ANTI-INFLAMMATORY EFFECT OF CARBON MONOXIDE ON THE NEURON-MICROGLIA BIDIRECTIONAL COMMUNICATION

NUNO RICARDO LUCAS SOARES

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

Doutoramento em associação entre:

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

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

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

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PROGRAMAS DE DOUTORAMENTO FCT

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Para a minha mãe

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Aos pais da Joana, por me fazerem sentir parte da família (espero continuar), pelos gelados e jantares, sei que dou muita despesa.

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ABSTRACT

Neurological disorders refer to any pathology that affects the brain, spinal cord, and peripheral nerves. These include stroke, epilepsy, psychiatric disorders, dementia, neuroinfections and tumours. The World Health Organization (WHO) estimates that, in 2030, almost 8 billion people will die because of neurological complications. Moreover, it reveals that formal care costs related to neuropathology morbidities will continue skyrocketing in the future.

The pathophysiology and progression of central nervous system (CNS) disorders are often multifactorial and poorly understood, which makes efficient treatment particularly difficult. There are several hallmark mechanisms of dysfunction: Uncontrolled neuroinflammation is a key driving force for damage propagation and homeostatic imbalances. Dysregulation of adult neurogenesis and loss of specific neuronal population are also neurological hallmarks of ageing and disease. Current day findings have helped to illuminate the underlying mechanisms of disease, but several questions remain unanswered. Therefore, there is an ongoing struggle to further disclose neurological disease aetiology and find new therapeutic alternatives in brain pathologies.

Carbon monoxide (CO) is an endogenous gaseous molecule, which confers protection in different tissues, including the CNS. The main aim of this PhD thesis was to better understand the mechanisms of CO-induced neuroprotection. The modulatory role of CO was addressed on two distinct processes: neurogenesis and neuroinflammation. Different models were employed. CO-releasing molecules (CORMs) were used to deliver CO in vitro.

Chapter I introduces concepts regarding the brain, neuron, and glial cells. Additionally, state-of-the art knowledge about molecular pathways of neurodifferentiation, microglia function in immunity and homeostasis, as well as microglia-neuron communication, phagocytosis, and cell death. There is a great focus on the biological role of CO.

In **Chapter II**, it is reported that CO enhances neuronal production yield in an SH-SY5Y neuroblastoma cell line differentiation model. The CO's stimulatory effect on neuronal differentiation had previously been linked to modulation of cell death and improvement of mitochondrial metabolism in other models. In here, CO improved SH-SY5Y neuroblastoma cell line neurogenesis by acting on the Glucose-6-phosphate dehydrogenase (G6PD) and enhancing Pentose Phosphate Pathway (PPP) flux. The reported effect was independent of *de novo* Glutathione (GSH) synthesis. Knocking down the expression of the rate-limiting enzyme G6PD blocked the CO-induced improvement of SH-SY5Y differentiation. In summary, CO-driven metabolic reprogramming has a central role in cell fate and neuronal differentiation processes.

Chapter III focuses on CO as a mediator of neuron-microglia remote communication. Neuron-microglia conditioned media protocol revealed that CO provides cytoprotection to neurons exposed to

microglia pro-inflammatory media. In fact, CO suppressed exacerbated microglia reactivity by limiting Tumour necrosis factor α (TNF-α) and nitric oxide (NO) secretion. Furthermore, the effect of CO on neuron-microglia communication was also assessed in the absence of an inflammatory context. CO-treated microglia conditioned media partially protected neurons against cell death induced by oxidative stress. Moreover, supernatant from CO-treated microglia improved neuronal morphological complexity. This neurotrophic effect generated by CO-treated microglia might be dependent on microglia increased secretion of Interleukin-10 (IL-10). This chapter demonstrates that CO has an anti-inflammatory non-cell autonomous role, providing neuroprotection *via* regulation of microglia function and unidirectional communication with neurons. It was demonstrated for the first time that CO enhances microglial neurotrophic functions.

After exploring CO's mediation of remote communication (secretome) between neurons and microglia, a microfluidic cell system is used in **Chapter IV**, to explore neuron-microglia direct interactome during inflammation, while also focusing on the phagocytic process. CO's administration inhibited microglia secretion of inflammatory mediators and limited neuronal apoptosis and morphological atrophy. Focusing on the direct cell contact, microglial clearance of synaptic material is decreased under inflammatory conditions, while CO reverted phagocytosis to control levels, indicating that CO improves microglial removal of synaptic content under stress conditions. Along with synaptic pruning, CO also regulates microglial phagocytosis in an inflammatory context. In fact, CO had a quality control effect on microglia engulfment of neuronal cells under inflammation by blocking removal of viable neurons but enhancing the phagocytosis of neuronal debris and apoptotic cells. This apparent homeostatic role of CO had never been described, and the underlying molecular pathways are under study.

Lastly, a final discussion is presented in **Chapter V**, which integrates information from all chapters. The novelty and overall impact of the thesis generated data are contextualized in the scope of the existing literature. Experimental shortcomings and future steps are discussed.

Altogether, this PhD thesis provides a strong contribution to comprehending key CNS mechanisms. Additionally, it contributes for the better understanding of CO as a potentially therapeutic cytoprotective and homeostatic molecule.

RESUMO

Doenças neurológicas refere-se a qualquer tipo de patologia que afecta o cérebro, espinal medula e sistema nervoso periférico. Entre outras, podemos identificar doenças como o acidente vascular cerebral (AVC), epilepsia, doenças psiquiátricas, demência, infecções neurológicas e tumores. A Organização Mundial da Saúde (OMS) estima que, em 2030, quase 8 mil milhões de pessoas irão morrer devido a este tipo de doenças.

A fisiopatologia e progressão dos distúrbios do Sistema Nervoso Central (SNC) são, na maior parte das vezes, multifatoriais e mal compreendidos, tornando um tratamento eficiente particularmente complicado. Porém, existem vários mecanismos característicos de disfunção e dano. Neuroinflamação descontrolada é uma das causas principais de propagação de dano e desequilíbrios homeostáticos. Desregulação de neurogénese adulta e morte de populações neuronais especificas são outra característica patológica. Descobertas recentes têm ajudado a esclarecer mecanismos moleculares de doença, mas muitas questões permanecem sem resposta. Desta forma, existe um esforço contínuo para melhor entender estas patologias e encontrar novas alternativas terapêuticas.

O monóxido de carbono (CO) é uma molécula gasosa endógena, que é protetora em vários tecidos, incluindo no SNC. O principal objectivo desta tese de Doutoramento foi entender de melhor forma os mecanismos de neuroprotecção do CO. O efeito modulatório do CO foi estudado em dois processos biológicos diferentes: neurogénese e neuroinflamação. Para isto, vários modelos experimentais foram implementados.

O **Capítulo I** introduz conceitos teóricos relativos ao cérebro, ao neurónio e às células da glia. É também feita uma descrição do estado-da-arte relativo às vias moleculares de diferenciação, função da microglia na imunidade e homeostasia, comunicação microglia neurónio, fagocitose e morte celular. Existe um foco nas propriedades biológicas do CO.

No **Capítulo II**, é reportado que o CO reforça a produção de neurónios num modelo de diferenciação neuronal usando a linha celular de neuroblastoma SH-SY5Y. O efeito estimulatório do CO na diferenciação neuronal havia sido relacionado com modulação de morte celular e melhoramento do metabolismo mitocondrial em outros modelos. Aqui, o CO melhorou a neurogénese na linha celular SH-SY5Y, ao actuar sobre o enzima Glucose-6-fosfato desidrogenase (G6PD) e aumentar o fluxo da via das pentoses fosfato. O efeito aqui reportado foi independente da síntese *de novo* de glutatião. *Knockdown* da expressão do enzima limitante da via, G6PD, bloqueou a estimulação da diferenciação neuronal induzido pelo CO. Sumariamente, o CO promove uma reprogramação metabólica que tem um papel importante no destino da célula e em mecanismos de diferenciação neuronal.

O **Capítulo III** foca-se no CO como mediador da comunicação remota neurónio-microglia. Utilizando um protocolo de meio condicionado, o CO protegeu os neurónios incubados com meio pro-inflamatório de microglia. O CO inibiu a elevada secreção de TNF-α e NO em microglia reactiva. Além disso, o envolvimento do CO na comunicação neurónio-microglia foi também avaliado na ausência de um estímulo inflamatório. O meio condicionado de microglia tratada com CO protegeu parcialmente os neurónios contra morte celular induzida *via* stress oxidativo. Adicionalmente, o sobrenadante de microglia tratada com CO promoveu um aumento na complexidade morfológica neuronal. Este efeito neurotrófico gerado pela microglia tratada com CO parece ser dependente de um aumento na secreção microglial de IL-10. Este capítulo indica que o CO tem um papel anti-inflamatório celular não-autónomo, promovendo neuroprotecção através da regulação da função microglial e comunicação unidirecional com os neurónios. Aqui, foi demonstrado pela primeira vez que o CO estimula a função neurotrófica da microglia.

Um sistema de cultura de células em microfluídicas é usado no **Capítulo IV** para explorar o interactoma directo neurónio-microglia no contexto neuroinflamatório, focando também na regulação da fagocitose. Administração de CO inibiu a secreção de mediadores inflamatórios por parte da microglia e limitou apoptose neuronal e atrofia morfológica. Sob condições inflamatórias, a remoção de conteúdo sináptico por parte da microglia decresceu. Porém, o CO reverteu este efeito, indicando que esta molécula melhora a fagocitose de sinapses sob condições de stress. Em conjunto com a poda sináptica, o CO também regulou a fagocitose microglial de neurónios durante a inflamação. Na verdade, o CO teve um efeito de controlo de qualidade na fagocitose de neurónios: bloqueou a remoção disfuncional de células viáveis, mas promoveu a fagocitose de resíduos neuronais e material apoptótico. Este mecanismo aparentemente homeostático, promovido pelo CO, não havia sido descrito e experiências estão a decorrer de modo a entender as vias moleculares que o regulam.

Por último, a discussão final é apresentada no **Capítulo V**, integrando a informação de todos os capítulos. Aqui, a inovação e impacto geral dos resultados são contextualizados tendo em conta a literatura existente. Melhorias experimentais e próximos passos são discutidos.

Em suma, esta tese de Doutoramento oferece uma forte contribuição para o entendido de mecanismos chave no funcionamento do sistema nervoso. Adicionalmente, contribui para a melhor compreensão do CO como molécula citoprotectora e homeostática no SNC.

THESIS PUBLICATIONS

1. Soares, N. L.; Almeida, A. S.; Sequeira, C. O.; Pereira, S. A.; Sonnewald, U.; Vieira, H. L. A.. ‘‘Improvement of neuronal differentiation by carbon monoxide: Role of pentose phosphate pathway’’. Redox Biol. 2018 Jul; 17: 338–347.

