação, diferenciação e morte celular durante a diferenciação neuronal foi estudada usando três modelos diferentes com complexidade crescente: linha celular humana SH-S5Y5 isolada de neuroblastoma, linha celular humana NT2 isolada de teratocarcinoma e culturas organotípicas de hipocampo. Aqui, provou-se que o CO melhora a neurogénese por prevenção da morte celular. No **Capítulo III**, a linha celular hMVbcl-X_L foi utilizado para avaliar a modulação de outros mecanismos pelo CO durante a diferenciação neuronal, para além de prevenção de morte celular. Este modelo celular foi gerado a partir da região do mesencéfalo ventral fetal humano, apresentando características genuínas de sua identidade regional mas estas células sobre-expressam a proteína anti-apoptótica Bcl-X_L. Assim, este modelo permitiu compreender que, para além da protecção da morte celular, o CO promove uma modulação metabólica durante o processo de diferenciação neuronal. Portanto, no **Capítulo IV**, a linha celular NT2 foi usada para compreender mais profundamente o efeito do CO na modulação do metabolismo, ou seja, a transição do metbolismo glicolítico para o metabolismo oxidativo. De facto, observou-se que o CO estimulou a produção neuronal, promovendo o metabolismo oxidativo. Finalmente, há que considerar que, durante a neurogénese, há a necessidade de (i) metabolitos específicos, tais como nucleótidos e (ii) defesas antioxidantes que necessitam de

dadores de eletrões como NADPH que vai permitir a reciclagem do glutationo. Assim, no **Capítulo V**, a modulação exercida pelo CO na via de pentoses fosfato (PPP) e no metabolismo do glutationo foi avaliada utilizando linha de células SH-SY5Y.

No **Capítulo VI** é apresentada uma discussão geral que integra os resultados desta tese no contexto científico atual. Em conclusão, esta tese destaca a modulação bioquímica durante neurogénese e apresenta o CO como uma molécula terapêutica promissora para a melhoria da produção neuronal para estratégias de terapia celular.

THESIS PUBLICATIONS

1. **Almeida AS**, Soares NL, Vieira M, Gramsbergen JB, Vieira HL. Carbon Monoxide Releasing Molecule-A1 (CORM-A1) Improves Neurogenesis: Increase of Neuronal Differentiation Yield by Preventing Cell Death, PLoS One. 2016 May 4; 11(5):e0154781.

2. **Almeida AS**, Sonnewald U, Alves PM, Vieira HL. Carbon monoxide improves neuronal differentiation and yield by increasing the functioning and number of mitochondria, J Neurochem. 2016 Aug; 138(3):423-35.

LIST OF ABBREVIATIONS

Abbreviation	Full form
ANLS	Astrocyte-neuron lactate shuttle
ATCC	American type culture collection
ATP	Adenosine triphosphate
Bax	Bcl-2-like protein 4
BCA	bicinchoninic acid
Bcl2	B-cell lymphoma 2
Bcl-X_L	B-cell lymphoma-extra-large
bFGF	*Basic fibroblast growth factor*
CNS	Central nervous system
CO	Carbon monoxide
CO_2	Carbon dioxide
CORM	Carbon monoxide-releasing molecule
COX	Cytochrome c oxidase
CRABP	Cellular retinoic acid-binding protein
CYS	Cysteine
CYS-GLY	Cysteine-glycine
DAPI	4',6-diamidino-2-phenylindole

DBH	Dopamine beta-hydroxylase
DCF	2',7'-dichlorofluorescein
DG	Dentate gyrus
DIV	Day *in vitro*
DMEM-HG	Dulbecco's Modified Eagle Medium – High Glucose
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGF	*Epidermal growth factor*
ESC	Embryonic stem cell
$FADH_2$	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FI	Fold increase
GABA	γ-Aminobutyric acid
GC-MS	Gas chromatography - mass spectrometry
Glc	Glucose
Glut	Glucose transporter
GSH	Glutathione
GSSG	Glutathione disulfide (oxidized glutathione)
GTP	Guanosine triphosphate

H_2DCFDA	2',7'-dichlorofluorescein diacetate
HBSS	Hank's Balanced Salt Solution
HO	Heme oxygenase
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
IFN-γ	Interferon γ
IGF-1	Insulin-like growth factor 1
IGFBP-4	Insulin-like growth factor binding protein 4
IL-15	Interleukin 15
Lac	Lactate
LDH	Lactate dehydrogenase
MAP2	Microtubule associated protein 2
MCT2	Monocarboxylate transporter 2
mRNA	Messenger RNA
MSTFA	N-metyl-N-(trimethylsilyl)trifluoroacetamide
MTBSTFA	N-metyl-N-(tert-butyldimethylsilyl)trifluoroacetamide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide

NADPH	Nicotinamide adenine dinucleotide phosphate
NF-L	Neurofilament light chain
NSC	Neural stem cell
NT	Neurotrophin
Nurr1	Nuclear receptor related 1 protein
O_2	Molecular oxygen
OHSC	Organotypic hippocampal slice culture
PBS	Phosphate buffered saline
PCA	Perchloric acid
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase
PGDH	Phosphogluconate dehydrogenase
PI	Propidium iodide
ppm	Part per million
PPP	Pentose phosphate pathway
Q-PCR	Quantitative polymerase chain reaction
RA	Retinoic acid
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RPL22	Ribosomal Protein L22
RT	Room temperature
RT-Q-PCR	Real time quantitative polymerase chain reaction
SBD-F	Ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacriylamide
SGZ	Subgranular zone
SVZ	Subventricular zone
t-BDMS-Cl	Tert-butyldimethylchlorosilane
TCA	Tricarboxylic acid
TCEP	Tris(2-carboxyethyl)phosphine
td	Doubling time
TH	Tyrosine hydroxylase
TKT	Transketolase
VEGF	Vascular endothelial growth factor
VM	Ventral mesencephalic
WB	Western blot

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INTRODUCTION

CONTENTS

Ana S. Almeida has written the whole chapter based on the referred bibliography.

1. STEM CELLS

Embryonic and adult stem cells have the capability to produce identical copies of themselves *via* cell division (self-renewal) and the ability to generate multiple functional differentiated cells types (multipotency). Thus, stem cells can proliferate into an increasingly undifferentiated stem cell population that possesses the same potential as the parent cell, or they can originate different tissues during embryonic and postnatal development, which is crucial for tissue repair [1]–[3].

Due to their potency or the diversity of cell types stem cells can generate, different types of stem cells can be identified. The best example of potency is the zygote, however it is not generally regarded as stem cell because of their transient feature, no self-renewing capacity, despite the ability to give rise to both embryonic and extraembryonic tissues [4]. Amongst true stem cells, embryonic stem cells (ESCs), isolated from the inner mass of the blastocyst, have the broadest potential. These cells are pluripotent stem cells that can generate all cell types during embryo development. However, stem cells are not restricted to development stages. Actually, adult individuals also have a large number of tissue-specific stem cells, which are capable of generating certain cell types but not those from unrelated tissues. The best-studied tissue-specific stem cell is the hematopoietic stem cell, which generates all blood cell types. Other

tissue-specific stem cells have been identified in numerous organs including muscle, skin, gut, liver, pancreas and brain. Herein it will be targeted neural stem cells that originate several cell types from the nervous system.

1.1.Neural stem cells (NSCs) and adult neurogenesis

In the last 20 years, several reports contradicted the dogma proclaiming that no new neurons are born in the adult brain [5]. Now it is broadly accepted that neural stem cells (NSCs) are present through live in neurogenic niches. Thousands of NSCs exist on the border of the lateral ventricle and striatum of the subventricular zone (SVZ) and on subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, where they differentiate and persist for long periods of time (Fig.1.1) [6], [7]. Neurons generated in the SVZ migrate to the olfactory bulb and differentiate into interneurons. Neurogenesis in the dentate SGZ gives rise to neurons in the granule cell layer (GCL) [8].

In addition to *in vivo* studies, NSCs have been isolated from various regions of the adult brain and cultured *in vitro* in order to extensively characterize them at different developmental stages [9]–[11]. NSCs can differentiate into neurons under defined culture conditions and are able to synapse with each other or with co-cultured neurons [1], [12]–[14]. Also these cells can generate

astrocytes, and oligodendrocytes [3], [15] depending on culture conditions.

Neural stem and progenitor cells (NSCs) in the lateral ganglionic eminence (LGE) and subventricular zone (SVZ) ensure neurogenesis during embryonic development and throughout adult life, which is called adult neurogenesis. However, NSC proliferation and neuroblast formation in SVZ are decreased in aged animals [2], [16], [17].

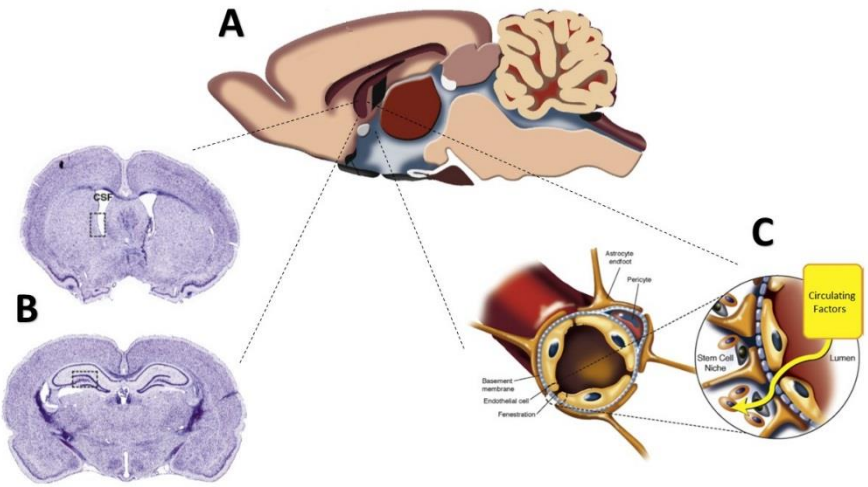


Figure 1.1 – Stem cell niches in the brain. (A) scheme of a sagittal section through adult rat brain; (B) coronal sections through adult rat brain showing the SVZ (upper panel) and SGZ of the hippocampus (lower panel); (C) left panel: enlargement of the vasculature in a niche site, depicting fenestrations between endothelial cells and gaps in the glial end feet; right panel: further enlargement at a fenestration site showing the permeability of the BBB and access of the niche to circulating blood factors (adapted from [18]).

Adult neurogenesis consists of generation of neurons from neural stem/precursor cells and occurs in specific brain regions called neurogenic zones. This process occurs in the hippocampus of several vertebrate species such as rodents [19], [20] and primates including humans [21]–[23].

At least five steps appear to be involved in the neurogenesis process: (i) proliferation of stem/progenitor cells, (ii) migration of newborn neurons, (iii) neuronal differentiation and maturation, (iv) integration into neuronal circuits and (v) survival of cells [3]. The integration of newly born neurons into the mature hippocampal network, as well as the physiological implications of this neurogenesis are far less understood. Anatomical studies have shown that the newly born neurons receive synaptic inputs on their dendritic arbors and send axonal projections toward their normal targets: the CA3 pyramidal cells [24]. Electrophysiological recordings suggest that CA3 pyramidal cells develop functional properties of mature dentate granule cells [25]. Furthermore, neurogenesis in the adult hippocampus has been correlated with learning and memory in some, but not all, hippocampus-dependent behavioral studies [1], [26], [27].

Neurogenesis process can be modulated by hormones, growth factors, neurotransmitters as well as by environmental factors and under pathologic conditions [1], [28]–[31]. Namely, EGF, IGF-1 and corticosteroids modulate neuronal production in hippocampus [1],

[28], [29], as well as scenarios of ischemia and exercise increase neurogenesis [30], [31].

1.2.Neurogenesis in pathological scenarios

Neurogenesis occurs through life, but there is a significant decline during aging [29], [32], [33]. Nevertheless, some studies with mice, rat and primates models showed that it is possible to treat old brains with infusion of growth factors [32], [34] or promoting an enriched environment [33], which stimulates the production of new neurons [2], [8], [32], [35], [36]. These strategies are particularly relevant in pathological scenarios such as ischemic stroke, seizures and neurodegenerative diseases development.

Ischemic stroke causes neuronal loss mainly in the striatum and cerebral cortex, whereas hippocampal formation is spared [37]. This injury leads to deficits in hippocampus-associated spatial memory [37]. Improvement of neurogenesis might counteract cognitive impairments and contribute to recovery of stroke-impaired motor function [8].

Following acute seizures there is an increased production of new neurons in the adult dentate gyrus (DG) by NSCs [38]. Seizure-induced enhancement of neurogenesis is decreased in aged rats [38], [39]. However, available data indicate that, despite diminished baseline levels, aged NSCs maintain the potential to respond to extrinsic cues similar to NSCs in adult animals, which is important in

the perspective of a potential future therapeutic use of neuronal replacement from endogenous NSCs in human neurodegenerative disorders [40], [41].

2. *IN VITRO* MODELS FOR NEUROGENESIS RESEARCH

The culture of NSCs is an essential tool for assessing the molecular mechanisms controlling differentiation in the nervous system [42], [43]. Human NPCs can be obtained from brain biopsy or can be differentiated from pluripotent stem cells. Regardless of their source, one of the main challenges in this field is to mimic *in vitro* neural development as similar as possible to the *in vivo* situation. Much has been done in order to confirm similarities between cells growing in dishes and cells in the *in vivo* context in the brain. Indeed, *in vitro* models have evolved as reliable tools for studying cellular and molecular aspects of neural differentiation [42], [43].

2.1.Neural Stem Cell lines

Mouse embryonic (ES) and carcinoma (EC) cell lines are *in vitro* models to study the neuronal differentiation process, allowing the assessment of the involved cellular mechanisms [44]. These immortal cell lines can be maintained and replicated keeping their

pluripotency, providing unlimited supply of cells capable of proliferating in culture for long periods and of differentiating into several cell types [44], [45], including post-mitotic neurons. Neuronal differentiation occurs upon removal of growth factors from the culture medium [46], [47] or treatment with retinoic acid (RA) [48]–[54]. RA is a derivative of vitamin A, which is essential for promoting normal patterning and neurogenesis during development. RA signaling pathway, leading to neuronal differentiation, is dependent on retinoic acid binding proteins (CRABP)-I and II, which in turn deliver RA into the nuclear RA receptors (RARs). Then RARs directly regulate the expression of specific RA-inducible genes and neuronal differentiation [45], [52]–[54].

2.1.1. hVMbcl-X_L cell line

The human ventral mesencephalic (VM) stem cell line hVMbcl-X_L was generated from a 10-week-old foetus (Lund University, Sweden) and immortalized by infecting the cells with a retroviral vector coding for *v-myc* (LTR-vmyc-SV40p-Neo-LTR), creating a multipotent cell line [46]. Derivatives of these cells were genetically modified (retrovirus) to overexpress the anti-apoptotic gene B-cell lymphoma-extra large ($BclX_L$), essentially as described by Liste *et al.* in 2007 [47]. hMVbcl-X_L cell line proliferates in the presence of growth factors (EGF and bFGF). Upon removal of growth factors, the cells readily differentiated into neurons, astrocytes and oligodendrocytes [46], [47].

hVMbcl-X_L cell line has been used in studies of microtransplantation into striatal slice cultures [55] and in validation of 3D systems that allow an efficient differentiation combined with real-time *in situ* confirmation of neuronal fate [56].

2.1.2. SH-SY5Y cell line

SH-SY5Y human neuroblastoma cells were derived from a thrice-cloned cell line, SK-N-SH [57], [58]. These cells were derived from neural crest [48], [49], [58] and represents a rapid and representative model for studying neuronal differentiation processes [50]. These cells differentiate into neuron-like cells that fulfill the morphological, biochemical and functional neuronal criteria [49], [50], [59]. Due to their neuronal characteristics, these cells have been used extensively to study neuron-like behavior [57] and constitute a valuable model for neuronal toxicity studies [60]–[63].

2.1.3. NT2 cell line

NT2 lineage is derived from human testicular embryonic teratocarcinoma and differentiates into functional post-mitotic neurons. Obtained NT2-derived-neurons express many neuronal markers [51], such as cytoskeletal proteins, secretory markers and surface markers. Also, NT2 cells express nestin and vimentin (neuroepitelial precursor cell markers), while NT2-derived-neurons

express MAP2, NF-L and α-internexin, among other neuronal specific proteins, and produce a variety of neurotransmitters, namely GABA and glutamine [64], [65]. Moreover, NT2-derived neurons can form functional synapses [66] have also been used in several transplantation studies in experimental animals [67], [68] and in human patients[69].

2.2.Ex vivo models

2.2.1. Hippocampal organotypic cell cultures

In order to increase the model complexity, organotypic cultures were used. Organotypic slice cultures are a great *in vivo*-like model for assessing cell proliferation, differentiation and migration in a tissue context. This model better mimics *in vivo* cerebral environment, which facilitates the study of new factors for improvement of neuronal differentiation [70], [71].

Continuous formation of new neurons and glial cells in some specific brain zones can be evaluated using this *in vivo*-like model of adult neurogenesis, which occurs mainly in the Subventricular zone and in the Hippocampus, specifically in both Subgranular region of the Dentate gyrus and in *Cornu Ammonis* region 1 (Fig.1.2). Contrary to what is generally thought, there is evidence that neurons can also be generated in mature brain parenchyma (cortical layer) [6], [72].

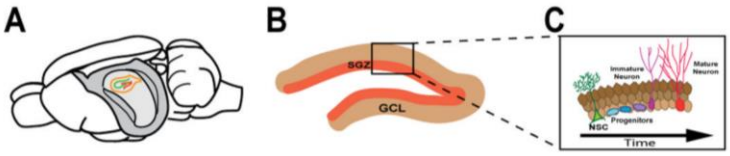


Figure 1.2 - Adult hyppocampal neurogenesis (A) Dissection of the rodent telecephalon. (B) Enlarged schematic of the dentate gyrus shows the superior and inferior blades of the granule cell layer (GCL, brown) and the subgranular zone (SGZ, red) where the hippocampal neurogenesis occurs in the postnatal period through adulthood. (C) Higher magnification of the boxed region from (B) displays the phases of adult hippocampal neurogenesis as a function of time. The neural stem cells (NSCs, green) putatively give rise to the transiently-amplifying progenitors (blue and violet) whose progeny differentiate into immature neurons (dark violet) and finally into fully mature dentate gyrus GC neurons (red) (adapted from [72]).

The organotipic brain tissue slice cultures are derived from postnatal mice and can be grown for several weeks *in vitro.* They mature into organotypically organized brain tissue with display of a basic cellular content and connective axonal network characteristic of the donor brain area *in vivo* [73].

3. CELL DEATH IN STEM CELLS NICHES

Adult stem cell proliferation and cell death appear to be coupled in many systems to control cell number, patterning and lineages [74]–[76]. Indeed, genetically modified *in vivo* models, where executor or regulatory apoptotic genes (caspase-3, caspase-9, Bak, Bax, among others) are knock out, resulted in supernumerary neurons in the brain [75]. However, the incidence and role of cell death in the different stages are yet not well understood. Despite being established that a fine-tuning of neuronal population occurs in

healthy brain and that is dependent on the balance between proliferation/differentiation and cell death[2], [75], [77]–[79], apoptosis of differentiating neurons during embryonic development has been intensively studied [74]–[76], whereas cell death affecting adult neural stem cells is much less characterized.

4. OXYGEN LEVELS, REDOX STATE AND MITOCHONDRIAL FUNCTION DURING NEUROGENESIS

Since embryonic life, oxygen (O_2) levels control crucial events such as formation of placenta, vascular system and skeleton. Furthermore, in adulthood O_2 demands, consumption and flow vary not only amongst organs but also between regions of same organ [80]–[83]. These variations influence cell fate in a gradient manner and, consequently O_2 has been considered as a morphogen, which promotes regulatory roles in diverse pathways controlling also adult neuronal differentiation [81], [84]–[86]. Actually, it was suggested that fluctuations in O_2 levels misbalances the intracellular redox control, impairing the balance between stem cell proliferation, differentiation and death [87].

The cellular redox potential depends on the balance between relative amounts of the reduced and oxidized forms of redox couples, such as $NADP^+$/NADPH, GSSG/GSH, superoxide anion radical/oxygen and hydrogen peroxide/water. It is known that the

accumulation of reactive redox species persists only during early stages of differentiation, whereas a reductive environment is acquired in terminally differentiated neurons [87], [88].

Reactive oxygen species (ROS) are key factors in neuronal differentiation since the impairment of ROS formation prevents neuronal differentiation in different *in vitro* models, such as embryonic stem cells (ESCs), teratocarcinoma stem cells and neuroblastoma cells [89]–[93]. Furthermore, growing evidences also indicate ROS as hub regulators of various processes and pathways during neurodevelopment, namely stem cell self-renewal and differentiation [90], [94]. Thus, one can speculate that the final decision whether the NSCs will proliferate or differentiate is mainly based on redox-sensitive factors [84], [87], [91], [95]–[97]. Although the exact mechanism involved in ROS-mediated neurogenesis remains unclear, ROS generation might be closely related to alterations in O_2 levels.

Besides being a result of alterations in oxygen levels, ROS can be endogenously produced by mitochondria during oxidative phosphorylation, mainly at complexes I and III, since about 1-2% of oxygen is not totally reduced into H_2O (Fig.1.6) [88], [98]. During neuronal differentiation, mitochondrial population not only increases through mitochondrial biogenesis [93], [99]–[102], but it also occurs a reprogramming of mitochondrial structure and bioenergetics: mitochondria shifts from a condensed matrix structure to the typical

functional cristae morphology [103]. Accordingly to Cho and colleagues, the modifications of mitochondrial population during differentiation might permit more cellular respiration and, therefore, an increase of cellular ATP production [93], which is needed for cell differentiation from progenitor to fully mature and functional cell. Moreover, one cannot disregard the central role of mitochondria in integrating survival and death signals in intrinsic pathways of apoptotic cell death [98].

5. BIOENERGETIC PATTERN DURING NEURONAL DIFFERENTIATION

Cell differentiation goes hand-in-hand with metabolic alterations needed to provide new bioenergetic, synthetic and catabolic requirements important for cell identity. Actually, it was showed by mass spectroscopy that, during differentiation, it occurs the modulation of several genes involved in redox hemostasis, energy metabolism, RNA processing, retinoic acid signaling and ubiquitin dependent proteolysis [104].

Stem cell differentiation is dependent on cell metabolic shifts, mitochondrial function and oxygen levels [105]–[110]. Thus, metabolism is an important indicator of cell function, since it shifts together with differentiation, growth or anabolic capacities. For example, both neuronal and hematopoietic stem cells preferentially

rely to a greater extent on glycolysis whereas differentiated cells up-regulate oxidative phosphorylation to generate ATP [111]–[114]. However, little information exists regarding stem cell metabolism during proliferation and differentiation.

5.1.Glycolysis

Glycolytic breakdown of glucose is the exclusive source of energy in some mammalian tissues and cell types, such as brain, thus this pathway assumes a central position in glucose catabolism [115].

In glycolysis, a molecule of glucose is metabolized to two molecules of pyruvate (Fig. 1.3). During the sequential conversion of a single glucose molecule into two pyruvate molecules, it is released energy that is conserved in the form of ATP and NADH. These reactions are not oxygen dependent [115], [116].

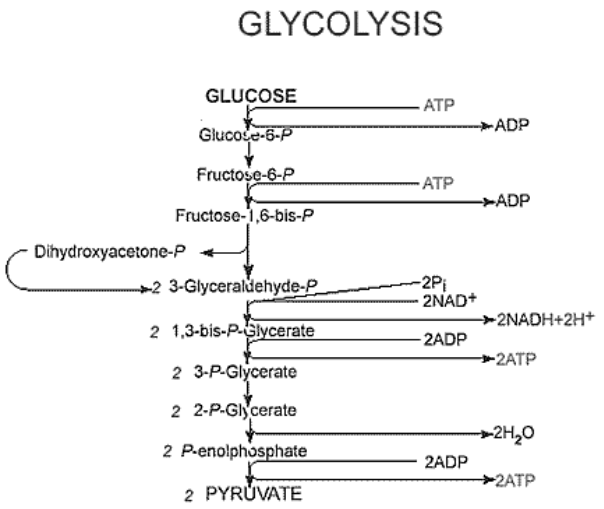


Figure 1.3 – Glycolytic pathway reactions (adapted from Metabolic MiniMaps).

In aerobic organisms or tissues, glycolysis is only the first stage of glucose degradation. The obtained pyruvate can be (reversibly) converted into lactate in the cytosol, which results in the production of NAD+ from NADH or it can be oxidized to yield the acetyl group of acetyl-coenzyme A that will be completely oxidized by the tricarboxylic acid cycle (Fig. 1.4) [115].

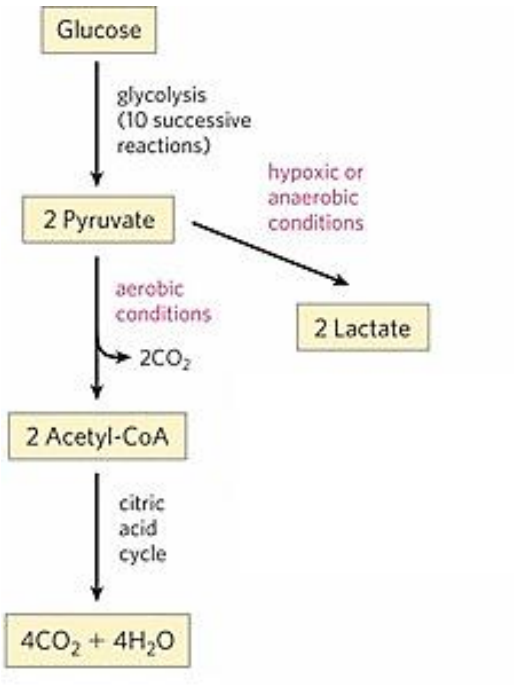


Figure 1.4 – Possible catabolic fates of the pyruvate formed in glycolysis (adapted from [115]). Glycolysis was the first metabolic pathway to be elucidated and, due to the hypoxic provenience of living organisms, anaerobic glycolysis is the probably the most ancient biological mechanism for obtaining energy [115], [117]. However, aerobic glycolysis can occur and it is a functional marker of dividing cells, whether they are stem or endothelial or tumor cells [114], [115].

5.2.Pentose Phosphate Pathway

Glucose metabolism can also have other catabolic fates, such as the particular important biosynthetic pathway termed pentose phosphate pathway (PPP), which leads to specialized products needed by the cell. PPP consists in a network of interconversion reactions between sugar phosphates and can be divided in two parts: the oxidative phase and the nonoxidative phase (Fig.1.5). The overall reaction catalyzed by the oxidative phase is the oxidation of glucose-6-phosphate to ribulose-5-phosphate and CO_2. Also, NADP+ is converted into nicotinamide adenine dinucleotide phosphate (NADPH) that acts as an important reducing agent that may participate in lipid and steroid synthesis or in the regeneration of glutathione and thioredoxin, which are involved in the cell's defense mechanism against oxidative stress. In the second phase of the PPP, 5-carbon sugars are nonoxidatively synthetized. The PPP is connected to the glycolytic pathway at the level of their common intermediates glyceraldehyde-3-phosphate and fructose-6-phosphate [115], [116], [118].

Rapidly dividing cells, like stem cells, use PPP to obtain NADPH (for maintaining an antioxidant status) and ribose-5-phosphate (for making RNA, DNA and coenzymes). Thus, during brain development PPP is extremely important. The prominent function of this pathway is supported by the increase in enzyme activity and flux rates of metabolites in the developing brain than in the adult brain [118].

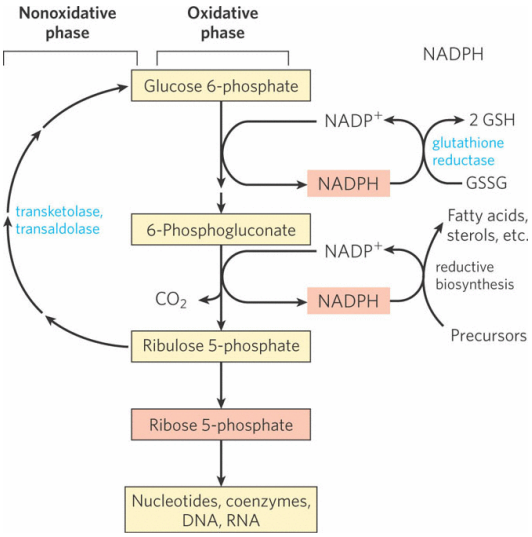


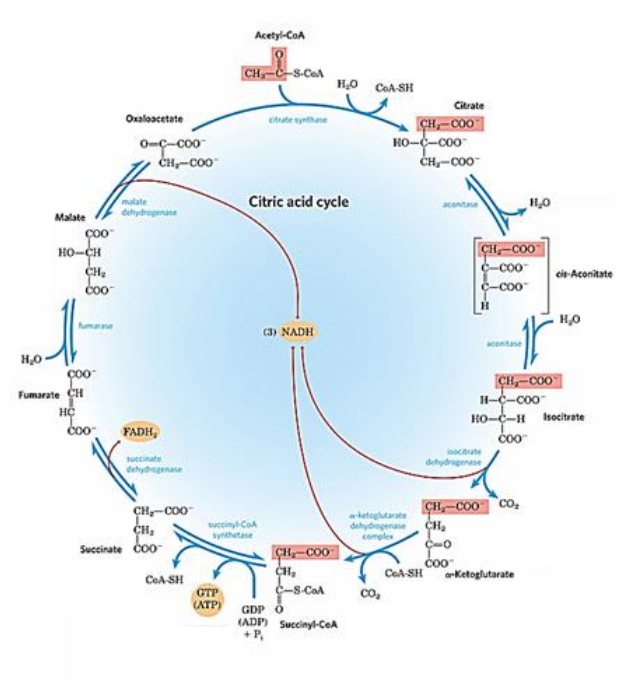
Figure 1.5 – General scheme of PPP (adapted from [115]).

5.3.TCA cycle and oxidative phosphorylation

In the presence of oxygen, the pyruvate produced by glycolysis or by the PPP can be converted to acetyl-CoA by the pyruvate dehydrogenase (PDH) complex, and subsequently metabolized in the TCA cycle (Fig. 1.6), to further produce ATP *via* coupling to the mitochondrial electron transport chain and cycle intermediates that will be precursors for a wide variety of products [115].

The oxidation of pyruvate to CO_2 in the TCA cycle generates energy-rich molecules such as GTP, NADH and $FADH_2$. The latter two will transfer electrons to oxygen in the electron transport chain, leading to the production of ATP in a process named oxidative phosphorylation (Fig. 1.6) [115], [116].

A



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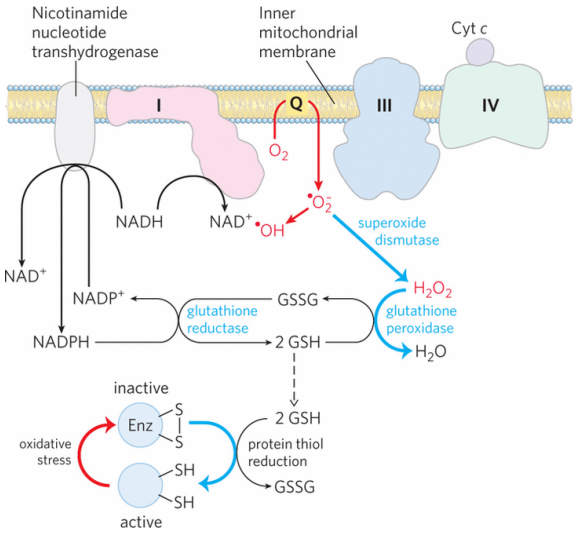

Figure 1.6 – TCA cycle and oxidative phosphorylation. (A) Reactions of the TCA cycle; (B) Mitochondrial electron-transfer reactions and coupled mechanisms of ROS formation and redox defense (adapted from [115]).

Recently, NSC dynamics studies had demonstrated that the activation of NSCs is accompanied by downregulation of glycolytic metabolism and upregulation of mitochondrial oxidation [119]. The currently accepted model defends that upon lineage differentiation, cell switch to oxidative metabolism, which is needed to support the growing energetic demands of specialized progeny. For instance, it is known that NSCs are more resistant to hypoxia and have lower requirement for oxidative metabolism but are more dependent on glycolysis than neurons [120] and that activation of NSCs is accompanied by a downregulation of glycolytic metabolism and an upregulation of mitochondrial oxidation [121], [122].

5.4.Other carbon sources

Although glucose is still considered the main substrate for brain, it is now known that lactate utilization by neurons also occurs. Actually both neurons and NPCs can survive with lactate as exclusive metabolic substrate [114], [123]. Moreover, in the developing brain lactate is a major substrate for oxidation metabolism in addition to being selectively utilized as an anabolic source for cell proliferation and differentiation. Álvarez and colleagues, showed that lactate intake and its subsequent oxidative metabolism direct progenitor commitment to a neuronal progenitor fate. These results support the

hypothesis of NSC and progenitor cells with different metabolic signatures coexisting in the neurovascular niche [114], [123].

6. CARBON MONOXIDE

Carbon monoxide (CO) is an endogenous product of heme degradation by heme oxygenase (HO), along with free iron and biliverdin (Fig. 1.7), which is rapidly converted into the anti-oxidant bilirubin [124]. Administration of CO at low concentrations produces several beneficial effects in distinct tissues, such as anti-inflammatory, anti-proliferative, vasodilator and anti-apoptotic [124], [125].

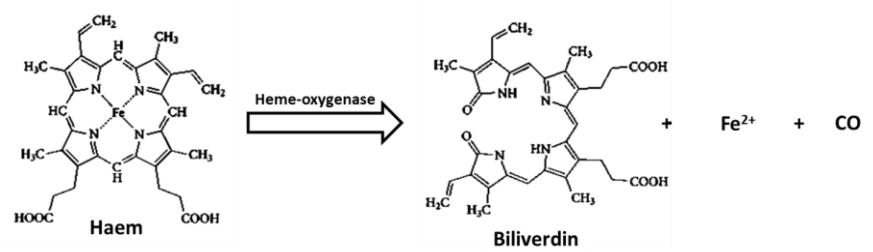

Figure 1.7 - The haem degradation pathway. Haem is catalysed into biliverdin by haem oxygenase with the release of a molecule of carbon monoxide (CO) and ferric iron.

CO is mostly known as a silent-killer due to its great affinity to hemoglobin, which compromises oxygen delivery and promotes high levels of intoxication and death. Furthermore, high concentrations of

CO are cytotoxic by inhibition of cytochrome c oxidase (COX), excessive ROS generation or uncoupling effect [126]. Nevertheless, CO is an endogenously produced gasotransmitter generated by the cleavage of heme group via the enzymatic activity of heme-oxygenase (HO) [124]. HO is a stress-related enzyme, whose expression or activity increases in response to several stresses, namely: oxidative stress, hypoxia, hyperoxia, hyperthermia, inflammation, UV, misfolded protein response, among others [124], [127], [128].

6.1.Carbon monoxide, cytoprotection and ROS signalling

Carbon monoxide has been reported as an anti-apoptotic agent in endothelial cells [129], pulmonary cells [130] and muscle [131]. Regarding the anti-apoptotic capacity of CO in the brain, it has been described in neurons and astrocytes [125], [127], [132]–[136] (for further review [137]). Although the beneficial effects of CO and its capacity to bind to transition metals, the physiological targets of this molecule are not identified. However, it is known that CO-induced cytoprotection can be dependent on generation of low amounts of ROS, which function as signaling molecules [125], [138]–[141]. Likewise, these low concentrations of CO promote mitochondrial biogenesis [141], increase COX activity [132], [142], [143], improve oxidative metabolism [144] and induce mild mitochondrial

uncoupling that protects mitochondria from oxidative stress [145], [146] (further reading [147]–[149]) (Fig. 1.8).

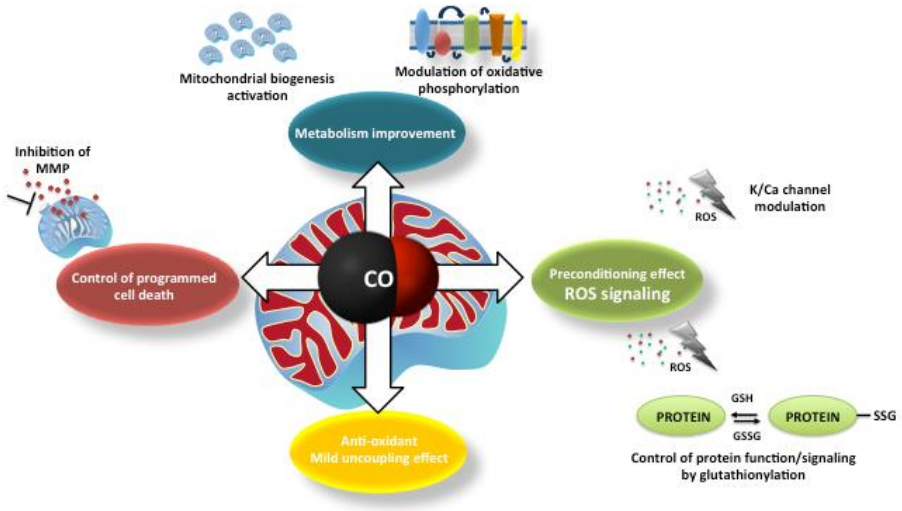

Figure 1.8 - The main described mechanisms of carbon monoxide on mitochondria: modulation of mitochondrial membrane permeabilization and cell death control; improvement of mitochondrial metabolism (modulation of cytochrome *c* oxidase activity and mitochondrial biogenesis), ROS generation and signaling (redox adaptive cell responses, alert signals) and mild uncoupling effect.

6.2.Carbon monoxide and cell differentiation

Although few data report CO as a factor involved in stem cell differentiation, several studies describe modulation of HO activity in different models of cell differentiation, such as T cells and mesenchymal stem cells [127], [150]–[157]. Endogenous CO was shown to stimulate differentiation of myeloid cells into functional macrophages [158] and CORM-A1 was used to modulate T-cell

proliferation and differentiation [159]. Recently, Suliman and colleagues showed that HO-1/CO system can modulate embryonic stem cell differentiation and maturation into functional cardiac cells, through enhancement of mitochondrial biogenesis [160].

6.3.CO and therapeutic application

Accumulating evidence of beneficial CO effects and its potential therapeutic application led to the development of CO releasing molecules (CORMs), which can be clinically more relevant approach to administer CO. The use of CORMs avoid some of the limitations of CO gas inhalation: need of hospital environment and devices, risk of high levels of carboxyhemoglobin and tissue/organ unspecific deliver of CO [161].

In this study it was used CORM-A1 (carbon-monoxide releasing molecule A1), which is a boronocarbonate compound containing a carboxylic acid for delivering CO [162]. CORM-A1 releases CO in a temperature and pH dependent manner, presenting a half-life of approximately 21 minutes for transfer of CO to myoglobin *in vitro* at pH of 7,4 and 37ºC [126], [162] (Fig.1.9).

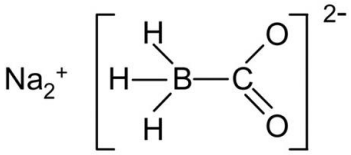

Figure 1.9 – Chemical structure of CORM-A1.

7. AIMS AND SCOPE OF THE THESIS

The main goal of this PhD thesis was to improve adult neurogenesis yield, using CO as modulator, and to disclose the associated cellular and biochemical pathways. To evaluate CO effect on adult neurogenesis, two strategies were adopted:

(i) **Cell lines** are simpler models to study neuronal differentiation process. They are good models for studies requiring a more controlled setting and cell line models allow the assessment of the underlying cellular and molecular mechanisms. In this thesis SH-SY5Y and NT2 cell lines were used due to their extensive representativeness for neuronal differentiation studies [48]–[51], [64]–[69], [163]. Nonetheless, they are tumour cells having mutagenic and oncogenic potential, being less representative of physiological conditions [48], [164], thus hMVbcl-x_L cell line was also studied. Although hMVbcl-x_L cells are derived from midbrain tissue, presenting genuine characteristics of their regional identity, they have been genetically modified in order to be cultured *in vitro* by over-expressing the anti-apoptotic protein Bcl-X_L [46], [47].

(ii) **Hippocampal organotypic slice cultures** (HOSCs) were used for validation of cell line generated data, because they represent a valuable model of adult neurogenesis, including neural stem cell proliferation, differentiation and migration within an intact neuronal circuitry [70], [71], [165].

The hypothesis that CO may play a role in modulating neuronal differentiation is based on three main correlations (Fig. 1.10). First, ROS are signaling molecules in several CO-induced pathways [166] and are also key players in neuronal differentiation [167]. Secondly, mitochondrial biogenesis is an important process during cell differentiation [93], [102] and CO promotes mitochondrial biogenesis [132], [141]. Third, CO has been described as anti-proliferative molecule in different cell types, namely smooth muscle, cancer and T cells [124], which can be involved in the balance between differentiation and proliferation that occurs during neurogenesis.

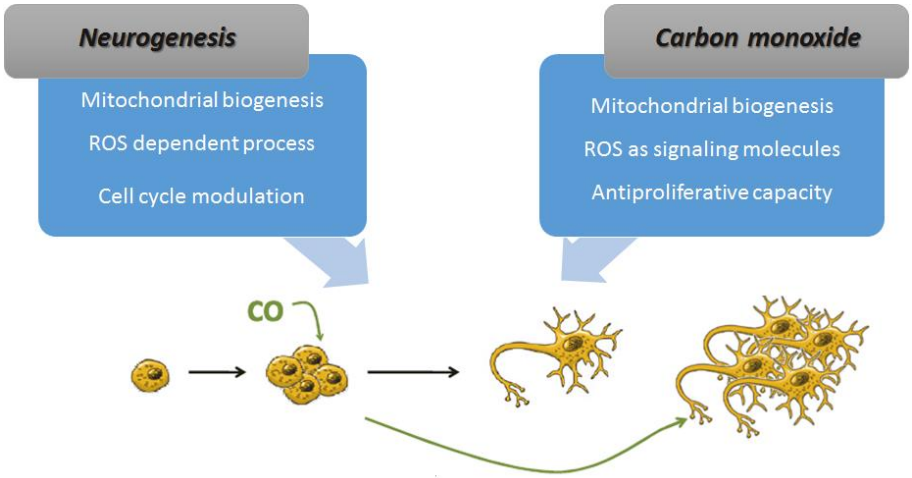

Figure 1.10 - The main hypothesis of this thesis. Correlation between adult neurogenesis process and carbon monoxide effect which were on the basis of this thesis hypothesis: CO could improve neuronal differentiation yield.

A schematic representation of the main questions of this thesis and model systems used is presented in **Figure 1.11**.

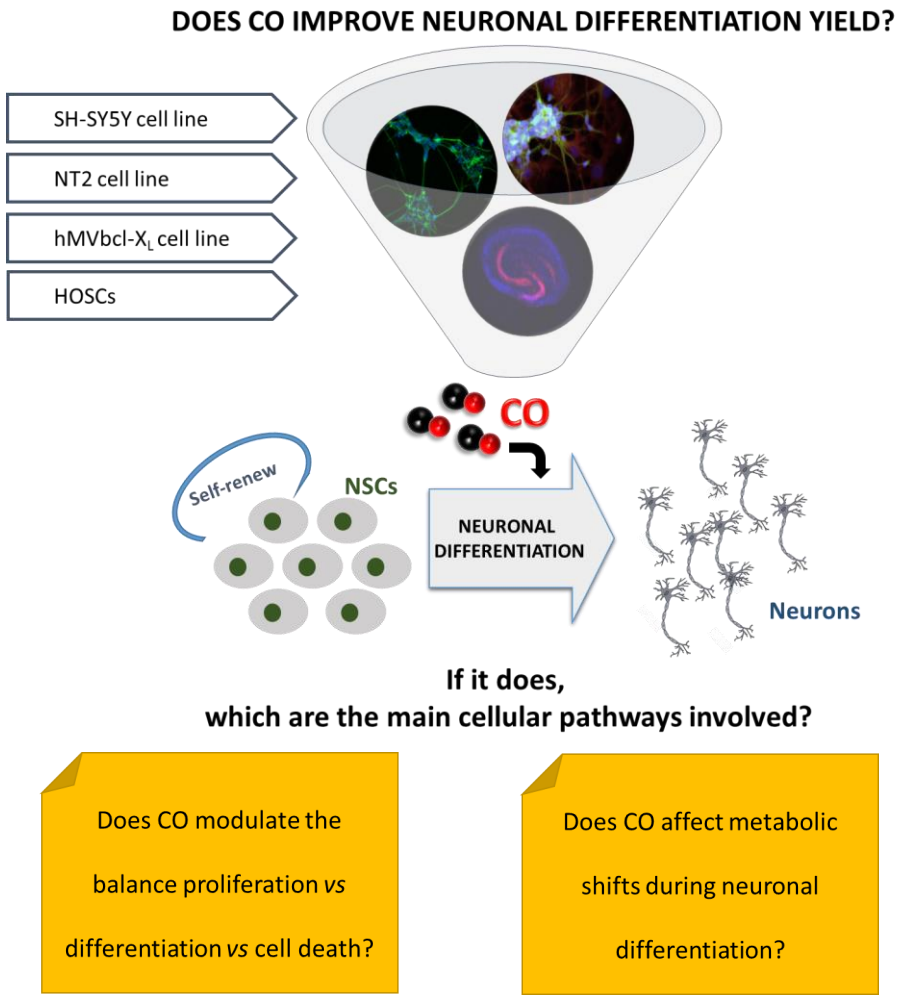

Figure 1.11 – Main questions and model systems of this thesis.

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II

Carbon monoxide releasing molecule-A1 (CORM-A1) improves neurogenesis: increase of neuronal differentiation yield by preventing cell death

This chapter is based on the following manuscript:

Carbon monoxide releasing molecule-A1 (CORM-A1) improves neurogenesis: increase of neuronal differentiation yield by preventing cell death

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ABSTRACT

Cerebral ischemia and neurodegenerative diseases lead to impairment or death of neurons in the central nervous system. Stem cell based therapies are promising strategies currently under investigation. Carbon monoxide (CO) is an endogenous product of heme degradation by heme oxygenase (HO) activity. Administration of CO at low concentrations produces several beneficial effects in distinct tissues, namely anti-apoptotic and anti-inflammatory. Herein the CO role on modulation of neuronal differentiation was assessed. Three different models with increasing complexity were used: human neuroblastoma SH-S5Y5 cell line, human teratocarcinoma NT2 cell line and organotypic hippocampal slice cultures (OHSC). Cell lines were differentiated into post-mitotic neurons by treatment with retinoic acid (RA) supplemented with CO-releasing molecule A1 (CORM-A1). CORM-A1 positively modulated neuronal differentiation, since it increased final neuronal production and enhanced the expression of specific neuronal genes: Nestin, Tuj1 and MAP2. Furthermore, during neuronal differentiation process, there was an increase in proliferative cell number (ki67 mRNA expressing cells) and a decrease in cell death (lower propidium iodide (PI) uptake, limitation of caspase-3 activation and higher Bcl-2 expressing cells). CO supplementation did not increase the expression of RA receptors. In the case of SH-S5Y5 model, small amounts of reactive oxygen species (ROS) generation emerges as important signaling molecules

during CO-promoted neuronal differentiation. CO's improvement of neuronal differentiation yield was validated using OHSC as *ex vivo* model. CORM-A1 treatment of OHSC promoted higher levels of cells expressing the neuronal marker Tuj1. Still, CORM-A1 increased cell proliferation assessed by ki67 expression and also prevented cell death, which was followed by increased Bcl-2 expression, decreased levels of active caspase-3 and PI uptake. Likewise, ROS signaling emerged as key factors in CO's increasing number of differentiated neurons in OHSC.In conclusion, CO's increasing number of differentiated neurons is a novel biological role disclosed herein. CO improves neuronal yield due to its capacity to reduce cell death, promoting an increase in proliferative population. However, one cannot disregard a direct CO's effect on specific cellular processes of neuronal differentiation. Further studies are needed to evaluate how CO can potentially modulate cell mechanisms involved in neuronal differentiation. In summary, CO appears as a promising therapeutic molecule to stimulate endogenous neurogenesis or to improve *in vitro* neuronal production for cell therapy strategies.

CONTENTS

Ana Sofia Almeida had carried out the majority of the experimental part and was involved on the decisions on how to execute the experiments, as well as on the interpretation and discussion of the results.

INTRODUCTION

Adult neurogenesis, which consists of generation of neurons from neural stem/precursor cells, occurs in specific brain regions called neurogenic zones. These niches are mostly located in the subventricular zone (SVZ), on the border of the lateral ventricle and striatum, and the subgranular zone of the dentate gyrus (DG) in the hippocampus [1]. At least five steps appear to be involved in the neurogenesis process: (i) proliferation of stem/progenitor cells, (ii) migration of newborn neurons, (iii) neuronal differentiation and maturation, (iv) integration into neuronal circuits and (v) survival of cells [2].

Programmed cell death is an important developmental cell process that occurs during neural development: from early embryonic proliferation stages until adult stages [3]–[5]. In the central nervous system (CNS), the majority of neuronal apoptosis is caused by an intrinsic program independent of external cues [6]. About half of the cortical interneurons are eliminated from CNS during neurogenic development [3]. It is possible that the terminal division of the interneuron progenitors gives rise to a pair of cells that have different propensity to initiate apoptosis [3]. Actually, cell death of differentiating neurons during embryonic development has been intensively studied [3]–[5], whereas cell death affecting adult neural stem cells is much less characterized. Adult stem cell proliferation and cell death appear to be coupled in many systems to control cell

number, patterning and lineages. Indeed, in genetically modified mouse models, where executor or regulatory apoptotic genes (caspase-3, caspase-9, Bak, Bax, among others) are knock out, resulted in supernumerary neurons in the brain [4].

Carbon monoxide (CO) is an endogenous product of heme degradation by heme oxygenase (HO), among with free iron and biliverdin, which is rapidly converted into the anti-oxidant bilirubin [7]. Administration of CO at low concentrations produces several beneficial effects in distinct tissues, such as anti-inflammatory, anti-proliferative, vasodilator and anti-apoptotic [7], [8]. In CNS, the anti-apoptotic capacity of CO has been described in neurons and astrocytes, using *in vitro* and *in vivo* models [8]–[13], for further review [14] . Many CO-induced effects are dependent on generation of small amounts of reactive oxygen species (ROS), which can signal different pathways [8], [15]–[18] and can promote tissue tolerance by stimulation of preconditioning [12], [19]–[21]. Although few data report CO as a factor involved in stem cell differentiation, several studies describe modulation of HO activity in different models of cell differentiation, such as T cells and mesenchymal stem cells [11], [22]–[29]. Recently endogenous CO was shown to stimulate differentiation of myeloid cells into functional macrophages [30] and CORM-A1 was used to modulate T-cell proliferation and differentiation [31]. The hypothesis that CO may play a role in modulating neuronal differentiation is based on two correlations.

First, ROS are signalling molecules in several CO-induced pathways [32] and are also key players in neuronal differentiation [33]. Secondly, mitochondrial biogenesis is an important process during cell differentiation [34], [35] and CO promotes mitochondrial biogenesis [9], [17].

Accumulating evidence of beneficial CO effects and its potential therapeutic application led to the development of CO releasing molecules, which can be clinically more relevant approach to administer CO. In this study it was used CORM-A1 (carbon-monoxide releasing molecule A1), which is a boronocarbonate compound containing a carboxylic acid for delivering CO [36]. CORM-A1 releases CO in a temperature and pH dependent manner, presenting a half-life of approximately 21 minutes for transfer of CO to myoglobin *in vitro* at pH of 7,4 and 37ºC [36], [37].

The main purpose of the present study was to assess the potential role of CO in modulating neuronal differentiation. Three different *in vitro* models with increasing complexity were used: (i) human neuroblastoma SH-SY5Y cell line [38]–[40], (ii) human teratocarcinoma NT2 cell line [41] and (iii) organotypic hippocampal slice cultures (OHSC). Cell lines are simpler models to study the neuronal differentiation process, allowing the assessment of the involved cellular mechanisms. Thus cell lines are good models for studies requiring a more controlled setting. SH-SY5Y cells are derived from neural crest [38], [40] and represents a rapid and representative

model for studying neuronal differentiation processes [39]. NT2 lineage is derived from human testicular embryonic teratocarcinoma and differentiates into functional post-mitotic neurons, which express many neuronal markers [41] and produce a variety of neurotransmitter phenotypes [42]–[44]. Moreover, NT2-derived neurons can form functional synapses [45] have also been used in several transplantation studies in experimental animals [46], [47] and in human patients[48]. Both immortal cell lines provide unlimited supply of cells capable of proliferating in culture for long periods and of differentiating into several cell types, including post-mitotic neurons upon treatment with retinoic acid (RA)[38]–[41], [49]–[51]. RA is a derivative of vitamin A, which is essential for promoting normal patterning and neurogenesis during development. RA signalling pathway leading to neuronal differentiation is dependent on retinoic acid binding proteins (CRABP)-I and II, which in turn deliver RA into the nuclear RA receptors (RARs). Then RARs directly regulate the expression of specific RA-inducible genes and neuronal differentiation[49]–[51].

Despite all previously described advantages of using NT2 and SH-SY5Y cell lines, the human origin of both used cell lines is a clear advantage to study human neuronal processes and cell manipulation for potential therapy. Furthermore, these cell lines have similar characteristics of human cells expressing a number of specific proteins, contrary to primary cultures of rodent precursor cells.

Nonetheless, they are tumour cells having mutagenic and oncogenic potential, being less representative of physiological neuronal differentiation [38], [52]. Therefore, for data validation we also studied the effect of CO supplementation in OHSC, which represent a valuable model of adult neurogenesis, including neural stem cell proliferation, differentiation and migration within an intact neuronal circuitry [53]–[55].

This study demonstrated that CO does increase the final yield of post-mitotic neurons in both human cell models of neuronal differentiation and improves neurogenesis in the *ex vivo* model of OHSC. In fact, during neuronal differentiation process, CO partially inhibits apoptosis in a ROS-dependent manner and simultaneously increases the number of proliferating precursor cells. Thus, a novel promising therapeutic role for CO can emerge: improvement of *in vitro* neuronal cell production and/or stimulation of endogenous neurogenesis.

MATERIAL AND METHODS

Materials

All chemicals used were of analytical grade and were obtained from Sigma unless stated otherwise. The mass spectrometry derivatization reagents MTBSTFA (*N*-methyl-*N*- (tert - Butyldimethylsilyl) trifluoroacetamide), MSTFA (*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide) and the t-BDMS-Cl (tert-butyldimethylchlorosilane) were purchased from Regis Technologies, Inc. (Morton Grove, IL, USA).

Plastic tissue culture dishes were acquired from Sarstedt (Germany); foetal bovine serum (FBS), penicillin/streptomycin solution (Pen/Strep), and Dulbecco's minimum essential medium (high glucose, L-glutamine and pyruvate) (DMEM-HG) were obtained from Invitrogen (United Kingdom); and BALB/c mice pups were purchased from Instituto Gulbenkian de Ciência (Oeiras, Portugal).

NT2 human teratocarcinoma cell line

Maintenance of undifferentiated cells

Undifferentiated NT2 cells from American Type Culture Collection (ATCC) were grown in DMEM-HG supplemented with 10%(v/v) FBS and 1%(v/v) Pen/Strep (growth medium). Cells were maintained in a humidified atmosphere of 5%(v/v) CO_2 at 37ºC. Undifferentiated cells *per* vial were grown in 75cm^2 T-flasks and subcultured with fresh

growth medium whenever high cell confluence was achieved (about 90-100% cell confluence). Cells were rinsed with phosphate-buffered saline (PBS) and then incubated with trypsin for 2 minutes at 37ºC for trypsinization and resuspended in growth medium in a 1:4 cell passage. Growth medium was changed every 2 to 3 days.

Neuronal differentiation protocol

Following trypsinization and resuspension in growth medium, cells were counted in trypan blue and split $2{,}3 \times 10^6$ cells per $75 cm^2$ T-flask. Neuronal differentiation was induced 24 hours after plating undifferentiated cells to ensure settle and attachment to flask surface and attain appropriate density. The NT2 cell line neuronal differentiation was induced in DMEM-HG with 10%(v/v) FBS and 1%(v/v) Pen/Strep, supplemented with 10µM *all-trans* RA (differentiation medium). CO effect was studied by using the same composition of differentiation medium supplemented with 25µM CORM-A1. Differentiation medium was replaced three times a week until reach 10 differentiation treatments (24 days). After neuronal differentiation and before neuronal enrichment, cells were collected for analysis. Protein from cell extracts was quantified using BCA assay (Pierce, Illinois).

Neuronal enrichment

After the 10th differentiation treatment, cells were replated at lower density to disperse the dense multilayer cell culture and start neuronal enrichment. On the following day, the culture medium was exchanged with fresh growth medium supplemented with mitosis inhibitors: 1μM Cytosine Arabinoside, 10μM Floxuridine and 10μM Uridine for neuronal enrichment. Growth medium supplemented with mitosis inhibitors was replaced, twice a week, for 10 days, making a total of 3 to 4 treatments. On the 10th day of neuronal enrichment, enriched culture was collected for different analysis. Protein from cell extracts proteins was quantified using BCA assay (Pierce, Illinois). The used protocol is schematically represented in Fig.2.1A.

SH-SY5Y neuroblastoma cell line

Maintenance of undifferentiated cells

The SH-SY5Y cell line was cultured in DMEM/F-12 supplemented with 10%(v/v) FBS and 2%(v/v) Pen/Strep (growth medium). Cells were maintained in a humidified atmosphere of 5%(v/v) CO_2 at 37ºC. Undifferentiated cells were grown in 75cm^2 T-flasks and subcultured with fresh growth medium whenever cell confluence achieved (about 80-90% cell confluence). Cells were detached by trypsinization at room temperature (R.T.) and slight shaking and hitting to drain down

cells with trypsin and resuspended in growth medium in a 1:4 cell passage. Growth medium was changed twice a week.

Neuronal differentiation protocol

Following trypsinization and resuspension in growth medium, cells were plated on 75cm^2 T-flasks in a 1:2 cell passage. Neuronal differentiation was induced 24 hours after plating undifferentiated cells to ensure settle and attachment to flask surface and attain appropriate density, approximately about 50% cell confluence in all 75cm^2 T-flasks.

Neuronal differentiation was induced with DMEM/F-12 medium, reduced serum to 1%(v/v) FBS, 2%(v/v) Pen/Strep and supplemented with 10μM of *all-trans* RA (differentiation medium). CO effect was studied by using the same composition of differentiation medium supplemented with 25μM CORM-A1. Differentiation medium was replaced twice (1st and 4th days) of the 7 days of treatment. On the 7th day, cells were collected for analysis. Protein from cell extracts proteins was quantified using BCA assay (Pierce, Illinois). Whenever it is the case, neuronal differentiation of SH-SY5Y cells was supplemented with 5mM of N-acetylcysteine (NAC).

Neuronal enrichment

After the 7th day of differentiation, cells were replated at lower density to disperse the cell culture for neuronal enrichment. On the following

day, the culture medium was exchanged with fresh growth medium supplemented with mitosis inhibitors: 1μM Cytosine Arabinoside, 10μM Floxuridine and 10μM Uridine for neuronal enrichment. Growth medium supplemented with mitosis inhibitors was replaced after 2 days. On the 5th day of neuronal enrichment, enriched cultures were collected for different analysis. The used protocol is schematically represented in Fig.2.1B.

Organotypic Hippocampal Slice Cultures (OHSC)

For mouse tissue collection, mice were rapidly decapitated with minimizing suffering procedures. The procedure was approved by the National Institutional Animal Care and Use Committee (Direção Geral de Alimentação e Veterinária with reference number 0421/000/000/2013) and accordingly with relevant national and international guidelines.

OHSC were prepared from eight days old BALB/c mice pups. Mice were decapitated and the brains were removed. The brain was cut along the midline and the hemispheres separated for the hippocampi to be exposed. The hippocampi were dissected out, cut in 350μm thick transverse slices on a McIlwain tissue chopper and transferred to a petri dish containing a balanced salt solution with 25mM glucose. The slices were separated under the stereomicroscope and placed in inserts with semipermeable membranes (Millipore, France),

six slices culture arranged in a circle per insert. The inserts were placed in 6-well culturing plates, each well containing culture medium (25% heart inactivated horse serum, 25% Hank's balanced salt solution, 50% OptiMEM medium and 25mM glucose). The plates were maintained in a humidified atmosphere of 5%(v/v)% CO_2 at 33ºC. Medium was changed twice a week throughout the growth period. Cultures were mature at 11 days *in vitro* (DIV)[54], [56]–[58]. After 11DIV, the culture medium was exchanged by a differentiation medium (98% Neurobasal medium, 2% B-27 supplement and 1mM L-glutamine) and slices were treated with 25μM CORM-A1 twice a week[57]–[60]. Whenever it is the case, OHSC was also supplemented with 0.5 or 5mM of N-acetyl-cysteine (NAC). To investigate effects on cell proliferation, cultures were fixed after 3 days of CORM-A1 treatment (at 14 DIV, ki67 was used as a marker for cell proliferation). To study on neuronal differentiation, matured OHSC were maintained for 12 days longer (until 26 DIV) to investigate the expression of neuronal markers (Tuj1). The effect of CORM-A1 treatments was assessed at the various time points by immunohistochemistry, cell counting and propidium iodide uptake (see below). The used protocol is shown in Fig.2.1C.

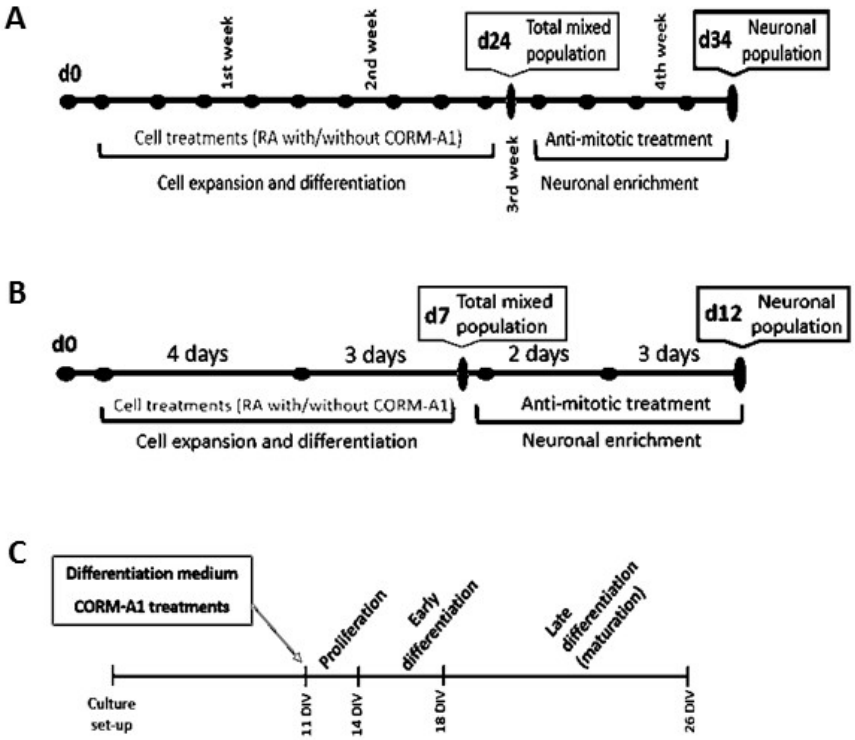


Figure 2.1 – Scheme of the different models for neuronal differentiation assessment. (A) Neuronal differentiation of NT2 cells was performed throughout 3 weeks, with medium exchanges three times a week in alternated days. After 24 days of differentiation (d24), the obtained mixed (undifferentiated and neuronal) cell population was treated with anti-mitotic compounds for neuronal enrichment during 10 days (d34). B) SH-SY5Y cells were induced to differentiate during 7 days (d7), subjected to a differentiation medium exchange at day 4. After 7 days of differentiation (d7), a mixed population of undifferentiated cells and post-mitotic neurons was obtained. In order to obtain an enriched neuronal population, cultures are treated with anti-mitotic compounds for 5 days (d12). C) After 11 days of *in vitro* culture, the differentiation medium is added to the OHSC (11 DIV). The medium is exchanged twice a week during 2 weeks. Then, fully mature and differentiated slice cultures were obtained (26DIV).

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 using a 0,2µM filter and stored at -20ºC. CORM-A1 reconstituted and stored at -20ºC was compared with iCORM-A1 and CO gas saturated solutions (Fig.2, S1 Fig. and S2 Fig.) and no evidences of loss of CO due to storage were found. Thus, for each use, a pre-prepared aliquot was thawed and immediately used.

Preparation of inactivated CORM-A1

CO-depleted inactive form (iCORM-A1) was generated to be used as negative control by initially dissolving CORM-A1 in 0.1 M HCl and then bubbling pure N2 through the solution for 10 min in order to remove the residual CO gas [36]. The solution of iCORM-A1 was finally adjusted to pH 7.4. Then, the solution was filtrated using a 0,2µM filter and stored at -20ºC. For each use, an aliquot was thawed and immediately used.

Preparation of CO Solutions

Fresh stock solutions of CO gas were prepared each day and sealed carefully. PBS was saturated by bubbling 100% of CO gas for 30 min to produce 10e-3 M stock solution. The concentration of CO in

solution was determined spectrophotometrically by measuring the conversion of deoxymyoglobin to carbon monoxymyoglobin as described previously [61]. 100% CO was purchased as compressed gas (Linde, Germany).

Cell counting and viability

Cell cultures were visualized using an inverted microscope with phase contrast (DM IRB, Leica, Germany). Total cell number was determined by counting cell nuclei using a Fuchs-Rosenthal hemacytometer, after digestion with 0,1M citric acid/1% Triton X-100 (wt/wt)/0,1% crystal violet (wt/v).

After differentiation treatments cells were harvested and the assessment of cell viability was performed using 1μM PI (15 minutes at 37ºC). Data acquisition was obtained in FACSCalibur and Cell Quest Software (BD Biosciences, San Jose, CA, USA). Flow cytometry data was analyzed by FlowJo software version 10.1.

PI uptake evaluation in OHSC

A final concentration of 2μM PI was added to the medium 24 hours before starting the differentiation process of the OHSC[54], [60]. PI uptake on the whole culture was assessed by fluorescent microscopy, at 5x magnification, every 24hours.

Immunofluorescence microscopy

NT2 and SH-SY5Y cells were plated at a density of $2x10^6$ cells/well in 24-well plates coated with Poly-D-lysine in 0,15M sodium borate buffer solution pH 8,4. Cells were fixed with 4%(v/v) PFA and 4%(w/v) sucrose solution (20 minutes at R.T.) and then permeabilized with 0,3%(v/v) Triton X-100 solution (15 minutes at R.T.). Later, cells were incubated 2 hours at R.T. with primary antibody: Tuj1 (Sigma-Aldrich, T8660); MAP2 (Sigma-Aldrich, M1406); ki67 (Millipore, AB9260) or active caspase-3 (Cell Signaling, #9664), following incubation for 1 hour at R.T. with secondary antibody: AlexaFluor 488 anti-mouse (A11001) or AlexaFluor 594 anti-rabbit (A11012). Primary and secondary antibodies were dilute in 1%(v/v) BSA and 0,1%(v/v) Triton X-100 solution. Cultures were mounted on Prolong mounting media (with DAPI - Invitrogen) and images were captured with Zeiss Axiovert 40 CFL microscope. All solutions were prepared in PBS (1X). Washes with PBS (1X) solution were performed between each step.

In a 6-well plate OHSC were washed with PBS and fixed with 4% (w/v) paraformaldehyde in PBS with 4% (w/v) sucrose for 30 minutes. After fixation, OHSC were washed and stored in PBS. The insert membrane, containing the cultures of interest, was cut and transferred to a 12-well plate well containing Tris-buffered saline buffer with 0,1-0,3% Triton X-100 (TBS+TX-100) for cell permeabilization. Then slices were pre-incubated for 30 minutes at R.T. in 0,05M TBS containing 10% serum to block non-specific binding sites. Afterwards, cultures were

incubated with primary antibody (Tuj1 (Sigma-Aldrich, T8660); MAP2 (Sigma-Aldrich, M1406); ki67 (Millipore, AB9260); active caspase-3 (Cell Signaling, #9664)) for 2 days at 4ºC, followed by incubation for 2 hours at R.T. with secondary antibody (AlexaFluor 488 anti-mouse, A11001; AlexaFluor 594 anti-rabbit, A11012). Whole mount samples were transferred to high adherence microscope slides and the insert membrane was removed. Slices were mounted using ProLong mounting (Invitrogen) medium with DAPI and visualized afterwards in a fluorescence microscpe (Leica).

Immunoblotting

Cell extracts were separated under reducing electrophoresis on a 1mm of NuPAGE Novex Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane (HybondTMC extra, Amersham Biosciences). Tuj1, Actin and cleaved caspase-3 proteins were stained with α-Tuj1 (Sigma-Aldrich, T8660), α-cleaved caspase-3 (Cell Signaling, #9664) and α-actin (Sigma-Aldrich, A4700) at 1/1000 dilution for 2h at room temperature. Blots were developed using the ECL (enhanced chemiluminescence) detection system after incubation with HRP-labeled anti-mouse IgG anti- body (Amersham Biosciences Bioscience), 1/5000, 1h of room temperature incubation.

Quantitative-Polymerase chain reaction (Q-PCR)

For evaluation of gene expression, mRNA was extracted from NT2 and SH-SY5Y cells using High Pure RNA isolation kit (Roche Diagnostics) and cDNA synthesis was performed using the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics). PCR was performed using specific forward and reverse primers designed for the Nestin gene (5′-CTGTGAGTGTCAGTGTCCCC-3′ and 5′-CTCTAGAGGGCCAGGGACTT-3′), Tuj1 gene (5′-GCAAGGTGCGTGAGGAGTAT-3′ and 5′-GTCTGACACCTTGGGTGAGG-3′), MAP2 gene (5′-GGAGCTGAGTGGCTTGTCAT-3′ and 5′-CTAGCTCCAGACAGACGCAG-3′), ki67 gene (5′-GAAGTCCCTGAAGACCTGGC-3′ and 5′-GTCTGGCTGTGAAGCTCTGT-3′), Bcl2 gene (5'-TCATGTGTGTGGAGAGCGTC-3' and 5'-TCAGTCATCCACAGGGCGAT-3'), Bcl-XL gene (5'-ACTCTTCCGGGATGGGGTAA-3' and 5'-TTGTCTACGCTTTCCACGCA-3'), CRABP1 gene (5'-CCAGGACGGGGATCAGTTCT-3' and 5'-CTAAACTCCTGCACTTGCGT-3'), CRABP2 gene (5'-CTCAAAGTGCTGGGGGTGAA-3' and 5'-TGATCTCCACTGCTGGCTTG-3'), RARα gene (5'-CCTGGACATCCTGATCCTGC-3' and 5'-CATCATCCATCTCCAGGGGC-3'), RARβ gene (5'-TGACAGCTGAGTTGGACGAT-3' and 5'-AGCACTGGAATTCGTGGTGT-3'), RARγ gene (5'-CTGTGCGAAATGACCGGAAC-3' and 5'-CTGCACTGGAGTTCGTGGTA-3') and RPL22 gene (5'-CACGAAGGAGGAGTGACTGG-3' and 5'-TGTGGCACACCACTGACATT-

3'), respectively. Fast Start DNA Master Plus SYBR Green I (Roche Diagnostics) was used with the experimental run protocol: denaturation program was 95 ˚C for 10 minutes, followed by 45 cycles of 95 ˚C for 10 seconds, 60˚C for 10 seconds and 72 ˚C for 10 seconds.

Gas Chromatography-Mass Spectrometry (GC-MS)

For analysis of ^{13}C percent enrichment in intracellular neurotranmitters, cell extracts obtained from total mixed populations of NT2 and SH-SY5Y cell lines were lyophilized and resuspended in 0.01M HCl followed by pH adjustment to pH<2 with HCl 6 M. Samples were dried under atmospheric air (50 ˚C), and metabolites were derivatised with MTBSTFA in the presence of 1% *t*-BDMS-Cl [62], [63]. The samples were analyzed on an Agilent 6890 gas chromatograph connected to an Agilent 5975B mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The parent ion (M) and atom percent excess for one ^{13}C atom (M+1) values for glutamate and GABA were calculated from GC-MS data using MassHunter software supplied by Agilent (Agilent Technologies, Palo Alto, CA, USA) and correcting for the naturally abundant ^{13}C by using non-enriched standards [64]. Data were normalized by total amount of protein.

Measurement of ROS Generation

ROS generation was followed by the conversion of 5μM 2',7'-dichlorofluorescein diacetate (H2DCFDA) (Invitrogen) to fluorescent 2',7'-dichlorofluorescein (DCF). While superoxide anion formation was quantified using 5μM MitoSOX Red mitochondrial superoxide indicator (Life Technologies, Scotland). After differentiation process, cells were incubated for 20 min with 5μM H2DCFDA or MitoSOX prepared in PBS. Cells were washed twice, and fluorescence was measured (λ_{ex} 485 nm/λ_{em} 530 nm and λ_{ex} 510 nm/λ_{em} 580 nm, respectively) using a TECAN infinite F200 PRO spectrofluorimeter. ROS generation was measured at the end of neuroranl differentiation process 24 days for NT2 cells and 7 days for SH-SY5Y cells. For SH-SY5Y cell ROS were also quantified 1h following RA treatment (with or without CORM-A1) for assessing CO-induced ROS generation and signaling. N-acetyl-cysteine (NAC) 5mM was added during all the neuronal differentiation (7 days for SH-SY5Y cell lines). ROS generation was calculated as an increase over base-line levels, determined for untreated cells (100%) and normalized by total cell count for each condition.