LIST OF ABBREVIATIONS

ABBREVIATION	FULL TEXT
5-TAMRA	5-(and-6)-carboxytetramethylrhodamine succinimidyl ester
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Aβ	Amyloid β
BAI1	Brain-specific angiogenesis inhibitor 1
Bax	Bcl-2-associated X protein
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BR	Bilirubin
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
BV	Biliverdin
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CO	Carbon monoxide
COHb	Carboxyhaemoglobin
CORM	CO-releasing molecule
COX	Cytochrome c oxidase
CR3	Complement Receptor 3
CX3CL1	CX3C motif chemokine ligand 1 (or fractalkine)
CX3CR1	CX3C chemokine receptor 1 (or fractalkine receptor)
DAPI	4',6-diamidino-2-phenylindole
DCF	2',7'-dichlorofluorescein
DG	Dentate gyrus

DIV	Days *in vitro*
dLGN	Dorsolateral geniculate nucleus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
Egr-1	Early growth response protein 1
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FBS	Foetal bovine serum
Fc	Fragment crystallizable region
G6PD	Glucose-6-phosphate dehydrogenase
GABA	γ-Aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas chromatography–mass spectrometry
GDNF	Glial cell-derived neurotrophic factor
GSH	Glutathione
GSSG	Glutathione disulfide
H2DCFDA	2',7'-dichlorofluorescein diacetate
HIF-1α	Hypoxia-inducible factor 1 α
HO	Haem oxygenase
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
HYCO	Hybrid CO-releasing molecule
I/R	Ischaemia/Reperfusion
Iba-1	Ionized calcium-binding adapter molecule 1
ICAM	Intercellular adhesion molecule
iCORM	Inactive CO-releasing molecule

IFN-γ	Interferon γ
Ig	Immunoglobulin
IGF-1	Insulin growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
Keap1	Kelch-like ECH-associated protein 1
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MerTK	MER proto-oncogene, tyrosine kinase
MFG-E8	Milk Fat Globule-Epidermal Growth Factor-Factor 8
MGC	Mixed glial cell
MMP	Mitochondrial membrane permeabilization
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
MTBSTFA	N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NLRP3	NLR Family Pyrin Domain Containing 3
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf-2	Nuclear factor erythroid 2-related factor 2
NSPC	Neural stem/progenitor cells

OB	Olfactory bulb
OBSC	Organotypic brain slice cultures
OGD	Oxygen-glucose deprivation
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase
PDL	Poly-D-Lysine
PDMS	Polydimethylsiloxane
PE/Cy7	Phycoerythrin/Cyanine
PFA	Paraformaldehyde
PGC-1α	Peroxisome proliferator-activated receptor gamma co-activator 1 α
PGDH	6-phosphogluconate dehydrogenase
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PPAR-γ	Peroxisome proliferator-activated receptor γ
PPP	Pentose phosphate pathway
PSD-95	Postsynaptic density protein 95
PtdSer	Phosphatidylserine
Q-PCR	quantitative Polymerase Chain Reaction
RA	Retinoic Acid
RhoA	Ras homolog family member A
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SAH	Subarachnoid haemorrhage

SBD-F	Ammonium 7- fluoro-2,1,3-benzoxadiazole-4-sulfonate
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SNAP-25	Synaptosomal-Associated Protein, 25kDa
STAT	Signal transducer and activator of transcription
SVZ	Subventricular zone
***t*-BHP**	*tert*-Butyl hydroperoxide
TBS	Tris-buffered saline
TCA	Tricarboxylic acid cycle
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TGF-β	Transforming growth factor β
TKT	Transketolase
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
TREM-2	Triggering receptor expressed on myeloid cells 2
VGLUT1	Vesicular glutamate transporter 1
VIP	Vasoactive intestinal peptide
WB	Western Blot
WHO	World Health Organization
α-syn	α-synuclein

LIST OF FIGURES

TABLE OF CONTENTS

1

INTRODUCTION

INDEX

Nuno L. Soares has written the whole chapter based on the referred bibliography

1. Brain

Neuroscience is an ever-growing, interdisciplinary field of study that focuses on understanding the structure and function of the nervous system.

The brain is the iconic and complex organ that is the central object of interest for neuroscientists. Making up the Central Nervous System (CNS), along with the spinal cord, the brain is responsible for interpreting and integrating information from all the body and has control over a large set of activities – motor function, breathing, blood pressure, food intake, balancing, memory formation, cognition, speech and sensory functions[1]. It is, however, susceptible to damage caused by homeostatic imbalances: several CNS disorders, such as Stroke, Neurodegenerative diseases and mental illnesses are increasingly prevalent and are a major cause of mortality, morbidity, decreased life expectancy and quality of living[1,2]. Despite this, much of their aetiology and pathological progression remains poorly understood.

The brain is also a cultural symbol, a representation of several aspects that individually define us as humans: reason, consciousness, empathy, desire. All of this makes CNS-centred research very prevailing and appealing. Numerous hallmark breakthroughs have led to a better understanding of brain anatomy, physiology, chemistry and molecular mechanisms, but many questions arise every day. Hence, modern neurosciences are under constant growth and mutation, in an evolving quest to understand the brain in all its complexity.

Anatomically speaking, the human brain is shielded by multiple barriers that afford physical and biochemical protection[1]. The outermost is the skull, a bone structure that absorbs and protects the soft tissue from physical impact. Inside the skull, the brain is suspended in a liquid substance, the cerebrospinal fluid, that is produced in the choroid plexus and cushions the brain from shocks[1,3]. Lastly, the blood-brain barrier (BBB), a specialized microvasculature composed of vascular epithelial cells, pericytes and astrocyte end-feet processes, isolates the brain parenchyma from the circulatory system. This barrier allows selective transport of nutrients, which is key to maintain homeostasis, and protect the brain from infection[1,3].

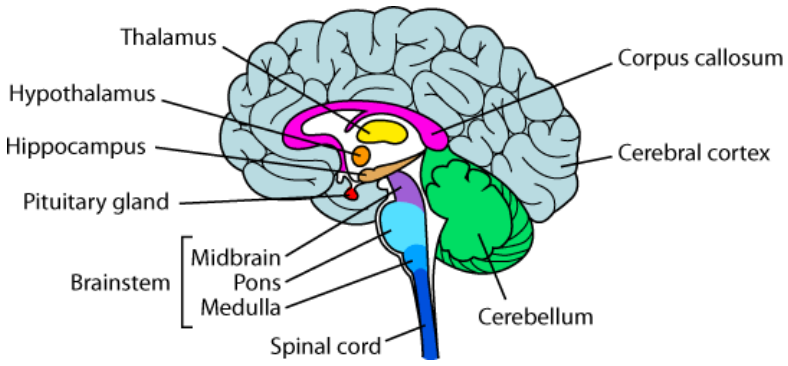


Figure 1 – Schematic representation of the human brain regions. Adapted from https://askabiologist.asu.edu/parts-of-the-brain

The human brain can be divided into three major regions: the brainstem, the cerebellum and the cerebrum[1] (Figure 1). The cerebrum is the largest region of the brain and is separated into symmetrical left and right hemispheres that are connected by the fibrous corpus callosum structure, which sits at the bottom of the cerebrum. The outer layer of the cerebrum, the cortex, has a distinct wrinkled aspect that significantly increases its surface area. The cortex is arranged into specific layer structures and has key role in sensory input, motor functions, signal integration, among others. It is generally subdivided into lobes: frontal, parietal, occipital and temporal, named after the cranial bones, that overlie these regions.

Underneath the cortex, lie several subcortical structures, such as the diencephalon (thalamus, epithalamus, subthalamus and hypothalamus), basal ganglia and the hippocampus. Subcortical structures participate on mechanisms of hormone production, cognitive, social and affective functions[1].

The brainstem region is contiguous to the spinal cord and adjacent to the cerebellum[1]. In the human brain, it is divided into the midbrain, pons and medulla oblongata. This region has a crucial role as a conducer of sensory information between the forebrain and motor neurons throughout the body. This brain region is also involved in the control of heart rate, respiration and blood pressure. The cerebellum sits at the base of the cerebrum[1]. This region is fundamental for sensorimotor coordination (balance, posture), learning and planning of movements. The cerebellum receives and integrates inputs from regions of the cerebral cortex related with planning and initiation of highly skilled movements[1].

1.1. Neurons

Neurons are highly specialized post-mitotic cells that make up the large bulk of the nerve tissue and have the critical role in transmitting and integrating information[4]. Neurons are very heterogeneous in nature and can be subdivided based on function (sensory, motor, interneurons), morphology (unipolar, bipolar, multipolar and pseudounipolar) and location[4,5]. Still, these cells are typically comprised by a compact soma (cellular body), dendrites and the axon, with respective terminals[5] (Figure 2). The axon, coated in myelin, protrudes out of the soma, carrying action potential that will reach the terminal and transmit information to adjacent cells. The specialized communication structure is called synapse[4,5].

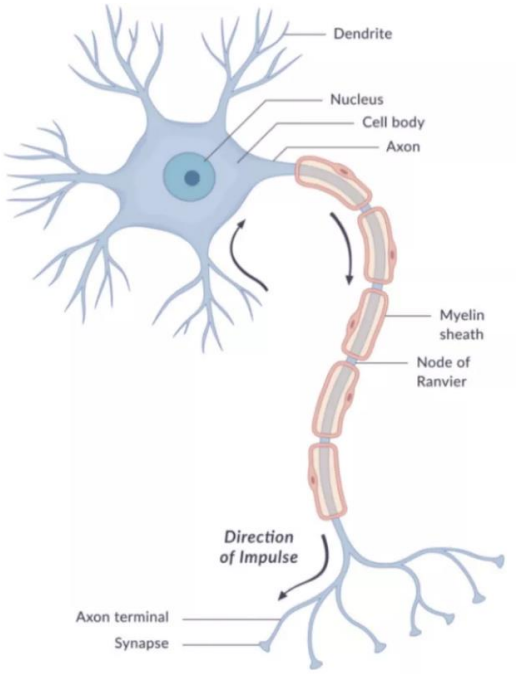


Figure 2 – Illustration of the neuron morphology. The cell body contains most organelles. Dendrites extend from the body and receive stimuli from upstream cells. Axons arise from the soma and conduct electrical impulses (action potentials) towards the terminals, where it signals to other neurons. Axons are typically myelinated, which increases the rate of electrical impulses. Adapted from https://www.thoughtco.com/neurons-373486

There are two types of synapses, chemical and electric[1,4,5] (Figure 3). In chemical synapses, the electrical impulse leads to the pre-synaptic neuron releasing neurotransmitters from synaptic vesicles into the synaptic cleft, a gap between pre- and post-synaptic cells[1]. The neurotransmitters will subsequently bind to receptor on the target cell, causing alterations in post-synaptic current flow transduction that will result in signal transmission. The nature of the chemical synapse is generally defined according to the nature of the neurotransmitter. Glutamate, Histamine and Acetylcholine are examples of excitatory neurotransmitters, whereas γ-aminobutyric acid (GABA) has an inhibitory effect. The biochemistry of the transmitters also varies, but they are generally subdivided into amino acids, amines, small-molecule neurotransmitters, purines, peptides and gasotransmitters[1].

In electrical synapses, current is carried through ions that passively flow from the pre-synaptic into the post-synaptic membrane, *via* specialized gap junctions that physically connect the communicating cells. Importantly, electrical synapse transmission is extremely fast, due to how quick the current flow across gap junction[1]. Hence, electrical synapses are present in *flight or fight* response or in the heart muscle, providing smooth and synchronized contraction[1,4,5]

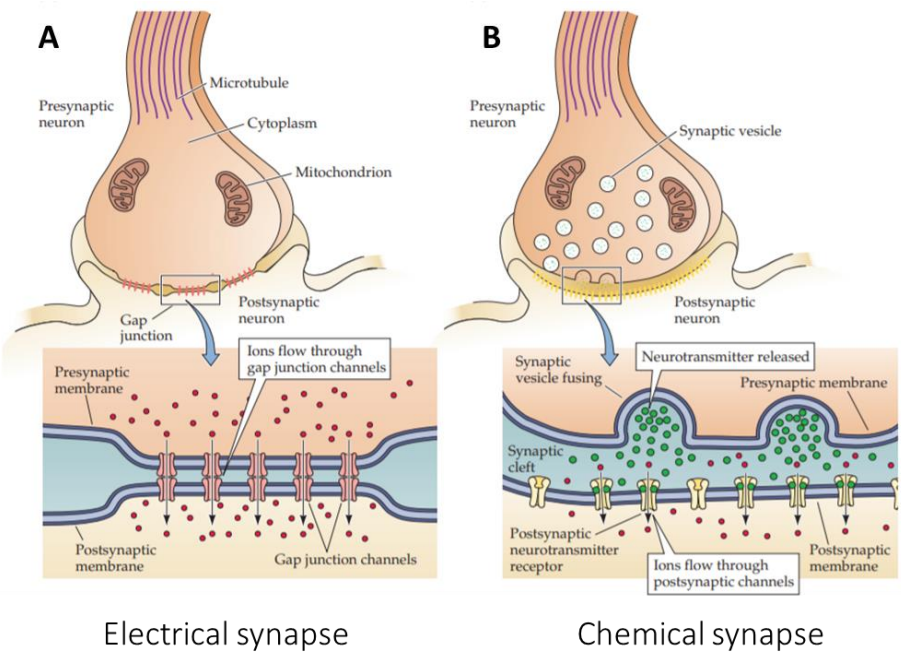


Figure 3 – Depiction of differences between electrical (A) and chemical synapses (B) mechanisms of transmission at the synaptic gap. Adapted from (Purves et al. 2004).

1.2. Neurons and neurogenesis

Neurogenesis consists on the process by which new, functional neurons are formed from neural stem cells. One of the central dogmas of modern neuroscience is centred on the notion that neurogenesis is absent in the adult brain, only occurring in pre-natal and restrict post-natal periods. It is now known that adult neurogenesis, while limited in number and to specific neurogenic niches (subgranular zone of the dentate gyrus (DG) and subventricular zone (SVZ) of the lateral ventricle), does occur[6,7].

Adult neurogenesis is a complex process that involves multiple steps: (I) Stem cell proliferation and asymmetric division, (II) newborn post-mitotic neuroblast translocation to a specific region, (III) physiological and phenotypic cell differentiation, (IV) neuronal circuitry integration and (V) cell survival[8–10]. The several steps of neurogenesis are actively dependent on a cascade of dynamic environmental and cellular cues. These cues, as well as the nature of the newly formed neurons and brain regions it integrates differs depending on the neurogenic niche they originate from[11,12] (Figure 4).

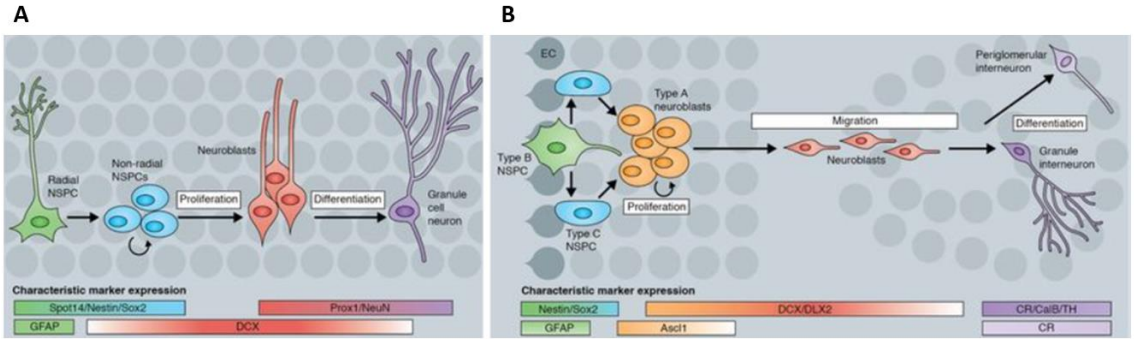


Figure 4 – Adult neurogenic in the DG (A) and SVZ (B). Mechanisms that govern cell activation, proliferation, migration and integration and cell population marker expression differ between zones. In the DG, radial Neural Stem/Progenitor cells (NSPC) generate an amplified population of non-radial NSPCs, which will form immature neurons which will differentiate into glutamatergic granule cells. In the SVZ, NSPCs generate proliferative type C progenitors, that give rise to immature neurons and glial populations. These cells will migrate to the olfactory bulb (OB) and there differentiate mostly into GABAergic and dopaminergic neurons. Adapted from (Braun and Jessberger et al. 2014).

Adult neural stem cells are characterized by self-renewal and multipotency, generating not only neurons but also astrocytes and oligodendrocytes[13–15]. Stem cell communication within neurogenic niches regulates population number and responsiveness to stimuli. Ephrins and Synapsins are families of proteins that are involved in neuroblast migration, differentiation and survival[16,17]. Other molecules involved in neuronal differentiation include epidermal growth factor (EGF), insulin growth factor (IGF), neurotransmitters and neuropeptides[18–20]. Environmental cues, like exercise, are also important for neurogenic signalling[21,22].

Apoptosis is a crucial counterbalance to proliferation/differentiation, acting as a cell number and lineage control system for the maintenance of tissue homeostasis. In fact, *in vivo* knock out of key apoptosis genes (caspase-3, Bax) resulted in supernumerary populations in the brain[23].

Metabolism and metabolic programming also have a key role in neurogenesis. Cell differentiation involves increased size, morphological complexity, motility, as well as organelle synthesis[8–10], all of which have robust bioenergetic requirements. Adult neural stem cells are highly glycolytic and require *de novo* lipid synthesis to sustain proliferation rate[24]. However, once they enter differentiation, NSPC progeny becomes increasingly more dependent on oxidative phosphorylation for energy consumption[25,26]. Consequently, cells on a later stage of differentiation also produce more mitochondria-derived reactive oxygen species (ROS), which can act as an important signalling mechanism[25,27]. Blockade of ROS formation inhibits neuronal differentiation in several *in vitro* models[28,29].

One important catabolic pathway is the Pentose Phosphate Pathway (PPP), which oxidizes glucose-6 phosphate. PPP generates important reducing agents (nicotinamide adenine dinucleotide phosphate, NADPH) that are involved in oxidative defences and lipid synthesis. The second phase of the PPP generates ribose-5-phosphate, an important nucleotide building block[30].

There are numerous remaining questions regarding adult neurogenesis, such as what determines stem cell programming, the nature, heterogeneity and role of newly formed cells and its impact on the neural tissue it integrates. Also, it is important to understand the mechanisms of neurogenic decline and disruption in ageing and disease[31,32]. Doing so, would allow for the potential modulation of neurogenesis and use it as a tool for therapeutic purposes, which is particularly relevant in the advent of neurodegenerative disease research. Answering these questions is an effort that is in motion, particularly in the advent of neurodegenerative disease research.

1.3. Glial cells

The work of 19th century neuroscientists Camillo Golgi, Santiago Ramón y Cajal and Rudolf Virchow were key scientists in introducing and characterizing the concept of a new cell type in the CNS: glial cells, the 'glue of the brain' (glia meaning 'glue' in Greek). This historical presumption of glia as a connective neural tissue has evolved. We know today that these cells display a wide range of paramount supportive mechanisms in the brain and spinal cord[33].

Glia can be subdivided into macroglia (which include Astrocytes, Oligodendrocytes and Schwann cells, among others) and microglia (Figure 5).

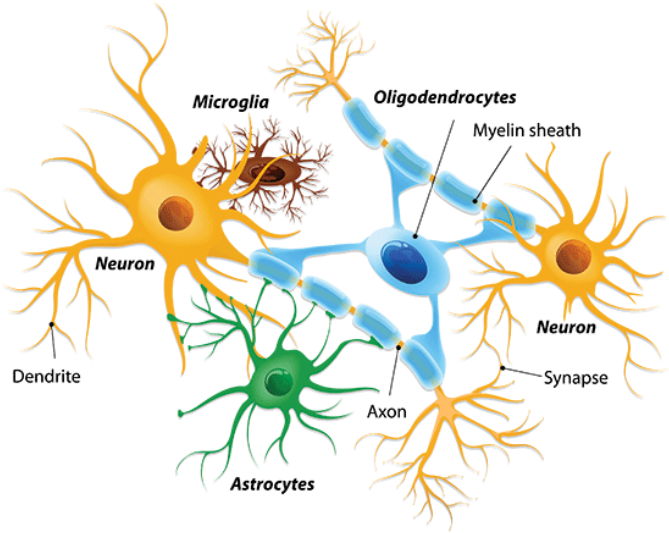


Figure 5 – Glial cell populations and interaction with neurons. Adapted from https://www.sciencenewsforstudents.org/article/scientists-say-glia.

Oligodendrocytes are responsible for the production of myelin, a lipid-rich substance that coats axons[34]. Myelin is crucial in facilitating the propagation of electrical transmission speed, permitting long-distances signalling [34,35]. Oligodendrocyte shape, with several extensions (Figure 5) allow them in wrapping a larger number of axons. In the peripheral nervous system, the cells responsible for this function are called

Schwann cells[1]. Astrocytes are the most abundant glia subtype in the human brain[35]. These cells are active in the maintenance of appropriate biological and chemical CNS homeostasis: ion regulation, metabolic support, extracellular matrix formation, neurotransmitter recycling and modulation of synaptic transmission[36,37]. Astrocytes also play a role in vasoconstriction/vasodilation and form part of the BBB, as priorly stated[36]. Their size and complex star-shape allow them to be in close contact with multiple neurons at same time.

2. Microglia

Microglia are the first line of defense for the CNS and make up for about 5-10% of total glia in the human brain[38]. Initially described by Rio-Hortega in the early 20th century, microglia are believed to have originated from a common haematopoietic precursor in the yolk sac during embryonic development, that migrates to and colonizes the brain tissue. There, microglial cell populations proliferate and self-sustain locally[38,39].

Microglia have a small body with a distinct ramified morphology for actively patrolling the brain parenchyma, scavenging for dead cells, debris, infectious and other potential noxious agents[38]. The 'resident immune cells of the brain' are armed with several surface molecules, including Toll-like receptors (TLR), cytokine and chemokine receptors, purinergic receptors, adhesion molecules, Fc receptors, ion channels, that allow recognition and response to homeostatic imbalances[38]. Microglia undergo physiological and morphological alterations (Figure 6), namely: (i) adopting an 'amoeboid' shape, (ii) overexpressing surface molecules, (iii) producing and secreting a wide range of mediators (cytokines, chemokines, ROS, proteases), (iv) priming other glial cells and (v) recruiting peripheral immune populations[38,40–44]. This robust response allows for the scavenging and phagocytic removal of pathogenic agents (bacteria, viruses, misfolded proteins, debris) and induce cell death in compromised neural tissue[38].

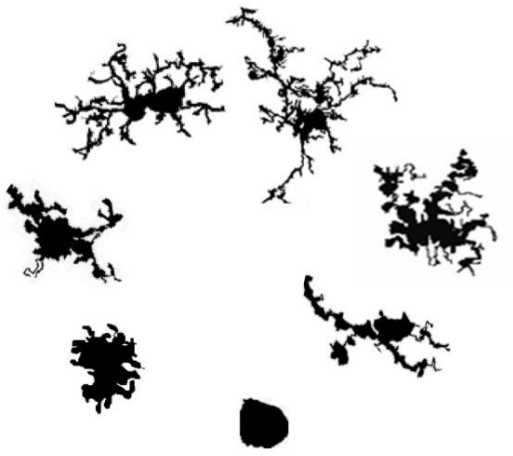


Figure 6 – Microglia morphological dynamics. Representation of adult human microglia under several stages of transformation between 'amoeboid' and ramified shape. Adapted from (Karperien et al. 2013).

Microglia have constant functional relevancy. Nevertheless, microglia activation is not a simplistic 'on/off switch'. In fact, microglial populations adopt heterogeneous natures, presenting a balance between a surveillance state and an 'active' one, which is the key feature for neuroinflammatory mechanisms and overall tissue homeostasis.

2.1. Microglia and neurotrophism

Microglia also play important roles in non-immune surveillance mechanisms (Figure 7). These cells are involved in homeostatic processes in brain development, trophic support, myelination and synaptic plasticity[45–47].

In early brain development, microglia is a key cell type in the regulation of cellular population number, as they selectively phagocyte dying neuronal precursor cells, while also inducing apoptosis *via* secretion of ROS and inflammatory factors and subsequently engulfing cells in specific brain regions[48,49]. Conversely, microglia also produce several factors, such as brain-derived neurotrophic factor (BDNF) and thrombospondin, which act as survival cues[50–55]. Neural fate in the layer V of the neocortex, for example, is actively dependent on IGF-1 locally produced by a microglial subpopulation[56]. Additionally, during brain development, breakthrough studies indicate the existence of an intimate connection between immunity and neuron wiring, as microglia orchestrate mechanisms of axonal outgrowth and the stimulation of cortical interneuron migration[57–59].

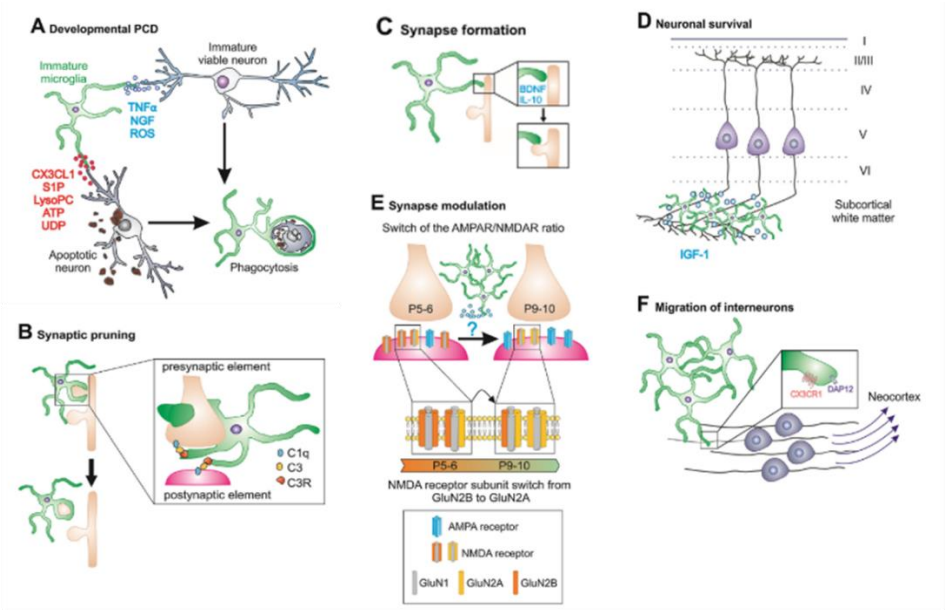


Figure 7 – Microglia functions during brain development and homeostatic support. Microglia are involved in removal of apoptotic neurons and induction of apoptosis signalling in early brain development (A), pruning of weak, immature or superfluous synapses (B) and synaptogenesis (C). Additionally, microglia are also involved in trophic support signalling, essential for layer 5 pyramidal neurons (D), regulation of expression of receptors at the synapses (E) and stimulation of interneuron migration. Adapted from (Mosser et al. 2017).