Statistical analysis

Data concerning cell culture were carried out at least in three independent culture preparations. For every immunocytochemistry

and immunohistochemistry assay a representative image is showed. All values are mean ± SD (standard deviation), n≥3. Error bars, corresponding to standard deviation, are represented in the figures. Statistical comparisons between two groups were made with an independent two-tailed Student's *t-test*. Between multiple groups, statistical comparisons were performed using one-way ANOVA single factor with replication and Bonferroni's multiple comparisons test for confidence intervals and significance correction. For all the data, *p*-value is indicated for each figure.

RESULTS

CO increases the final neuronal differentiated cell population

The effect of CO on modulation of neuronal differentiation was tested in both NT2 and SH-SY5Y cell lines. These cells were differentiated in the presence of RA supplemented with CORM-A1 (Fig.2.1A and B). Of note, CO cannot induce neuronal differentiation *per se* since NT2 and SH-SY5Y cells were not able to differentiate into neurons without RA. Actually, NT2 cells treated only with CORM-A1 did not differentiate and died following 7 days of procedure (data not shown). Thus, CO increased the RA-induced neuronal differentiation, but this gasotransmitter is not a differentiating factor.

After differentiation process and neuronal enrichment procedure, cells were harvested and their nuclei were counted. It was observed that RA treatment supplemented with CORM-A1 at 25μM yielded a duplication of the final number of NT2 post-mitotic neurons (Fig. 2.2A) and a 6-fold increase on SH-SY5Y post-mitotic neuronal population when compared to RA treatment without supplementation (Fig. 2.2B). Immunocytochemistry analysis was performed in order to evaluate neuronal morphology and the expression of neuronal specific protein Tuj1 in post-mitotic NT2 (Fig. 2.2C) and SH-SY5Y neurons (Fig.2.2D). Neurons obtained from treatment with RA supplemented with CORM-A1 were comparable to neurons obtained from RA treatment only (Fig. 2.2C-D). Furthermore, neuronal functionality was assessed by quantification of two neurotransmitters, glutamate and GABA, in cell extracts at the end of neuronal differentiation process. For NT2 model, the levels of glutamate were similar in the presence or absence of CORM-A1 (Fig. 2.2E), while GABA was too low for precise quantification in both treatments. In SH-SY5Y cells, the intracellular levels of GABA and glutamate were comparable (Fig. 2.2F). In conclusion CORM-A1 increased neuronal yield production. Co-treatment with CORM-A1 did not cause (i) any cell morphology alteration, (ii) any change on the expression of neuronal specific protein Tuj1 nor (iii) any change on intracellular neuronal transmitters' level.

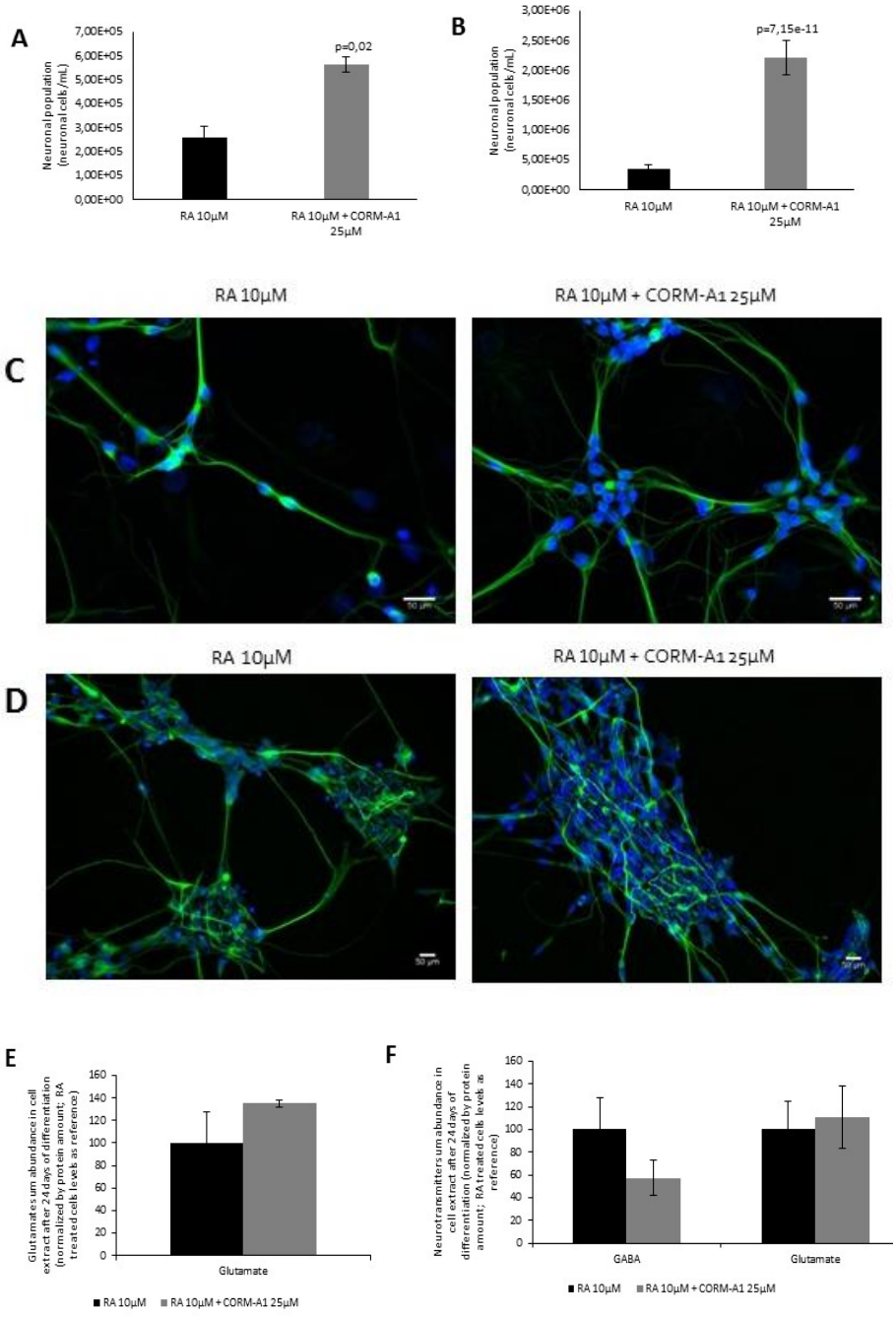


Figure 2.2 – CORM-A1 increases final yield of enriched neurons. (A) Neuronal yield is calculated based on nuclei count *per* volume of NT2 derived post-mitotic neurons (neuron/mL) after 24 days of differentiation and 10

days of neuronal enrichment; (B) Nuclei count per volume of SH-SY5Y derived post-mitotic neurons (neuron/mL) after 7 days of differentiation and 5 days of neuronal enrichment (C) Characterization of NT2 derived post-mitotic neurons by immunocytochemistry (green staining: Tuj1; blue staining: DAPI; magnification 200x); (D) Characterization of SH-SY5Y derived post-mitotic neurons by immunocytochemistry (green staining: Tuj1; blue staining: DAPI; magnification 100x); (E,F) Characterization of neuronal functionality by neurotransmitter quantification (glutamate and GABA) in mixed cell populations of NT2 and SH-SY5Y cells after 24 and 7 days of differentiation, respectively. Glutamate and GABA quantification is normalized by total protein amount.

In order to validate that CORM-A1 effect is dependent on CO release, NT2 cells were also differentiated using CO gas supplementation *via* CO-saturated PBS solutions (Fig. 2.3A). CO gas also increased the final yield of post-mitotic neurons in a dose-dependent manner (Fig. 2.3A). Thus, CORM-A1 improvement of final neuronal production is due to its CO molecule and not due to CORM-A1 chemical structure.

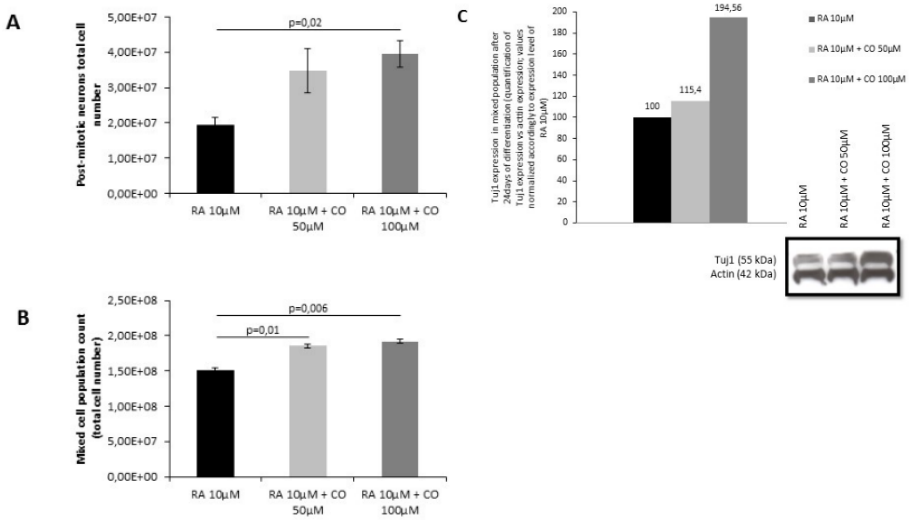


Figure 2.3 – Effect of CO gas saturated solution supplementation on NT2 cells neuronal differentiation. (A) Post-mitotic neurons total cell count after 24 days of differentiation and 10 days of neuronal enrichment; (B) Total mixed population cell count after 24days of differentiation; (C) Tuj1 protein analysis in total mixed cell population after 24 days of differentiation (specific Tuj1 expression quantification normalized by Actin expression).

CO increases total cellular population during differentiation process: precursor cells, early stage neurons and mature neurons

The CO enhancement of neuronal production yield indicates that this gasotransmitter might modulate the differentiation process. CO's modulation of neuronal differentiation process can be due to three main hypotheses: (i) increased cell proliferation, (ii) protection against cell death and/or (iii) by facilitating the neuronal differentiation *per se*. In order to clarify these hypotheses, the mixed cell populations (containing progenitor cells and post-mitotic neurons) were evaluated at the end of differentiation process and before neuronal enrichment.

In the beginning of the differentiation process, the inoculated cell number was the same for RA treatment and RA treatment supplemented with CORM-A1 for both NT2 and SH-SY5Y cell lines. At day 24, the total number of cells in the mixed population of NT2 cells treated with CORM-A1 and RA was higher than only with RA supplementation (Fig.2.5A), whereas inactive CORM-A1 (iCORM-A1) rendered a lower total number of cells in the mixed population (Fig. 2.4 A). The same effect was observed in the SH-SY5Y cells, whose cell

growth increase was higher than 50% (Fig. 2.4B) and CO's effect was lost when cells were differentiated with iCORM-A1 as supplement (Fig. 2.4 C and D). Therefore, CO increased total cellular population (including precursor proliferating cells, early stage neurons and mature neurons) at the end of differentiation procedure. Moreover, CORM-A1 effect is due to the release CO gas and not to its chemical structure, since the cell population increase was not observed when inactive CORM-A1 was applied.

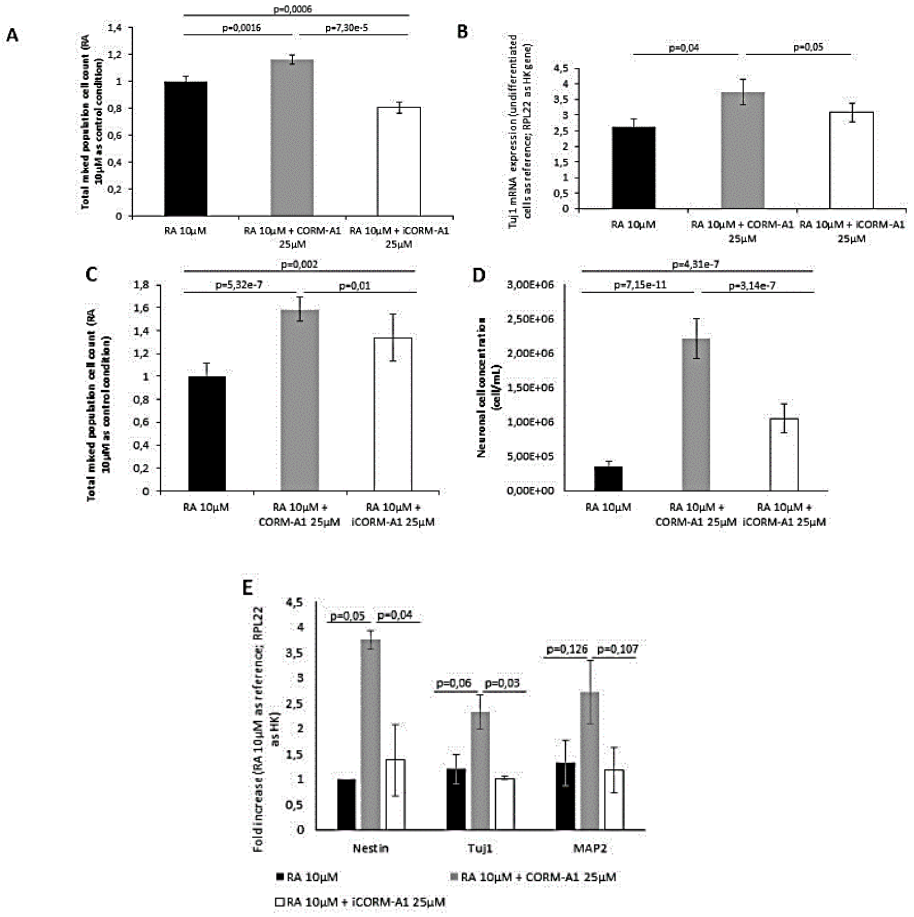


Figure 2.4 – Effect of iCORM-A1 supplementation neuronal differentiation. (A) NT2 total mixed population cell count (total cell number after 24days of differentiation); (B) NT2 cells Tuj1 expression of total mixed cell population after 24 days of differentiation; (C) SH-SY5Y total mixed population cell count (cell concentration after 7 days of differentiation); (D) SH-SY5Y neuronal cell count (cell concentration after 7 days of differentiation followed by 5 days of anti-mitotic treatment); (E) mRNA expression quantification of specific neuronal differentiation markers (Nestin for neuronal precursors, Tuj1 for early differentiated neurons and MAP2 for mature neurons) for SH-SY5Y mixed cell population after 7 days of differentiation.

For characterization of mixed cell population at the end of neuronal differentiation process, the expression of specific genes (Nestin, Tuj1 and MAP2) was assessed by mRNA quantification using RT-Q-PCR. Neuronal precursor cells express Nestin, while Tuj1 is expressed in early stage of neuronal differentiation and MAP2 is expressed by mature neurons. Cells obtained from neuronal differentiation of NT2 cells upon supplementation with CORM-A1 present some increased expression of Nestin, Tuj1 and MAP2 (Fig. 2.5C). In other hand, SH-SY5Y presented higher levels of Nestin, Tuj1 and MAP2 mRNA, when cultured in the presence of CORM-A1 (Fig. 2.5D).

In contrast, NT2 cell neuronal differentiation process supplemented with iCORM-A1 did not increase Tuj1 expression. Indeed, its levels were similar to the ones obtained from cells that were differentiated only in presence of RA (Fig. 2.5B). Accordingly, NT2 cells treated with CO gas saturated solutions presented the same effects already described for CORM-A1: increased mixed cell population with higher expression of Tuj1 per cell (Fig. 2.5B and C). Taking all together, we

can claim that CORM-A1 role on neuronal differentiation is due to CO gas that is released from CORM-A1.

Moreover, the increased expression of specific marker of neuronal precursor cells (Nestin) indicates that population of neuronal-committed proliferative cells is positively modulated by CORM-A1. At this point one can speculate that CORM-A1 increases the number of cells to be differentiated into neurons, expressing Tuj1 and MAP2 proteins. Nevertheless, it cannot be excluded that CO can also modulate cell death and/or increasing number of differentiated neurons process *per se*.

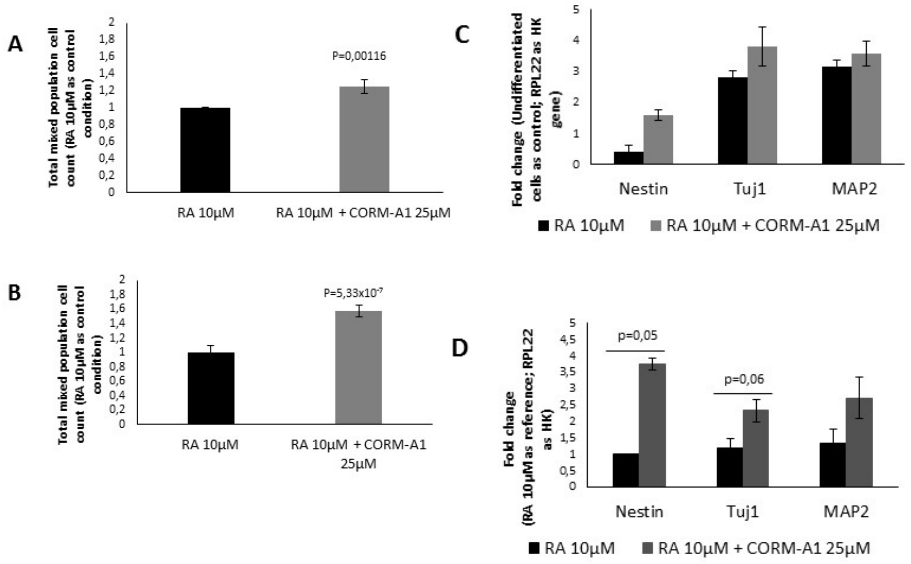


Figure 2.5 – CO increases total mixed cellular population during differentiation process: precursor cells, early stage neurons and mature neurons. Characterization of mixed cell population was assessed following neuronal differentiation and before neuronal enrichment. (A) Nuclei count of NT2 mixed population after 24 days of differentiation; (B) Nuclei count of SH-SY5Y mixed population after 7 days of differentiation. mRNA expression of specific neuronal differentiation markers (Nestin for neuronal precursors,

Tuj1 for early-differentiated neurons and MAP2 for mature neurons) was quantified for (C) NT2 mixed population and for (D) SH-SY5Y mixed population.

CORM-A1 does not increase the expression of retinoic acid receptors

As showed above, CO improved the RA-induced neuronal differentiation. RA signalling pathway leading to neuronal differentiation is dependent on retinoic acid binding proteins (CRABP)-I and II, which in turn deliver RA into the nuclear RA receptors (RARs). Then RARs directly regulate the expression of specific RA-inducible genes and neuronal differentiation [49]–[51]. Once CO is not a differentiating factor *per se*, but a modulator of neuronal differentiation, CO effect in RA signaling pathway was assessed in both NT2 and SH-SY5Y cell lines (Fig.2.6).

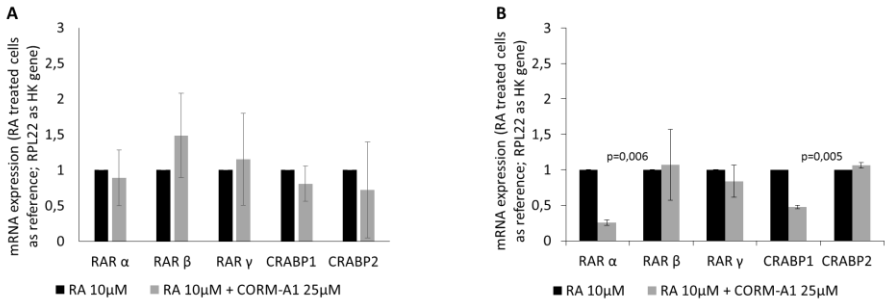


Figure 2.6 – CORM-A1 does not increase the expression of retinoic acid receptors in mixed cell population (after neuronal differentiation procedure). (A) mRNA expression of cellular and nuclear retinoic acid receptors were measured in NT2 mixed population after 24 days of differentiation; (B) Quantification of mRNA of cellular and nuclear retinoic acid receptors in SH-SY5Y mixed population after 7 days of differentiation.

CORM-A1 did not cause any significant difference in expression of RA's receptors in total mixed population, after 24 days of differentiation of NT2 cells (Fig. 2.6A). On other hand, for SH-SY5Y total mixed population, there was a decrease in RAR α and CRABP1 expression (Fig. 2.6B). Because mixed population obtained from SH-SY5Y differentiation for 7 days is mainly composed of post-mitotic cells (Fig. 2.2 and 2.5), one can speculate that the diminished expression of RAR α and CRABP1 is due to the decreased need of this kind of receptors. In conclusion, improved number of differentiated neurons by CO is not due to a positive modulation of retinoic acid receptors in the cells.

CO and cell proliferation

CO's effect on modulation of cell proliferation can be partially assessed by mRNA ki67 expression [65], which was quantified by RT-Q-PCR in mixed cell populations of NT2 and SH-SY5Y cells (Fig. 2.7A and B). Concerning NT2 cell line (Fig.2.7A), ki67 mRNA quantification was done during differentiation process at two different time points. It was observed that CORM-A1 supplementation highly increased ki67 mRNA expression at day 17, while at the end of differentiation process (day 24), the amount of ki67 expressing cells decreased significantly in the presence of CORM-A1. Thus, these data indicate that CO might increase the number of cells with activate cell cycle

during the neuronal differentiation process. However this effect decreases by the end of neuronal differentiation procedure.

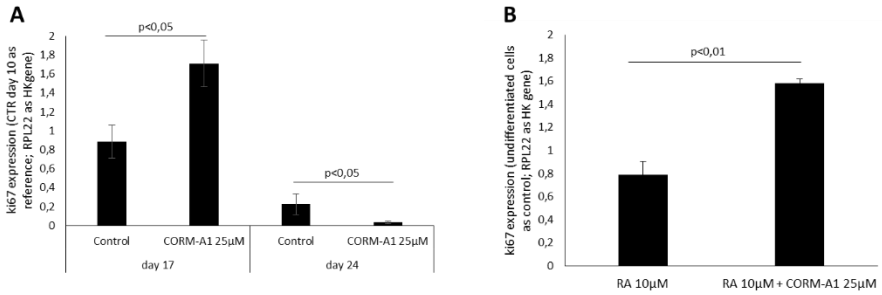


Figure 2.7 – CORM-A1 promotes cell proliferation. (A) Ki67 mRNA expression was assessed in NT2 mixed cell population at two distinct time points, day 17 and day 24, during neuronal differentiation process; (B) quantification of ki67 mRNA expression in SH-SY5Y mixed population after 7 days of differentiation.

The levels of ki67 mRNA expression in SH-SY5Y cells (Fig.2.7B) were higher in the case of CORM-A1 supplementation at the end of differentiation process (day 7). Taking all together, these data suggest that CORM-A1 treatment enhanced proliferative cell population, although further experiments are needed to effectively assess cell proliferation. Both cell lines presented different patterns of ki67 expression at the end of differentiation process, which can be due to the intrinsic differences between cell models. Indeed, NT2 cells yield a mixed population at day 24 that contains higher levels of Tuj1 and MAP2, both specific markers for neurons (Fig. 2.7C). While, SH-SY5Y cells have a shorter procedure of differentiation, which can be the reason for the relative high levels of ki67 and Nestin

expression, and low levels of Tuj1 at day 7 (Fig. 2.5D). Hence, CORM-A1 supplementation increased the proliferative population of both cell models during differentiation process.

CO prevents cell death during neuronal differentiation

Still, one cannot disregard that CO is a well-accepted anti-apoptotic molecule in several distinct tissues [7]–[9], [12], [32], [66]. Based on the work developed by Boya and colleagues, proliferation and cell death appear to be coupled in many systems, in particular during cell differentiation for controlling cell number, patterning and lineages [4]. Taking these facts into account, one can speculate that the CORM-A1 increases mixed cell population and final neuronal population by also inhibiting cell death in a ROS dependent manner *via* a preconditioning effect.

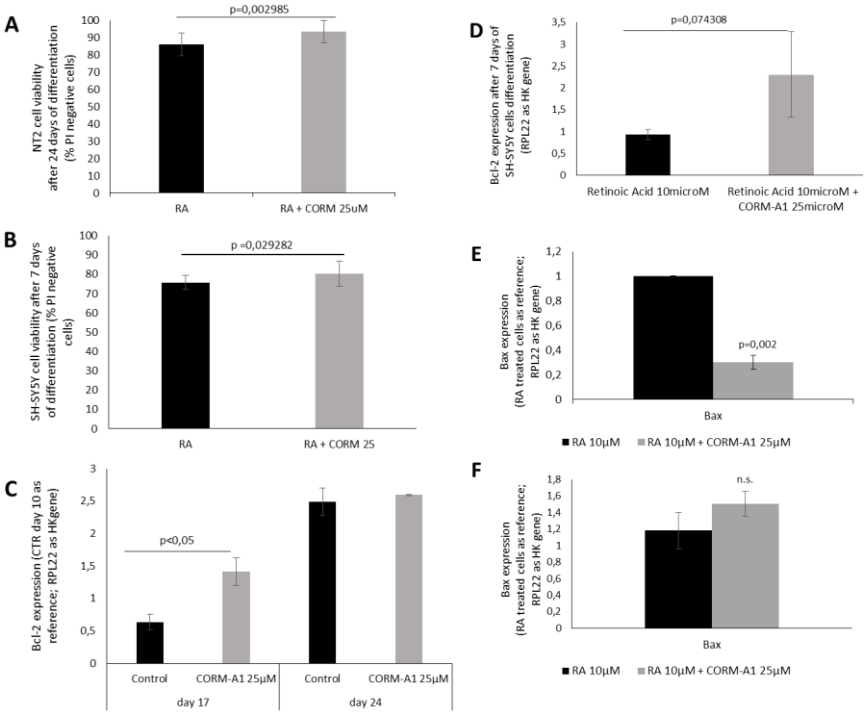
To disclose the anti-apoptotic effect of CO during neuronal differentiation, cellular viability of NT2 and SH-SY5Y cell lines were analyzed by flow cytometry using propidium iodide (PI) at the last day of differentiation (day 24 and 7, respectively) (Fig.2.8A and B). Cells treated with CORM-A1 presented slightly but significant less incorporation of PI, meaning that there was less cell death in culture at the end of differentiation process for both cell models. Furthermore, the expression of the anti-apoptotic gene Bcl-2 was quantified by RT-Q-PCR (Fig.2.8C and D). CORM-A1 increases Bcl-2

mRNA expression during the differentiation process (day 17) in NT2 cells (Fig. 2.8C). These data directly correlate with the results for ki67 expression presented in the previous section (Fig.2.7A). Therefore it seems that because CORM-A1 prevents cell death by increasing the expression of anti-apoptotic Bcl-2, there is a robust cell proliferation process ongoing, which is reflected by the increased levels of ki67. On the other hand, at day 24, NT2 cells express as much Bcl-2 with or without CORM-A1 supplementation (Fig.2.8C). Also, this can be correlated with ki67 mRNA amount since the levels of proliferation is lower in this point of the process (Fig.2.7A). CORM-A1 treated SH-SY5Y cells displayed an increased level of Bcl-2 expression in the last day of differentiation (Fig. 2.8D). Once more, this correlates with the increased ki67 mRNA expression at the same time point (Fig.2.7B).

For further investigate cell death modulation by CORM-A1, expression of the pro-apoptotic gene Bax and activation of caspase-3 were also assessed. In the case of NT2 cell line, the total mixed population at day 24 of differentiation presented lower levels of Bax mRNA expression. Bax is a pro-apoptotic protein that interacts with BCL-2 family leading to cytochrome c release from mitochondria, and posterior initiation of caspase cascade. Increased levels of Bcl-2 expression and decreased Bax expression are corroborated by the lower amount of cleaved and activated caspase-3 protein present in NT2 cell extract after 24 days of differentiation (Fig. 2.8G). In SH-SY5Y cell model, despite the increased level of Bcl-2 mRNA

expression in the presence of CORM-A1, non-significant difference in Bax mRNA expression was found due to CORM-A1 treatment after 7 days of differentiation process (Fig. 2.8F). Also, a decrease on caspase-3 activation was found in SH-SY5Y cells (Fig.2.8H).

Taking all together, it seems that CORM-A1 improved neuronal yield by limiting cell death during neuronal differentiation process. Thus, higher amounts of post-mitotic generated neurons are achieved in the presence of CORM-A1.



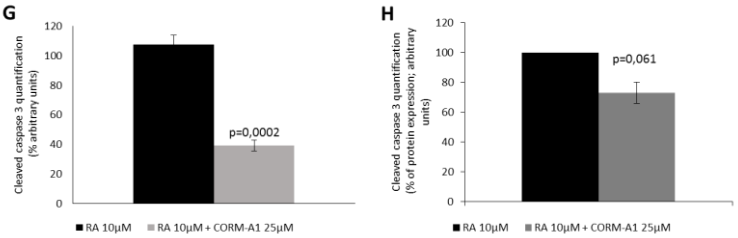
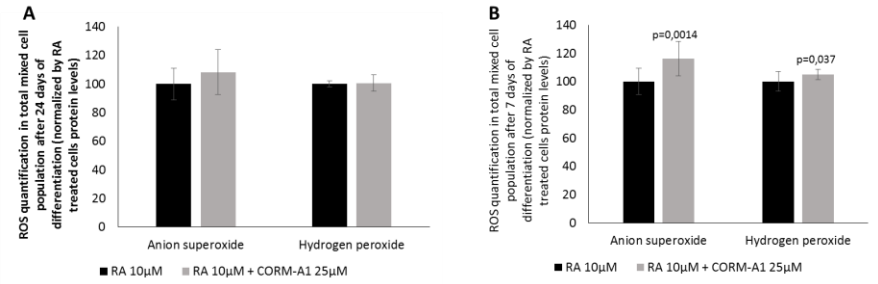


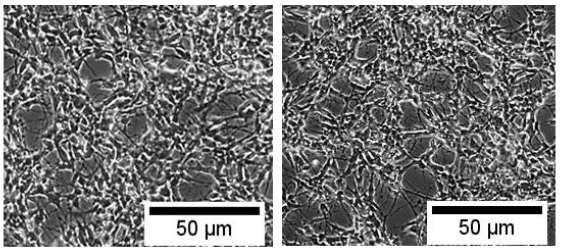
Figure 2.8 – CORM-A1 prevents cell death in mixed cell population (following neuronal differentiation procedure). (A) NT2 PI incorporation evaluation after 24 days of differentiation; (B) SH-SY5Y PI incorporation evaluation after 7 days of differentiation; (C) NT2 mixed population's Bcl-2 expression at two distinct time points, day 17 and day 24, during neuronal differentiation process; (D) SH-SY5Y mixed population's Bcl-2 expression after 7 days of differentiation; (E) NT2 mixed population's Bax expression after 24 days of differentiation; (F) SH-SY5Y mixed population's Bax expression after 7 days of differentiation; (G) cleaved caspase-3 protein quantification by western blot analysis of cell extracts obtained after 24 days of NT2 neuronal differentiation; (H) cleaved caspase-3 protein quantification by western blot analysis of cell extracts obtained after 7 days of SH-SY5Y neuronal differentiation.

Reactive oxygen species role in CORM-A1 modulation of neuronal differentiation

Small amounts of CO stimulate endogenous mechanisms of cellular defense and maintenance of tissue homeostasis in a ROS dependent manner [12], [15], [19]–[21], [32]. Furthermore, ROS signaling also modulates neuronal differentiation. Thus, ROS generation was quantified in mixed cell populations following neuronal differentiation process (Fig. 2.9). For NT2 cell line, no significant difference was observed in the levels of ROS generation (Fig. 2.9A). In contrast, for SH-SY5Y cells there was an increase on ROS levels in the

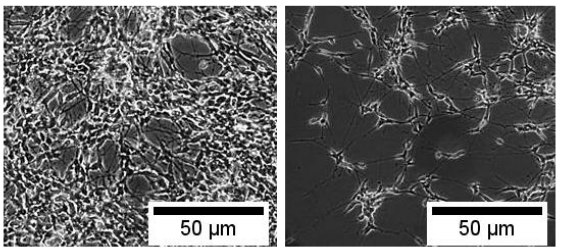
presence of CO treatment. At day 1 of neuronal differentiation process, CO promotes ROS generation 1h after treatment (Fig. 2.9B).





DAY 7
RA 10µM

DAY 7
RA 10µM + CORM-A1 25µM



DAY 7
RA 10µM + NAC 5mM

DAY 7
RA 10µM + CORM-A1 25µM + NAC 5mM

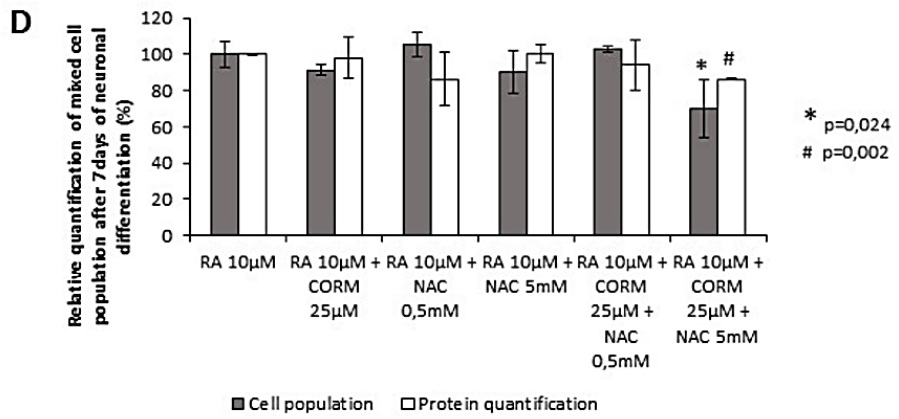


Figure 2.9 – Role of reactive oxygen species (ROS) in CORM-A1 modulation of neuronal differentiation. (A) Quantification of intracellular ROS in NT2 mixed cell populations after 24 days of neuronal differentiation (values normalized by total cell count); (B) Intracellular ROS quantification in SH-SY5Y mixed population 1h after treatment with RA 10µM ± CORM-A1 25µM and after 7 days of differentiation (values normalized by total cell count); (C) Phase contrast images of SH-SY5Y cells after 7 days of differentiation under different conditions (including N-acetylcysteine treatment); (D) Quantification of cell number and protein levels of SH-SY5Y cells after 7 days of differentiation under different conditions.

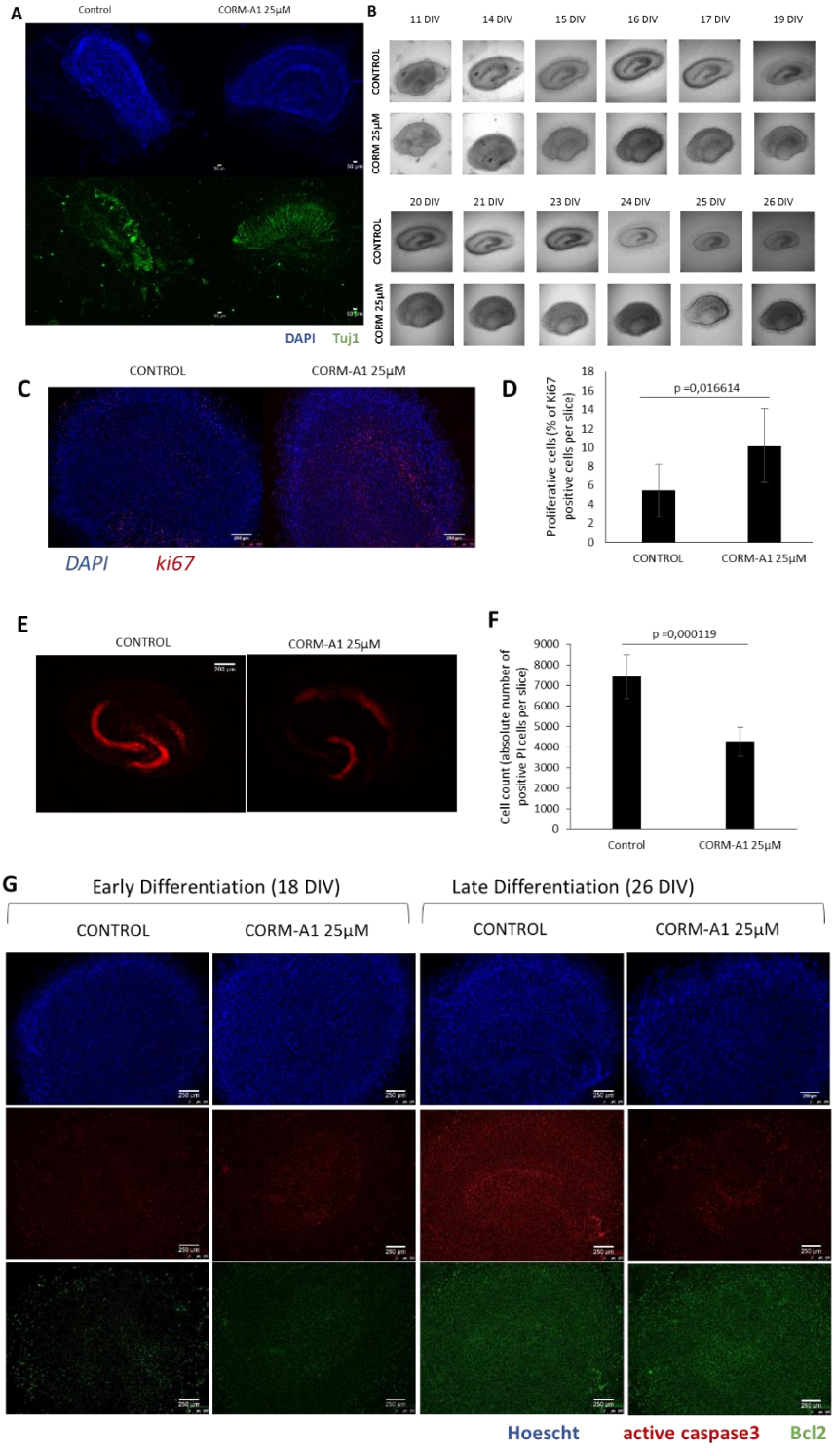
These data are in accordance with the fact that CO generates ROS as signaling molecules at short periods after treatment [8], [12], [15]. Furthermore, at the end of neuronal differentiation process, CO-treated SH-SY5Y cells presented higher levels of ROS, compared to the levels of cell population treated only with RA, which suggests an increase on cellular oxidative state in the presence of ROS (Fig. 2.9B). Moreover, when SH-SY5Y differentiation procedure was done in the presence of the anti-oxidant N-acetyl-cysteine (NAC), CORM-A1 positive modulation (increased yield) of neuronal differentiation is lost (Fig. 2.9C). Indeed, total amount of cell population at day 7 of neuronal differentiation procedure was lower in the presence of NAC

compared to RA and CORM-A1 treatment, cell population was quantified by cell counting and total protein amounts (Fig. 2.9D). Thus, CO modulation of neuronal differentiation is a ROS dependent process, at least for SH-SY5Y cell model.