These myeloid cells are also active synaptic modulators[59–61]. For example, in post-natal period, microglia selectively eliminate 'weak' or deficient pre-synapses in specific regions, namely in the dorsolateral geniculate nucleus (dLGN) of the thalamus, hippocampal CA1 region and spinal cord[60,62]. Its activity in synaptic pruning is actively regulated by neuron-astrocyte-microglia tripartite communication, as astrocytic derived Interleukin-33 (IL-33) has recently been shown to drive microglia synapse removal[62]. Similarly, specific neuron-microglia communication pathways are required for microglia synaptic refinement. Examples of this are the CX3CL1-CX3CR1 pair and the immune surface molecule Triggering receptor expressed on myeloid cells 2 (TREM-2)[63]. Disruption of these signalling mechanisms lead to long-term consequences in brain network connectivity and signal transmission[48,56]. Inversely, microglia can also induce synaptogenesis and spine formation[46,64]. The identified microglia synaptogenic factors include soluble BDNF and IL-10[65–68]. Current *in vivo* data suggest that microglia contact triggers neuron Ca^{2+} transients and subsequent actin accumulation and filopodia formation[64]. In summary, microglial function is very heterogeneous, playing a central role not only in CNS immunity, but also development and overall homeostasis[69].

2.2. Inflammatory modulation

As priorly stated, microglia shift between a surveillance 'resting' mode and a 'reactive' state, which initiates robust innate immune functions and is pivotal for CNS homeostasis. However, this balance is very delicate and needs constant regulation. Loss of equilibrium results in the creation of a sustained inflammatory milieu with a damaging nature.

For regulating neuroinflammation, CNS innate immune system is exposed to ubiquitous microenvironmental cues[70–76]. Microglia cells are in constant communication with themselves, other glial cells and neurons. Production of anti-inflammatory factors (such as IL-10, IL-4, IL-13 and TGF-β - Transforming growth factor β) and of neurotransmitter and neuropeptides (like Vasoactive intestinal peptide – VIP and Pituitary adenylate cyclase-activating polypeptide – PACAP) is key in the resolution of inflammation[77–84]. Likewise, several neuron-microglia specific pathways are relevant for microglial activity fine-tuning, including CD200-CD200R, CD45-CD45L, CX3CL1-CX3CR1 (fractalkine – fractalkine receptor), CD172a-CD47 and TREM-2[85–96], as illustrated in Figure 8.

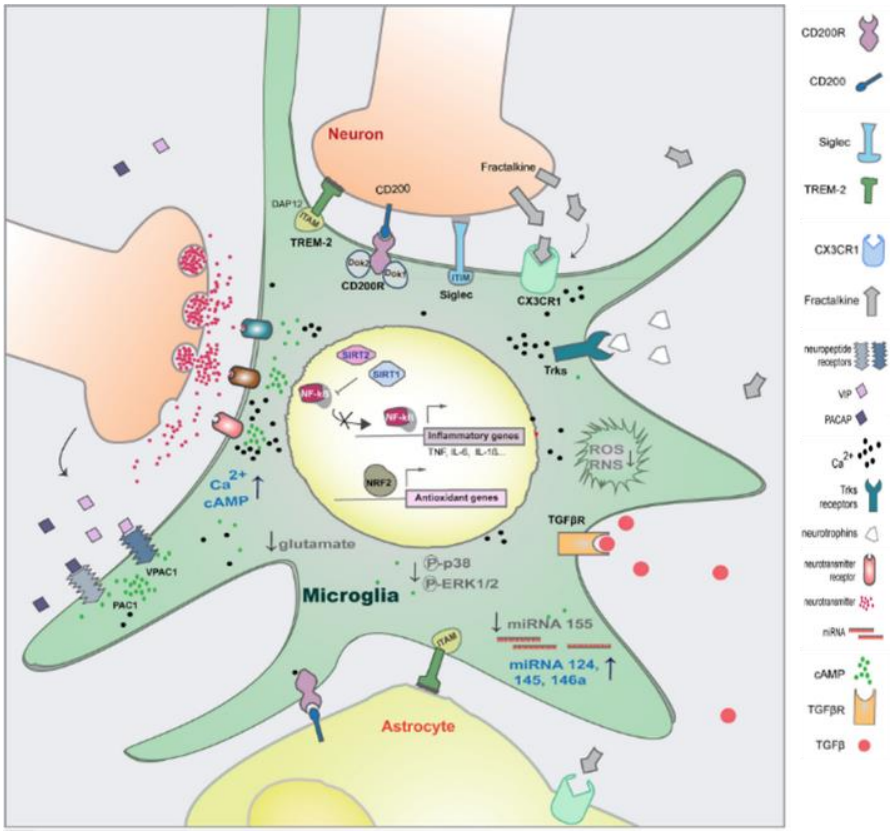


Figure 8 – Microglia communication mechanisms that regulate neuroinflammatory activity. Direct cell-to-cell pathways between neuron and microglia (TREM-2, CD200/CD200R, CX3CL1/CX3CR1), soluble mediators like TGF-β, neuropeptides VIP and PACAP and neurotrophic factors such as BDNF and NT-3, are key in the creation of a regulatory milieu that partially control microglial transcription of inflammatory mediators. Adapted from (Fernandes et al. 2014).

CD200-CD200R is a pair of membrane Immunoglobulin superfamily glycoproteins[97,98]. Microglia express CD200R whose direct interaction with neuronal CD200 acts as a 'calming' input on inflammatory activity. Interruption of this pathway leads to an enhanced microglia response to activation stimuli[91,99]. Low levels of the CD200-CD200R pair has been registered in brains of Alzheimer's Disease (AD) patients, particularly in the hippocampus[100]. Moreover, CD200-CD200R disruption in an *in vivo* model of experimental autoimmune encephalomyelitis (EAE) increases tissue inflammation and disease severity. This is reversed by overexpressing CD200 on neurons[101]. Additionally, recent data show that CD200-CD200R pathway could have a significant impact on microglia phagocytosis as well[102,103].

Similarly, CX3CL1 expression in neurons can provide a 'calming cue' to microglia populations, regulating engulfment of synaptic material[104,105]. Ransohoff and colleagues published that CX3CL1 depletion results in exacerbated microglia inflammatory function *in vivo*[106]. Still, other data show conflicting information: this communication pair is overexpressed in samples from EAE rodents and its effect is even more ambiguous in AD models[107,108]. It could be argued that this heterogeneity is related to the fact that neuronal CX3CL1 has both a membrane bound form and a soluble one, which may have varying regulatory consequences on the receptor.

TREM-2, another membrane microglial Ig superfamily receptor, is an additional inhibitory 'check-point' for microglial inflammatory response[87,109]. Additionally, TREM-2 has a crucial role in phagocytosis, as its depletion causes an impairment of apoptotic cell clearance[110].

Overall, microglial cells are in constant crosstalk with a dynamic regulatory milieu, which actively regulates several of microglial functions[71]. Understanding and describing these inter-cellular communication pathways is an important step for comprehension of the brain and offers potential therapeutic tools and approaches in the future.

2.3. Microglia dysfunction and Disease

Both acute and low-grade, chronic inflammatory mechanisms perturb the CNS equilibrium and can irreversibly damage brain tissue. At a molecular level, several mediators can be neurotoxic and cause cell death, namely reactive oxygen and nitrogen species (RNS), glutamate, cytokines such as TNF-α (tumour necrosis factor α) and a wide range of proteases [40,111,112]. BBB integrity can also be eroded, which causes a consequent leakage of peripheral immune populations and other circulating molecules into the brain parenchyma[113], further amplifying the noxious nature of the response. Aberrant phagocytosis and lower levels of nourishing factors are also a common causes of local damage[114–117].

CNS inflammation is a predominant feature of neurological diseases, both chronic (Parkinson's, AD, Multiple Sclerosis, Schizophrenia)[40,118–120] and acute (Brain ischaemia, Traumatic brain injury)[43,121]. While

these disorders are very heterogeneous in nature, they all present similar local inflammatory features and targeting neuroinflammation can contribute to limit pathological severity[122–124].

In AD, systemic inflammation is a pathological driving force[124,125]. In brains of both human patients and AD mouse models, microglial cells proliferate and accumulate around Amyloid β (Aβ) deposits[125]. These cells display stark increase in reactive markers and pro-inflammatory cytokines[126–130]. Vast literature has demonstrated that this inflammatory phenotype occurs as consequence of exposure to fibrillary forms of Aβ[131,132]. Microglia recognize, bind to and degrade soluble forms of Aβ via micropinocytosis[133,134]. These cells also engulf aggregates *via* phagocytosis mediators like scavenger and TLR families[126,127]. Findings have implied that microglial cells which engulf Aβ are unable to efficiently degrade fibrils, and that this chronic deficient clearing mechanism contributes significantly to progression of disease[131]. Some have also argued that the pre-existence of a low-grade inflammatory state leads not only to functional damage to neurons but also contribute to limit plaque clearance[135,136].

In other chronic neurological disorders, there are similar hallmarks of microglia dysregulation: In tissue samples from Parkinson's Disease (PD) human brains, microglia overexpress cytokines and reactive markers. Additionally, phagocytic dysregulation also appears to be involved in pathogenicity, as α-synuclein (α-syn) can disrupt engulfment[137,138]. In brain ischaemia, a prevalent acute pathology, microglia migrate to the injury site and locally engulf and degrade compromised cells, in order to return the tissue to homeostasis. However, sustained inflammatory response carried by these cells can have an opposite effect and act as a secondary damage inducer[112,121].

Similarly, microglia (dys)function during ageing is an object of great interest. Several studies in aged brains of humans and other mammals show that microglia have considerable physiological alterations and present a constant 'primed state', with higher expression of several surface receptors and pro-inflammatory mediators[139–143]. Morphological dynamics are also altered, as microglia present reduced processes, similar to the inflammatory 'amoeboid' phenotype, become less motile and phagocytic[144–146]. These cells are more responsive to stimuli and elicit an exaggerated and prolonged immune response. This hyper reactive milieu correlates with and accompanies increasing tissue cell death and cognitive decline[147,148]. Understanding the mechanisms of microglia senescence can be a steppingstone to better comprehend the biological significance of its dysfunction in the context of neurodegeneration.

Microglia have a central role in the CNS homeostasis. However, current literature highlights how microglial function is a double edged-sword under a constant and tight control network[71]. Such control network is comprised by the other brain populations, microglia itself and the environmental milieu[71]. Loss of this equilibrium can lead to exacerbated microglial activation, which can turn its function from damage response to a damage perpetuation[111].

3. Phagocytosis

Phagocytosis is one of the key cellular functions of microglia. It is a complex, multi-step process by which cells recognize, bind, engulf and digest large particles (≥0.5 μm), which can be pathogens, cellular debris, senescent cells or foreign bodies of a varied nature. It is a fundamental part of innate immunity and key in the initiation of adaptative response[149,150]. Likewise, phagocytosis is also crucial for tissue homeostasis by eliminating apoptotic cells[151]. Cells with capacity to phagocyte are also referred to as 'professional phagocytes' and include, other than microglia, several immune populations: macrophages, neutrophils, monocytes and dendritic cells[152].

Given the heterogeneity of phagocytic cell type, the target nature, environment and cellular interactions, all the major steps in phagocytosis have a large degree of complexity and variety. Particle recognition is mediated by an array of receptors: Foreign bodies express surface pathogen-associated molecular patterns (PAMPs), which are recognized by TLRs and others, like Dectin-1 and scavenger receptor A[153–156]. Potential pathogens can also be recognized by circulating antibodies, which can be subsequently bound to a cascade of complement system components. Phagocytes can then bind, through specific surface receptors, to the Fc region of Igs that coat the foreign body or to specific complement regions, and initiate phagocytosis[157–159].

Phagocytes also have specific molecular mechanisms for the detection and engulfment of dying cells (Figure 9). Cells undergoing cell death release soluble 'find me' signals, such as nucleotides and lysophosphatidylcholine (LPC), that facilitate recruitment[160–163]. Also, apoptotic cells express surface 'eat me' signals (phosphatidylserine, oxidized LDL, Intercellular adhesion molecule 3)[164–167], which are in turn recognized by phagocyte surface receptors, as are MerTK (MER proto-oncogene, tyrosine kinase), $\alpha_v\beta_3$-integrin (vitronectin), CD36 or brain-specific angiogenesis inhibitor 1 (BAI1)[165,168,169], either directly or via adaptor molecules. Inversely, healthy cells express surface receptors, like CD31 and CD47, which have been identified as 'don't eat me' signals[170,171].

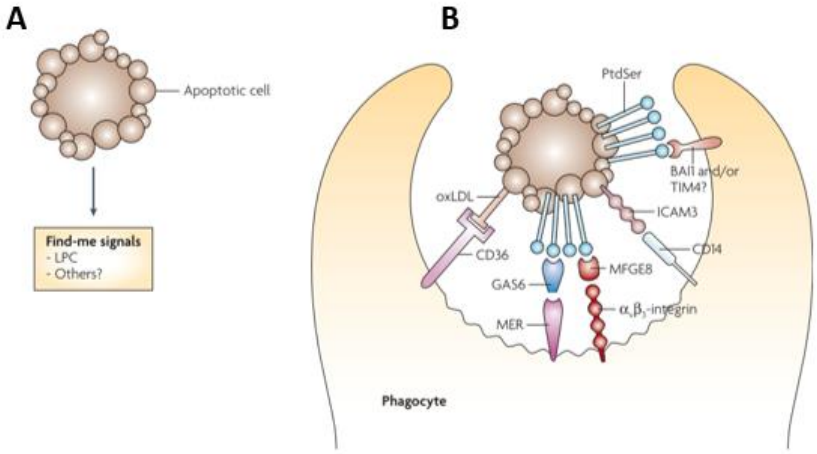


Figure 9 – 'Find-me' (A) and 'eat-me' (B) signalling between apoptotic cell and phagocyte. Dying cells secrete extracellular factors that stimulates the recruitment of phagocytic cells, like the well described LPC. Release of nucleotides has also been considered by some as a 'find-me' signal. 'Eat-me' signals in the target cell and respective receptors and adaptor molecules in the phagocyte mediate specific cell recognition and initiate engulfment. Elevated expression of surface Phosphatidylserine (PtdSer) is key for this mechanism. Adapted from (Ravichandran and Lorenz, 2007).

Particle recognition triggers signalling pathway cascades which mediate actin remodelling and lead to particle internalization, phagosome sealing and formation[172–177]. Subsequently, the nascent phagosome becomes increasingly acidic, as it undergoes four stages of maturation in succession[178–180]. This multistep process potentiates the degradative nature of the organelle. Phagosome lumen acidification occurs due to the action of a proton-pumping vacuolar ATPase, oxidative species, anti-microbial peptides and other enzymes [178–180].

In the brain, microglia are the major professional phagocyte cell type[181,182]. These cells are equipped with most of the surface receptors expressed by other tissue phagocytes and have similar physiological functions (Figure 10), namely: detecting and degrading potentially pathogenic agents; removing dying or superfluous cells and acting as synaptic modulators[181–184]. Additional examples of microglia phagocytosis in physiology are mentioned in subsections 2.1.

Being a major part of the brain immune response, microglia phagocytosis is tightly controlled, and in fact several 'checkpoints' regulate the extent of microglia phagocytic activity.

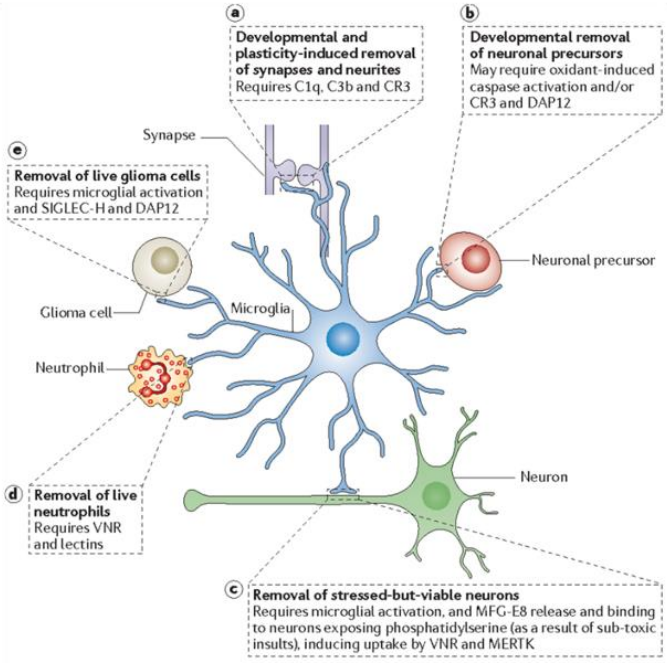


Figure 10 – Microglia phagocytosis and implications in the CNS. Microglial cells are involved in the engulfment of synapses and neuronal processes (A), as well as targeted removal of neuronal precursors (B), key mechanisms during brain development. These cells can also remove other populations, such as neutrophils, as well as glioma cells, through cell-specific signalling (E,D). In pathology, stressed neurons (exposed to oxidative stress, inflammation or other sublethal stimulus) can express low levels of membrane PtdSer, which microglia will recognize, engulfing live cells and causing cell death. Adapted from (Brown and Neher 2014).

3.1 Phagoptosis

In 2014, Brown and colleagues have described 'phagoptosis' as a novel cell death form: death by phagocytosis of otherwise viable cells[185]. Also known as 'primary phagocytosis', it differs from 'regular' phagocytosis, where engulfment and removal are a consequence of target cell apoptosis and not the reason. Phagoptosis mediates turnover of erythrocytes, tumours, and other populations[48,49,186,187], and thus is quantitatively one of the main forms of cell death in the body. In the brain, phagocytosis of live neurons has also been referred to as 'neurophagy', and is a key event to several brain physiological processes[188].

Phagoptosis can have, however, a pathological role[185]. Interaction between phagocyte and target cell is finely mediated by phagocytic receptors recognizing aforementioned 'eat me' and 'don't eat me' signals (Figure 9). Imbalances in this cross-talk can cause phagocytosis of healthy cell populations. In the brain, several sub-lethal insults can lead to activation of microglia, which engulf viable neurons and cause subsequent pathological neuronal loss[116]. Sustained microglial production of ROS and RNS causes adjacent viable neurons to expose PtdSer at their membrane surface, disrupting 'eat-me' signalling and initiating engulfment and consequent neuronal death[181,189]. *In vivo* injection of lipopolysaccharides (LPS) into rat brains caused neuroinflammation, which in turn promoted neuronal loss and microglia engulfment. These events were partially reverted by MFG-E8 (Milk Fat Globule-Epidermal Growth Factor-Factor 8) genetic ablation, as

well as vitronectin chemical inhibition[117]. Phagoptosis is also involved in damage propagation in stroke, particularly in the penumbra area, where stressed neurons reversibly expose PtdSer. Again, microglia overexpression of phagocytosis receptors can directly influence the extent of damage in this key region. In fact, MerTK deficient and MFG-E8 knockout mice experienced reduced neuronal loss and motor deficit in the weeks after the ischaemic event[190]. Similar results have been registered in PD animal models, where knockout of complement C3 can limit loss of dopaminergic neurons in the substantia nigra[191].

Therefore, phagoptosis is an extremely dynamic and delicate process, where the target cell ceases to exist, and as such, cannot be analysed. Modulation of these mechanisms currently seem like an enticing prospect, but one needs to better understand the molecular players and interactions that govern phagoptosis, as the mechanisms that lead to deleterious phagoptosis are the same involved in mechanisms relevant for tissue homeostasis, such as removal of protein aggregates and turnover of particular cell populations.

4. Cell Death

Cell death is an incredibly complex biological process in which cells irreversibly cease to function. It is pivotal in tissue development, homeostasis, innate and adaptative immunity and other crucial biological processes. Types of cell death can be characterized based on different features: molecular mechanisms (involvement of proteases, nucleases and other enzymes), physiology ('programmed' or 'non-programmed') or morphological alterations (loss of membrane integrity, cell fragmentation, engulfment).

4.1. Apoptosis

Apoptosis (Greek for 'falling off') is a programmed, caspase-dependent cell death type which occurs in multicellular organisms. This regulated process presents several morphological hallmarks: retraction of cellular processes, nuclear shrinkage ('pyknosis'), chromatin condensation, DNA fragmentation ('karyorrhexis') and plasma blebbing, with formation of numerous mall, organelle-containing apoptotic bodies[192,193]. Phagocytic cells engulf and degrade these membrane structures, avoiding spill out of apoptotic material and potential damage perpetuation[192,193]. Phagocytic cells recognize apoptotic targets by specific surface 'eat me' signals, such as the exposure of PtdSer on the plasma membrane[164].

Apoptosis has decisive homeostatic importance: It is a mechanism to remove ageing, superfluous, mutated or harmful cells[193]. It occurs basally during ageing, tissue development, as a 'counterbalance' to mitosis and cell population control[194].

Apoptosis can be subdivided into three different pathways, depending on the stimulus that triggers the response and intracellular regulatory pathways that carry it out: extrinsic, intrinsic and perforin/granzyme pathways[193] (Figure 11).

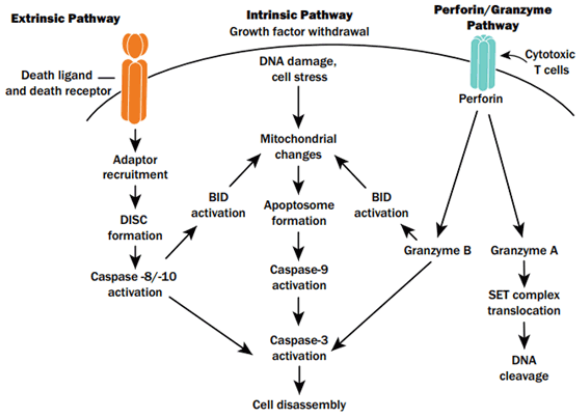


Figure 11 – Schematic representation of apoptosis initiation and signalling. Extrinsic, Intrinsic and Perforin/Granzyme pathways are triggered by distinct stimuli, present distinct molecular machinery but lead to a common execution pathway (caspase-3 activation). Adapted from https://www.novusbio.com/research-areas/apoptosis/death-receptor-signaling-pathway

5. Haem oxygenase

Haem oxygenase (HO) is an enzyme responsible for recycling haem [195,196]. This enzyme binds to and catalyses haem group degradation into carbon monoxide (CO), free iron (Fe^{2+}) and biliverdin (BV), which is readily converted to bilirubin (BR) by biliverdin reductase (Figure 12) [195,196].

There are two known HO isozymes: HO-1 (inducible form), which is highly expressed in tissues that degrade red blood cells (the spleen, liver, bone marrow) but rarely detected at basal levels in other tissues, and HO-2 (constitutive form), which is detected in several tissues, such as the brain, testes, liver, kidney and gut[197–199]. Being the only known enzyme capable of haem degradation, HO plays a very important function in tissue redox maintenance[198]. Hence, the gene that encodes for HO-1 (HMOX) is activated by an array of chemical and physical stressors, such as oxidative agents, inflammatory signalling and several environmental imbalances (heavy metal, heat, radiation, hypoxia, hyperoxia or infection)[200–207]. HO-1 expression is dependent on the Keap1/Nrf-2 (Kelch-like ECH-associated protein 1, Nuclear factor erythroid 2-related factor 2) pair[208]. Keap1 is a cytoplasmic protein that binds the transcription factor Nrf-2, restraining it to the cytoplasm and promoting proteasomal degradation. Upon redox imbalance, Keap1 thiol groups are oxidized, leading to the release of Nrf-2 transcription factor, which in turn migrates to the nucleus and initiates HO-1 expression[209].