In vivo-like validation of CO modulation of neuronal differentiation

In order to validate the in vitro data (NT2 and SH-SY5Y cell lines), CORM-A1 modulation of neuronal differentiation was also assessed in OHSC. This is an advantageous *ex vivo* model for assessing cell proliferation, differentiation and migration in a tissue context, since it mimics the *in vivo* cerebral tissue structure [53]–[55]. Continuous formation of new neurons can be evaluated using this *ex vivo* model of adult neurogenesis, which occurs, particularly in both Subgranular region of the Dentate Gyrus and in *Cornu Ammonis* region 1 [1], [55], [67]. Cultures were mature at 11 days *in vitro* (DIV) [54], [56]–[58]. After that, the culture medium was exchanged by a differentiation medium and slices were treated with 25μM CORM-A1 twice a week [57]–[60]. To investigate effects on cell proliferation, cultures were fixed after 3 days of CORM-A1 treatment (at 14 DIV, ki67 was used as a marker for cell proliferation). To study neuronal differentiation, matured OHSC were maintained for 12 days longer (until 26 DIV) to investigate the expression of neuronal markers (Tuj1) (Fig.2.1C).

In OHSC, no difference in cell morphology was found in the slices supplemented or not with CORM-A1. CORM-A1 treatment increased Tuj1 expression following the differentiation process at 18DIV (Fig. 2.10A). Interestingly at 26DIV it is visible a shrinkage in the control hippocampal slices that is abolished in the case of CORM-A1 supplemented cultures (Fig.2.10B), indicating a cytoprotective effect of CORM-A1. Furthermore, in OHSC expression of ki67 was assessed by immunohistochemistry for following proliferative cell fate. It was observed that CORM-A1 promoted increased expression of ki67 among whole hippocampal slice (Fig. 2.10D), in particular in dentate gyrus (Fig. 2.10C), indicating an improvement on proliferative cell population by CO. In conclusion, CORM-A1 stimulates increasing number of differentiated neurons in OHSC without causing any morphological alteration in hippocampal tissue, consolidating the effect already observed in cell line models.


A
Control
CORM-A1 25µM
DAPI Tuj1
B
11 DIV
14 DIV
15 DIV
16 DIV
17 DIV
19 DIV
CONTROL
CORM 25µM
20 DIV
21 DIV
23 DIV
24 DIV
25 DIV
26 DIV
C
CONTROL
CORM-A1 25µM
DAPI ki67
D
Proliferative cells (% of Ki67 positive cells per slice)
p =0,016614
CONTROL
CORM-A1 25µM
E
CONTROL
CORM-A1 25µM
F
Cell count (absolute number of positive PI cells per slice)
p =0,000119
Control
CORM-A1 25µM
G
Early Differentiation (18 DIV)
Late Differentiation (26 DIV)
CONTROL
CORM-A1 25µM
CONTROL
CORM-A1 25µM
Hoescht
active caspase3
Bcl2

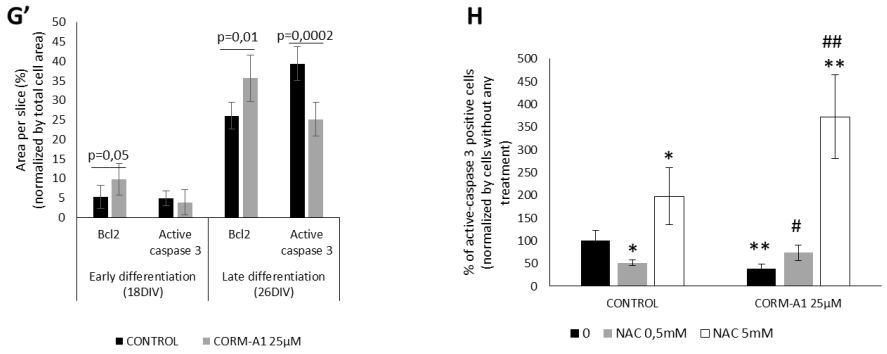


Figure 2.10 – Validation of CORM-A1 role in neuronal differentiation in *ex-vivo* model of OHSC. (A)OHSC immunohistochemistry at 26 DIV (green staining: Tuj1; blue staining: DAPI; magnification 50x); (B) phase contrast images during neuronal differentiation process (magnification 40x); (C) OHSC immunohistochemistry at 14 DIV (red staining: ki67; blue staining: Hoechst33342; magnification 50x); (D) Proliferative cells (ki67 positive cells) per slice. Ratio calculated taking in account the area positively stained for ki67 and Hoechst33342; (E) OHSC PI uptake (red staining) at 26 DIV (magnification 50x); (F) PI uptake per slice – count of the positively marked cells in each slice; (G) OHSC Active caspase-3 and Bcl-2 expression at two distinct time points, 18 DIV and 26 DIV, during neuronal differentiation process (red staining: active caspase-3; blue staining: Hoescht33342; green staining: Bcl2) (magnification 40x); Area per slice. Ratio calculated taking in account the area positively stained for active caspase-3 and Bcl2 at two distinct time points, 18 DIV and 26 DIV, during neuronal differentiation process; (H) Immunohistochemistry quantification of active caspase-3 positive cells in HOSC after 26DIV, treated with N-acetyl-cysteine and CORM-A1 during neuronal differentiation process.

For assessing cell death in this model, PI uptake was measured in OHSC after 26 DIV accordingly with Noraberg [60] and it was observed that CORM-A1 supplementation prevented the uptake of this dye in the internal part of the slice (Fig. 2.10E-F). Concerning apoptosis assessment, it was observed by immunohistochemistry, an increase in Bcl-2 expression in slices treated with CORM-A1, both in early (18 DIV) and late (26 DIV) differentiation stages. Likewise, the occurrence of cleaved and active caspase3 was lower in CORM-A1

treated slices at late differentiation stage (Fig. 2.10G). Moreover, whenever HOSC differentiation procedure was done in the presence of the anti-oxidant NAC, CORM-A1 modulation of cell death (assessed by decreased active caspase 3 positive cells) during neuronal differentiation was lost (Fig. 2.10H). Thus, CO inhibition of cell death and consequently its modulation of neuronal differentiation is a ROS dependent process in HOSC model.

The data obtained in OHSC validate CO's increasing number of differentiated neurons in a more physiological model. Furthermore, these results reinforce the hypothesis that CO increases number of differentiated neurons by (i) limiting apoptosis in a ROS signaling dependent manner and by (ii) enhancing cell proliferation.

DISCUSSION

The increasing prevalence of aging related diseases, such as neurodegenerative diseases and ischemic stroke, boosts the investigation of stem cell based therapies. Better understanding the molecular mechanisms underlying neuronal differentiation opens new windows for potential replacement therapies against neuronal impairment or death in the CNS. Regarding this, our aim was to study whether low doses of CO can improve neuronal production yields in three models with different complexity levels: human neuroblastoma

SH-SY5Y cell line, human teratocarcinoma NT2 cell line and mice organotypic hippocampal slice culture (OHSC). SH-SY5Y and NT2 cell lines are derived from neural crest and embryonic carcinoma cells, thus the stem cell characteristics are preserved. RA treatment stimulates neuronal differentiation, giving rise to neurons expressing neuronal markers [43], neurotransmitters [42], [44] and also able to form synapsis [47], [48]. However, one cannot disregard the fact that they also maintain some mutagenic and oncogenic potential [38], [52]. These issues can reduce the physiological representativeness of these cell line models concerning neuronal differentiation. Therefore CO modulatory effect was also validated in OHSC, which represents a more physiological model for studying adult neurogenesis, where its neuroanatomy and cell-to-cell interactions are partially preserved. In OHSC model, neurogenesis is stimulated by addition of growth factors [53]–[55]. In all studied models, CORM-A1 supplementation during neuronal differentiation procedures yielded higher levels of neuronal cell population and preserved tissue structure and homeostasis in the particular case of HOSC.

It is increasingly accepted that CO acts in a ROS-dependent manner; activating endogenous mechanisms of defense and maintaining tissue homeostasis, either *via* a rapid boost of CO (such as the use of CO saturated solutions) or slower administration of CO (for instance CORM-A1 presenting a half-life of 21 minutes) [8], [15], [16], [18], [68]. Actually, there are several examples that CO acts by activation

of a preconditioning state *via* ROS signaling [12], [19]–[21]. Furthermore, CO is intimately related to cell metabolism modulation [9], [69], which is also a sort of conditioning of cells to a oxidative metabolic profile, which is tightly connected to cell differentiation process. Thus, in the tested models, only two CORM-A1 treatments *per* week (amongst with RA or growth factors supplementation) can in fact change cell fate and promote increased number of differentiated neurons by boosting cell survival that, in turn, promote the continuous improvement of neuronal differentiation yield. One cannot disregard that different expositions of CO could change its biological effect. Indeed, it can be speculated that continuous CO treatment, even at low concentrations, could become cytotoxic. Further studies are necessary to clarify this issue.

Furthermore, ROS generation appear to be key signaling factors in CO-induced neuronal differentiation in SH-SY5Y cell line and in HOSC model. Increased levels of ROS were found in the presence of CORM-A1. Most importantly, the use of the anti-oxidant molecule (N-acetyl-cysteine) reverted the CO-induced prevention of cell death and improvement on differentiated neurons number.

There are three hypotheses for the mechanisms underlying CO-induced improvement of neuronal yield: (i) increased proliferative cell population, (ii) protection against cell death and/or (iii) direct facilitation of neuronal differentiation process. Our data indicate that CO increase proliferative cell population; nevertheless there is a great

amount of literature demonstrating that CO has an anti-proliferative role. In the late 90's Morita and colleagues found that CO had the capacity to decrease proliferation of vascular smooth muscle cells [70]. Since then, several studies pointed out the anti-proliferative ability of CO in different models: airway smooth muscle, pancreatic cancer cells, breast cancer cells and human umbilical vein endothelial cells [71]–[74]. Furthermore, Chen and colleagues were the first correlating the well-established anti-apoptotic capacity [8], [9], [12], [14], [21], [37] of HO-1/CO pathway with the increased proliferation of olfactory receptor neurons [75]. Also, it is known that cellular proliferation and programmed cell death are directly related with neuronal differentiation from early embryonic proliferating stages until late adult stages [3], [4]. The incidence and role of cell death in the different stages are yet not well understood. For instance, death of neuronal cells during development is much better characterized than the cell death occurring during adult neurogenesis. Furthermore, a fine-tuning of neuronal population is dependent on the balance between proliferation/differentiation and cell death [4], [76]–[79]. Thus, we have shown the importance of cell death modulation during adult neuronal differentiation. Our data demonstrate that CO improvement of neuronal differentiation yield is clearly related to the control of cell death in a ROS signaling dependent manner. CORM-A1 treatment increased cell viability in the three tested models. CORM-A1 increased the expression of anti-

apoptotic gene Bcl2, while limited caspase-3 activation. In the particular case of NT2 cells, CORM-A1 decreased the expression of pro-apoptotic gene Bax. Because there are strong evidences showing CO as anti-proliferative factor and our data are apparently controversial since CO increased proliferative cell population; one can speculate that this increase might be a consequence of a cytoprotective role of CO. Indeed by limiting cell death, CO would increase the amount of proliferating cells, which in turn generates more neuronal precursors and mature neurons, as it was observed in NT2 and SH-SY5Y models.

CO emerges as a promising therapeutic molecule that increases the number of differentiated neurons by preventing cell death during the proliferation of stem/progenitor cells. Although the apparent increase on proliferating cell population might be due to the anti-apoptotic capacity of CO, the hypothesis of CO-improved proliferative capacity cannot be excluded since proliferation was not deeply studied. Also, one cannot disregard that CO can also facilitate neuronal differentiation *per se*, by modulating cellular processes involved in neurogenesis. Nevertheless, it can be excluded any CO's involvement on the increased expression of retinoic acid receptors. Further studies are needed to clarify CO mode of action in stem cells differentiation, namely through cell metabolic adaptations, during neuronal differentiation and maturation. Migration of newborn

neurons, integration into neuronal circuits and subsequent cell survival should be addressed using organotypic brain slice cultures.

Finally, the novelty of this work consists in establishing CO as a promising cytoprotective molecule for neuronal differentiation processes. Potentially two scenarios can be envisaged for the use of CO: (i) improving *in vitro* neuronal production for cell therapies, such as transplantation or (ii) modulation of endogenous neurogenesis *via,* for instance, systemic effect of CO or direct perfusion through blood-brain barrier.

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III

A novel class of carbon monoxide-releasing molecules enhances dopaminergic differentiation of human neural stem cells

This chapter is based on data to be published as:

A novel class of carbon monoxide-releasing molecules enhances dopaminergic differentiation of human neural stem cells

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unpublished data

ABSTRACT

Exploratory studies using human fetal tissue have suggested that intrastriatal transplantation of dopaminergic neurons may become an effective treatment for patients with Parkinson's disease (PD). However, the use of human fetal tissue is compromised by ethical, regulatory and practical concerns. Human stem cells constitute an alternative source of cells for transplantation in PD patients. Nevertheless, efficient protocols for controlled dopaminergic differentiation need to be developed.

Short-term low-level carbon monoxide (CO) exposure has been shown to affect cell signaling in several tissues, resulting in both cytoprotection and anti-inflammation. The present study investigated the role of CO on dopaminergic differentiation of human neural stem cells (NSCs). In addition, a new system for CO production and delivery was used, based on decarbonylation reaction of methyldiphenylsilacarboxylic acid, a novel CO-releasing molecule. Short-term cell exposure to 25 parts per million (ppm) CO at days 0 and 4 significantly increased the relative content of β-tubulin III immature neurons and tyrosine hydroxylase (TH) expressing catecholaminergic neurons, as assessed 6 days after differentiation. Also the number of microtubule associated protein 2 (MAP2) mature neurons had increased significantly. Moreover, the content of apoptotic cells (active Caspase 3 positive cells) was reduced, whereas the expression of a cell proliferation marker (Ki67) was left

unchanged. Increased VEGF in cultures exposed to CO may suggest a mechanism involving mitochondrial alterations and metabolic modulation. In conclusion, the present procedure using controlled, short-term CO exposure allows efficient dopaminergic differentiation of human NSCs at low cost and may as such contribute to the future development of donor cells for transplantation in PD.

CONTENTS

Ana S. Almeida had carried out all the experiments concerning mRNA expression and metabolic assessment, as well as data analysis and interpretation and discussion of results from all the study.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder affecting more than six million people worldwide [1]. The disease is associated with a progressive loss of midbrain dopaminergic neurons and subsequent depletion of striatal dopamine. Cardinal symptoms include bradykinesia, rigidity, tremor and postural instability, but non-motor symptoms also occur, such as loss of sense of smell, constipation and sleep disturbances [2].

Several explorative clinical studies using human fetal ventral mesencephalic tissue have indicated that intrastriatal transplantation may become a future treatment for PD [3]–[8]. However, the use of human fetal tissue is hampered by ethical concerns, suboptimal survival of grafted dopaminergic neurons, development of postgrafting dyskinesias in some patients, and the complex logistics related to collection and storage of the donor tissue [3], [7], [9]–[13]. Pre-differentiated induced pluripotent stem cells, embryonic stem cells and neural stem cells (NSCs) represent potential alternative sources of cells for cell replacement therapy in PD. NSCs are self-renewable multipotent cells that can be isolated from the developing and mature nervous system. Such cells may have significant advantages compared to human fetal tissue as they can be propagated to almost unlimited numbers of relatively homogenous cells *in vitro* and can be frozen without significant loss of viability [14], [15]. Nevertheless, efficient, simple and cost-effective protocols

for controlled generation of functional dopaminergic neurons are still not available.

Carbon monoxide (CO) is an endogenous product of heme degradation, a reaction catalyzed by the enzyme heme oxygenase (HO) [16]. This gasotransmitter shows several beneficial biological activities and has been the target of extensive studies related to cardiovascular diseases, inflammatory disorders and organ transplantation [17]. The great potential of CO in biomedical applications has prompted development of several delivery strategies of CO for therapeutic or research purposes. Gas inhalation is the most simple strategy and has been greatly used in pre-clinical *in vivo* experiments [18]–[20]. Cell cultures can also be exposed to CO in gas chambers as described for neurons [21] and macrophages [22]. Another possible strategy for *in vitro* application of CO gas is the use of CO-saturated solutions [23], [24]. Nevertheless, for all these approaches CO gas bottles are handled with the potential risk of leaking the odorless and highly toxic gas. Furthermore, gas inhalation is not the most appropriate method for CO administration in a clinical context, since it promotes increased carboxyhaemoglobin levels, as well as CO delivery occurs to both healthy and diseased tissues. Therefore, CO-releasing molecules (CORMs) have been developed for providing controlled CO delivery [25]. The most studied non-metal based CORM is boranocarbonate $[H_3BCO_2]Na_2$ (CORM-A1), which has been shown to modulate cytoprotection,

hormesis and inflammation in several studies [26]–[28]. There are also many metal-based compounds studied in biological systems, and the most explored is the water-insoluble dimer $[Ru(CO)_3Cl_2]_2$ (CORM-2) and its water soluble derivative $Ru(CO)_3Cl(\kappa^2\text{-}H_2NCH_2CO_2)$ (CORM-3). CORM-2 and CORM-3 have been tested in pre-clinical studies of cardioprotection [29], [30], inflammation [31]–[33], neuroprotection [34], transplantation [35] and pain [36].

In the CNS, the CO/HO axis is a key player in processes involved in cytoprotection, vasomodulation, neuroinflammation, neural cell death, metabolism and cellular redox responses [37]. CO was first recognized as a neurotransmitter by Verma and colleagues in 1993 [38], and their work led to extensive research on CO and HO in the nervous system. Interestingly, both HO activation or induction and exogenous administration of CO were reported to stimulate neuroprotection and maintenance of tissue homeostasis in response to various pathophysiological conditions; including cerebral ischemia [19], [39]–[41], cerebrovasodilation [27], [42], [43], neuroinflammatory [20], [44], [45] and neurodegenerative diseases [46]–[48].

The CO-induced pathways and putative targets are a matter of debate. Nevertheless, it is well accepted that CO activates soluble guanylyl cyclase and nitric oxide synthase, increasing the cGMP and nitric oxide (NO) levels respectively, whose best described effects are modulation of vasodilation [49]. In neurons, CO-induced cGMP

production is involved in protection against cell death [21], [50].

In the present study two major novelties are approached. Firstly, it is assessed the role and the molecular mechanisms of CO on modulation of dopaminergic differentiation of human NSCs. Secondly, a new strategy for delivering CO gas is tested. In this new system, CO is generated by a decarbonylation reaction using the new CORM methyldiphenylsilacarboxylic acid ($MePh_2SiCO_2H$), along with the non-transition-metal activator potassium fluoride and dimethyl sulfoxide [51]. This strategy avoids the use of CO gas bottles, thus being safer and more cost-effective than previously described methods.

MATERIAL AND METHODS

Carbon monoxide releasing molecules (CORMs)

Crystalline silacarboxylic acid was synthesized from the corresponding chlorosilane *via* reduction with metallic lithium, and allowed it to react with CO_2 [51]. By mixing methyldiphenylsilacarboxylic acid ($MePh_2SiCO_2H$) with the non-transition-metal activator potassium fluoride (KF; Sigma) and the solvent dimethyl sulfoxide (DMSO; Sigma) a decarbonylation reaction results in CO-release (Fig. 3.1A) [51]. For the present study, a plexiglass chamber was developed (Fig. 3.1B). The amount of solids required to achieve a predefined level of CO (12,5-100 ppm) were

placed in a glass vial (Supelco) and transferred to the exposure chamber together with the culture plates/flasks and a CO monitor (Dräger). The chamber was placed at 36 ºC, 5% CO_2 and 95% humidified air. To start CO release, DMSO was lead through a separator in the wall of the chamber and into the vial with silacarboxylic acid/KF. A ventilator homogenized the concentration of gas in the closed atmosphere (Fig. 3.1B). The CO concentration was measured throughout all experiments (Fig. 3.2).

Culturing and passaging of NSCs

Two human ventral mesencephalic (VM) stem cell lines generated in previous studies were used (hVMbclX_L). In brief, VM cells were derived from a 10-week-old foetus and immortalized using a retroviral vector coding for *v-myc* (LTR-vmyc-SV40p-Neo-LTR), creating a multipotent cell line (hVM1) [52]. The hVM1 cells were genetically modified (MLV-based retroviral vector) to over-express the anti-apoptotic gene BclX_L (LTR-Bcl-X_L-IRES-rhGFP-LTR), essentially as described by Liste *et al.* [53].

Cells were propagated in poly L-lysine (PLL/10 µg/ml; Sigma)-coated culture flasks containing HNSC100 medium (DMEM/F12 w. Glutamax (Gibco), 2% (v/v) 30% glucose (Sigma), 0.5% (v/v) 1 M Hepes (Gibco), 2.5% (v/v) AlbuMAX-I (Gibco), 1% (v/v) N2 supplement (Gibco), 1% (v/v) NEAA (Sigma) and 1% penicillin/streptomycin (Gibco))

supplemented with 20 ng/ml epidermal growth factor (EGF, R&D Systems) and 20 ng/ml basic fibroblast growth factor (bFGF, R&D Systems) at 36 ˚C, 5% CO_2/95% humidified air. Medium was changed every third day, and cells passaged at 80% confluence.

Neuronal differentiation protocols

NSCs were passaged and plated into PLL-coated 24-/96-well trays or T75 culture flasks (Nunc, Sigma) with HNSC100 medium. hVMbclX_L cells were exposed to CO for 30 min. Untreated cultures served as controls. hVMbclX_L cultures received CO treatment at day 0 followed by differentiation for 1, 6 and 10 days or were exposed to CO at days 0 and 4 and differentiated until day 6 or 10 (Fig. 3.1C).

Fixation and immunocytochemistry

Monolayer cultures were fixed (20 min) in 4% paraformaldehyde/0.15M phosphate buffer. For immunocytochemistry cultures were washed in 0.05M tris-buffered saline (TBS) containing 0.1% triton X-100 (Sigma) and pre-incubated (30 min) in TBS/10% donkey or sheep serum (Gibco). Primary antibodies (24 hrs; 4 ˚C) were diluted in TBS/10% donkey or sheep serum: Tyrosine hydroxylase (TH; polyclonal rabbit; Chemicon) 1:600; β-tubulin III (β-tubIII; monoclonal mouse; Sigma) 1:2000; human nuclei (HN; monoclonal mouse; Chemicon) 1:500; microtubule

associated protein 2ab (MAP2; monoclonal mouse; Sigma) 1:2000; Ki67 (monoclonal mouse; BD Pharmigen) 1:500; Caspase3 (Casp3; polyclonal rabbit; R&D Systems) 1:5000.

Cultures were then incubated for 1 hr with biotinylated anti-rabbit or anti-mouse antibodies (GE Healtcare) diluted 1:200 in TBS/10% donkey or sheep serum followed by 1 hr with horseradish peroxidase-conjugated streptavidin (GE Healthcare) diluted 1:200 in TBS/10% donkey or sheep serum. For development/visualization 3,3´-diaminobenzidine (DAB; Sigma) was used.

Western blotting

Western blotting was performed as described by Krabbe *et al.* [54]. Membranes were incubated (overnight/4 ºC) with anti-TH (1:2000; monoclonal mouse; Chemicon) or anti-β-tubIII antibody (1:2000; monoclonal mouse; Sigma) diluted in TBS/Tween-20, washed, incubated (1 hr) with HRP-conjugated anti-mouse antibody (1:2000; DAKO) diluted in TBS/Tween-20, developed with chemiluminiscence (SuperSignal®Extended duration substrate; Thermo Scientific), and visualized using a CCD camera. Loading control: alpha-actin antibody (1:6000; mouse; Chemicon).

Quantitative-Polymerase chain reaction

Messenger RNA was extracted using the High Pure RNA isolation kit (Roche Diagnostics), and cDNA synthesis was performed using the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics). PCR was performed using specific forward and reverse primers designed for: TH (5′-CGGGCTTCTCGGACCAGGTGTA-3′ and 5′-CTCCTCGGCGGTGTACTCCACA-3′), Nurr1 (5′-CTGCAAAAGGAGACAATATAGACCA-3′ and 5′-ATCGTAGACCCCAGTCACATAA-3′), DAT (5′-TTCCTCAACTCCCAGTGTGC-3′ and 5′-AGGATGAGCTCCACCTCCTT-3′), DBH (5′-CTTCCTGGTCATCCTGGTGG-3′ and 5′-TCCAGGGGGATGTGATAGGG-3′), Glut1 (5'-TTATAGGACCCCGGCCATTG-3′ and 5′-CTGAGCGAGGCAGTGGTTA-3′) and RPL22 (5'-CACGAAGGAGGAGTGACTGG-3' and 5'-TGTGGCACACCACTGACATT-3'). Fast Start DNA Master Plus SYBR Green I (Roche Diagnostics) was applied using the following protocol: denaturation program, 95 °C for 10 min followed by 45 cycles of 95 °C for 10 sec, 60 °C for 10 sec and 72 °C for 10 sec.

MTS cell viability assay

Cell viability was determined using a MTS kit (CellTiter 96®Aq$_{ueous}$One Solution; Promega) according to the manufacturer's instructions and a Vmax kinetic microplate reader with SoftMax®Pro software

(Molecular Devices).

High-performance liquid chromatography (HPLC)

Dopamine and homovanillic acid (HVA) were assessed in culture medium/extracts derived from cells differentiated (14 days) according to our standard protocol supplemented by 25 ppm CO (30 min) at days 0 and 4.

Sample preparation; medium: Cells were washed twice in Hank's balanced salt solution (HBSS; Life Technologies), followed by incubation (2 hrs/36 °C) in 200 µl of HBSS containing 10µM nomifensine (Research Biochemicals International). A 100 µl sample was transferred to HPLC vials containing 50 µl of mobile phase (10% methanol (v/v), 20 g/l citric acid monohydrate, 100 mg/l octane-1-sulfonic acid sodium salt, 40 mg/l EDTA dissolved in Milli-Q water and pH adjusted to 4.0; all from Merck/VWR Chemicals) and stored at -20°C until HPLC analysis with electrochemical detection [55], [56].

Sample preparation; extracts: After removing the culture medium, 150 µl/well of 0.1 M perchloric acid (PCA; Merck) with antioxidants (0.2 g/L $Na_2S_2O_5$, 0.05g/L Na_2-EDTA; Merck) was added. Cells were resuspended in PCA, transferred to dark eppendorf vials on ice, briefly sonicated and centrifugated (20.000 x *g*/20 min/4 °C). The supernatant was stored at -20 °C until analysis.

Multi cytokine array

Conditioned culture medium was frozen (-20 °C), and cells were collected as described for Western blotting but with the cell pellets dissolved in RayBio® Cell Lysis Buffer (RayBioech). Protein concentrations were determined using a protein assay (BioRad). Four membranes (Human Cytokine Antibody Array-5; RayBiotech) were incubated (30 min/RT) with Blocking Buffer (BB) (RayBiotech), and 1 ml conditioned culture medium or 160 µg cell lysate (diluted to 1 ml in BB) was added (incubation; 1 hr/RT followed by 12 hrs/4 °C). After washing, membranes were incubated with biotin-conjugated antibody diluted in BB (2 hrs/RT and 12 hrs/4 °C). Membranes were then incubated with HRP-conjugated streptavidin diluted in BB (2 hrs/RT), washed, developed with chemiluminiscence (RayBiotech), and visualized using a CCD camera (Carestream). Densitometric analysis was performed using Image J software (NIH). Changes >50% relative to control were taken into consideration.

Lactate/Glucose Ratio

Total glucose and lactate concentrations in the culture supernatant were determined with automated enzymatic assays (YSI 7100 Multiparameter Bioanalytical System; Dayton, OH). The rate between lactate production and glucose consumption was obtained by linear regression of the metabolites concentrations. The ratio between

lactate production and glucose consumption rate was determined for each time point.

Cell counting

Quantification of cells was performed using bright field microscopy (Olympus). Cells with an extensive immunostaining and a well-preserved cellular structure were counted in 16 randomly selected areas/well (X200) using an ocular grid (0.5x0.5 mm^2).

Statistical analysis

Statistical analysis was performed using Prism GraphPad Software. Cell numbers were compared by one way analysis of variance followed by Dunnett's multiple comparisons test. Student's t-test was used when comparing only two groups. $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

RESULTS

Carbon monoxide release system

To characterize and validate decarbonylation reaction from the new CO-releasing molecule (CORM) $MePh_2SiCO_2H$ (Fig. 3.1A), the CO concentration was measured in the gas chamber every minute throughout a 30 min exposure period (Fig. 3.1B). The CO level increased rapidly after mixing $MePh_2SiCO_2H$, potassium fluoride and dimethyl sulfoxide, reaching the required concentrations after 5 min and maintaining a constant level during the entire exposure period (Fig. 3.2).

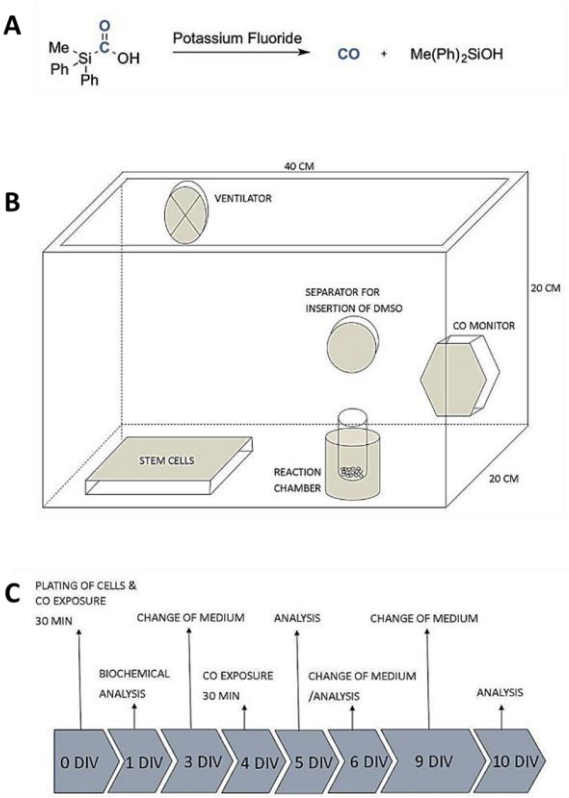


Figure 3.1 - Chemical reaction releasing carbon monoxide (CO) and experimental setup. (A) The chemical reaction releasing CO when mixing $MePh_2SiCO_2H$, KF and DMSO. (B) Illustration of the CO gas chamber. (C) Human neural stem cells were plated at day 0, cultured for 4 hrs followed by one or two 30 min CO treatments. All culture medium was changed at days 4, 6 and 9. For experiments with $hVMbclX_L$ cells: 1) cultures received CO treatment at day 0 followed by immunocytochemistry at days 1, 6 and 10, or 2) cultures received CO treatment at days 0 and 4 and were used for cytokine profiling (day 5), immunocytochemistry (day 6 and 10), Western blotting (day 6) or MTS assay (day 6). Untreated control cultures were included in all experiments. DIV=days *in vitro*.

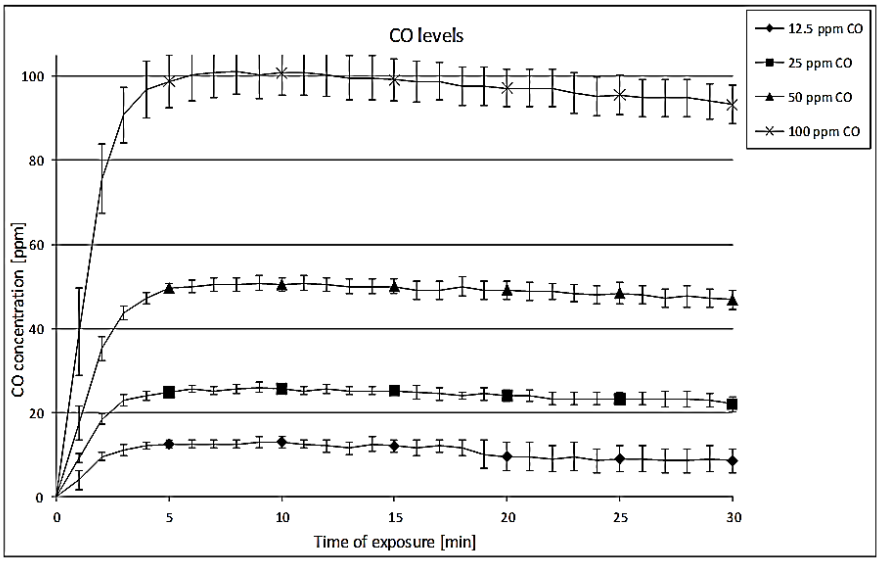


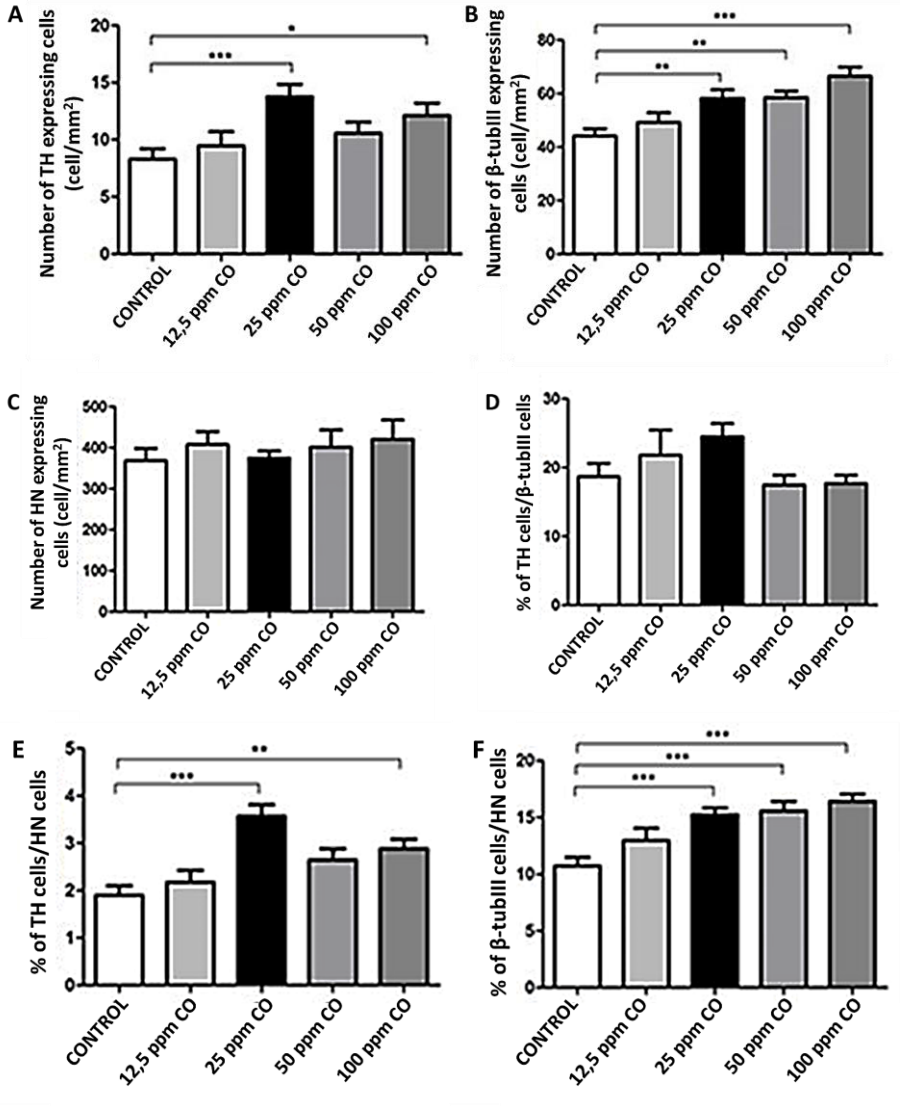
Figure 3.2 - Repeated measure of carbon monoxide (CO) levels in the CO chamber during the 30 min exposure period. Measurements visualized in the figure represent data from the analysis of 4 different CO concentrations (12.5-100 parts per million (ppm)). Data are expressed as mean±SEM.