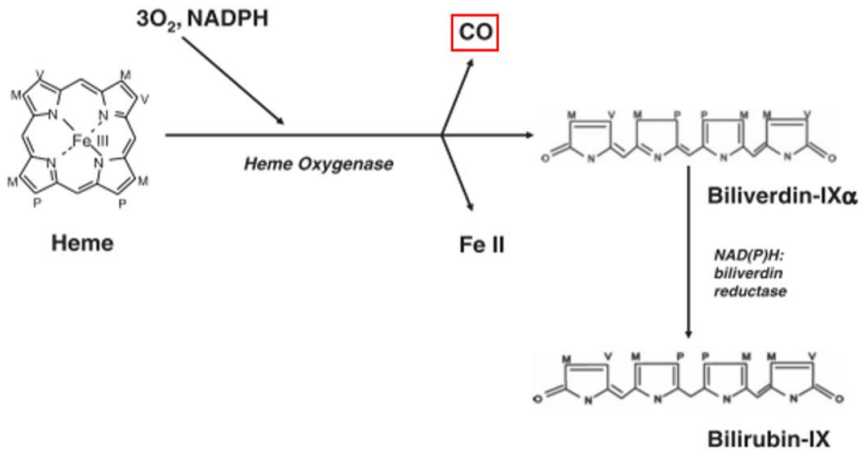


Figure 12 – HO enzymes catalyze haem degradation. These enzymes catalyze the rate-limiting step of haem metabolism. O2 and NADPH:cytochrome P450 reductase act as cofactors and haem is both the substrate and works as the prosthetic group. Adapted from (Ryter, Alam and Choi, 2006).

HO has several reported cytoprotective properties, namely vasoregulatory, anti-inflammatory, anti-apoptotic and anti-proliferative[200,210–214]. These findings have been described in many distinct *in vivo* disease models (Table 1). Moreover, HMOX1$^{-/-}$ mice develop chronic inflammation that ramps up as the animals age[215]. The only two humans reported to have suffered from HO-1 activity deficiencies died due to inflammatory-related complications at young age[216,217]. HO-1's major cytoprotective functions led to many researchers considering the prospects of modulating its expression as a therapeutic tool.

Table 1 – Protective effect of HO-1 expression in several disease models.

Disease Model	Effect	Reference
Cerebral ischaemia	Increase neuronal viability in cortex and striatum	218
Aneurysmal subarachnoid haemorrhage	Decreased cerebral vasospasms	219
Ocular inflammation	Anti-inflammatory effect	220
Pulmonary hypertension	Vasodilating and anti-proliferative effect on pulmonary vessels	221
Cardiac transplantation	Prevent graft rejection	222
Inflammatory bowel disease	Cytoprotective and anti-inflammatory	223
Liver ischaemia	Hepatocyte cytoprotection	224
Hypertension	Attenuates blood pressure	225

It has been postulated that HO-1 protective effects are a functional consequence of the main products of its catalytic reaction, combined with the active recycling of haem, a potentially toxic molecule. BV/BR are strongly anti-oxidant, as well as anti-nitrosative and scavenge and quench singlet molecular O_2[226–228]. *In vitro* administration of BR protects HeLa cells against H_2O_2 toxicity and *in vivo* ischaemia/reperfusion (I/R) experiments with hyperbilirubinemic rat models show higher resistance to oxidative stress[229,230]. The relevancy of free iron is a regular debate topic: authors have hypothesized that production of this transition

metal increases the expression of ferritin, which is necessary for redox balance[231]. On the contrary, some have argued that the production of free Fe^{2+} alone induces an imbalance in redox homeostasis which is ultimately deleterious. Lastly, CO is also described as a major 'executor' of HO-1 mediated cytoprotection, as CO presents potent modulatory properties in inflammation, cell death, metabolism, vasoregulation and cell cycle[198].

In 2003, Otterbein and Soares have posited that HO-1 acts as a 'therapeutic funnel', suggesting that several protective molecules exert their effect through downstream activation of HO-1 and that the involvement of this enzyme is crucial for overall protection[232]. This, in turn, has led to more research oriented towards the cytoprotective properties of this enzyme and the products of the reaction it catalyses. Therefore, CO, one of the products of haem degradation, has emerged as a potential candidate for HO-conferring cytoprotection.

5.1. Carbon monoxide: An historical perspective

CO is a diatomic molecule widely known due to being poisonous. An odourless, colourless, tasteless gas, the toxic nature of the 'the silent killer' stems from its very high affinity to bind to transition metals in biological systems (Iron, Copper, Molybdenum, Manganese and others), which are an integral functional part of haemproteins and other metal complexes.

CO is chemically inert and binds to haemoglobin[233] (Hb), forming carboxyhaemoglobin (COHb), with an ≈240-fold greater affinity than oxygen, displacing it from the haem complex and compromising oxygen delivery to tissue, resulting in quick and deadly systemic hypoxia[234]. At cellular level, it also binds to cytochrome c oxidase (COX), disturbing electron transfer in mitochondrial chain reaction, which affects ATP synthesis and overall mitochondrial function[235]. Other molecular targets of CO include soluble guanylyl cyclase (sGC), inducible nitric oxide synthase (iNOS), cytochrome P450 and NADPH oxidase[236–238].

Despite the overt toxicity, CO is a true proxy for the popular Paracelsus saying, 'The dose makes the poison'. In fact, CO is produced endogenously (about 14mL per day in the human body)[239] by the reaction that degrades free haem groups[195,196]. Although it was considered a by-product of this reaction, in the last two decades it has been shown that this is not the case. In fact, in 1993, it was first reported that CO could act as a neurotransmitter by inducing cyclic guanosine monophosphate (cGMP) production in the brain *via* sGC modulation[240]. At the end of 1990s, subsequent results also showed that regulation of cGMP levels was associated with vasodilation in different tissues[214,241–244]. This elicited increasing interest from the scientific community and has helped to understand the protective potential of CO.

5.2. Carbon monoxide: biological effects and molecular targets

CO has possibly become the most extensively studied product of HO activity. CO has heterogeneous protective properties that include vasodilatory, anti-apoptotic, anti-inflammatory, anti-proliferative and metabolic modulator in various complex *in vivo*[245–249] and *in vitro* models[213,250–252] (for more, see Table 2).

Table 2 – Protective actions of CO.

Disease Model	Effect	Reference
Lung ischaemia	Cytoprotection effect. Reduced lung endothelial cell apoptosis	[253]
Liver ischaemia	Cytoprotection and improved hepatic function	[254]
Vascular injury (arteriosclerotic)	Anti-inflammatory effect and inhibition of smooth muscle proliferation	[255]
Kidney transplantation	Anti-inflammatory effect and improvement of allograft success	[256]
Bacteraemia	Bactericidal effect, survival improvement in immunocompromised animals	[257]
EAE	Anti-neuroinflammatory and cytoprotective. Amelioration of pathological severity	[258]
Brain ischaemia	Cytoprotection effect. Reduced focal ischaemia injured and ameliorates neurological score	[259]
Perinatal brain ischaemia	Cytoprotection effect. Reduced focal ischaemia injured.	[260]

CO has a chemical affinity for transition metals, which allows it to bind to haemproteins, such as Hb or COX. Likewise, CO also modulates other proteins, namely sGC[236], nitric oxide synthase (NOS)[237], NADPH Oxidase[261], as well as molecular players such as mitogen activated protein kinases (MAPK p38, ERK1/2 and JNK)[307,308,317,318].

Mitochondria are one of the most well accepted CO cellular targets and are very important signalling intermediaries[264] (Figure 13). Binding to the haemprotein COX (complex IV) limits the electron transport chain[235], leading to electron accumulation (mainly at the complex III), prolonging the partially reduced state (ubisemiquinone) of coenzyme Q[265,266]. O_2 is consequently reduced to superoxide O_2^-, which can be converted into hydrogen peroxide and other ROS[265,266]. Low CO concentrations only partially and transitorily inhibit mitochondrial respiration and low levels of ROS are produced[250]. This shift into a mild pro-oxidant environment acts via ROS signalling as a pre-conditioning stimulus that triggers anti-apoptotic and anti-oxidant molecular machinery, such as transcription factors PPAR-γ (Peroxisome proliferator-activated receptor γ) and HIF-1α (Hypoxia-inducible factor 1 α)[265,267]. Interestingly, in mitochondria deficient cells it has been shown that the cytoprotection is abrogated[265].

CO also activates mitochondrial biogenesis. Administration correlates with increased mitochondrial DNA and protein content in cardiac cells[268]. CO upregulates expression of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α) and nuclear respiratory factor-1 and 2, key for expression of

mitochondrial proteins[268]. Likewise, CO is also involved in other mitochondrial quality control mechanisms (mitophagy, fusion and fission), indicating an active role as a homeostatic modulator in response to environmental changes and stress[269]. CO-mediated cytoprotection is dependent on cell metabolism modulation[269]. There is also strong evidence that low levels of CO enhance mitochondrial oxidative phosphorylation and decrease glycolysis: In isolated cortical mitochondria and primary astrocytes, administration of CO improved activity of ADP/ATP translocase and blocked mitochondrial membrane permeabilization (MMP)[270] in a ROS-mediated manner (*via* protein glutathionylation).

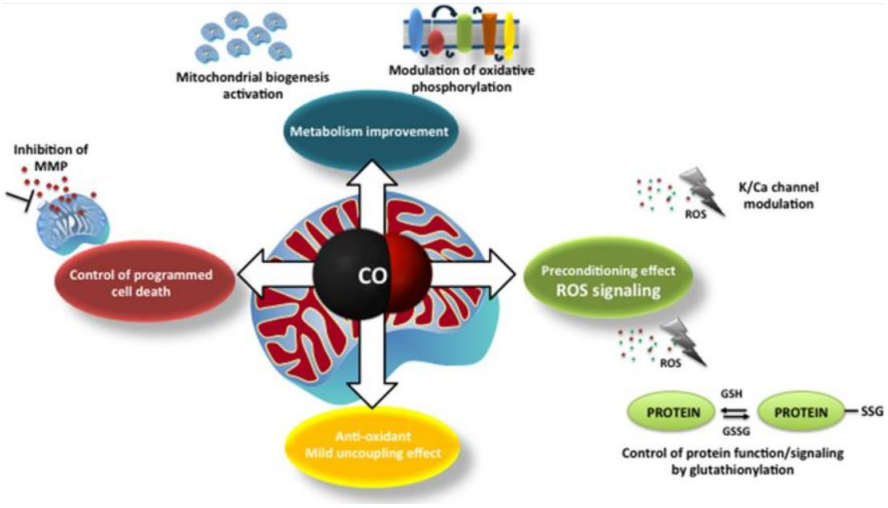


Figure 13 – Described mechanisms for CO as a modulator of the mitochondria. Adapted from (Almeida et al. 2015).

Altogether, there is not just a unique pathway or target for CO to modulate. CO acts on mitochondria, modulating metabolism and cell survival, but also generating mitochondrial ROS, which is a secondary cell signalling mechanisms that affects a vast molecular target network.

5.3. The CO/HO-1 system in the CNS

The importance of the CO/HO axis in the CNS is subject of interest in the field. Occurrence of HO-2 activity is particularly predominant in neuronal populations in the OB, hippocampus, cerebellum and others[271]. Unlike HO-1, HO-2 transcription is not stress-inducible, but in the developing brain, it has been demonstrated that the HMOX2 promoter functionally responds to adrenal glucocorticoid signalling[272]. Interestingly, HO-2 expression is protective against apoptosis in *in vitro* cultures[273,274] and *in vivo* models of brain ischaemia[275,276]. In fact, HO-2$^{-/-}$ animals appear to be more susceptible to tissue damage and brain oedema after ischaemic injury[275,276].

HO-1 is prevalent in the brain, specifically in glial cells[277]. Contrary to other tissues, this inducible enzyme can be detected at considerable levels in several brain regions[278]. Importantly, the HMOX1 gene is a

stress responsive enzyme whose expression increases as a consequence of a wide array of homeostatic imbalances[200–207]. The neuroprotective effects of the HO-1 system have been reported by multiple authors and include regulation of redox homeostasis, anti-inflammation and anti-apoptosis (see Table 1).