Effect of CO on stem cell differentiation

To investigate the effect of CO on the dopaminergic differentiation, hVMbclX$_L$ cells were differentiated for 6 days and received CO at days 0 and 4 (Fig. 3.1C). At the end of the differentiation process, the density of neurons increased significantly, when the cells were exposed to CO at 25 and 100 ppm compared to control (Fig. 3.3A). Moreover, the percentage of TH neurons relative to human nuclei cells (total cells) was significantly higher for cultures exposed to CO at 25 and 100 ppm compared to control (Fig. 3.3E). Representative digital images visualizing the content and morphology of neurons are shown in Fig. 3.3H.

The density of β-tubulin III neurons increased significantly in cultures treated with 25-100 ppm CO compared to control (Fig. 3.3B). Furthermore, the percentage of β-tubIII-expressing neurons of total cells was significantly higher (Fig. 3.3F). No differences in total cells were detected (Fig. 3.3C). Representative images of these cells are shown in Fig. 3.3H.

TH and β-tubIII expression was also investigated by Western blotting (WB) showing increased TH expression at 25 and 100 ppm CO compared to control. Moreover, there was indication of increased β-tubIII expression in all CO treated groups (Fig 3.3G). In summary, short-term CO exposure during stem cell differentiation has the capacity to increase both density and relative content of cells expressing TH and β-tubIII.


A
Number of TH expressing cells (cell/mm²)
20
15
10
5
0
*
***
CONTROL
12,5 ppm CO
25 ppm CO
50 ppm CO
100 ppm CO
B
Number of β-tubIII expressing cells (cell/mm²)
80
60
40
20
0
***
**
**
CONTROL
12,5 ppm CO
25 ppm CO
50 ppm CO
100 ppm CO
C
Number of HN expressing cells (cell/mm²)
500
400
300
200
100
0
CONTROL
12,5 ppm CO
25 ppm CO
50 ppm CO
100 ppm CO
D
% of TH cells/β-tubIII cells
30
20
10
0
CONTROL
12,5 ppm CO
25 ppm CO
50 ppm CO
100 ppm CO
E
% of TH cells/HN cells
5
4
3
2
1
0
**
***
CONTROL
12,5 ppm CO
25 ppm CO
50 ppm CO
100 ppm CO
F
% of β-tubIII cells/HN cells
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CONTROL
12,5 ppm CO
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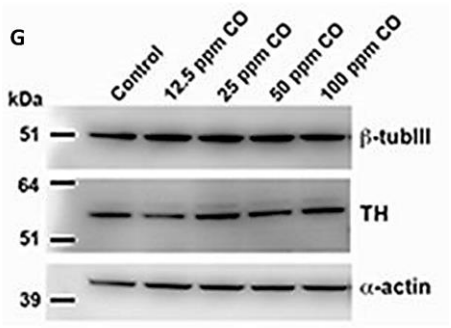
100 ppm CO



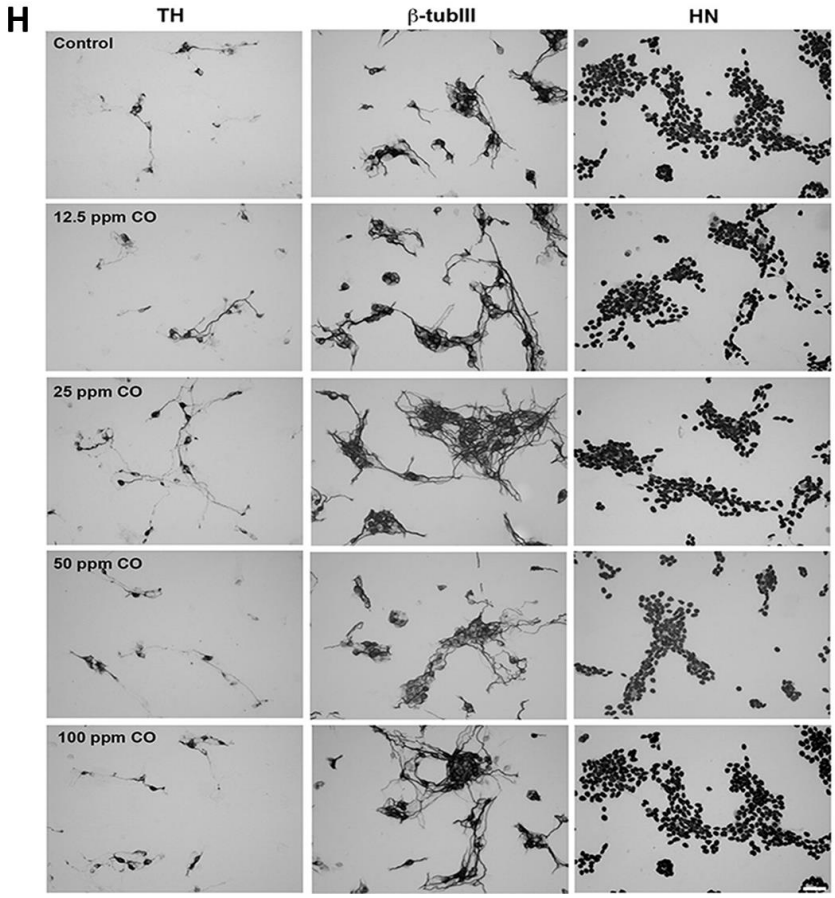


Figure 3.3 - Dose response effects of short-term carbon monoxide (CO) treatment on neuronal differentiation of human neural stem cells (hVMbclX$_L$). Quantitative analysis of total cells (human nuclei cells), cells differentiated into TH and β-tubIII neurons in 6-day-old cultures. (A) Quantification of TH neurons showed a significant increase for 25 and 100 ppm CO compared to control; (B) Quantification of β-tubIII neurons revealed a

significant increase for cells treated with 25, 50 and 100 ppm CO; (C) No differences between numbers HN cells were seen; (D) The percentage of TH neurons of β-tubIII neurons did not differ between the groups; (E) Exposure to 25 and 100 ppm CO resulted in a significant increase in the percentage of TH neurons of total cells, and (F) 25-100 ppm CO resulted in a significant increase in the percentage of β-tubIII neurons of total cells as compared to untreated controls. Data are based on four independent experiments and expressed as mean±SEM (*p<0.05, **p<0.01, ***p<0.001); (G) Western blotting for β-tubIII showed an increase in signal intensities for all CO treatment groups compared to control; (H) Digital images of cultures treated with CO expressing TH, β-tubIII and HN. Scale bar = 50 μm.

Influence of cell differentiation time

To address if the effect of CO was transient or long-lasting hVMbclX$_L$ cells received CO treatment (25 ppm; 30 min) at days 0 and 4 and were differentiated for 6 or 10 days (Fig. 3.4). At day 6 and 10, the content of TH neurons relative to β-tubIII neurons had increased significantly in the CO treated groups (Fig. 3.4B). Furthermore, the relative yields of TH neurons of total cells had increased (Fig. 3.4C). At day 6 and 10, WB analysis indicated increased signal intensities for β-tub III, and at day 10 TH expression was slightly increased for cultures exposed to CO compared to control (Fig. 3.4A). In summary, CO-induced improvement on neuronal differentiation lasts up to 10 days. Representative photomicrographs of TH and β-tubIII neurons are shown in Figs. 3.4D and E.

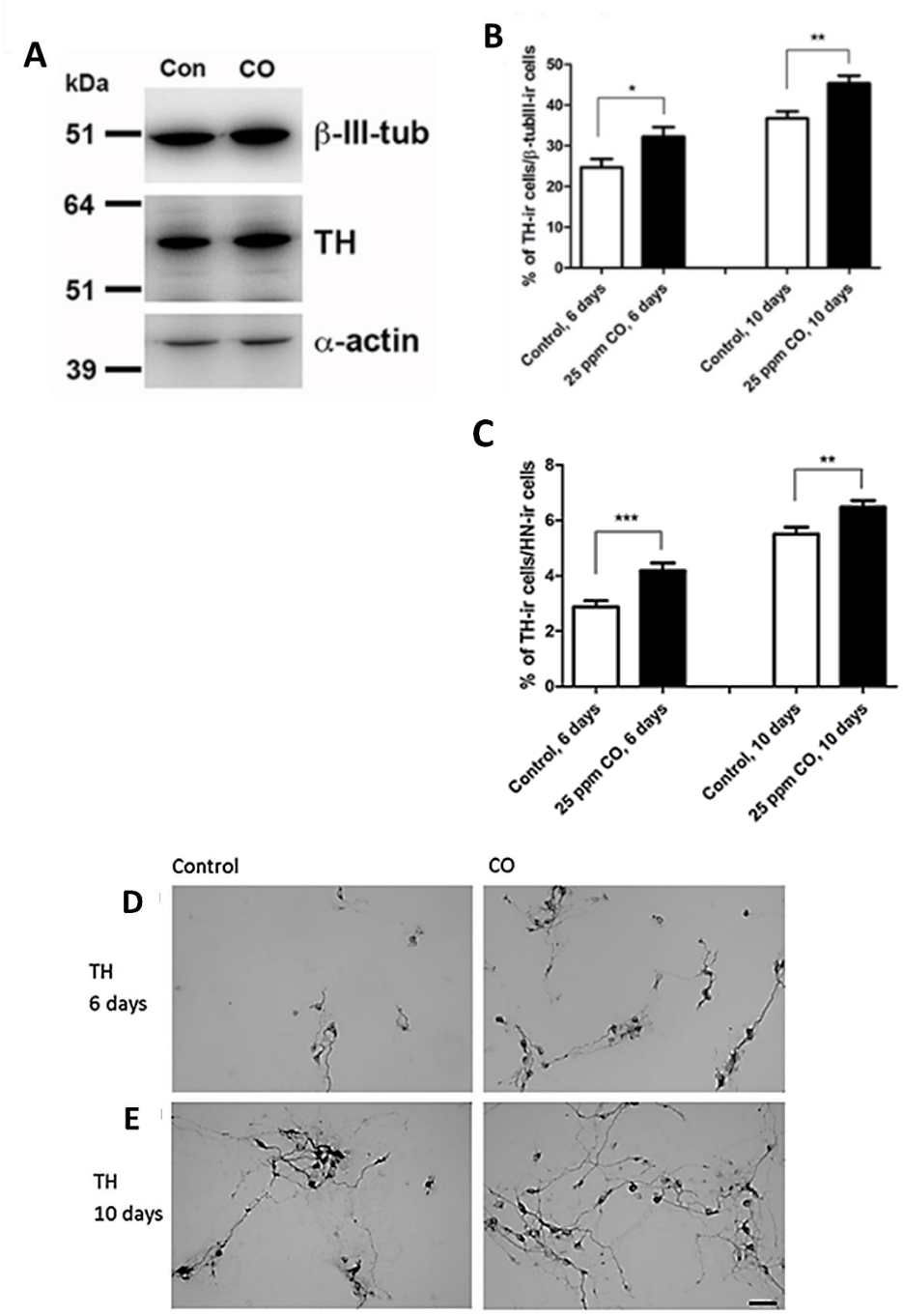


Figure 3.4 - Effects of carbon monoxide (CO) on neuronal and dopaminergic differentiation. Quantitative analysis of 6- and 10-day-old cultures (hVMbclX$_L$) differentiated into dopaminergic neurons by exposure to

25 ppm CO at days 0 and 4. Control cells followed the same protocol but received no CO treatment. (A) Western blotting for β-tubIII and TH showed a slight increase in band intensities after CO treatment compared to controls; (B) At day 10, the percentages of TH neurons of total neurons (β-tubIII) were significantly higher for the CO treatment groups compared to control; (C) At days 6 and 10 also the percentages of TH neurons of total cells (HN cells) were significantly increased for the CO treatment groups. Data are expressed as mean±SEM (*p<0.05, **p<0.01, ***p<0.001); (D, E) Representative digital images of TH neurons displaying a mature neuronal morphology with long processes. Scale bar = 50 μm. Con=control.

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To investigate whether a single dose of CO would be sufficient to elevate the content of TH cells, a group of cultures were exposed to 25 ppm CO at day 0 followed by differentiation for 1, 6 and 10 days. No difference was found between CO treatment and control cultures at day 1, whereas a significant increase in TH neurons was seen at 6 and 10 days after CO treatment (data not shown). The number of HN cells did not differ between CO treatment and control cultures at any time point. Consequently, the relative content of TH neurons had increased significantly at day 6 and 10 in CO exposed cultures compared to controls. In summary, the positive effect of CO on the relative content of TH cells was not transient, and it could be obtained even with a single dose of CO.

Effect of CO on neuronal maturation and dopaminergic capacity

To investigate the potential effect of CO on neuronal maturation, the amount of mature MAP2 neurons were quantified in 6-day-old

cultures (25 ppm CO; 30 min; day 0 and 4 versus control). The percentage of MAP2 neurons of total cells was significantly higher for CO-treated cultures (Fig. 3.5A). Moreover, CO treated cells displayed a more mature morphology with longer and branching processes. Representative images of MAP2 neurons can be seen in Fig. 3.5B.

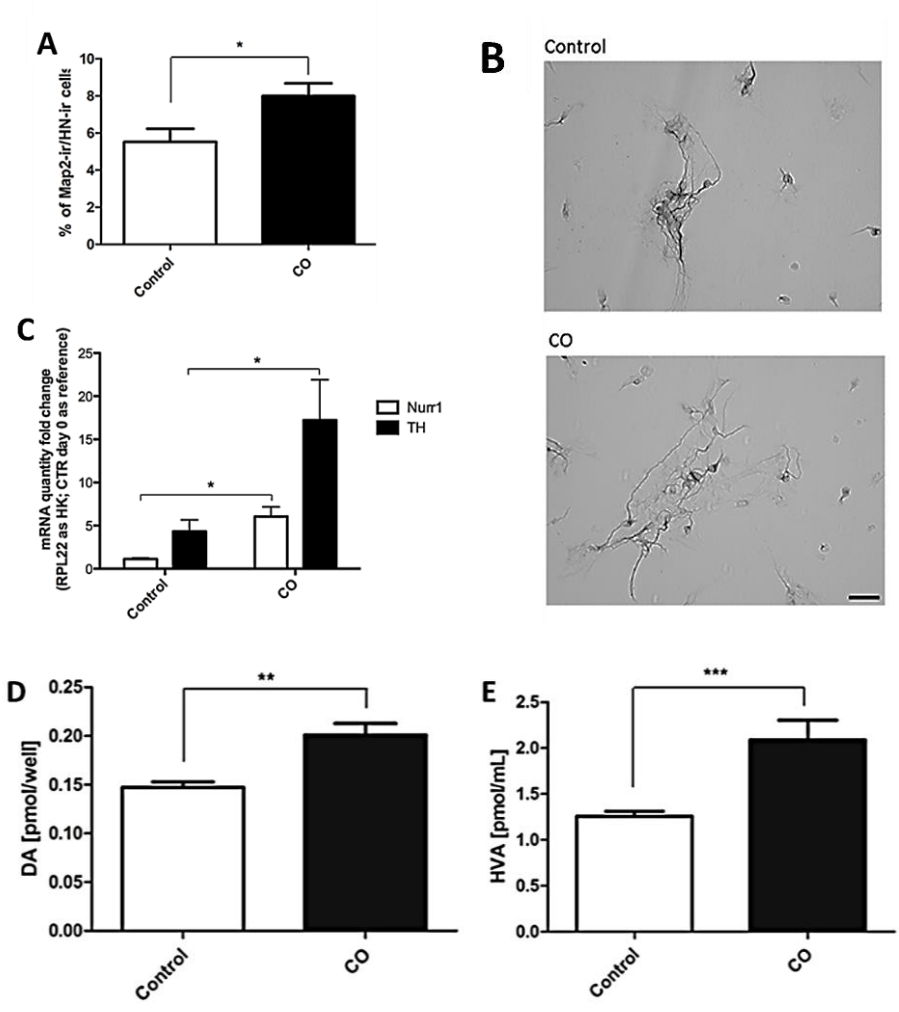


Figure 3.5 - Characterization of neuronal cells in differentiated cultures. Assessment of neuronal maturation of hVMbclX$_L$ cells receiving 25 ppm CO treatment at days 0 and 4 and differentiated for 6, 10 and 14 days. Control

cultures followed the same protocol but received no CO treatment. (A) Percentage of MAP2 neurons of total cells showed a significant increase for cultures treated with CO compared to controls; (B) Digital images of MAP2 neurons showing mature neuronal morphology with long processes. Scale bar = 50 µm; (C) Quantitative mRNA analysis of hVMbclX$_L$ cells receiving 25 ppm CO treatment at days 0 and 4 and differentiated for 10 days. Control cells followed the same protocol but received no CO treatment. Quantities of mRNA were compared with mRNA levels at day 0. TH mRNA levels were significantly increased for cultures treated with CO compared to controls; (D,E) HPLC analysis for DA in cell extracts (**$p<0.01$) and the DA metabolite HVA in culture medium (***$p<0.001$) from untreated controls and cultures receiving 25 ppm CO at days 0 and 4 and differentiated for 14 days. The analyses revealed significant elevations in both DA and HVA for cultures treated with CO compared to controls.

The expression of catecholaminergic/midbrain-specific genes (TH, Nurr1 DAT and DBH) was assessed by mRNA quantification using real-time Q-PCR. TH and Nurr1 (Fig. 3.5C) were increased significantly after CO treatment, whereas DAT levels were lower. DBH mRNA levels were also increased after CO treatment (not shown). In addition, HPLC analysis revealed a significant elevation of dopamine levels in cell extracts (Fig. 3.5D) and significantly increased homovanillic acid (HVA) levels in culture medium from cells exposed to CO compared to controls (Fig. 3.5E). However, noradrenaline could not be detected under the chromatographic conditions used. In summary, CO treatment stimulates neuronal maturation and formation of neurons from hVMbclX$_L$ present catecholaminergic/midbrain-specific gene expression.

Molecular mechanisms of action: Effects of CO on cell proliferation, apoptosis and cytokine profile

To address the effect of CO on cell proliferation, hVMbclX_L cells received a single dose of CO at day 0 (25 ppm; 30 min) and were differentiated for 1, 6 or 10 days. The relative content of proliferative cells did not differ between the groups at any time-point (Fig. 3.6). However, the overall percentage of Ki67 cells was found to increase during the differentiation from day 1 to 10 (Fig. 3.6A). Representative photomicrographs of Ki67 cells are shown in Fig. 3.5B. Further evaluation of cell proliferation was performed by measuring MTS reduction in 6-day-old cultures receiving CO treatment compared to control. The analysis revealed no difference between the groups (Fig. 3.6C). To evaluate the potential effect of CO on apoptosis, 6-day-old cultures receiving CO treatment at days 0 and 4 were immunostained for active Caspase3 (Casp3). The relative content of Casp3 cells was significantly reduced after CO treatment (Fig. 3.6D). In summary, CO is not increasing hVMbclX_L proliferation but, by preventing cell death, is improving neuronal yield during neuronal differentiation.

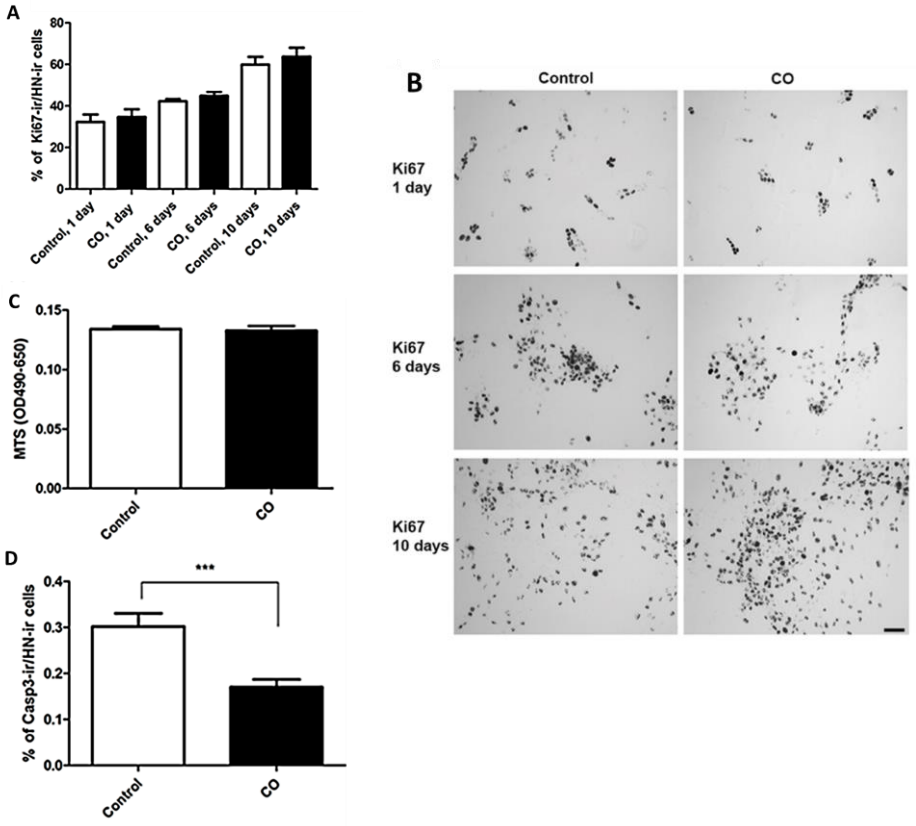
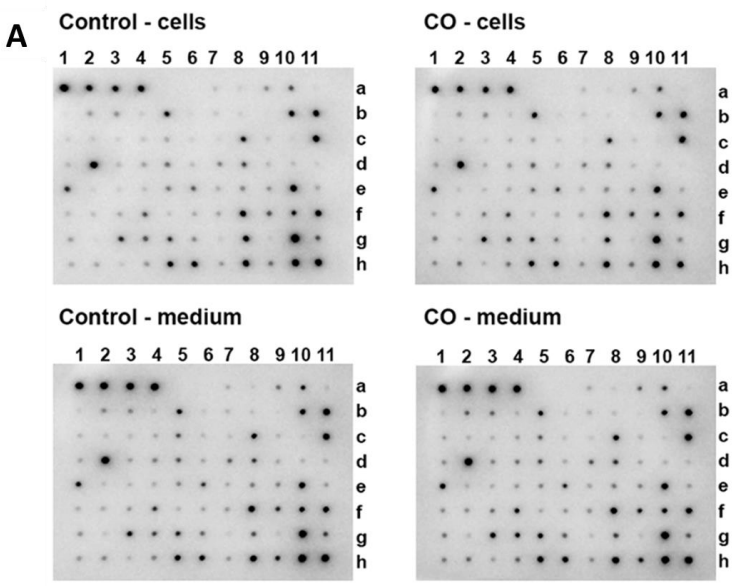


Figure 3.6 - Effects of carbon monoxide (CO) treatment on proliferation and apoptosis. Assessment of proliferation in hVMbclX$_L$ cells receiving 25 ppm CO at day 0 (30 min) and differentiated for 1, 6 and 10 days and apoptosis in cultures receiving 25 ppm CO treatment (30 min) at days 0 and 4 and differentiated for 6 days. Control cells followed the same protocol but received no CO treatment. (A) The percentage of Ki67 cells of HN cells showed no difference between CO treatment and control cultures at any time-point; (B) Representative digital images of Ki67 cells receiving 25 ppm CO treatment at day 0 and differentiated for 1, 6 and 10 days; (C) Analyses of MTS reduction in cultures receiving 25 ppm CO treatment at days 0 and 4 and differentiated for 6 days did not differ between the groups; (D) The percentage of Casp3 apoptotic cells of total cells was significantly reduced for cultures receiving CO compared to controls. Data are expressed as mean±SEM (***$p<0.001$); (E) Digital images of Casp3 cells. Scale bar = 50 µm.

To address whether CO had an effect on cytokine profiles (conditioned culture medium/cell lysates), multi-cytokine analysis was performed using hVMbclX$_L$ cells receiving 25 ppm CO (30 min) at days 0 and 4 and differentiated for 5 days versus untreated controls (Fig. 3.7). Densitometric analysis revealed a reduction in the release of neurotrophin-3 (NT-3) and an increase in neurotrophin-4 (NT-4) and vascular endothelial growth factor (VEGF) in cells receiving CO (Fig. 3.7). The culture medium revealed a reduction in interleukin-15 (IL-15) and interferon-γ (IFN-γ) levels and an increase in insulin-like growth factor binding protein-4 (IGFBP-4) after CO treatment (Fig. 3.7).



B

1	2	3	4	5	6	7	8	9	10	11	
Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GROα	a
I-309	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	b
IL-12p40p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-4	MCSF	MDC	MIG	MIP-1β	c
MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-1β	TNF-α	TNF-β	EGF	IGF-1	Angiogenin	d
Oncostatin M	Trombopoitin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ckβ 8-1	Eotaxin	Eotaxin-2	Eotaxin-3	e
FGF-4	FGF-6	FGF-7	FGF-9	Flt3-Ligand	Fractalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2	f
IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3	g
NT-4	Ostepontin	Osteoprotegerin	PARC	PIGF	TGF-β2	TGF-β3	TIMP-1	TIMP-2	Pos	Pos	h

Figure 3.7 - Effects of CO treatment on cytokine profiles. Semi-quantitative expression profile of cytokines in cells (hVMbclX_L) and medium from cultures receiving 25 ppm CO treatment (30 min) at days 0 and 4 and differentiated for 5 days compared to untreated controls. (A) Digital images of signal intensities of 80 different cytokines plus positive and negative staining controls; (B) Schematic overview illustrating the different cytokines. Comparison of intensities revealed an increase for VEGF, NT-4 and ostepontin and a relative reduction for NT-3 in cells receiving CO compared to controls. The semi-quantitative expression of cytokines in the medium revealed an increase for IGFBP-4 and a reduction in IFN-γ and IL-15 for cultures treated with CO compared to control.

Glucose Metabolism Assessment

CO increases VEGF, a cytokine described to be responsible for metabolism regulation [57], [58]. For instance, both *in vivo* and in endothelial cell line, Domigan and colleagues showed that whenever knocking-out VEGF occurs an alteration in mitochondrial homeostasis [58]. Also, a complete ablation of VEGF in brown adipocytes decreases oxidative capacity of mitochondria [57]. Thus, it is important to evaluate metabolic status of hVMbclX_L cells during dopaminergic differentiation (Fig. 3.8).

Characterization of cell metabolism, in particular the balance between glycolytic and oxidative metabolism, can be assessed by

extracellular quantification of lactate production and glucose consumption, whose ratio is calculated over time. Whenever this ratio (lactate concentration/glucose concentration) is close to 2, it means a fully glycolytic metabolism, since through glycolysis one molecule of glucose gives rise to two molecules of lactate. Thus, as much as this ratio decreases higher levels of oxidative phosphorylation might occur. In order to assess how CO modulates glucose metabolism, culture supernatants were collected during differentiation (days 3, 6 and 9) and the levels of glucose and lactate were quantified. In Table 3.1, one can find the values for lactate production *per* glucose consumption, for hVMbclX$_L$ cells exposed to CO and control cells. In both cases during the differentiation process cells progressively change their metabolism, as ratios increase over time. However, cells exposed to CO present a decreased ratio of qLac/qGlc, thus one can conclude that CO exposure stimulates oxidative phosphorylation during neuronal differentiation, comparing to control conditions (Table 3.1).

Table 3.1 – Metabolic characterization of glucose utilization during differentiation of hVMbclX$_L$. Lactate production per glucose consumption (qLac/qGlc) ratios calculated between treatments with CO 25 ppm (n≥3).

Differentiation process (day)	Ratio qLac/qGlc	
	Control	CO
3	0.91	0.80
6	1.46	1.14
9	1.86	1.32

In summary, the complex mechanisms underlying the observed effects of CO on stem cell differentiation into dopaminergic neuronal cells involve a reduction of apoptotic cell death, a changed cytokine profile and metabolism modulation, but CO does not influence cell proliferation.

DISCUSSION

To our knowledge, this is the first study demonstrating a positive effect of CO on dopaminergic neuronal differentiation of human NSCs. In brief, hVMbclX$_L$ cell line was exposed to low levels of CO during their differentiation. Short-term CO treatment significantly increased both the numbers and relative yields of β-tubIII neurons, suggesting that CO treatment stimulates neurogenesis. Moreover, the relative content of TH neurons was significantly increased after CO exposure (Fig. 3.3), which indicates that CO treatment favors induction or survival of the catecholaminergic phenotype. Exposure of hVMbclX$_L$ cells to CO also increased the number of MAP2 neurons (Fig. 3.5A), indicating that CO promotes generation of mature neurons. Control experiments confirmed that the observed effects were due to the release of CO and not the reagents used to initiate the chemical reaction (data not shown).

In our study, CO treatment did not alter the total number of cells. Furthermore, the MTS analysis used to assess cell proliferation and

viability revealed no change after CO treatment, which was in line with the unchanged content of HN cells (Fig. 3.6). Thus, it is unlikely that the applied CO concentrations influenced cell proliferation or were toxic to the cells. This is to some extent in accordance with studies showing an anti-proliferative effect of CO in other tissues, including vascular smooth muscle cells and T-lymphocytes, through activation of mitogen-activated protein kinases and the cell cycle inhibitor p21 [16], [67], [68]. Interestingly, studies exposing NSCs to low oxygen tension have reported both increased cell proliferation and dopaminergic differentiation [15], [69]. In the present study using CO exposure under normoxia, dopaminergic differentiation was increased without an improvement of cell proliferation, which suggests other underlying mechanisms than those triggered by low oxygen.

Many biological effects of CO are associated with generation of low levels of reactive oxygen species (ROS), which act as signaling molecules [21], [59]–[63]. CO-induced ROS generation is mainly due to partial inhibition of cytochrome C oxidase [64]–[66]. Interestingly, stimulation of ROS production is also important for cell signaling during neuronal differentiation and/or survival of embryonic stem cells, mesenchymal stem cells and neuronal progenitor cells [21], [23], [64], [65].

Previous studies have shown an anti-apoptotic effect of CO treatment on fibroblasts, endothelial cells, astrocytes, cerebellar granule cells

and neural stem cells [21], [24], [60], [70]–[72]. To address whether a similar effect was present in hVMbclX_L cells, cultures were immunostained for active Casp3. The relative content of Casp3 cells was significantly reduced after CO, indicating that CO has an anti-apoptotic effect. However, the number of Casp3 positive cells was very low most likely due to the over-expression of the anti-apoptotic protein BclX_L, which should be also taken into account.

For characterization of the catecholaminergic cell population obtained after CO treatment, the expression of catecholaminergic/midbrain-specific genes (TH, Nurr1, DAT and DBH) was assessed by real-time Q-PCR (Fig. 3.5C). Nurr1 is involved in maintenance of midbrain dopaminergic activity and is related to dopaminergic differentiation since Nurr1-null mouse-derived NSCs fail to differentiate and express TH [73], [74]. In the present study, Nurr1 expression was increased after CO exposure, which is in accordance with the observed increase in TH expression and release of dopamine and HVA. This could indicate that a substantial fraction of the neurons were dopaminergic.

The levels of dopamine in neuronal cells can be modulated by the activity of DAT and DBH. DAT is responsible for dopamine transport from the synaptic cleft, and surprisingly it was down-regulated after CO treatment. This may be due to the significant increase in the pool of free dopamine after CO treatment and/or due to the artificial *in vitro* conditions. The observed increase in DBH following CO

treatment, which catalyses the conversion of dopamine into noradrenaline, may simply reflect the rise of intracellular dopamine levels as a result of TH up-regulation.

Interestingly, a reduction in NT-3 and an increase in NT-4 expression levels were found in cell lysates from cultures receiving CO treatment. No studies have investigated the effect of CO treatment on NT-3 and NT-4, but both neurotrophins have been reported to be involved in neuronal growth, synapse formation, maturation and plasticity. Moreover, NT-3 is expressed in NSCs, stimulating their neuronal differentiation and survival [75]–[77]. The down-regulation of NT-3 may reflect the observed stimulatory effect of CO on neurogenesis and cell maturation leading to a reduction in the pool of NSCs. On the other hand the observed up-regulation of NT-4 could potentially stimulate further maturation and growth of cells, which, at day 5, are still undergoing differentiation.

The cytokine profiling of conditioned culture medium revealed changes for IL-15, IFN-γ and IGFBP-4 after CO treatment (Fig. 3.7). The decrease in IL-15 observed for CO-treated cultures could indicate that CO exhibit an anti-inflammatory effect on NSCs. Indeed, previous studies have demonstrated that IL-15 is a pro-inflammatory cytokine present in both NSCs and differentiated neurons during inflammation. Nevertheless, it has also been reported that decreased levels of IL-15 *in vivo* lead to an increase in cell differentiation and reduction in cell proliferation [78]–[80]. Interestingly, a study

culturing rat NSCs showed that IL-15 treatment reduced the number of MAP2 neurons, thus inhibiting neuronal maturation [81]. The observed decrease of IL-15 found in our study may therefore have contributed to increased cell maturation as shown by the increased number of MAP2 neurons found after treatment. The reduction in IFN-γ in cultures receiving CO also suggests an anti-inflammatory role of CO since existing literature describe pro-inflammatory characteristics of IFN-γ [82].

Previous studies have shown that IGFBP-4 plays a role in the developing brain by stimulating neuronal differentiation of NSCs. In the present study the increase in IGFBP-4 in cultures receiving CO could indicate that CO signals through IGFBP-4 to promote cell differentiation [83], [84].

Also, semi-quantitative cytokine profiling of cell lysates and conditioned culture medium showed an increase in VEGF for CO-treated cultures (cell lysates) compared to controls (Fig. 3.7). This is consistent with other studies demonstrating that CO elevates VEGF levels in astrocytes and cardiomyocytes [85]–[87].Moreover, VEGF is described to be responsible for metabolism regulation [57], [58]. Thus, metabolic status of hVMbclX$_L$ cells was evaluated during dopaminergic differentiation (Fig. 3.8). Lower ratios of lactate production *per* glucose consumption in cells differentiated after CO exposition suggest an increase in glucose oxidation, which lead one to speculate that CO pushes cell metabolism towards oxidative

phosphorylation. In conclusion, one possible mechanism for CO to promote neuronal differentiation is by metabolism modulation and reinforcement of oxidative phosphorylation, however further studies are needed.

One of the novelties of this study consists in the new strategy for delivering CO gas. In this new system, CO is generated by a decarbonylation reaction using the new CORM $MePh_2SiCO_2H$, along with potassium fluoride and DMSO [51]. This strategy avoids the use of CO gas bottles, being safer and easier to use in the benchtop. Also, it is more cost-effective than previously described methods and a great strategy for the laboratory scale studies, however one cannot disregard the lack of potential to be used in human therapeutics.

Short-term treatment of human NSCs during neuronal differentiation process with a low dose of CO, represents an efficient, simple and safe method for *in vitro* derivation of dopaminergic neurons with midbrain characteristics that may as such contribute to the future development of donor cells for transplantation in PD.

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IV

CARBON MONOXIDE IMPROVES NEURONAL DIFFERENTIATION AND YIELD BY INCREASING THE FUNCTIONING AND NUMBER OF MITOCHONDRIA

This chapter is based on the following manuscript:

Carbon monoxide improves neuronal differentiation and yield by increasing the functioning and number of mitochondria

Ana S. Almeida, Ursula Sonnewald, Paula M. Alves and Helena L.A. Vieira (2016) *Journal of Neurochemistry*, 138(3): 423-435

ABSTRACT

The process of cell differentiation goes hand-in-hand with metabolic adaptations, which are needed to provide energy and new metabolites. Carbon monoxide (CO) is an endogenous cytoprotective molecule able to inhibit cell death and improve mitochondrial metabolism. Neuronal differentiation processes were studied using the NT2 cell line, which is derived from human testicular embryonic teratocarcinoma and differentiates into post-mitotic neurons upon retinoic acid treatment. CO-releasing molecule A1 (CORM-A1) was used do deliver CO into cell culture. CO treatment improved NT2 neuronal differentiation and yield, since there were more neurons and the total cell number increased following the differentiation process. CO supplementation enhanced the mitochondrial population in post-mitotic neurons derived from NT2 cells, as indicated by an increase in mitochondrial DNA. CO treatment during neuronal differentiation increased the extent of the classical metabolic change that occurs during neuronal differentiation, from glycolytic to more oxidative metabolism, by decreasing the ratio of lactate production and glucose consumption. The expression of pyruvate and lactate dehydrogenases was higher, indicating an augmented oxidative metabolism. Moreover, these findings were corroborated by an increased percentage of ^{13}C incorporation from [U-^{13}C]glucose into the tricarboxylic acid cycle metabolites malate and citrate, and also

glutamate and aspartate in CO-treated cells. Finally, under low levels of oxygen (5%), which enhances glycolytic metabolism, some of the enhancing effects of CO on mitochondria were not observed. In conclusion, our data show that CO improves neuronal and mitochondrial yield by stimulation of tricarboxylic acid cycle activity, and thus oxidative metabolism of NT2 cells during the process of neuronal differentiation.

CONTENTS

Ana Sofia Almeida had carried out the majority of the experimental part and was involved on the decisions on how to execute the experiments, as well as on the interpretation and discussion of the results.

INTRODUCTION

Embryonic and adult stem cells have the capability to produce at least one daughter stem cell upon division (self-renewal) and the ability to generate differentiated cells (potency). Thus stem cells can proliferate into an increasingly, undifferentiated stem cell population, or they can originate all tissues during embryonic and postnatal development, which is crucial for tissue repair. This process of cell differentiation goes hand-in-hand with metabolic alterations needed to provide new bioenergetic, synthetic and catabolic requirements important for cell identity. In 2013, Göttle and colleagues demonstrated the role of purine metabolism and purine pools during neuronal differentiation [1]. Actually, they show that dysfunctions in purine metabolism can lead to specific abnormalities, such as decreased dopamine levels [1]. Thus, regulation of stem cell's metabolic pathways must be considered in what regards stem cell fate, namely promotion and regulation of cell differentiation [2]–[8]. The modulation of stem cell differentiation is dependent on cell metabolic shifts, mitochondrial function and oxygen levels [9]–[14]. Stem cells appear to rely to a greater extent on glycolysis than on oxidative phosphorylation to generate ATP [15]–[17]. Consistently, there is lower levels of mitochondrial population in human ESCs than in their differentiated progeny [18]–[21]. Furthermore neuronal differentiation process promotes mitochondrial biogenesis. Accordingly to Cho and colleagues, the increase of mitochondrial

population during differentiation might permit more cellular respiration and, therefore, an increase of cellular ATP production [18], which is needed for cell differentiation from progenitor to fully mature and functional cell. Stem cell dependency on glycolysis raises as an adaptation to the low oxygen levels, which indeed are present *in vivo* during development and in an adult stem cell microenvironment or 'niche'. Adult neural stem cells (NSCs) reside in niches that are characterized by low oxygen levels (1–6%) [23], [24]. Accumulating evidence supports the idea that the fate of embryonic and adult stem cells can be controlled by oxygen signaling [5], [25]–[30]. For instance, oxygen gradients in the niche might help to direct stem cells to differentiate into specific cell lineages. Likewise, low oxygen levels can specify the fate of differentiating NSCs, promoting the production of dopaminergic neurons and oligodendrocytes [29], [30].

NT2 cell line is derived from human testicular embryonic teratocarcinoma and is able to differentiate into neurons upon retinoic acid treatment. NT2 cells differentiate into functional post-mitotic neurons, which express many neuronal markers [31] and produce a variety of neurotransmitter phenotypes [32]–[34]. Moreover, NT2-derived neurons can form functional synapses [35] and have also been used in several transplantation studies in experimental animal models [36], [37] and in human patients [38].

CO is mostly known as a silent-killer due to its great affinity to hemoglobin, which compromises oxygen delivery and promotes high levels of intoxication and death. Furthermore, high concentrations of CO are cytotoxic by inhibition of cytochrome c oxidase (COX), excessive ROS generation or uncoupling effect [39]. Nevertheless, CO is an endogenously produced gasotransmitter generated by the cleavage of heme group via the enzymatic activity of heme-oxygenase (HO)[40]. HO is a stress-related enzyme, whose expression or activity increases in response to several stresses, namely: oxidative stress, hypoxia, hyperoxia, hyperthermia, inflammation, UV, misfolded protein response, among others [40]–[42]. Likewise, it has been demonstrated that low levels of exogenous CO promote cytoprotection, limit inflammation and prevent cell death [40], [41], [43]–[46]. Moreover, CO-induced cytoprotection can be dependent on generation of low amounts of ROS, which function as signaling molecules [43], [47]–[50]. Likewise, low concentrations of CO promote mitochondrial biogenesis [51], [52], increase COX activity [44], [53]–[56], improve oxidative metabolism [51], [57] and induce mild mitochondrial uncoupling that protects mitochondria from oxidative stress [58], [59]. For further reading, please read the following reviews [51], [60], [61].

Recently we have shown that CO improves neuronal differentiation by preventing cell death [62]. Because modulation of cell metabolism and oxygen signaling are involved in neuronal

differentiation, the main purpose of the present work is to assess the role of CO in promoting neuronal differentiation through metabolism modulation, in particular oxidative phosphorylation. This study demonstrated that CO increases NT2 neuronal yield production through stimulation of oxidative metabolism.

MATERIAL AND METHODS

Materials

All chemicals were of analytical grade and were obtained from Sigma unless stated otherwise. Plastic tissue culture dishes were from Sarstedt (Germany); fetal bovine serum, penicillin/streptomycin solution, and Dulbecco's minimum essential medium (high glucose, L-glutamine and pyruvate) were obtained from Invitrogen (United Kingdom). ^{13}C-labelled glucose was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

The mass spectrometry derivatization reagents MTBSTFA (*N*-methyl-*N*-(tert - Butyldimethylsilyl) trifluoroacetamide), MSTFA (*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide) and the t-BDMS-Cl (tert-butyldimethylchlorosilane) were purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). All other chemicals were of the purest grade available from regular commercial sources.

NT2 human teratocarcinoma cell line

Maintenance of undifferentiated cells

Undifferentiated NT2 cells from American Type Culture Collection (ATCC) were grown in DMEM-HG supplemented with 10%(v/v) FBS and 1%(v/v) Pen/Strep (growth medium). Cells were maintained in a humidified atmosphere of 5%(v/v) CO_2 at 37ºC. Undifferentiated cells *per* vial were grown in 75cm^2 T-flasks and subcultured with fresh growth medium whenever high cell confluence was achieved (about

90-100% cell confluence), cells were rinsed with phosphate-buffered saline (PBS) and then incubated with trypsin for 2 minutes at 37ºC for trypsinization and resuspended in growth medium in a 1:4 cell passage. Growth medium was changed every 2 to 3 days.

Neuronal differentiation protocol

Following trypsinization and resuspension in growth medium, cells were counted in trypan blue and split $2,3x10^6$ cells per $75cm^2$ T-flask. Neuronal differentiation was induced 24 hours after plating undifferentiated cells to ensure that they settle and attach to flask surface and attain appropriate density. The NT2 cell line neuronal differentiation was induced in DMEM-HG with 10%(v/v) FBS and 1%(v/v) Pen/Strep, supplemented with 10µM *all-trans* retinoic acid (RA), which is the differentiation medium. CO effect was studied by using the same composition of differentiation medium supplemented with 25µM CORM-A1. Differentiation medium was replaced three times a week until reaching 10 differentiation treatments (24 days). For hypoxic experiments, cells were maintained in a humidified atmosphere of 5% (v/v) O_2, 5%(v/v) CO_2 and 90% (v/v) N_2 at 37ºC.

Neuronal enrichment

After the 10^{th} differentiation treatment, cells were replated at lower density to disperse the dense multilayer cell culture and start neuronal enrichment for obtaining neurons. On the following day, the

culture medium was exchanged for fresh growth medium supplemented with mitosis inhibitors: 1μM Cytosine Arabinoside, 10μM Foxuridine and 10μM Uridine for neuronal enrichment. Growth medium supplemented with mitosis inhibitors was replaced twice a week for 10 days, making a total of 3 to 4 treatments. On the 10th day of neuronal enrichment, enriched neurons were collected for different analysis. The used protocol is schematically represented in Figure 4.1.

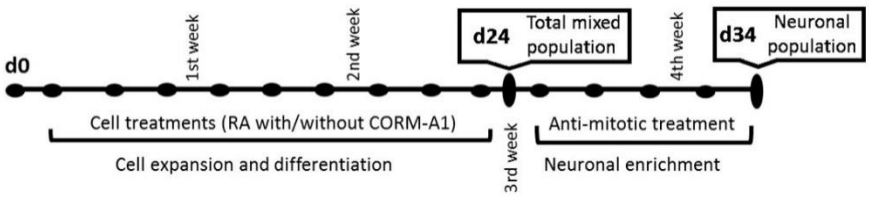


Figure 4.1 – Scheme of NT2 cells neuronal differentiation. Neuronal differentiation of NT2 cells is performed over 3 weeks, with medium exchanged three times a week on alternate days. After 24 days of differentiation, the obtained mixed (undifferentiated and neuronal) cell population is treated with anti-mitotic compounds for neuronal enrichment during 10 days.

Carbon monoxide releasing molecule A1 (CORM-A1)

The solution of CORM-A1 (Sigma, Germany) was prepared in milli-Q water with a final concentration of 5mM. Then, solution was filtrated with 0,2μM filter and stored at -20ºC. For each use, an aliquot was thawed and immediately used. CO-depleted inactive form (iCORM-A1)

was generated to be used as negative control by initially dissolving CORM-A1 in 0.1 M HCl and then bubbling pure N2 through the solution for 10 min in order to remove the residual CO gas. The solution of iCORM-A1 was finally adjusted to pH 7.4 [63]. Then, the solution was filtrated using a 0,2µM filter and stored at -20ºC. For each use, an aliquot was thawed and immediately used.

Cell counting and viability

Cell cultures were visualized using an inverted microscope with phase contrast (DM IRB, Leica, Germany). Total cell number was determined by counting cell nuclei using a Fuchs-Rosenthal hemacytometer, after digestion with 0.1M citric acid/1% Triton X-100 (wt/wt)/0.1% crystal violet (wt/v).

Quantitative-Polymerase chain reaction (Q-PCR)

Mitochondrial population assessment

Genomic DNA was extracted from cells after differentiation using the High Pure PCR Template preparation kit (Roche Diagnostics, Mannheim, Germany). PCR was performed using specific forward and reverse primers designed for the mitochondrial COXII gene (5'-ACAGACGAGGTCAACGATCC-3' and 5'-AGATTAGTCCGCCGTAGTCG-3') and for the nuclear GAPDH gene (5'-GCATCCTGGGCTACACTGAG-3' and 5'-GTCAAAGGTGGAGGAGTGGG-3'), respectively. GAPDH gene was used as housekeeping for mitochondrial DNA quantification. Fast

Start DNA Master Plus SYBR Green I (Roche Diagnostics) was used with the experimental run protocol: denaturation program was 95° C for 10 min, followed by 45 cycles of 95° C for 15'', 60 °C for 6'' and 72° C for 20''.

Evaluation of gene expression (mRNA quantification)

For evaluation of gene expression, mRNA was extracted from NT2 cells using High Pure RNA isolation kit (Roche Diagnostics), and cDNA synthesis was performed using the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics). PCR was performed using specific forward and reverse primers:

Gene	Forward Primer	Reverse Primer
Nestin	5′-CTGTGAGTGTCAGTGTCCCC-3′	5′-CTCTAGAGGGCCAGGGACTT-3'
Tuj1	5′-GCAAGGTGCGTGAGGAGTAT-3′	5′-GTCTGACACCTTGGGTGAGG-3′
MAP2	5′-GGAGCTGAGTGGCTTGTCAT-3′	5′-CTAGCTCCAGACAGACGCAG-3′
Pyruvate dehydrogenase	5′-AGGGTGGTTTCTATCTGTCTTGT-3′	5′-TCATGCTTCTTTTATCCTCTTGCT-3′

Lactate dehydrogenase	5'-GGCTATTCTTGGGCAACCCT-3'	5' -TGGAAGTGGTACCAATACAACTCA-3'
MCT2	5'-GTAGAACGCCAGAGCACAGA-3'	5'-AGGTGTTCTCCTCAAGACTATGC-3'
Glucose transporter 1	5'-TTATAGGACCCCGGCCATTG-3'	5'-CTGAGCGAGGCAGTGGTTA-3'
RPL22	5'-CACGAAGGAGGAGTGACTGG-3'	5'-TGTGGCACACCACTGACATT-3'

Fast Start DNA Master Plus SYBR Green I (Roche Diagnostics) was used with the experimental run protocol: denaturation program was 95 °C for 10 min, followed by 45 cycles of 95 °C for 10'', 60°C for 10'' and 72 °C for 10''.

Immunoblotting

Cell extracts were separated under reducing electrophoresis on a 1mm of 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel, for 1 hour under 130-150V. Samples were transferred to a nitrocellulose membrane (HybondTMC extra, Amersham Biosciences) for 1 hour under 500mA. PDH or Actin protein was stained with α-PDH (abcam, ab110330) or α-actin (Sigma-Aldrich, A4700) at 1/1000

dilution for 2h at room temperature. Blots were developed using the ECL (enhanced chemiluminescence) detection system after incubation with HRP-labeled anti-mouse IgG anti- body (Amersham Biosciences Bioscience), 1/5000, 1h of room temperature incubation.

Lactate/Glucose Ratio

Total glucose and lactate concentrations in the culture supernatant were determined with automated enzymatic assays (YSI 7100 Multiparameter Bioanalytical System; Dayton, OH). The rate between lactate production and glucose consumption was obtained by linear regression of the metabolites concentrations, then the ratio between lactate production and glucose consumption rate was determined for each time point.

Gas Chromatography-Mass Spectrometry (GC-MS)

For analysis of ^{13}C percent enrichment in intracellular metabolites, NT2 cells differentiated for 24 days were incubated with [U-^{13}C]glucose for 24 hours. After the incubation period, cells were washed twice with cold PBS and the intracellular metabolites extracted with 70% ethanol and lyophilized. Cell extracts were resuspended in 0.01M HCl followed by pH adjustment to pH<2 with HCl 6 M. Samples were dried under atmospheric air (50 °C), and metabolites were derivatised with MTBSTFA in the presence of 1% *t*-

BDMS-Cl (Mawhinney et al. 1986) (see Amaral et al. 2014 for further details). The samples were analyzed on an Agilent 6890 gas chromatograph connected to an Agilent 5975B mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The parent ion (M) and atom percent excess for ^{13}C atoms (M+1, M+2 and M+3) values for citrate, malate, aspartate and glutamate were calculated from GC-MS data using MassHunter software supplied by Agilent (Agilent Technologies, Palo Alto, CA, USA) and correcting for the naturally abundant ^{13}C by using non-enriched standards [64].

Statistical analysis

The data concerning cell culture were carried out at least in three independent preparations. All values are mean ± SD, n≥3. Error bars, corresponding to standard deviation, are represented in the figures. Statistical comparisons between multiple groups were performed using ANOVA single factor with replication and between two groups were made with an independent two-tailed Student's *t-test*. For all the data, *p*-value is indicated for each figure.

RESULTS

CORM-A1 modulates neuronal differentiation

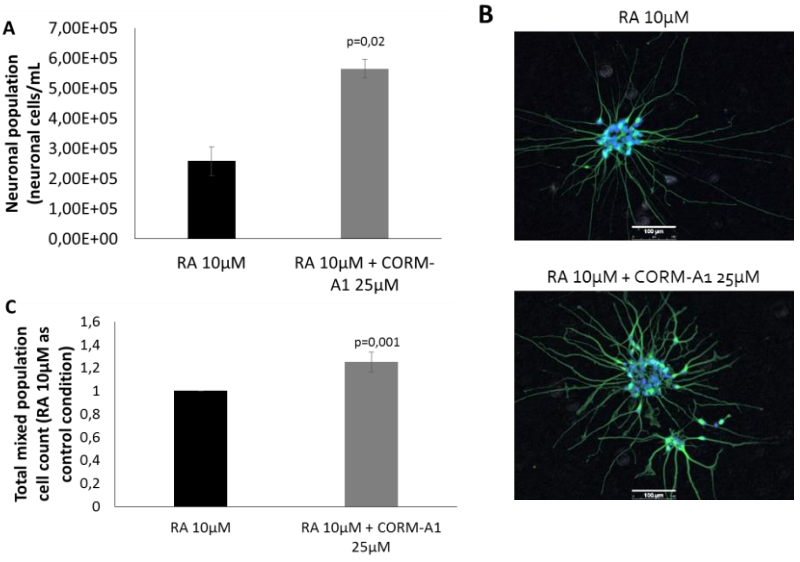
In order to characterize the effect of CORM-A1 in neuronal differentiation of NT2 cell line, cells were differentiated with retinoic acid (RA) supplemented with CORM-A1 (Fig. 4.1). RA is the factor that allows these cells to differentiate into post-mitotic neurons and CORM-A1 is only capable of modulating the process when administered jointly with RA. CO *per se* is not an inducer of neuronal differentiation, since NT2 cells died whenever treatment was done only with CORM-A1 without RA supplementation (data not shown). During the differentiation process, culture supplementation with 25μM CORM-A1 yielded a duplication of the final number of post-mitotic neurons (Fig.3.2A). Likewise, in the first two columns of Table 3.1 the kinetic parameters for cell growth demonstrated an increase on growth rate (μ), doubling time (td) and fold increase (FI) in the presence of CORM-A1.

Furthermore, neuronal morphology and expression of the neuronal protein Tuj1 (assessed by immunocytochemistry) are similar between neurons derived from RA treatment alone and derived from RA supplemented with CORM-A1 (Fig. 4.2B). Moreover, supplementation with inactivated CORM-A1 did not increase total mixed cell population nor did it stimulate Tuj1 expression.

Table 4.1 - Growth rate (μ), doubling time (td) and fold increase (FI) values of NT2 cells differentiated in normoxic conditions (21% O_2) and hypoxic conditions (5% O_2).

Cell Growth Kinetic Parameters

	Normoxia		Hypoxia
	RA 10μM	RA 10μM + CORM-A1 25μM	RA 10μM + CORM-A1 25μM
μ (day^{-1})	0,08±0,03	0,09±0,03	0,07±0,01
DT (day)	9,62±3,44	8,27±2,91	10,23±1,29
FI	6,53±3,29	8,03±4,49	5,29±1,00



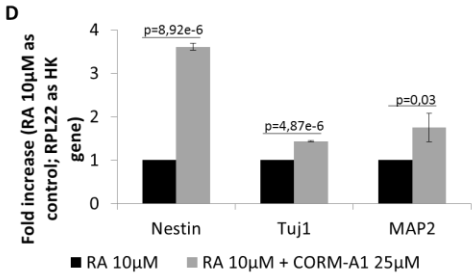


Figure 4.2 – CORM-A1 improves the neuronal differentiation process. (A) Relative quantification of neuronal cells obtained after 34 days of neuronal differentiation and enrichment processes; (B) Immunocytochemistry of NT2 derived post-mitotic neurons (green staining: Tuj1; blue staining: DAPI; magnification 200x); (C) Relative quantification of cells obtained in the mixed cell population after 24 days of neuronal differentiation; (D) mRNA expression of specific neuronal differentiation markers (Nestin for neuronal precursors, Tuj1 for early differentiated neurons and MAP2 for mature neurons) in NT2 mixed cell population after 24 days of neuronal differentiation.

The enhancement in the final yield of post-mitotic neurons promoted by CORM-A1 supplementation can be due to increased precursor cell proliferation or a facilitation of neuronal differentiation *per se*. To clarify this issue, the total mixed cell population (composed of progenitor cells and post-mitotic neurons) was evaluated before neuronal enrichment with anti-mitotic agents. For the same number of inoculated undifferentiated NT2 cells, the total number of cells in the mixed population after 24 days of differentiation was higher in the presence of CORM-A1 (Fig. 4.2C). The mixed cell population obtained by treatment with only RA and RA supplemented with CORM-A1 were characterized by mRNA

quantification of neuronal related genes. The neuronal precursor cell marker Nestin, as well as Tuj1 and MAP2, which are expressed in the early stage of neuronal differentiation and by mature neurons, respectively, presented higher levels of mRNA in the presence of CORM-A1 (Fig. 4.2D). In summary, accordingly to our previously our data [62], CO released by CORM-A1 improves the neuronal differentiation process in the NT2 cell model.

Mitochondrial population assessment

Cellular differentiation leads to mitochondrial biogenesis [18], [22]. According to Cho and colleagues, the increase of mitochondrial population during differentiation should permit more cellular respiration and, therefore, an increase in ATP concentration [18], which is needed for cell differentiation to progress from progenitor to fully functional cell. Mitochondrial population of NT2 cells under differentiation was assessed by mitochondrial DNA quantification by Q-PCR. It was observed that during neuronal differentiation with RA, as expected, there was a significant increase in mitochondrial population compared to undifferentiated cells (data not shown). However, CORM-A1 supplementation did not increase mitochondrial DNA content in the mixed cell population obtained after 24 days of differentiation (Fig. 4.3A). In order to understand whether progenitor and neuronal cells had different levels of mitochondrial population,

mitochondrial DNA was quantified in the post-mitotic NT2 derived neurons following neuronal enrichment (Fig. 4.3B). Indeed, post-mitotic neurons obtained from the differentiation medium supplemented with RA and CORM-A1 presented higher levels of mitochondrial DNA than post-mitotic neurons derived from differentiation medium supplemented only with RA (Fig. 4.3B). Thus, CORM-A1 enhances the mitochondrial population of post-mitotic neurons. Because CO is known as the modulator of cell metabolism [44], [57], one can speculate that CORM-A1 supplementation increases neuronal yield due to its capacity to raise mitochondrial metabolism and, subsequently, ATP production.

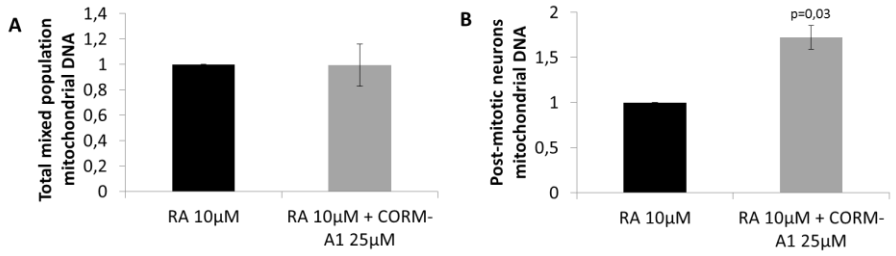


Figure 4.3 – Mitochondrial population assessment.
Mitochondrial DNA quantification by Q-PCR in (A) mixed population of NT2 cells after 24 days of differentiation; (B) post-mitotic neurons obtained from NT2 cells differentiation following enrichment process.

Cellular metabolism assessment

In order to understand more deeply whether CORM-A1-increased mitochondrial population reflects a change in cell metabolism, the expression of some metabolic enzymes and transporters was evaluated at the end of the neuronal differentiation process (Fig. 4.4A). Glucose transporter 1 (Glut1) is a transporter responsible for the glucose entrance in the cell, while MCT2 is the monocarboxylate transporter present in neurons, in particular lactate. Pyruvate dehydrogenase (PDH) is involved in the conversion of pyruvate to acetyl-CoA that then enters into Tricarboxylic Acid Cycle (TCA), and is an irreversible reaction. Finally, lactate dehydrogenase (LDH) is the enzyme catalyzing pyruvate into lactate. There is a tendency of CORM-A1 to increase oxidative metabolism: the expression of both membrane transporters (Glut1 and MCT2) mRNA is increased. Likewise, there are higher levels of mRNA expression of PDH and LDH (Fig. 4.4A). PDH protein levels were also quantified by immunoblot, presenting a significant increase in its expression in the presence of CORM-A1 (Fig. 4.4B).

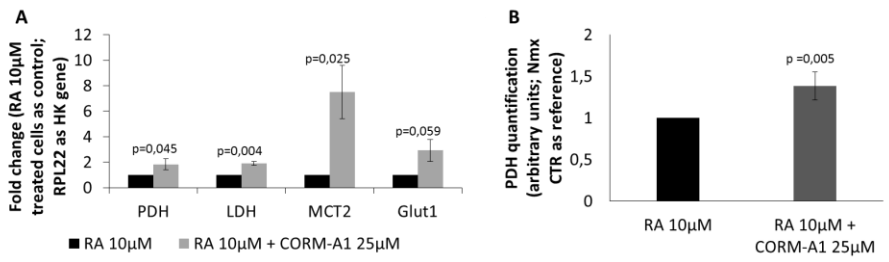


Figure 4.4 – Oxidative metabolism from glucose assessment. (A) mRNA expression of specific metabolic markers (PDH for pyruvate dehydrogenase, LDH for lactate dehydrogenase, MCT2 for monocarboxylate transporter 2 and GLUT1 for glucose transporter 1) in NT2 mixed cell population after 24 days of neuronal differentiation; (B) PDH protein quantification by immunoblotting.

Characterization of cell metabolism, in particular the balance between glycolytic and oxidative metabolism, can be assessed by extracellular quantification of lactate production and glucose consumption, whose ratio is calculated over time. Whenever this ratio (lactate concentration/glucose concentration) is close to 2, it means a fully glycolytic metabolism, since through glycolysis one molecule of glucose gives rise to two molecules of lactate. Thus, as much as this ratio decreases oxidative phosphorylation might occur. In order to understand how CORM-A1 modulates oxidative metabolism, supernatants of NT2 cells during differentiation were collected and glucose and lactate levels were quantified. In Table 3.2, one can observe the ratios of lactate production per glucose consumption in the case of cells treated with RA only and cells treated with RA supplemented with CORM-A1.

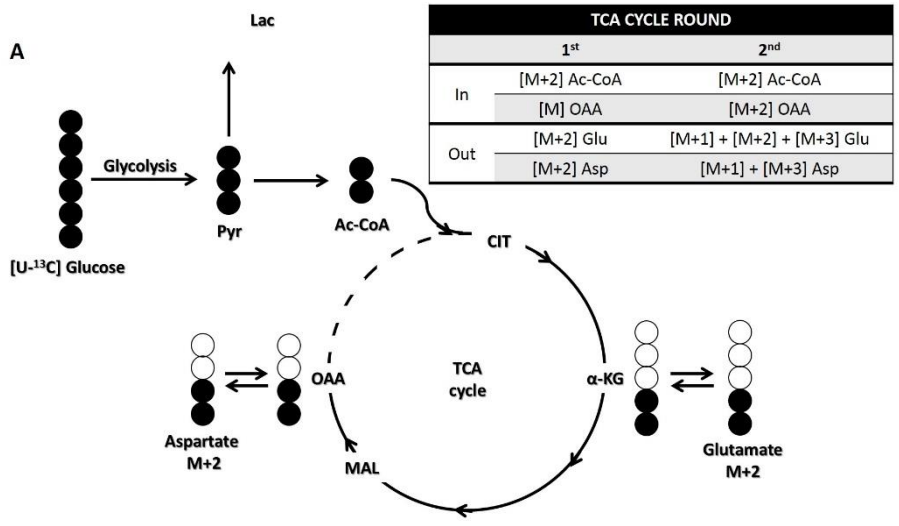
Table 4.2 - Metabolic characterization of NT2 neuronal differentiation. Lactate production per glucose consumption (qLac/qGlc) ratios were calculated between treatments (n≥3).

Differentiation process (day)	Ratio qLac/qGlc	
	RA 10µM	RA 10µM + CORM-A1 25µM
2	2,00 ± 0,19	2,00 ± 0,19 (p=0,96)
4	1,97 ± 0,18	1,80 ± 0,16 (p=0,41)
7	1,56 ± 0,14	1,40 ± 0,06 (p=0,17)
13	1,34 ± 0,05	1,26 ± 0,03 (p=0,03)
17	1,27 ± 0,12	0,90 ± 0,08 (p=0,08)
24	1,05 ± 0,03	0,92 ± 0,08 (p=0,08)

In both cases it is clear that during the differentiation process cells progressively change their metabolism from glycolytic to oxidative, as the ratios decrease over time, which is fully in accordance with the shift between more proliferative to differentiated cells. Furthermore, when neuronal differentiation process occurs in the presence of CORM-A1, the decrease in the ratio occurs early on and is always lower for the cultures supplemented with CORM-A1. Thus, we can conclude that CORM-A1 supplementation stimulates oxidative phosphorylation (Fig.4.4, Table 4.2).

Metabolic profile

Mitochondrial metabolism was assessed by measuring % ^{13}C enrichment above the natural 1.1% using GC-MS. To study ^{13}C enrichment in intracellular metabolites, NT2 cells differentiated for 24 days were incubated with [U-^{13}C]glucose for 24 hours in the presence and absence of CORM-A1 supplementation and cell extracts were prepared for GC-MS analysis. % ^{13}C enrichment in TCA cycle metabolites (citrate and malate) and aspartate and glutamate were quantified.



TCA CYCLE ROUND		
	1st	2nd
In	[M+2] Ac-CoA	[M+2] Ac-CoA
	[M] OAA	[M+2] OAA
Out	[M+2] Glu	[M+1] + [M+2] + [M+3] Glu
	[M+2] Asp	[M+1] + [M+3] Asp

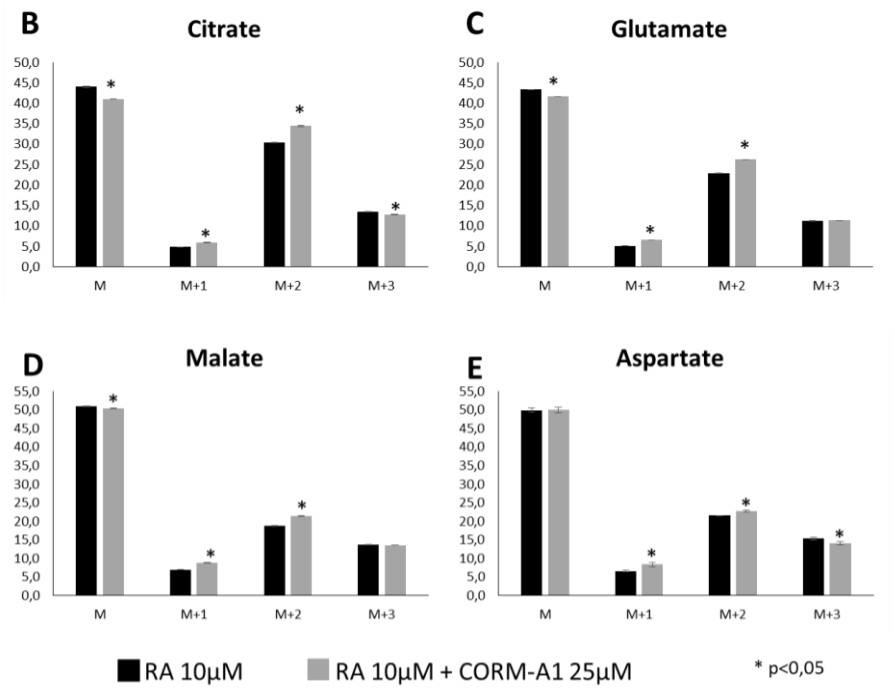


Figure 4.5 – Metabolic profile. (A) Labelling patterns derived from [U-^{13}C]glucose metabolism; The % enrichment with ^{13}C leading to the formation of molecules with masses: M+1, M+2 and M+3 for (B) citrate, (C) glutamate, (D) malate and (E) aspartate, determined by GC-MS analysis of NT2 cell extracts differentiated for 24 days and subjected to 24h incubation with medium containing [U-^{13}C]glucose.

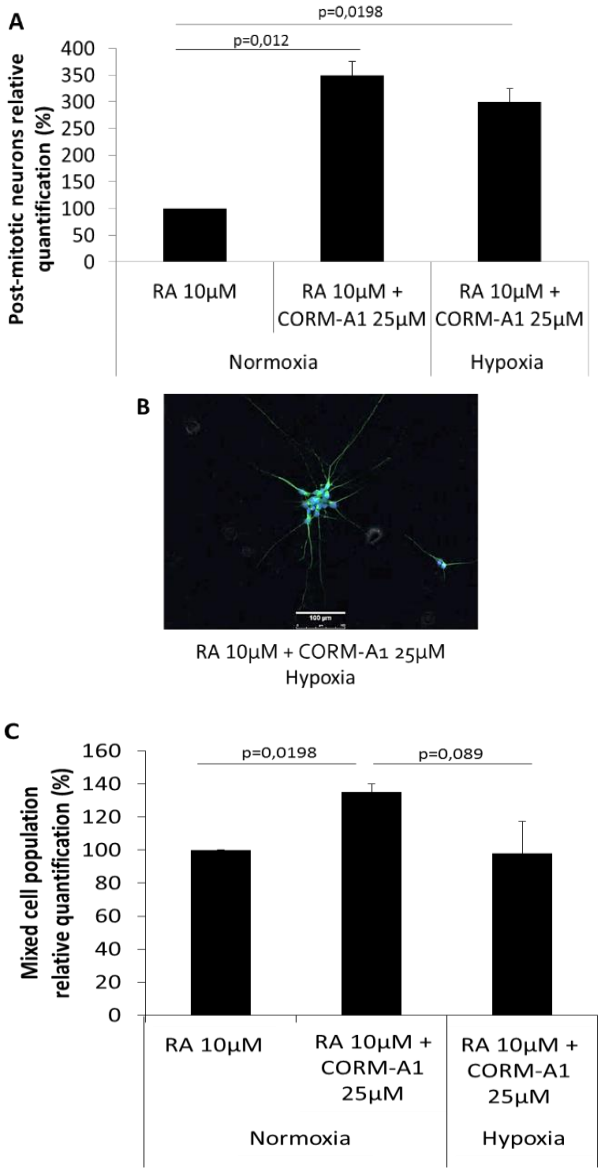
Metabolism of [U-^{13}C]glucose yields [U-^{13}C]pyruvate which is then converted into [1,2-^{13}C]acetyl CoA. This molecule condenses with non-labelled oxaloacetate to form double-labelled (M+2) compounds in the first turn of the TCA cycle (Fig. 4.5A) and monolabeled (M+1) isotopologues in the second turn if mono labeled oxaloacetate condenses with unlabeled acetyl CoA. Furthermore, in a combination of the first and second turn of the TCA cycle, [1,2-^{13}C]acetyl CoA can condense with labelled oxaloacetate and give rise to the formation of

diversely labelled compounds (Fig. 4.5A). GC-MS analysis of NT2 cell extracts incubated with [U-^{13}C]glucose showed that % ^{13}C labelling of glutamate, aspartate, malate and citrate was increased for M+1, M+2 (Fig. 4.5B-E). These increases in labelling corroborate our hypothesis: CO improves neuronal differentiation through stimulation of mitochondrial metabolism.

CORM-A1 supplementation under hypoxic conditions

Oxygen levels are extremely important for oxidative phosphorylation, thus the assessment of CORM-A1 effect on neuronal differentiation under hypoxia is important to validate CO modulation of cell metabolism during differentiation. NT2 cells were differentiated under 5% O_2 levels and it was observed that, although there were no morphological differences in the final post-mitotic neurons (Fig. 4.6B), some of the reported effects of CORM-A1 during neuronal differentiation are lost under hypoxia. The number of post-mitotic neurons obtained is lower under hypoxia, but still higher than neurons differentiated with RA alone (Fig. 4.6A). In addition, the number of total mixed cell population following 24 days of differentiation is decreased to the levels of control, RA supplementation only (Fig. 4.6C). Hypoxia partially reverted the increased mRNA expression of the specific neuronal genes (Nestin and Tuj1) due to the presence of CORM-A1 (Fig. 4.6D). Under

hypoxia, the CORM-A1-increased mitochondrial population of the post-mitotic enriched neurons is reverted to control levels (Fig. 4.6E). Furthermore, growth rates (μ), doubling times (DT) and fold-increase (FI) of NT2 cell cultures during neuronal differentiation were calculated and compared (Table 4.1).