The inducible nature of the HO-1 gene, in conjunction with the vastly described cytoprotective properties, have led this enzyme to be seen as a promising pharmacological target for several disorders. CO, both endogenous and exogenous, can also stimulate HO-1 expression[198]. Thus, in the last two decades, authors have used this information to explore new routes to study the CO/HO-1 axis in neurological pathologies. In a model of EAE using $HMOX^{-/-}$ mice, HO-1 induction or CO's exposure presented reduced demyelination, paralysis and mortality rate after disease onset, by reducing systemic autoimmune inflammation[258]. Similarly, exogenous administration of CO protects against exacerbated inflammation in rat model of haemorrhagic stroke, limiting delayed apoptosis in the ischaemic penumbra[279]. Administration of exogenous CO to the brain of new-born piglets is anti-apoptotic and limits cerebrovascular injury caused by bicuculline-induced epileptic seizures[280]. Other cerebral disease models in which the HO-1/CO axis has a protective effect, include animal models of cerebral malaria and neuropathic pain[281,282].

Neuronal apoptosis is prevented by CO administration *in vitro*, which selectively inhibits $K_v2.1$ potassium channels protecting against oxidative apoptosis in primary hippocampal neurons[283]. Likewise, CO protects cerebellar granule cells from excitotoxicity by opening of ATP dependent-mitochondrial K channels *via* ROS generation[250]. The CO/HO-1 axis also confers cytoprotection in primary cultures of astrocytes, by metabolic modulation, reinforcing oxidative phosphorylation and limiting oxidative stress-induced cell death[251].

5.4. Carbon monoxide and neuroinflammation: Molecular mechanisms

While CO/HO-1 exhibit a wide range of biological effects and molecular targets, probably the most well established is their anti-inflammatory role. CO's modulation of neuroinflammation has been studied using BV2 microglia cell cultures, reducing secretion of inflammatory mediators (TNF-α, NO) in cells exposed to activation stimuli[284,285]. CO also participates in the regulation of microglia dynamics (migration and cell structure)[286].

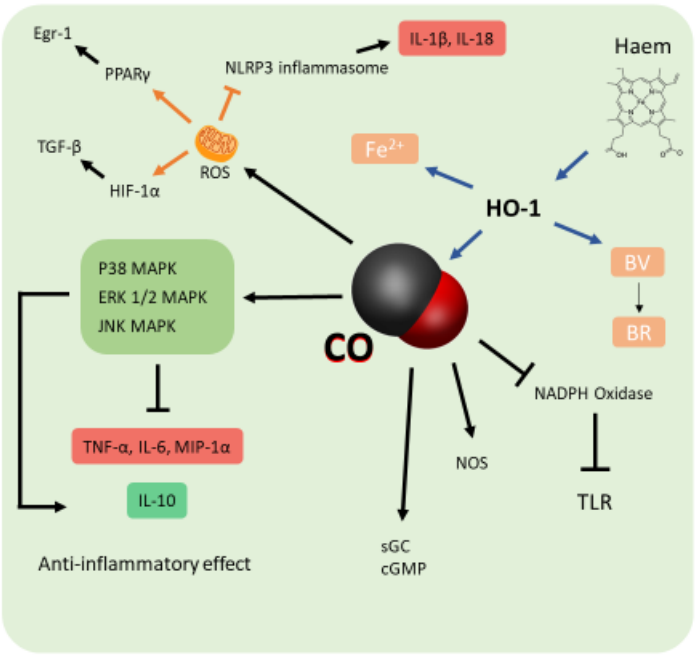


Figure 14 – Regulatory effect of the HO-1/CO axis in molecular players involved in immune response. Adapted from (Ryter and Choi, 2015).

At a molecular level, CO regulates several players involved in the neuroinflammatory response (Figure 14). This gas alters the activity of mitogen activated protein kinases (p38 MAPK kinase hyperphosphorylation, among others), pivotal mediators of inflammatory and stress response, leading to decreased expression of key cytokines (TNF-α, IL-6, IL-1β, Macrophage inflammatory protein-1α) and increased expression of anti-inflammatory compounds (IL-10, TGF-β)[252,284,285,287–289]. CO stimulates mitochondrial ROS production, priming transcription factors like HIF-1α and PPAR-γ[247,265], which blocks Egr-1 (Early growth response protein 1), an inflammatory transactivator. Other regulatory mechanisms include possible modulation of the NLRP3 (NLR Family Pyrin Domain Containing 3) inflammasome[290] and inhibition of TLR signalling via binding to the NADPH Oxidase[261]. In BV2 microglia, CO-mediated anti-neuroinflammation is partially regulated by promoting a shift towards a more pro-respiratory profile, connecting inflammatory control with metabolism[291].

Altogether, there is robust evidence highlighting CO as anti-neuroinflammatory factor that impacts on microglia function *via* regulation of several molecular pathways. Still, much information is yet to be known regarding how CO mediated-modulation impacts the surrounding CNS environment and how microglia communicate, regulate, and are regulated by other neural cells.

5.5. Carbon monoxide and cell differentiation: Molecular mechanisms

The CO/HO-1 axis has also shown capacity to modulate cell growth and differentiation. CO exerts anti-proliferative effects in *in vitro* smooth muscle cell cultures, with sGC/cGMP and p38 MAPK being involved[214]. Another recent *in vitro* study has also shown that exogenous CO administration can have immunomodulatory effects on rodent lymph node T cell maturation, programming differentiation into Th2 cells and suppressing generation of Th1/Th17 populations[292].

Our lab has demonstrated that CO improves neuronal differentiation. SH-SY5Y and NT2 cell lines displayed a higher neuronal yield at final endpoint when supplemented with exogenous CO, due to inhibition of apoptosis[293]. We have also published that NT2 improvement of neuronal differentiation and yield is partially dependent on CO metabolic modulation, specifically by increasing mitochondrial number and promoting a shift towards oxidative phosphorylation[294]. CO's effects appear to be partially governed by ROS mediated signalling[293]. In fact, it has also been shown that low-levels of CO modulate differentiation of human neural stem cells into dopaminergic populations, in a mitochondrial-generated ROS dependent manner[295].

Hence, CO has emerged as a neurogenic agent in several experimental models. Understanding how it acts on stem cell survival and metabolic programming is a subsequent step to use this gas as an enticing tool in the cell therapy field.

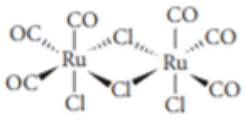
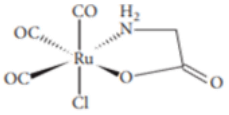
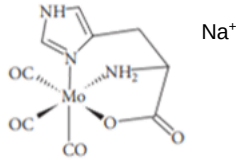
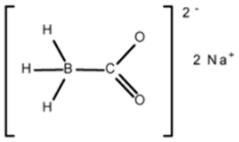
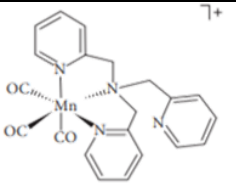
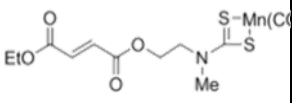
5.6. Carbon monoxide delivery strategies

As it became evident that the CO/HO-1 axis has strong beneficial properties, there is a new focus on translating this fundamental knowledge into potential therapeutic strategies and pushing for the development of CO delivery alternatives.

CO's administration via gas mixtures was first utilized as a delivery technique. There is a precedent for inhalation therapies: NO has been used in this fashion as a vasodilator in pulmonary disorders and preterm babies[296,297]. CO, unlike NO, is chemically inert and non-expensive. Some tests using inhaled CO in animal models of disease have shown moderate success[298–300]. More recently, several clinical trials have been taking place, addressing the therapeutic potential of inhaled CO gas, mainly in respiratory disorders such as idiopathic pulmonary fibrosis (NCT01214187), acute respiratory distress syndrome (NCT02425579) and respiratory distress syndrome (NCT00094406). However, inhaled CO presents many limitations, which make it inviable for therapeutic approaches, namely lack of tissue specificity and, most importantly, toxicity related problems which are particularly difficult to overcome[301,302].

One pharmacological approach that has emerged as an alternative for inhaled administration is the usage of CO-releasing molecules, or CORMs[303]. These synthetic molecules aim to deliver CO into biological systems under a controlled fashion, allowing for the circumvention of toxicity and unlock new therapeutic possibilities[303–305]. The chemical nature of CORMs is relatively heterogeneous (Table 3): The first CORMs to be developed were complex metal carbonyl compounds (M(CO)y) in which the carbonyl groups function as coordinated ligands of a central transition metal (Fe, Mo, Co, Ru, Mn)[303], but other subsequent candidates have differed considerably from a chemical standpoint[306]. CORM-A1, for example, is a sodium boranocarbonate with distinct physical and chemical properties[307]. More recently, Motterlini and colleagues have synthetized a new family of Hybrid CO-releasing molecule (HYCOs), which merge the chemical structure of CORMs with an inducer of Nrf-2 (dimethyl fumarate has been used for this purpose), the transcription factor that regulates HO-1 expression[308]. Pharmacokinetic and pharmacodynamic parameters, like as water solubility, stability, toxicity, molecule half live and CO release kinetics vary significantly depending on the chemical nature of the molecule and there is an ongoing challenge to obtain candidates increasingly more fitting for biological systems and, ultimately, pharmacological application in humans. Traceability and tissue specificity are desired characteristics, and PhotoCORMs (CO release is photochemically induced)[309] and ET-CORMs (enzyme triggered)[310] are being developed with that intent.

Table 3 – Chemical and pharmacological properties of carbon monoxide-releasing molecules.

Compound name	Chemical structure	Chemical properties	Pharmacological properties
CORM-2		Soluble in DMSO, Ethanol, olive oil $t_{1/2}$ of 1 min of CO release. CO is released by ligand substitution	Bactericide[311], Anti-cancer[312], anti-apoptotic[313,314], anti-inflammatory[315,316], vasodilator[311], anti-angiogenic[317], anti-proliferative[317].
CORM-3		Soluble in acidic water (pH ≈3) $t_{1/2}$ of ≈ 1 min of CO release (at pH 7.4, 37°C). CO is released by ligand substitution	Bactericide[318], anti-apoptotic[319,320], anti-inflammatory[285,321], vasodilator[322],
ALF-186		Water-soluble $t_{1/2}$ of ≈ 24 min of CO release (at pH 7.4, 37°C). CO is released under aerobic conditions	Anti-apoptotic[323], anti-inflammatory[324]
CORM-A1		Water-soluble $t_{1/2}$ of ≈ 21 min of CO release (at pH 7.4, 37°C). CO's release is pH dependent	Bacteriostatic[325], anti-apoptotic[326,327], anti-inflammatory[328], anti-coagulator[329], inducer of neuronal differentiation[332,333]
PhotoCORM		Soluble in aqueous solutions CO is released upon irradiation at 325nm	Bactericide[309], Anti-cancer[330]
HYCO-3		Soluble in DMSO, sesame oil	Anti-inflammatory[331]

CORMs are widely used today as a premier CO delivery method in research, for both *in vitro* and *in vivo* studies, where its administration has been able to recapitulate the cytoprotective properties of the gas (see Table 3). There are ongoing questions regarding the physicochemical nature of these molecules: To what extent are the CORM 'skeletons' responsible for the biological effects? Will the molecules cross biological membranes, like the BBB? What are the best administration routes? Can the body metabolize and excrete the structure? While these are valid questions, which need answering soon, the outlook for CO as a therapeutic alternative and CORMs as a fitting delivery solution, seems to be bright and increasingly real. In fact, several P1 and P2 clinical trials for inhaled CO gas in lung inflammation, pulmonary hypertension, lung vascular injury and others have undergone completion in the recent decade.

6. Final remarks and thesis aims

Carbon monoxide (CO) is a promising molecule with proven therapeutic potential in the central nervous system. The aim of this PhD thesis is to unveil and better understand the molecular mechanisms of CO-derived cytoprotection in brain cells. Two key biological processes were focused: neurogenesis and inflammation. Several specific aims were addressed and are organized by chapters:

I. **CO role in modulation of the pentose phosphate pathway and how metabolic programming affects neuronal differentiation.** Cell line SH-SY5Y was used in a neuronal differentiation model for assessing CO's role during neurogenesis. The impact of CO on oxidative metabolism, particularly on the pentose phosphate pathway, was analysed during differentiation process. This chapter was published as: *"Improvement of neuronal differentiation by carbon monoxide: Role of pentose phosphate pathway''*.