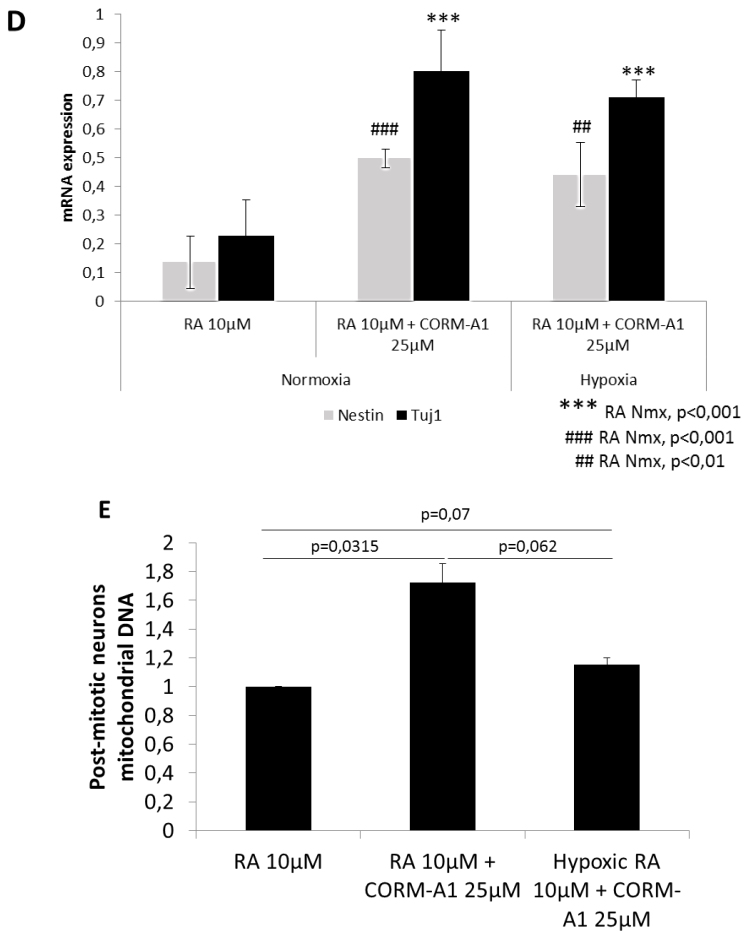


Figure 4.6 – Effect of hypoxia (5% O_2) on CORM-A1 modulation of neuronal differentiation. (A) Relative quantification of neuronal cells obtained after 34 days of neuronal differentiation and enrichment; (B) Immunocytochemistry of NT2 derived post-mitotic neurons (green staining: Tuj1; blue staining: DAPI; magnification 200x); (C) Relative quantification of neuronal cells obtained in the mixed cell population after 24 days of neuronal differentiation; (D) mRNA expression of specific neuronal differentiation markers (Nestin for neuronal precursors and Tuj1 for early differentiated neurons) in NT2 mixed cell population after 24 days of neuronal differentiation; (E) Mitochondrial DNA quantification by Q-PCR in post-mitotic neurons obtained from NT2 cells differentiation.

CORM-A1 supplementation increased cellular growth rate and decreased doubling time, which translates into higher fold-increase after the neuronal differentiation process under normoxic conditions. Upon hypoxia, CORM-A1 effect in growth rate and doubling time is reverted. Thus, one can speculate that, under lower levels of oxygen, CORM-A1 partially loses some capacities to positively modulate neuronal differentiation, namely: lower levels of post-mitotic neurons; mixed cell population after differentiation process presents lower levels of neuronal markers (mRNA expression of Nestin and Tuj1) and decreased mitochondrial population in post-mitotic neurons. Thus, in low levels of oxygen, CO is not able to improve neuronal differentiation.

DISCUSSION

Emerging evidence suggests that molecular regulators of energy metabolism play essential roles in stem cell fate, particularly in the decision to self-renew or differentiate [4], [5]. Due to the increasing prevalence of neurodegenerative diseases and ischemic stroke, modulation of neurogenesis is a promising strategy to regenerate tissue and improve the outcome of those devastating disorders related to aging. It is extremely important to understand the molecular mechanism underlying neuronal differentiation and also how these mechanisms can be modulated in order to stimulate

an efficient replacement of impaired neurons in the central nervous system. Therefore, cell metabolism manipulation can be a key tool for neurogenesis modulation. In the present study, our aim was to assess the metabolic regulation achieved by CO supplementation during the neuronal differentiation process, which in turn can improve neuronal production.

CO is intimately related to cell metabolism modulation [51], [65] by reinforcing oxidative phosphorylation: (i) in the context of cancer cells, which present an anti-Warburg effect [66] and (ii) in the primary cultures of astrocytes for promoting cytoprotection [44]. Furthermore, CO rescues mice from dying in the context of sepsis by supporting mitochondrial energy metabolism and promoting mitochondrial biogenesis [49], [67]. Actually, in the present study it was shown that CO improved neuronal differentiation rates by modulating cell metabolism, in particular reinforcing oxidative metabolism. In the presence of CORM-A1, there is an increase on mitochondrial population in post-mitotic neurons derived from NT2 cells. Increased glucose oxidation is also supported by decreased ratios of lactate production *per* glucose consumption upon supplementation with CORM-A1, which suggests that CO pushes cell metabolism towards oxidative phosphorylation. Neuronal metabolism is mostly oxidative and neurons also use glucose for maintaining their anti-oxidant status by generation of reduced glutathione via pentose-phosphate pathway (PPP). Moreover, whenever glycolysis is

forced in neurons (gene overexpression or inhibition), there is a great reduction PPP and consequently of anti-oxidant defence [68]. Therefore, for further study, the role of PPP must be taken into account during CO manipulation of neuronal differentiation.

New insights into the modulation of metabolic pathways have challenged the long-held assumption that all metabolic enzymes performing housekeeping functions, are being expressed at constant levels, in all cells [69]. During neuronal differentiation of NT2 cells supplemented with CORM-A1, mRNA expression of the enzymes PDH and LDH and the transporters Glut1 and MCT2 were higher than control, indicating an increased oxidative metabolism. Furthermore, it cannot be disregarded that neurons can take up and use lactate as oxidative substrate [70]. In particular under physiological conditions, there is the astrocyte-neuron lactate shuttle (ANLS) [70]–[72]. Because of their glycolytic metabolism, astrocytes produce and release lactate, which can be taken up and consumed by neurons. Although there are no astrocytes present in the neuronal differentiation culture, the differentiation process is not synchronized, and there are cells with different levels of maturation. Thus, it can also be speculated that the remaining progenitor cells could function as astrocyte-like cells by releasing lactate, since they have more glycolytic metabolism. Therefore, the increased expression of MCT2 in the presence of CO can also be related to an improved oxidative consumption of lactate by neurons. Likewise, the increase of PDH expression (mRNA and

protein) is coupled to a higher entrance of pyruvate into the TCA cycle and oxidative metabolism, which can be synthesized from both glucose and lactate. Finally, conversion of pyruvate into lactate by LDH is a reversible reaction, thus the higher levels of oxidative metabolism can also explain the increased expression of LDH. In conclusion, these data indicate that CORM-A1 positively modulates oxidative metabolism. The increased expression of Glut1 in the total mixed populations after 24 days of neuronal differentiation leads to a global increased oxidative metabolism [73]. Of note, Glut1 is expressed only *in vitro* neuronal cultures, while for *in vivo* models Glut3 must also be considered. Due to some residual mutagenic and oncogenic potential of the NT2 cells [74], one cannot disregard that aerobic glycolysis (Warburg effect) is more efficient in supporting rapid cell growth when compared with normal glycolysis linked to the TCA cycle and oxidative phosphorylation [69]. Thus, CO seems to favour neuronal differentiation at different levels: (i) supplying glucose needs for cellular growth and (ii) pushing metabolism from glycolytic to oxidative in order to stimulate differentiation. MCT2 is a proton symporter responsible for the transmembrane transport of mono-carboxylates in neurons [75], [76]. CORM-A1 also increased the expression of the monocarboxylate transporter MCT2, which is mostly present in neurons, more specifically in glutamatergic synapses [76], indicating the potential link between MCT2 function and glutamatergic transmission in functional neurons. These data

correlate with the fact that NT2-derived neurons are mostly glutamatergic [34]. Thus, upon supplementation with CORM-A1, differentiating NT2 cells yield a higher number of post-mitotic neurons that are metabolically more active. Moreover, one can speculate that these cells are more prepared to survive episodes of energy deprivation, such as stroke, in the re-oxygenation phase when they can take up lactate to support their mitochondrial activity. PDH and LDH were also quantified and, upon supplementation with CORM-A1, neuronal differentiation yielded cells with increased expression of both enzymes. Thus, the overexpression of LDH in differentiating neuronal cells induces an improved feeding of TCA cycle and consequently oxidative phosphorylation. The improvement in mitochondrial metabolism during NT2 neuronal differentiation was corroborated by isotopic enrichment studies, which showed that ^{13}C labelling of TCA cycle metabolites and amino acids derived for TCA cycle metabolites glutamate and aspartate was increased in the presence of CO. Finally, under hypoxic conditions (5% O_2), CORM-A1 supplementation did not yield the same beneficial effects on mitochondria as observed during normoxia (21% O_2). Thus, one can speculate that, under hypoxia the lack of oxygen impaired the ability of CO to increase mitochondrial metabolism and oxidative phosphorylation.

In summary, these data validate: (i) the role of cell metabolism, in particular oxidative metabolism, during the neuronal

differentiation process and (ii) that CO-improvement of neuronal differentiation is dependent on cell metabolism modulation. This study contributes to clarify the pathways involved in neurogenesis, promoting the establishment of new strategies to stimulate neuronal differentiation. The increasing knowledge of stem cell biochemistry can further research in areas such as regeneration and healing.

ACKNOWLEDGEMENTS

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V

IMPROVEMENT OF NEURONAL PRODUCTION BY CARBON MONOXIDE: ROLE OF PENTOSE PHOSPHATE PATHWAY

This chapter is based on the following manuscript:

Improvement of neuronal production by carbon monoxide: role of Pentose Phosphate Pathway

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unpublished data

ABSTRACT

Over the last decades, the silent-killer carbon monoxide (CO) has been shown as an endogenous cytoprotective molecule able to inhibit cell death and to modulate mitochondrial metabolism. Neuronal metabolism is mostly oxidative and neurons also use glucose via pentose-phosphate pathway (PPP) for maintaining their anti-oxidant status by generation of reduced glutathione. Moreover, whenever glycolysis is forced in neurons (gene overexpression or inhibition), there is a great reduction PPP and consequently of anti-oxidant defence. It is well known that neuronal differentiation process is related to ROS generation and signalling, however there is a lack of information about modulation of PPP during adult neurogenesis. Thus, the main goal of this study is to disclose CO modulation of neuronal differentiation and how it can be related with PPP regulation for increasing antioxidant defence.

It was used human neuroblastoma SH-S5Y5 cell line, which differentiate into post-mitotic neurons by treatment with retinoic acid (RA), as model for adult neurogenesis. CO-releasing molecule A1 (CORM-A1) was used do deliver CO into cell culture. SH-SY5Y cell differentiation process supplemented with CORM-A1 prompted an increase in neuronal yield. However, this improvement was not related to the classical metabolic shift that occurs during differentiation: (i) CORM-A1 did not alter glycolytic metabolism since

levels of lactate produced per glucose consumed was not altered and (ii) oxidative metabolism slightly decreased upon CORM-A1 supplementation as verified by the levels of labeled metabolites. Nevertheless, due to the fact that lactate dehydrogenase expression was increased by CORM-A1 but without any apparent effect on glycolysis, pentose phosphate pathway was assessed. The expression of PPP key enzymes (phosphogluconate dehydrogenase and transketolase) increased in the presence of CORM-A1. Furthermore, the ratio between reduced and oxidized glutathione increased, which is an indirect evaluation of this pathway. Moreover, CORM-A1 promotes the modulation of glutathione metabolism, once it increases protein glutathionylation levels. In summary, CO improves neuronal yield by modulating PPP in SH-SY5Y cells during neuronal differentiation.

CONTENTS

Ana Sofia Almeida had carried out the majority of the experimental part and was involved on the decisions on how to execute the experiments, as well as on the interpretation and discussion of the results.

INTRODUCTION

Stem cell fate can be regulated by various factors, namely cellular energy metabolism, which is capable of modulating the decision of stem cells to self-renew or to differentiate [1], [2]. Thus, manipulation of cell metabolism can be a key tool for neurogenesis modulation, in order to stimulate a replacement of impaired neurons in central nervous system (CNS), particularly in cases of neurodegenerative diseases and ischemic stroke.

CO is mostly known as a silent-killer due to its great affinity to hemoglobin, which compromises oxygen delivery and promotes high levels of intoxication and death. Nevertheless, CO is an endogenously produced gasotransmitter generated by the cleavage of heme group via the enzymatic activity of heme-oxygenase (HO)[3]. HO is a stress-related enzyme, whose expression or activity increases in response to several stressful stimuli, namely: oxidative stress, hypoxia, hyperoxia, hyperthermia, inflammation, UV and misfolded protein response, among others [3]–[5]. Likewise, it has been demonstrated that low levels of exogenous CO promote cytoprotection, limit inflammation, prevent cell death and improve neuronal differentiation [3], [4], [6]–[11]. Moreover, CO-induced cytoprotection can be dependent on generation of low amounts of ROS, functioning as signaling molecules [6], [12]–[15]. Low concentrations of CO promote mitochondrial biogenesis [16], [17], increase COX activity [7], [18]–[21], improve oxidative metabolism [16], [22] and induce mild

mitochondrial uncoupling that protects mitochondria from oxidative stress [23], [24]. For further reading, please consult the following reviews [16], [25], [26]. Herein, it was used CORM-A1 (carbon-monoxide releasing molecule A1), which able to release CO in a temperature and pH dependent manner, presenting a half-life of approximately 21 minutes for transfer of CO to myoglobin *in vitro* at pH of 7,4 and 37ºC [27], [28].

Pentose Phosphate Pathway (PPP) is an important route of glucose oxidation, divided in two branches, the oxidative and non-oxidative phase, where, by multiple reactions, sugar phosphates are interconverted. The oxidative phase of PPP is linked to glycolysis at the level of glucose-6-phosphate and catalyses its conversion into ribulose-5-phosphate and CO_2. Also, in this phase there is the reduction of $NADP^+$ into NADPH, the major reducing compound, which is required for regeneration of reduced glutathione (GSH) from its oxidized form (GSSG). On the other hand, the non-oxidative phase converts pentose phosphates into phosphorylated aldoses and ketones. This branch is linked to glycolysis by their common intermediates glyceraldehyde-3-phosphate and fructose-6-phosphate and it also produces ribose-5-phosphates, which are precursors for nucleotide synthesis [29], [30]. The activity of this non-oxidative phase of PPP is mainly used to support the active cellular proliferation during neurogenesis [31]. Moreover, modulation of PPP flux can allow cells to obtain the same amount of energy *per* glucose molecule,

when compared to glycolysis, but with an associated increase in nucleotide synthesis and in availability of electron donors, such as NADPH.

For assessing PPP modulation by CO, human neuroblastoma SH-S5Y5 cell line was chosen as cell model. This is a simple model to study neuronal differentiation process [32], allowing the assessment of the involved cellular mechanisms, which requires more controlled settings. SH-SY5Y cells are derived from neural crest [33], [34] and present the ability to differentiate into neuron-like cells that fulfil the morphological, biochemical and functional neuronal criteria [32], [34], [35], constituting a valuable model for neuronal toxicity studies [36]–[39].

Also, GSSG/GSH recycling is dependent on PPP and it has been already described that CO modulates GSH recycling [6]. Thus, one can speculate that CORM-A1 can stimulate PPP flux, which in turn can facilitate the cellular machinery rearrangement needed during neuronal differentiation.

The main goal of this study was to assess metabolic regulation of neuronal differentiation achieved by CO, in particular at the level of PPP. CORM-A1 supplementation increases neuronal yield of SH-SY5Y neuronal differentiation and it was observed that the presence of CORM-A1 increases PPP flux and modulates GSH metabolism, favouring protein glutathionylation. Herein was proved that adult neurogenesis may be differently metabolically modulated

accordingly to the characteristics of the different *in vitro* model chosen, which open routes to improved strategies to overcome neurodegenerative scenarios.

MATERIAL AND METHODS

Materials

All chemicals were of analytical grade and were obtained from Sigma unless stated otherwise. Plastic tissue culture dishes were from Sarstedt (Germany); fetal bovine serum, penicillin/streptomycin solution, and Dulbecco's minimum essential medium (high glucose, L-glutamine and pyruvate) were obtained from Invitrogen (United Kingdom).

The mass spectrometry derivatization reagents MTBSTFA (*N*-methyl-*N*- (tert-Butyldimethylsilyl) trifluoroacetamide), MSTFA (*N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide) and the t-BDMS-Cl (tert-butyldimethylchlorosilane) were purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). All other chemicals were of the purest grade available from regular commercial sources.

SH-SY5Y human neuroblastoma cell line

Maintenance of undifferentiated cells

The SH-SY5Y cell line was cultured in DMEM/F-12 supplemented with 10%(v/v) FBS and 2%(v/v) Pen/Strep (growth medium). Cells were maintained in a humidified atmosphere of 5%(v/v) CO_2 at 37ºC. Undifferentiated cells were grown in 75cm^2 T-flasks and subcultured with fresh growth medium whenever cell confluence achieved (about 80-90% cell confluence). Cells were detached by trypsinization at room temperature (R.T.) and slight shaking and hitting to drain down cells with trypsin and resuspended in growth medium in a 1:4 cell passage. Growth medium was changed twice a week.

Neuronal differentiation protocol

Following trypsinization and resuspension in growth medium, cells were plated on 75cm^2 T-flasks in a 1:2 cell passage. Neuronal differentiation was induced 24 hours after plating undifferentiated cells to ensure settle and attachment to flask surface and attain appropriate density, approximately about 50% cell confluence in all 75cm^2 T-flasks.

Neuronal differentiation was stimulated using DMEM/F-12 medium, reduced serum to 1%(v/v) FBS, 2%(v/v) Pen/Strep and supplemented with 10μM of *all-trans* RA (differentiation medium). CO effect was studied by using the same composition of differentiation

medium supplemented with 25μM CORM-A1. Differentiation medium was replaced twice (1st and 4th days) during 7 days of treatment. On the 7th day, cells were collected for analysis.

Neuronal enrichment

After the 7th day of differentiation, cells were replated at lower density to disperse cell culture for neuronal enrichment. On the following day, the culture medium was exchanged with fresh growth medium supplemented with mitosis inhibitors: 1μM Cytosine Arabinoside, 10μM Floxuridine and 10μM Uridine for neuronal enrichment. Growth medium supplemented with mitosis inhibitors was replaced after 2 days. On the 5th day of neuronal enrichment, enriched cultures were collected for different analysis. The used protocol is schematically represented in Figure 5.1B.

Carbon monoxide releasing molecule A1 (CORM-A1)

The solution of CORM-A1 was prepared in milli-Q water with a final concentration of 5mM. Then, solution was filtrated with 0,2μM filter and stored at -20ºC. For each use, an aliquot was thawed and immediately used.

Preparation of inactivated CORM-A1

CO-depleted inactive form (iCORM-A1) was generated to be used as negative control by initially dissolving CORM-A1 in 0.1 M HCl and then bubbling pure N2 through the solution for 10 min in order to remove all residual CO gas [28]. The solution of iCORM-A1 was adjusted to pH 7.4, filtrated using a 0,2µM filter and stored at -20ºC. For each use, an aliquot was thawed and immediately used.

Cell counting and viability

Cell cultures were visualized using an inverted microscope with phase contrast (DM IRB, Leica, Germany). Total cell number was determined by counting cell nuclei using a Fuchs-Rosenthal hemacytometer, after digestion with 0.1M citric acid/1% Triton X-100 (wt/wt)/0.1% crystal violet (wt/v).

Quantitative-Polymerase chain reaction (Q-PCR)

Genomic DNA was extracted from cells after differentiation using the High Pure PCR Template preparation kit (Roche Diagnostics, Mannheim, Germany). PCR was performed using specific forward and reverse primers designed for the mitochondrial COXII gene (5'-ACAGACGAGGTCAACGATCC-3' and 5'-AGATTAGTCCGCCGTAGTCG-3') and for the GAPDH gene (5'-GCATCCTGGGCTACACTGAG-3' and 5'-GTCAAAGGTGGAGGAGTGGG-3'), respectively. Fast Start DNA Master Plus SYBR Green I (Roche Diagnostics) was used with the

experimental run protocol: denaturation program was 95 °C for 10 min, followed by 45 cycles of 95 °C for 15'', 60 °C for 6'' and 72 °C for 20''.

For evaluation of gene expression, mRNA was extracted from NT2 and SH-SY5Y cells using High Pure RNA isolation kit (Roche Diagnostics), and cDNA synthesis was performed using the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics). PCR was performed using specific forward and reverse primers designed for the Phosphogluconate dehydrogenase gene (5'-ACCAGCAGACAATGCACGTA-3' and 5'- AGGGATGAAGACAGCCACAC-3'), Transketolase gene (5'- CATGCCAGTGACCGCATCAT-3' and 5'-ATGCGAATCTGGTCAAAGGC-3'), Pyruvate Dehydrogenase gene (5′-AGGGTGGTTTCTATCTGTCTTGT-3′ and 5′-TCATGCTTCTTTTATCCTCTTGCT-3′), Lactate Dehydrogenase gene (5′-GGCTATTCTTGGGCAACCCT-3′ and 5′-TGGAAGTGGTACCAATACAACTCA-3′) and RPL22 gene (5'-CACGAAGGAGGAGTGACTGG-3' and 5'-TGTGGCACACCACTGACATT-3'), respectively. Fast Start DNA Master Plus SYBR Green I (Roche Diagnostics) was used with the experimental run protocol: denaturation program was 95 °C for 10 min, followed by 45 cycles of 95 °C for 10'', 60°C for 10'' and 72 °C for 10''.

Lactate/Glucose Ratio

Total glucose and lactate concentrations in the culture supernatant were determined with automated enzymatic assays (YSI 7100 Multiparameter Bioanalytical System; Dayton, OH). The rate between lactate production and glucose consumption was obtained by linear regression of the metabolites concentrations.

Gas Chromatography-Mass Spectrometry (GC-MS)

For analysis of ^{13}C percent enrichment in intracellular metabolites cell extracts were lyophilized and resuspended in 0.01M HCl followed by pH adjustment to pH<2 with HCl 6 M. Samples were dried under atmospheric air (50 °C), and metabolites were derivatised with MTBSTFA in the presence of 1% *t*-BDMS-Cl (Mawhinney et al. 1986) (see Amaral et al. 2014 for further details). The samples were analyzed on an Agilent 6890 gas chromatograph connected to an Agilent 5975B mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). The parent ion (M) and atom percent excess for one ^{13}C atom (M+1) values for 3PG, PEP, alanine, aspartate, lactate, citrate and glutamate were calculated from GC-MS data using MassHunter software supplied by Agilent (Agilent Technologies, Palo Alto, CA, USA) and correcting for the naturally abundant ^{13}C by using non-enriched standards [40].

High Performance Liquid Chromatography (HPLC)

Cell extracts were performed by lysing cells with 250 μL of Triton X-100 (0.01 %) in PBS 1× (v/v) followed by centrifugation at 13000 rpm for 5 min, 4 ˚C. The aminothiols in cell extracts and culture media (supernatants) (50 μL) were reduced with tris(2-carboxyethyl)phosphine (TCEP); the proteins were precipitated with trichloroacetic acid; and reduced thiols were derivatized with ammonium 7- fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), according to Nolin et al. [41]. Samples were analyzed by HPLC system (Shimadzu) with a RF 10AXL fluorescence detector, operating at 385 nm (λ excitation) and 515 nm (λ emission). The aminothiols CYS, CysGly, and GSH were separated on a LiChrospher 100 RP-18 (250×4 mm, 5 μm; Merck), with a mobile phase consisting on a mixture of 0.1 M acetate buffer (pH 4.5, adjusted with acetic acid): methanol (99:1v/v) at a flow rate of 0.8 mL/min at 29 ˚ C. The run time was 20 min.

Immunofluorescence microscopy

SH-SY5Y cells were plated at a density of $2x10^6$ cells/well in 24-well plates coated with Poly-D-lysine in 0,15M sodium borate buffer solution pH 8,4. Cells were fixed with 4%(v/v) PFA and 4%(w/v) sucrose solution (20 minutes at R.T.) and then permeabilised with 0,3%(v/v) Triton X-100 solution (15 minutes at R.T.). Later, cells were incubated 2 hours at R.T. with primary antibody: Tuj1 (Sigma-Aldrich,

T8660) and ki67 (Millipore, AB9260), following incubation for 1 hour at R.T. with secondary antibody: AlexaFluor 488 anti-mouse (A11001) or AlexaFluor 594 anti-rabbit (A11012). Primary and secondary antibodies were dilute in 1%(v/v) BSA and 0,1%(v/v) Triton X-100 solution. Cultures were mounted on Prolong mounting media (with DAPI - Invitrogen) and images were captured with Zeiss Axiovert 40 CFL microscope. All solutions were prepared in PBS (1X). Washes with PBS (1X) solution were performed between each step.

Statistical analysis

The data concerning cell culture were carried out at least in three independent preparations. All values are mean ± SD, n≥3. Error bars, corresponding to standard deviation, are represented in the figures. Statistical comparisons between multiple groups were performed using ANOVA single factor with replication and between two groups were made with an independent two-tailed Student's *t-test*. For all the data, *p*-value is indicated for each figure.

RESULTS

CORM-A1 increases neuronal differentiation yield

CORM-A1 modulatory effect on neuronal differentiation was assessed using SH-SY5Y cells, which have the ability to differentiate into neurons upon treatment with retinoic acid (RA) (Fig. 5.1A). CORM-A1 was used as a supplement of the classical differentiation procedure. Treatment with only CO without RA is not enough to induce neuronal differentiation, since there was no neuronal differentiation (data not shown). The neuronal differentiation process of SH-SY5Y cells takes 7 days, followed by 5 days of neuronal enrichment with anti-mitotic agent treatment (Fig. 5.1A).

After 7 days of differentiation, it was obtained a mixed cell population composed of fully differentiated neurons and precursors cells that did not differentiate. This occurs mainly because cells are not synchronized in the beginning of the process. When SH-SY5Y cells were differentiated for 7 days in the presence of 25μM CORM-A1, the total number of cells of the mixed cell population increased significantly (Fig. 5.1B). As expected, this population was composed of neurons and proliferative cells, expressing Tuj1 and ki67 proteins, respectively, as assessed by immunocytochemistry (Fig. 5.1B). In order to understand whether this increase was due to an effect on cellular proliferation or differentiation, the mixed SH-SY5Y cell population was treated with anti-mitotic agents for neuronal

enrichment, and it was characterized by immunocitochemistry (Fig. 5.1C). For the same amount of cells in the beginning of neuronal differentiation process, enriched neuronal population increased about 4,5 times in the presence of CORM-A1 (Fig. 5.1C). Thus, CO released by CORM-A1 presents a positive modulatory role in the neuronal yield of SH-SY5Y cell line differentiation, as already demonstrated in Almeida et al 2016 [10].

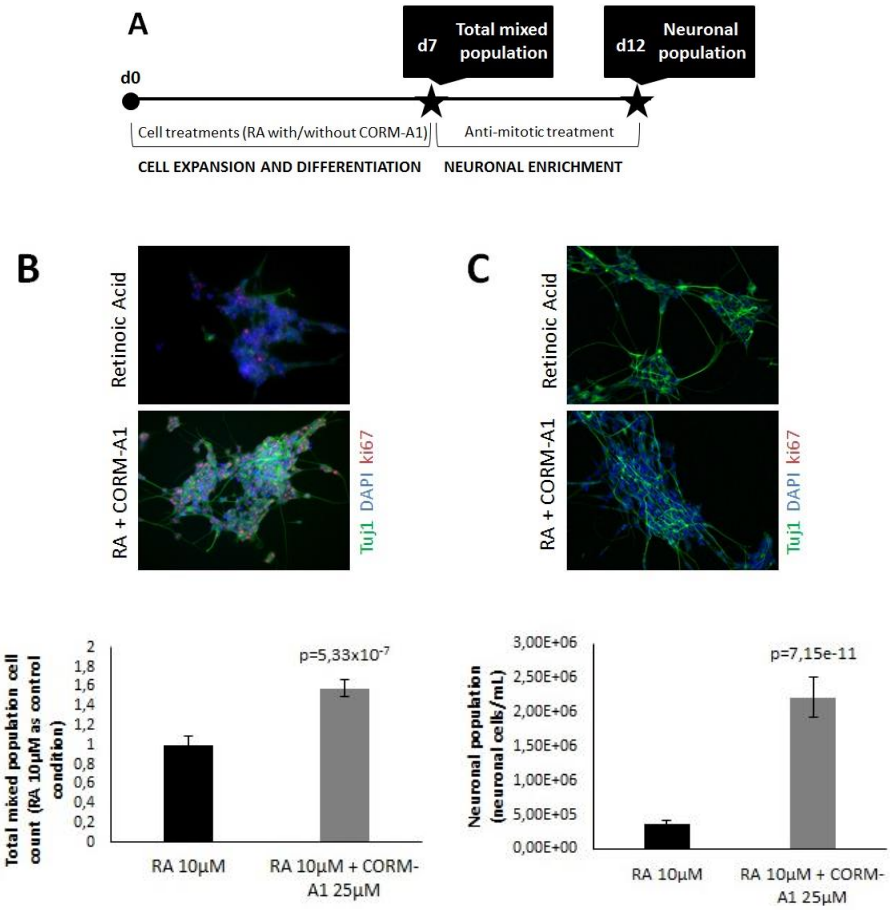


Figure 5.1 – CORM-A1 improves neuronal differentiation yield. (A) SH-SY5Y neuronal differentiation procedure: SH-SY5Y cells were induced to differentiate during 7 days (d7), subjected to a differentiation medium exchange at day 4. After 7 days of differentiation (d7), a mixed population of

undifferentiated cells and post-mitotic neurons was obtained. In order to obtain an enriched neuronal population, cultures are treated with anti-mitotic compounds for 5 days (d12); Characterization of SH-SY5Y cells by immunocytochemistry (green staining: Tuj1; blue staining: DAPI; red staining: ki67; magnification 100x) and nuclei count per volume of SH-SY5Y cells (B) after 7 days of differentiation and (C) after anti-mitotic treatment during 5 days. All values are mean ± SD, n≥3 and *p-value* is indicated in each panel.

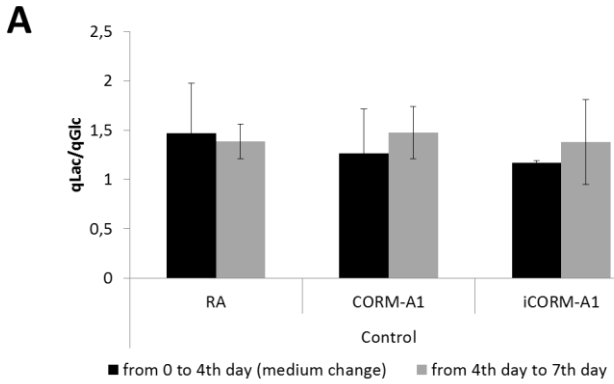
CORM-A1 effect on glycolytic metabolism

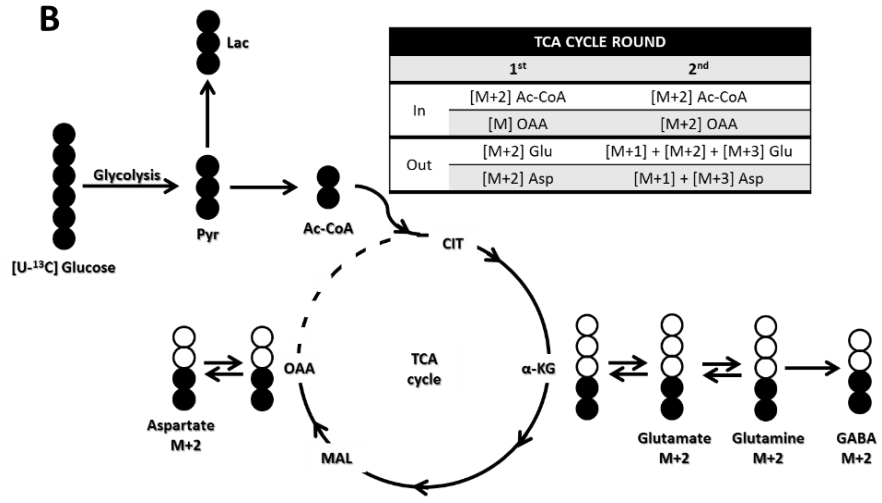
Because neuronal differentiation involves modulation of glycolytic metabolism [42]–[44] and CO also regulates cell metabolism [22], CORM-A1 effect on glycolysis was assessed (Fig. 5.2A). It was observed that the levels of lactate production *per* glucose consumption remain unchanged with or without CORM-A1 supplementation during neuronal differentiation process (Fig. 5.2A), which indicates that there might be no change on glycolysis due to CO presence.

During glycolysis, glucose is converted into pyruvate which can follow two distinct pathways: it can be transformed (i) in lactate by lactate dehydrogenase (LDH) or (ii) in acetyl-CoA, by pyruvate dehydrogenase (PDH) action followed by coenzyme A ligation, which will feed tricarboxylic acid cycle (TCA). Thus, glycolysis progression in the presence of CORM-A1 was evaluated using labelled glucose (^{13}C) and measuring the percentage of ^{13}C enrichment using GC-MS. To study ^{13}C enrichment in intracellular metabolites, SH-SY5Y cells differentiated for 7 days in the presence and absence of CORM-A1 supplementation were incubated with [U-^{13}C]glucose for 24 hours and

cell extracts were prepared for GC-MS analysis. The percentage of ^{13}C enrichment was quantified in lactate, in glutamine and glutamate and in TCA cycle metabolites citrate and aspartate. Metabolism of [U-^{13}C]glucose generates [U-^{13}C]lactate and [U-^{13}C]pyruvate. As shown in Fig. 5.2C, there is no significant difference of M+3 lactate levels between control cells and CORM-A1 supplemented ones. This data corroborate the ratios of lactate production *per* glucose consumption presented in Fig. 5.1A, meaning that there is no increase on glycolysis. In turn, [U-^{13}C]pyruvate is then converted into [1,2-^{13}C]acetyl CoA. This molecule condenses with non-labelled oxaloacetate to form double-labelled (M+2) compounds in the first turn of the TCA cycle (Fig. 5.2B). Furthermore, in a combination of the first and second turn of the TCA cycle, [1,2-^{13}C]acetyl-CoA can condense with labelled oxaloacetate and give rise to the formation of diversely labelled compounds (Fig. 5.2B). GC-MS analysis of SH-SY5Y cell extracts incubated with [U-^{13}C]glucose showed that the percentage of ^{13}C labelling of citrate, aspartate, glutamine and glutamate was decreased for M+2 (Fig. 5.2D-H) in the presence of CORM-A1. In summary, CORM-A1 supplementation of SH-SY5Y cells neuronal differentiation seems to not interfere with glycolysis but leads to a slight decrease in mitochondrial metabolism. Moreover, mRNA expression of pyruvate dehydrogenase (PDH) and lactate dehydrogenase (LDH) were quantified. CORM-A1 induces a decrease in PDH and an increase in LDH expression. This result correlates with

the decreased mitochondrial metabolism observed in CORM-A1 supplemented cells (Fig. 5.2 D-H) and with the unaltered ratio and quantity of lactate obtained during differentiation in the presence of CORM-A1 (Fig. 5.2 A and C). Consequently, one may speculate that, upon CORM-A1 supplementation, glucose is going to feed other pathways than glycolysis, such as PPP, which is parallel but interconnects to glycolysis and is very important during cellular differentiation.





TCA CYCLE ROUND		1st	2nd
In		[M+2] Ac-CoA	[M+2] Ac-CoA
		[M] OAA	[M+2] OAA
Out		[M+2] Glu	[M+1] + [M+2] + [M+3] Glu
		[M+2] Asp	[M+1] + [M+3] Asp

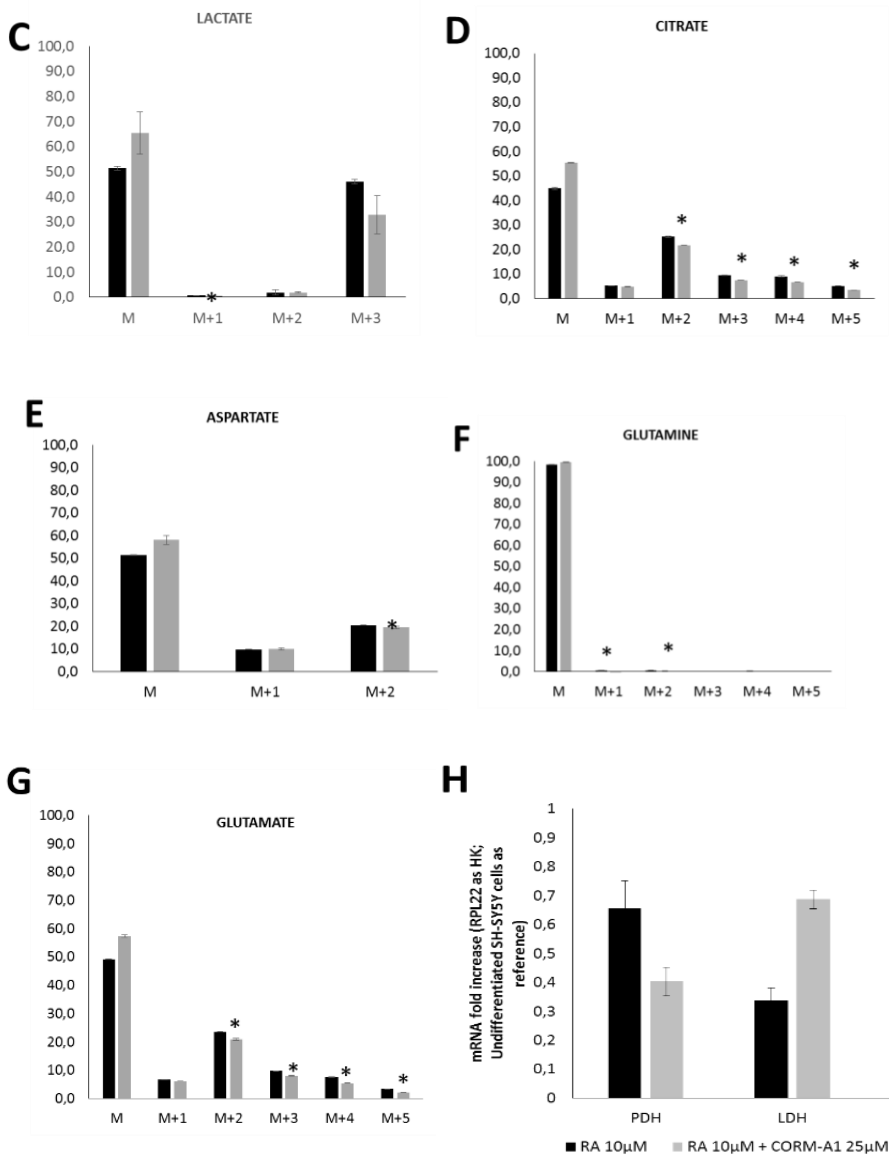


Figure 5.2 – Glycolytic metabolism profile. (A) Lactate production per glucose consumption (qLac/qGlc) ratios calculated between treatments (n≥3); (B) Labelling patterns derived from [U-^{13}C]glucose metabolism; The % enrichment with ^{13}C leading to the formation of molecules with masses: M+1, M+2 and M+3 for (C) lactate, (D) citrate, (E) aspartate, (F) glutamine and (G) glutamate, determined by GC-MS analysis of SH-SY5Y cell extracts differentiated for 7 days and subjected to 24h incubation with medium containing [U-^{13}C]glucose; (H) mRNA expression of specific metabolic markers (PDH for pyruvate dehydrogenase and LDH for lactate dehydrogenase) in SH-

SY5Y mixed cell population after 7 days of neuronal differentiation. All values are mean ± SD, n≥3 and *p-value* is indicated in each panel.

CORM-A1 modulates Pentose Phosphate Pathway

To study PPP, two key enzymes were assessed. mRNA expression of phosphoglucoronate dehydrogenase (PGDH) and transketolase (TKT), from oxidative and non-oxidative phases of PPP respectively (Fig. 5.3A). Actually, the expression of both enzymes increased in the presence of CORM-A1 in neuronal differentiated cells. Moreover, PPP assessment can be indirectly done by glutathione quantification using HPLC because PPP is coupled to GSSG/GSH recycling system, which is crucial for cellular anti-oxidant defense. Note that GSH reacts directly with radicals and it is an electron donor in reactions catalysed by glutathione peroxidases, generating, in turn, glutathione disulphide (GSSG). GSH is regenerated from GSSG in the reaction catalysed by glutathione oxidases, which transfers electrons from NADPH to GSSG [45], [46]: GSH recycling coupled to PPP.

Cell extracts of differentiated SH-SY5Y cells with or without CORM-A1 supplementation were analysed at 2h following the first treatment (Fig. 5.3B). Intracellularly, CORM-A1 supplementation leaded to a slight increase in the ratio GSH/GSSG. Although the difference between treated and non-treated cells with CORM-A1 is not

statistically relevant, one may speculate that the increase in GSH/GSSG ratio directly correlates with more quantity of NADPH. This electron donor is mainly provided by PPP, thus the increase of NADPH can be related with an increased PPP flux. In summary, the increase in GSH/GSSG ratio corroborates the result obtained from mRNA expression (Fig. 5.3 A and B): CORM-A1 supplementation appears to stimulate PPP.

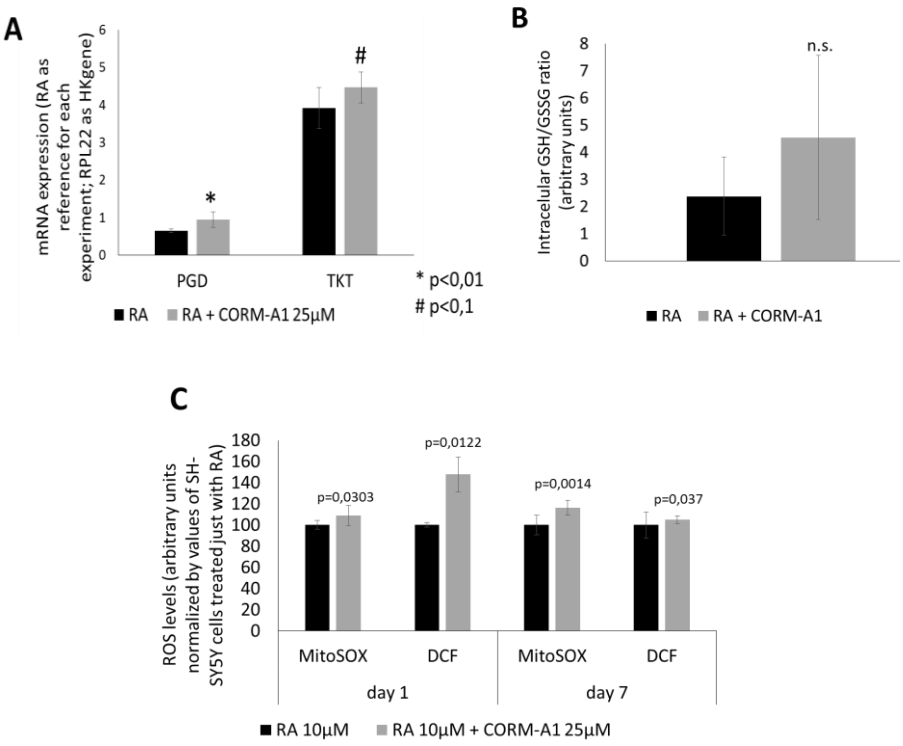


Figure 5.3 – CO modulation of pentose phosphate pathway. (A) mRNA expression of specific PPP enzymes (PGDH for phosphogluconate dehydrogenase and TKT for transketolase) in SH-SY5Y mixed cell population after 7 days of neuronal differentiation; (B) GSSG/GSH ratios 2 hour after CORM-A1 supplementation; (C) Reactive oxygen species (ROS) quantification 2hour after CORM-A1 supplementation (day 1) and in the end of neuronal differentiation (day 7).

Moreover, it is already known that CO effect occurs via ROS signaling [6], [7], [10] which promotes the oxidation of GSH into GSSG [6]. Thus, the reinforcement of PPP (Fig. 5.3A) and improvement of GSH recycling (Fig. 5.3B), can be a response to ROS production during CORM-A1 supplementation. ROS intracellular levels were quantified 2h after CORM-A1 supplementation and after 7 days of neuronal differentiation (Fig. 5.3C). It was observed that CORM-A1 increases ROS levels, immediately after its supplementation, and the increased levels are maintained at the end of neuronal differentiation process (Fig. 5.3C). Hence, CORM-A1 effect on neuronal differentiation of SH-SY5Y cells might be mediated by ROS as signaling molecules, which would require an improvement of GSSG recycling in order to control the redox state of the cell that is only possible due to an increase in NADPH levels. The electron donor NADPH is obtained by a boost in PPP. However, the contribution of anabolic and catabolic pathways of glutathione also should be taken in account.

CORM-A1 modulates Glutathione metabolism

An increase in the ratio GSH/GSSG can occur by three processes: (i) increase in glutathione catabolism; (ii) decrease in glutathione anabolism and/or (iii) increase of protein glutathionylation (Fig. 5.4A). In order to evaluate these processes, three different strategies were adopted: (i) quantification of Cys-Gly

peptide in extracellular environment [47]; (ii) quantification of the intracellular aminoacid Cysteine, which is the rate-limiting factor for GSH synthesis [47], [48]; and (iii) estimation of bound glutathione by difference in the quantification of total glutathione in the cell and free glutathione in the cell [48].

Glutathione is synthetized intracellularly through the condensation of three aminoacids: cysteine, glutamate and glycine (Fig. 5.4A). In Fig. 5.4A is schematized glutathione metabolism and recycling coupled to PPP. In the previous section (Fig. 5.3B) it was observed a tendency of CORM-A1 to improve the recycling of GSSG in GSH, however one cannot disregard that the proportion of glutathione within the cell can be affected by other mechanisms such as anabolism, catabolism and protein glutathionylation. Thus, the quantities of aminoacid cysteine and the peptide Cys-Gly were determined (Fig. 5.4 B-C). In order to assess protein glutathionylation, the percentage of the total glutathione that is not free intracellularly was calculated (Fig. 5.4D).

Glutathione anabolism (Fig. 5.4 B) seems to be unchanged by CORM-A1 supplementation: intracellular levels of total and free cysteine are similar between control cells (only treated with RA) and cells treated with CORM-A1 (RA + CORM-A1). On other hand, CORM-A1 slightly decreases glutathione catabolism (Fig. 5.4 C) because cells treated with CORM-A1 presented less extracellular levels of Cys-Gly peptide. Accordingly, CORM-A1 promotes an accumulation of

glutathione intracellularly. Inside the cell, glutathione can exist in the reduced form (free) or bound to other proteins (protein glutathionylation). In Fig. 5.4 D is possible to observe that the quantity of glutathione in the bounded form is significantly higher in cells treated with CORM-A1 than in control cells. In conclusion, the accumulation of intracellular glutathione is associated with the increase of protein glutathionylation caused by CORM-A1 supplementation.

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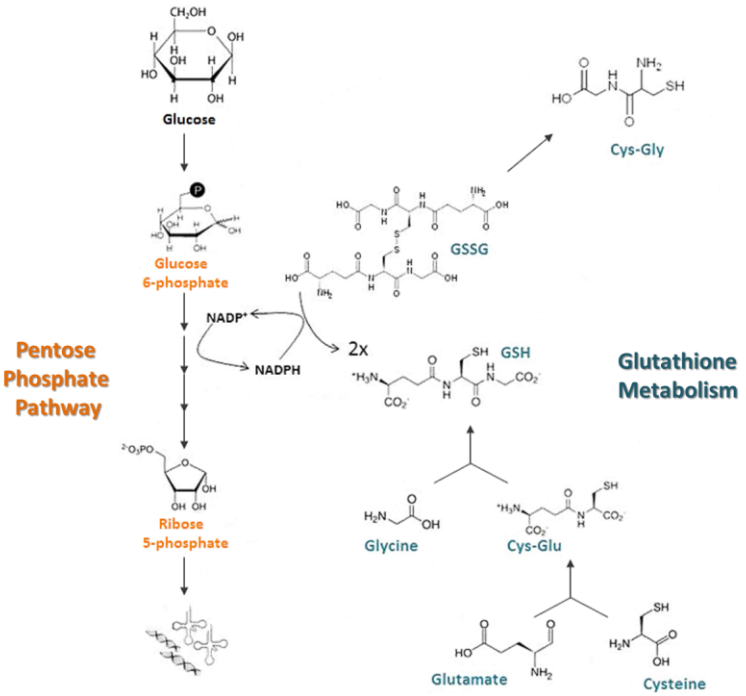

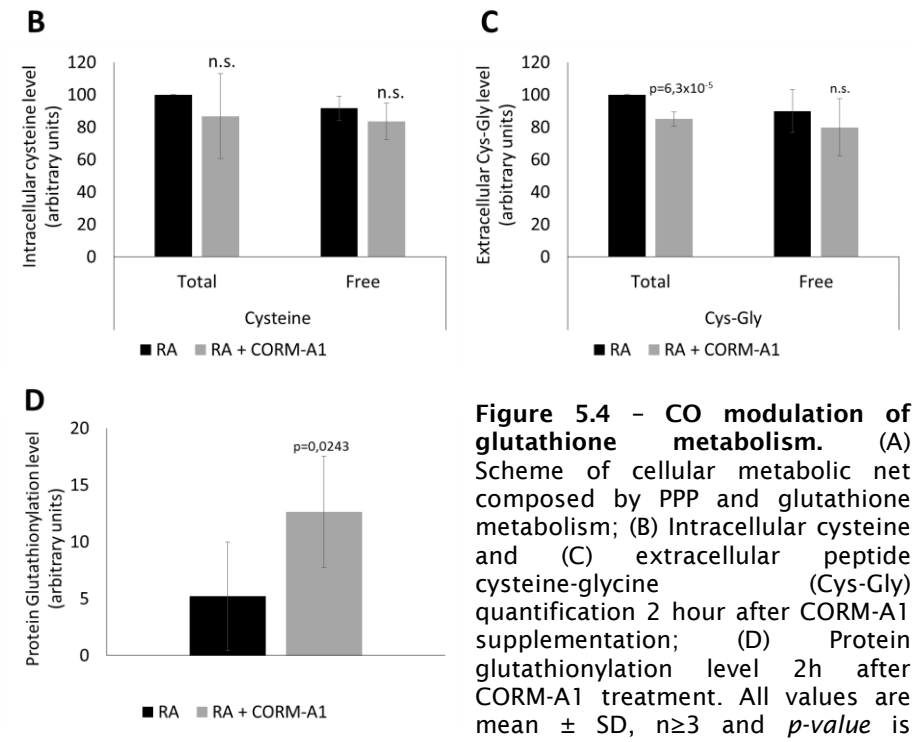

Figure 5.4 – CO modulation of glutathione metabolism. (A) Scheme of cellular metabolic net composed by PPP and glutathione metabolism; (B) Intracellular cysteine and (C) extracellular peptide cysteine-glycine (Cys-Gly) quantification 2 hour after CORM-A1 supplementation; (D) Protein glutathionylation level 2h after CORM-A1 treatment. All values are mean ± SD, n≥3 and *p-value* is indicated in each panel.

DISCUSSION

CO promotes cytoprotection, limits inflammation, prevents cell death and modulates neuronal differentiation [3], [4], [6]–[10], through generation of low amounts of ROS, which function as signalling molecules, and increase mitochondrial oxidized glutathione [6], [12]–[15].

In this study, human SH-SY5Y neuroblastoma cell line was used in order to understand whether the CO's modulatory effect in

neuronal differentiation was related to cell metabolism. Because glucose oxidation was not altered by CORM-A1 supplementation, PPP flux was targeted as potential involved pathway. Actually, it was observed that not only glycolysis was not affected by CORM-A1 supplementation, but also mitochondrial metabolism is slightly diminished. These results are not in accordance with data previously published by our group [11], where it was shown that CORM-A1 promotes neuronal differentiation of NT2 cells by reinforcing mitochondrial metabolism. In fact, the discrepancy between results can be due to the different cell models used. NT2 are teratocarcinoma-derived cells that present a pluripotent phenotype, while SH-SY5Y cells present already a neuronal predisposition given its morphology and the ability to differentiate along the neuronal lineage. Thus, one can speculate that NT2 cell line differentiation represents an early stage of neurogenesis process and SH-SY5Y cell line differentiation is a better model to characterize end phases of the process.

In addition to the results previously published by our group; neurogenic process is widely described to be accompanied to a metabolic shift, where cells become more dependent on oxidative metabolism than on glycolysis, resulting in neuronal population relying mostly on oxidative metabolism. In the present study, upon treatment with CORM-A1, we observed that glycolysis seems not to be altered, although there was an increase on LDH expression. On

other hand, mitochondrial metabolism diminished and even PDH expression decreased. Thus, one can speculate that CORM-A1 modulates other pathways of glucose metabolism, such as PPP.

Although PPP is a minor contributor to total glucose oxidation, it is essential for cell due to the importance of their products: (i) the electron donor NADPH and (ii) ribose-5-phosphate, both essential for biosynthetic processes, as neurogenesis. It is known that, in the brain, the flux through PPP is higher in the oxidative part than in the non oxidative one, thus the yield of the whole pathway is made taking in account the non oxidative part [49]. In order to understand if PPP was improved by CORM-A1, two key enzymes were chosen to assess the pathway: (i) 6-phosphogluconate dehydrogenase (PGDH) that catalyses the first step of PPP in the oxidative part and (ii) transketolase (TKT), an enzyme from the nonoxidative part, responsible for the production of fructose-6-phosphate and glyceraldeyde-3-phosphate, both intermediates that connect PPP to glycolysis. These are key enzymes that catalyse non-reversible reactions in PPP. Both PGDH and TKT mRNA expression increased in cells treated with CORM-A1, leading us to conclude that PPP is being reinforced by CO supplementation during neuronal differentiation of SH-SY5Y cells. Also, PGDH and TKT are expressed in the various cell types in the brain [50]–[52], but PGDH activity decreases after birth [53] while TKT activity increases during postnatal development [54], [55]. Taking in account the present data

(low mRNA expression of PGDH and higher mRNA expression of TKT) we can claim that SH-SY5Y cells mimic adult neurogenesis.

NADPH synthesis in PPP is extremely important in what regards to cell's antioxidant defences [56]–[61]. Moreover, in embryonic stem cells, it was observed that oxidative part of PPP was essential to generate NADPH to protect cells against oxidative stress but dispensable for the synthesis of ribose-5-phosphate (due to the interconnection to glycolysis) [62], [63]. Therefore, cellular ROS and glutathione levels were quantified in order to assess the relationship between CO supplementation, ROS as signalling molecules and glutathione as a measurement of antioxidant defences, which is also an indirect way to assess PPP. As expected, CORM-A1 supplementation increased cellular ROS levels that were not enough to cause oxidative stress (data not shown) but acted as signaling molecules, slightly increasing GSH/GSSG ratio.

Neuronal differentiation supplemented with CORM-A1 did not altered the intracellular levels of cysteine compared to control cells but decreased the extracellular levels of Cys-Gly, what indicates that glutathione synthesis does not increase and glutathione catabolism is reduced by CO. Concomitantly, CORM-A1 promotes protein glutathionylation. Glutathionylation is an oxidative post-translational modification that consists in the formation of disulphide bonds between protein thiols and glutathione. This process ensures protection of protein thiols against irreversible over-oxidation,

operates as a biological redox switch in both cell survival and cell death, acting as signalling regulator at organelle level. Further studies are needed concerning this issue during neuronal differentiation; but it is already known that glutathionylation in prone to occur in modulation of metabolism, cystoskeletal remodelling, apoptosis and DNA modifications [48], [64], [65]. Concerning our study, further validation is needed, such as (i) ROS scavenger prior to CORM-A1 supplementation in order to verify that CO-induced ROS are promoting PPP and (ii) gene expression silencing of glucose-6-phosphate dehydrogenase, once this enzyme controls PPP flux and will allow the confirmation that CO boosts this pathway and the concomitant modulation of glutathione metabolism.

In summary, our results (i) corroborate the importance of antioxidant defences during neuronal differentiation, (ii) present PPP as one more important pathway for modulation of neurogenesis and (iii) validate CO to improve neuronal yield by modulating PPP and glutathione metabolism. Altogether, this study helps to elucidate the integrated metabolism involved in adult neurogenesis.

ACKNOWLEDGEMENTS

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VI

DISCUSSION AND CONCLUSION

Ana S. Almeida has written the whole chapter based on the referred bibliography and her own results described in chapters II to V.

CONTENTS

1. DISCUSSION

In Neurosciences, the dogma that no new neurons can be born after the end of embryonic and early postnatal development was accepted for decades [1], [2]. However, since 1960s, some reports started to suggest that adult brain retains the capacity to generate new neurons in particular brain zones [3], [4]. In 1970, The Boulder Committee claimed the existence of multipotent progenitor cells in ventricular zone (VZ) and subventricular zone (SVZ) [5], [6]. Despite the existence of several studies about this issue [6]–[8], the acceptance of lifelong neurogenesis in mammalian brain just became broadly accepted in the beginning of the 21st century [9], [10].

The study of adult neurogenesis is extremely important due to the fact that brain can potentially restore some functions after acute or chronic injuries. Nevertheless, the endogenous mechanisms of repair have clear limitations, originating a large percentage of patients with brain injuries that have to daily lead to permanent functional deficits. Consequently, it is imperative to continue searching for strategies to enhance the course of degenerative or traumatic brain diseases. The main targets for brain injury repair are the modulation of endogenous neurogenesis for improving it and/or cell therapy by *in vitro*

production of neural cells for transplantation and their integration in existing neuronal circuits [11].

Carbon monoxide (CO) is an endogenous anti-apoptotic and anti-proliferative molecule, which can modulate several cellular pathways by ROS signaling (**Chapter I**). Thus, this thesis has explored the potential application of CO to improve neuronal differentiation process. Two distinct objectives can be reached by CO-induced increase on neuronal production yield: (i) improvement of neuronal cell production for potential future transplantation and (ii) disclosing the molecular mechanisms underlying this process for developing improved *in vivo* modulation of neurogenesis.

As presented in **Figure 1.11** (**Chapter I**), the general aim of this thesis is the evaluation of CO effect in different *in vitro* models of neuronal differentiation, with particular regard to the balance between cell proliferation, cell differentiations, cell death and metabolic shifts. The main achievements concerning CO modulation of specific pathways that lead to an increased neuronal yield are summarized in **Figure 6.1** and are discussed throughout this chapter. The achievements clearly contribute to spread knowledge about adult neurogenesis and to open a new therapeutic opportunity for CO.

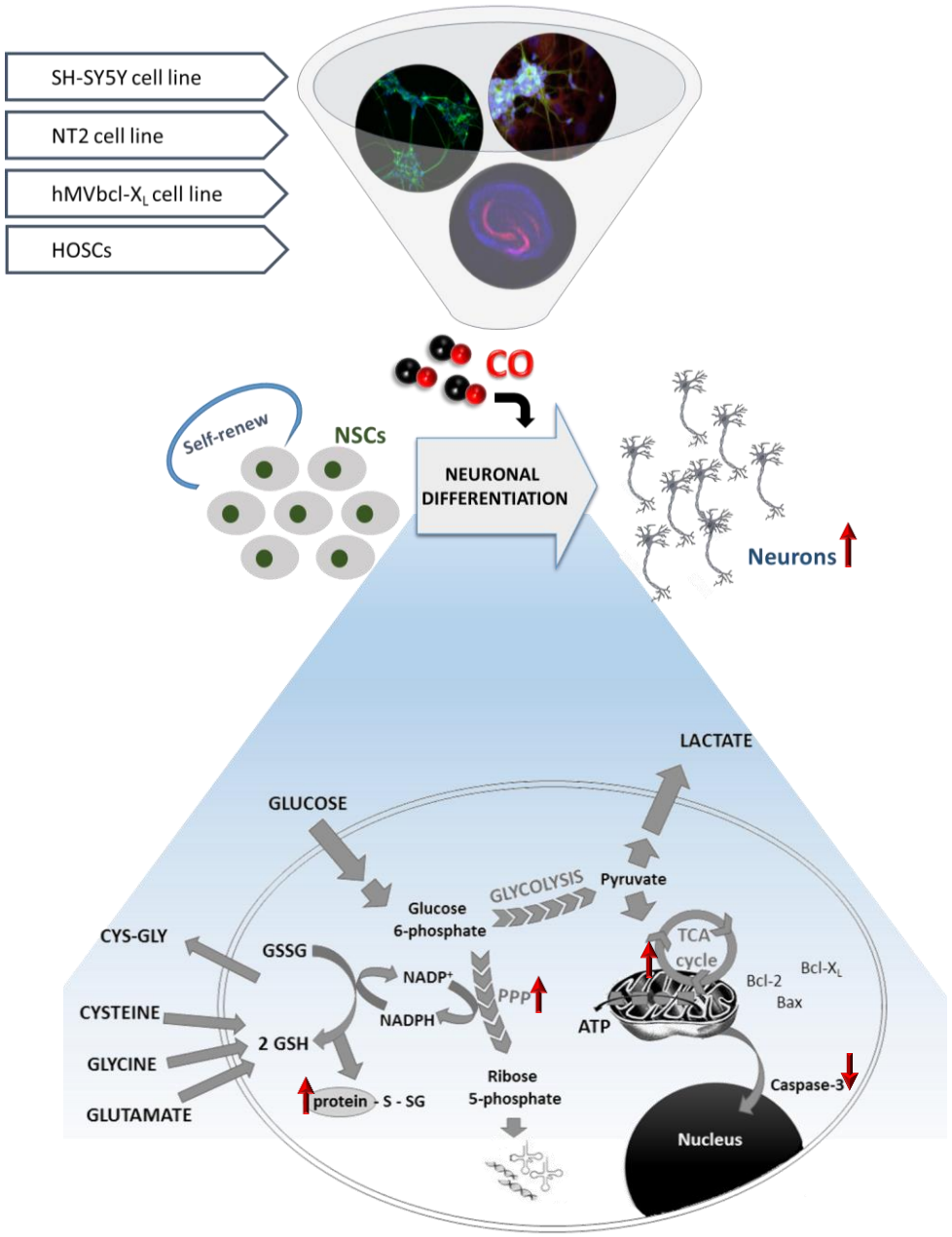

Figure 6.1 – Main achievements of this PhD thesis. CO does increase final yield of neuronal differentiation, through prevention of cell death (Chapter II and III) and modulation of cell metabolism: increase of mitochondrial metabolism (Chapter IV), increase of PPP flux and stimulation of glutathione recycling and protein glutathionylation (Chapter V).

1.1. *In vitro* neuronal production

In the adult brain, NSCs can be found in stem cell niches. These are anatomical compartments that includes cellular and acellular components that integrate the signalling network to regulate stem cells biology [12]–[14]. The ability to isolate and culture stem cells *in vitro* has greatly contributed for understanding the role, structure and regulation of stem cells niches and neuronal differentiation process [14], [15].

1.1.1. Perspective on the different models used in this thesis

In this thesis four different models were used: two cancer cell lines (NT2 and SH-SY5Y), one human neural stem cell line (hVMbcl-X_L) and organotipic cultures of hippocampal slices. The human origin of NT2 and SH-SY5Y cell lines is a clear advantage to study human neuronal differentiation processes and cell manipulation for potential use in cell therapy. Furthermore, these cell lines have similar characteristics of human cells expressing a number of specific proteins, contrary to primary cultures of rodent precursor cells. Nonetheless, they are tumor cells presenting mutagenic and oncogenic potential, being less representative of physiological neuronal differentiation [16], [17].

In contrast, hVMbcl-XL cells were generated from ventral mesencephalic region of a human fetus, presenting genuine characteristics of their regional identity, which exempt the need of complex patterning procedures. However, in order to be maintained in culture, these cells over-express the anti-apoptotic protein $BclX_L$. This features should be taken into consideration when evaluating proliferation and differentiation, since cell death is extremely related with neurogenesis [18], [19].

Cell lines are a powerful tools that offer several advantages: they are easy to use, provide an unlimited supply of material, provide consistent samples and reproducible results and bypass ethical concerns associated with the use of animal and human tissues. However, cell lines are genetically manipulated which can alter their phenotype, native functions and their responsiveness to stimuli. Moreover, it is important to consider physiologically relevant models that allow the neurogenesis assessment within an intact neuronal circuitry [20]–[22]. Thus, organotypic hippocampal slice cultures (OHSCs) represent a valuable model of adult neurogenesis, allowing the assessment of three key steps: neural stem cell proliferation, differentiation and migration [20]–[22]. The use of slice cultures is more common in studies of excitotoxicity and ischemia. However OHSCs can be used for assessing neurogenesis since the SGZ is a neurogenic zone preserving its property *in vitro* (reviewed in [20], [21]). Since 2004, these slice cultures have been used as

representative models for neurogenesis studies, allowing the follow-up of two different cells fate processes: proliferation and differentiation [23]–[28].

1.2. Cellular mechanisms behind adult neurogenesis

To improve the outcome in the injured brain, one must acquire knowledge about the potential of the endogenous stem cells for neural repair. Although all the efforts, the knowledge about physiological adult neurogenesis and about their regulatory mechanisms are scarce [11].

Data generated in this thesis demonstrated that CO increases neuronal production in different *in vitro* models of neuronal differentiation. Because it is known that in other biological systems CO limits cell death and modulates cell metabolism (**Chapter I**), these features were the main targets for studying the mechanisms behind the improvement of neuronal yield promoted by CO (**Chapters II** to **V**).

1.2.1. Programmed cell death

Cell death during embryonic development of the nervous system mainly occurs in order to regulate the size of progenitor populations and to remove erroneous or unwanted neural populations [29]. Also, adult neurogenesis and integration of young neurons into mature

neural circuits are regulated by cell death events [30]. In adult brain, survival and integration of young neurons is dependent on the competition of these cells with a diversity of neuronal cells in different stages of maturation. Thus, cell death role in adult neurogenesis may have unique regulatory mechanisms that are not properly studied yet [29]. Identification and quantification of cell death in adult brain is not easy because the presence of dead cells are transient as a result of active clearance by immune cells [31].

Because programmed cell death is tightly associated with neuronal differentiation *in vivo*, it can also originate limitations in *in vitro* neuronal production approaches. In **Chapter II** and **III**, it was demonstrated that CO improves adult neurogenesis yield by preventing apoptotic cell death. Thus, CO appears as an agent capable of increasing *in vitro* neuronal production for cell therapy strategies. Additionally, the use of genetically modified models that limit to execute cell death, such as hMVbxl-X_L cell line (**Chapter III**), is essential to assess not only the extent and distribution of cell death but also to identify cellular changes responsible for cell death modulation. Using this cell line one is able to keep the majority of cells alive in order to observe the intracellular changes occurring during neuronal differentiation. In the context of this thesis, this approach allowed the detection that, besides preventing cell death (**Chapter II**), CO can modulate cytokine expression and glucose metabolism (**Chapter III**). Based on this data, the work on metabolic

modulation was carried on and is now presented in **Chapters IV** and **V**.

Despite the challenges about assessing cell death during neuronal differentiation, it was demonstrated that CO is able to reduce apoptosis in the different chosen models of adult neurogenesis (**Chapter II**), promoting an increased neuronal population suitable for potential cell therapies, such as (i) cell transplantation in the case of neuronal production using cell lines and (ii) stimulation of endogenous neurogenesis occurring in hippocampus and mimicked by HOSCs. However, further studies are needed to establish the applicability of CO to stimulate endogenous neurogenesis by cell death prevention, once it was observed that knocking out regulatory apoptotic genes lead to supernumerary neurons in mouse brains [32]. Since this kind of cell death has the final aim of eliminating dysfunctional cells, one must consider that probably the modulation of neurogenesis by CO could not be applied *in vivo*, otherwise one can obtain brains with abnormal and dysfunctional cell populations which can cause more harm than beneficial effects. Nevertheless, CO supplementation appears as a promising factor for cell production regarding transplantation applications.

1.2.2. Bioenergetics and metabolism

Metabolic alterations are essential for stem cell proliferation and differentiation. Although existing few information about intermediary metabolism of stem cells, it is known that NSCs are more dependent on glycolytic metabolism than neurons [33]. Also, recent data showed that activation of quiescent NSCs is accompanied by downregulation of glycolytic metabolism and upregulation of mitochondrial oxidation [34], [35].

In **Chapter IV,** NT2 cell line was used to disclose the relationship between the improvement of neuronal production by CO and the effect of CO in the shift from glycolysis to oxidative metabolism during neuronal differentiation process. In fact, it was observed that CO stimulated neuronal production by promoting mitochondrial metabolism over glycolysis. These data strongly support the currently accepted model in which stem cells predominantly utilize glycolytic metabolism in order to maintain self-renew and lineage potency; while differentiated cells mainly use oxidative metabolism to support the high energetic demands of specialized progeny [36], [37].

Nevertheless, one cannot disregard that during neurogenesis there is the need of building blocks such as nucleotides and electron donors like NADPH, both acquired in PPP. Also, the known improvement of oxidative metabolism during differentiation lead to an increase in ROS levels that are controlled by antioxidant defenses,

such as glutathione recycling coupled with PPP. In **Chapter V**, it was demonstrated that CO improves the yield of SH-SY5Y cell line neuronal differentiation by increasing PPP flux that boosts glutathione metabolism.

Despite the important role of PPP by providing building blocks and electron donors that support the active cellular proliferation during neurogenesis, its role in maintaining the redox status is crucial. Signaling ROS mediate the loss of self-renewal capacity through control of mitochondrial biogenesis and metabolic shifts [38]–[42]. It has been suggested that low levels of ROS mediate cell proliferation, while higher levels (but still in signaling, non harmful concentrations) promote an activated state of NSCs [43]. This is line with the described mechanism behind CO effect: CO uses ROS as signaling molecules. Despite the fact that CO increases ROS levels (**Chapter II)**, further studies with ROS scavengers would be necessary to fully support the hypothesis that CO modulation of neuronal differentiation is dependent on ROS signaling.

2. FUTURE PROSPECTS

In this thesis it was demonstrated that CO has the ability to improve neuronal production by modulating cell death and some metabolic pathways. Nevertheless, it is imperative to understand the potential applicability of CO as modulator of endogenous

neurogenesis. Thus, *in vivo* approaches should be taken in account in further studies.

CORMs are promising molecules that increase the potential application of CO in therapeutics, once they can have the ability to release CO under appropriate conditions in a controlled-directed fashion, which can be tissue specific [44]–[46]. However, their capacity to cross the blood-brain barrier can be a drawback. Moreover, the hypothesis of increasing endogenous CO by modulation of HO-1 activity should be explored in order to avoid the presence and subsequent metabolization of foreigner chemical structures in the biological system during neuronal differentiation. HO-2, the constitutive form of heme oxygenase only present in brain and testis, can also be targeted in order to increase CO concentration in these areas.

By assessing the mechanisms underlying CO improvement of neuronal differentiation, it was possible to identify key pathways involved in neuronal differentiation. However, other pathways from the cell integrated metabolism should also be explored. For instance, it is important to understand whether the observed increased flux through TCA cycle could be related to any CO modulation in the contribution of fatty acids and aminoacids oxidation in this pathway. Particularly in *in vivo* approaches, one must consider the β-oxidation of fatty acids (i) in acetyl-CoA and further distribution through bloodstream to the brain and posterior entrance of this metabolite in

TCA cycle and (ii) in ketone bodies that can be directly consumed by the brain. Also, the NADH and $FADH_2$ produced in the process of β-oxidation are an added-value for the bioenergetics of the organism. Likewise, aminoacid catabolism should be assessed due to the Krebs bicycle that links the Urea cycle with TCA cycle. Basically, the carbon structure of the aminoacid can be transformed into α-ketoacids and feed TCA cycle. On the other hand, the ammonia group will enter Urea cycle. Through the Malate-aspartate shuttle and/or the Aspartate-arginino-succinate shunt the two cycles can communicate and feed themselves.

Moreover, the crosstalk between programmed cell death and autophagy is an essential topic in further studies. Over the last years some studies claimed that CO can improve autophagy in lung and heart models, through modulation of mitochondrial function [47]–[49]. Additionally, the association of apoptosis and autophagy in quality control of NSCs has been assessed. Autophagy may be critical not only for controlling self-renewal and proliferation, but also for regulating differentiation and maintenance of pluripotency in stem cells [50]. Finally, one cannot disregard that NSCs proliferation and differentiation require a dynamic turnover of intracellular components and cellular remodeling. This places autophagy in a crucial position because it can modulate both cellular metabolic directions: (i) catabolic activity through degradation and elimination of intracellular components and aggregates and (ii) anabolic activity

through supplying the energy recycling intermediates and providing the building blocks for the repair of intracellular structures [51], [52]. In conclusion, it would be very interesting to study whether CO modulation of neuronal differentiation is dependent on autophagy regulation.

All the experiments done in the scope of this thesis were done under ambient air conditions, considered normoxic by conventional standards of cell-culture practice. However, it is known that by the time inspired air reaches organs and tissues, pO2 levels have dropped to 2%-9% [53] and, consequently, oxygen concentration between 2%-9% have recently been appreciated by some scientists to constitute physiologic normoxia [15], [54]. Moreover, low oxygen tension is known to modulate the balance between proliferation and differentiation [54]–[56]. One cannot disregard also the fact that, *in vivo,* CO competes with O_2 for bounding to hemoglobin, leading to less oxygen in the brain. It is known that, in neurogenic zones, pO_2 can be in the range of 0,5%-8% [57], thus it is extremely important to mimic this hypoxic or even anoxic conditions when studying adult neurogenesis and in particular when using CO as modulator. Therefore, CO effect on adult neurogenesis should be assessed in low levels of oxygen in order to guarantee the evaluation in a more physiological environment. It is important to keep in mind that optimal concentrations for CO supplementation and even the mechanistic effect of CO can be different under low oxygen levels.

3. CONCLUSION

This thesis demonstrated that CO has potential as therapeutic molecule to improve neuronal production for cell therapy strategies. During neuronal differentiation, CO (i) modulates cell death promoting NSCs proliferation and differentiation, (ii) boosts the natural metabolic shift from glycolytic to oxidative metabolism and (iii) improves PPP flux, leading to a modulation of glutathione metabolism and promotion of protein glutathionylation. Moreover, disclosing molecular mechanisms underlying adult neurogenesis has the potential to facilitate novel cell therapies aiming at improving the outcome of neurodegenerative scenarios by modulation of *in vivo* endogenous neurogenesis.

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