II. **CO's modulation of microglia-neuron communication: anti-neuroinflammatory and neurotrophic role.** A neuron-microglia conditioned media approach (primary cultures and BV2-CAD cell lines) was used as a remote (secretome) communication model. The primary goals were to understand if and how CO modulates microglial production and release of soluble factor, both in the presence and absence of an inflammatory stimulus, and what consequences this had on neuronal function and survival. This chapter is currently under final preparation to be submitted as *"Carbon monoxide modulation of microglia-neuron communication: anti-neuroinflammatory and neurotrophic role''.*

III. **CO's effect on microglia-neuron immunoregulatory communication mechanisms: modulation of inflammation and phagocytosis.** Here, a primary microglia-neuron microfluidic culture system was the selected model to investigate the physical interactome between the two cell types. The major scientific questions were whether CO can regulate this intimate bi-directional contact and if so, how it affects cell survival and function. Emphasis was given to how CO regulates microglia phagocytosis mechanisms. Chapter III is under preparation to be published in the near future as: "*Carbon monoxide and neuron-microglia direct communication: role of phagocytosis and inflammation*"

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II

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

This chapter is based on the following published article:

'Improvement of neuronal differentiation by carbon monoxide: Role of pentose phosphate pathway'

Nuno L. Soares, Ana S. Almeida, Catarina O. Sequeira, Sofia A. Pereira, Ursula Sonnewald and Helena L.A. Vieira (2018), Redox Biology; 17: 338-347

Nuno L. Soares 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.

ABSTRACT

Over the last decades, the silent-killer carbon monoxide (CO) has been shown to also be an endogenous cytoprotective molecule able to inhibit cell death and modulate mitochondrial metabolism. Neuronal metabolism is mostly oxidative, and neurons also use glucose for maintaining their anti-oxidant status by generation of reduced glutathione (GSH) *via* the pentose phosphate pathway (PPP). It is established that neuronal differentiation depends on reactive oxygen species (ROS) generation and signalling, however there is a lack of information about modulation of the PPP during adult neurogenesis. Thus, the main goal of this study was to unravel the role of CO on cell metabolism during neuronal differentiation, particularly by targeting PPP flux and GSH levels as anti-oxidant system. A human neuroblastoma SH-S5Y5 cell line was used, which differentiates into post-mitotic neurons by treatment with retinoic acid (RA), supplemented or not with CO-releasing molecule-A1 (CORM-A1). SH-SY5Y cell differentiation supplemented with CORM-A1 prompted an increase in neuronal yield production. It did, however, not alter glycolytic metabolism, but increased the PPP. In fact, CORM-A1 treatment stimulated (i) mRNA expression of 6-phosphogluconate dehydrogenase (PGDH) and transketolase (TKT), which are enzymes for oxidative and non-oxidative phases of the PPP, respectively and (ii) protein expression and activity of Glucose-6-phosphate dehydrogenase (G6PD) the rate-limiting enzyme of the PPP. Likewise, whenever G6PD was knocked-down CO-induced improvement on neuronal differentiation was reverted, while pharmacological inhibition of GSH synthesis did not change CO's effect on the improvement of neuronal differentiation. Both results indicate the key role of PPP in CO-modulation of neuronal differentiation. Furthermore, at the end of SH-SY5Y neuronal differentiation process, CORM-A1 supplementation increased the ratio of reduced and oxidized glutathione (GSH/GSSG) without alteration of GSH metabolism. These data corroborate with PPP stimulation. In conclusion, CO improves neuronal differentiation of SH-S5Y5 cells by stimulating the PPP and modulating the GSH system.

INDEX

BACKGROUND

Stem cell fate can be regulated by various factors, namely cellular energy metabolism, which is capable of modulating stem cell decision between self-renewing or differentiation[1–3]. Thus, manipulation of cell metabolism can be a key tool for neurogenesis modulation. In fact, stimulation of endogenous neurogenesis can be particularly important for replacement of impaired neurons in central nervous system (CNS), in future potential applications against neurodegenerative diseases including ischaemic stroke and psychiatric disorders.

The Pentose Phosphate Pathway (PPP) is an important route of glucose oxidation, divided into 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, $NADP^+$ is reduced into NADPH, the major reducing compound, which is required for regeneration of reduced glutathione (GSH). 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[4,5]. The activity of this non-oxidative phase of PPP supports cellular proliferation during neurogenesis by the production of building blocks[6]. In addition, during neuronal differentiation process, there is an enhancement of mitochondrial and oxidative cell metabolism[3,7], which, in turn, increases ROS production. Thus, one can speculate oxidative phase of PPP can be critical during neuronal differentiation for reinforcing cellular anti-oxidant defence. Indeed, PPP generates reducing molecules NADPH, which facilitates recycling of oxidized GSSG into reduced GSH, the first line of cell anti-oxidant defence[8].

CO is mostly known as a silent-killer due to its great affinity to haemproteins, such as haemoglobin or cytochrome c oxidase (COX). Thus, high levels of CO can compromise systemic oxygen delivery or cellular mitochondrial function, promoting high levels of intoxication and even death. Nevertheless, CO is an endogenously produced gasotransmitter generated by the cleavage of haem group *via* the enzymatic activity of haem oxygenase (HO)[9]. HO is a stress-related enzyme, whose expression or activity increases in response to several stressful stimuli including oxidative stress, hypoxia, hyperoxia, hyperthermia, inflammation, UV and misfolded protein response[9–11]. Furthermore, it has been demonstrated that low levels of exogenous CO promote cytoprotection, limit inflammation, prevent cell death and improve neuronal differentiation[9,10,12–18]. The molecular mechanisms underlying CO-induced cytoprotection are associated with improvement of mitochondrial function and are dependent on generation of low amounts of ROS, as signalling molecules[12,19–22]. Likewise, low concentrations of CO promote mitochondrial biogenesis[23,24], increase COX activity[13,25–28], improve oxidative metabolism[23,29] and induce mild mitochondrial uncoupling that protects mitochondria

from oxidative stress [30,31]. For further reading, there are reviews[23,32,33]. Because of the great potential use of CO as a therapeutic gas, several strategies to deliver CO under biological context have been developed. CO-releasing molecules (CORM) are small organic or organometallic molecules able to release CO under a more physiologically relevant way than CO gas applications[34]. CORM-A1 (carbon monoxide-releasing molecule A1) is a boron-based molecule that has been often studied because it slowly releases CO under a controlled manner. CO-releasing is dependent on temperature and pH, with optimal release at pH of 7.4 and 37 °C and it presents a half-life of approximately 21 min to transfer CO to myoglobin *in vitro*[35,36]. Recently, it has been demonstrated that CO promotes neuronal differentiation[16] and increases dopaminergic differentiation[18]. The underlying molecular mechanisms of neuronal differentiation involve CO-induced improvement of mitochondrial metabolism[17]. Likewise, CO modulates cellular GSH levels in astrocytes[12] and GSSG/GSH recycling is dependent on PPP. Thus, it is hypothesized that CO can stimulate PPP flux, which in turn, facilitates the cellular machinery rearrangement needed during neuronal differentiation. For assessing PPP modulation by CO, a human neuroblastoma SH-S5Y5 cell line was used as cell model. This is a simple model to study neuronal differentiation process[37], allowing the assessment of the associated cellular mechanisms. SH-SY5Y cells are derived from neural crest[38,39] and present the ability to differentiate into neuron-like cells that fulfil the morphological, biochemical and functional neuronal criteria[37,39,40], constituting a valuable model also for neuronal toxicity studies[41–44].

The main goal of this study was to assess the metabolic regulation of neuronal differentiation achieved by CO, in particular, the role of PPP. CORM-A1 supplementation increased the yield of neuronal production following SH-SY5Y neuronal differentiation, along with an increase of ROS generation, PPP flux and a change in GSH availability. Likewise, whenever the limiting PPP enzyme Glucose-6-phosphate dehydrogenase (G6PD) was knocked down, the CO-induced increase of neuronal yield was reverted, while pharmacological inhibition of GSH synthesis had no effect on neuronal differentiation. In conclusion, in the SH-SY5Y model of adult neurogenesis, CO improves neuronal differentiation in a PPP dependent manner.

METHODS

Materials

All chemicals were of analytical grade and were obtained from Sigma unless stated otherwise. Plastic tissue culture dishes were from Sarstedt (Germany); foetal 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.

For liquid chromatography, the reduction reagent TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) and derivatization reagent SBD-F (7-fluorobenzofurazan-4-sulfonic acid ammonium salt) and the standards used, namely Cys (cysteine), CysGly (cysteinylglycine), GluCys (glutamylcysteine) and GSH (*γ*-glutamyl-cysteinylglycine) were purchased from Sigma-Aldrich.

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 37 °C. Undifferentiated cells were grown in 75 cm^2 T-flasks and sub-cultured with fresh growth medium, whenever cell confluence achieved (about 80-90% cell confluence). Cells were detached by trypsinization at room temperature (RT) 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 75 cm^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 75 cm^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 10 µM of *all-trans* RA (differentiation medium). CO effect was studied by using the same composition of differentiation medium supplemented with 25 µM CORM-A1. Whenever necessary, buthionine sulfoximine (BSO) at 50 µM was added in differentiation medium for preventing GSH synthesis. Differentiation medium was replaced twice (day 1 and day 4) during 7 days of treatment (Figure 1A). On day 7, cells were collected for analysis.

Neuronal enrichment

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

Carbon monoxide-releasing molecule A1 (CORM-A1)

The solution of CORM-A1 was prepared in milli-Q water with a final concentration of 5 mM. Then, the solution was filtrated with 0.2 µM filter and stored at - 20 °C. 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 a negative control by initially dissolving CORM-A1 in 0.1 M HCl and then bubbling pure N_2 through the solution for 10 min in order to remove all residual CO gas[36]. The solution of iCORM-A1 was adjusted to pH 7.4, filtrated using a 0.2 µM filter and stored at - 20 °C. 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 haemacytometer, after digestion with 0.1 M 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 by automated enzymatic assays (YSI 7100 Multiparameter Bioanalytical System; Dayton, OH). The rate of 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 6 M HCl. Samples were dried under atmospheric air (50 °C), and metabolites were derivatized with MTBSTFA in the presence of 1% *t*-BDMS-Cl, see references[45,46] for further details. The samples were analysed 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 ^{13}C atoms (M+1, M+2, etc) 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[47].

Immunofluorescence microscopy

SH-SY5Y cells were plated at a density of $2x10^6$ cells/well in 24-well plates coated with Poly-D-lysine (PDL) in 0.15M sodium borate buffer solution pH 8.4. Cells were fixed with 4% (v/v) PFA and 4% (w/v) sucrose solution, for 20 min at R.T., and then permeabilized with 0.3% (v/v) Triton X-100 solution, for 15 min 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 (Bovine Serum Albumin) 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.

Immunoblotting

SH-SY5Y cell samples were collected with lysis buffer, which consisted of 50 mM Tris-HCl, pH 6.8, 10% glycerol (v/v) and 2% SDS (w/v). Protein concentration was determined using the Pierce BCA Protein Assay Kit and was measured at 540 nm. Total protein extract (30 µg) was mixed with 10 mM DTT, 10% (v/v) and 0.005% (w/v) bromophenol blue, loaded into 12% polyacrylamide gels and electrically transferred to a nitrocellulose membrane (HybondTM-C extra, Amersham Biosciences). The membranes were blocked with 5% BSA in TBS with 0.1% Tween-20 (TBS-T) and subsequently incubated with primary antibodies in 5% BSA in TBS-T. Antibodies were against G6PD (Santa Cruz Biotechnology) used at 1/200 dilution and against α-actin (A4700; Sigma- Aldrich) used at 1/1000 dilution for 2 h at R.T. Blots were developed using the ECL (BioRad)

detection system after incubation with horseradish peroxidase (HRP)-labeled anti-mouse or anti-rabbit IgG antibody (Amersham Bioscience), 1/5000, 1 h of incubation at R.T. These experiments have been repeated three times.

G6PD siRNA transfection

G6PD protein expression was silenced by G6PD coding siRNA transfection according to the instructions of the manufacturer (Santa Cruz Biotech). SH-SY5Y cells at 40-60 % confluence were transfected using Lipofectamine™ RNAiMAX and 10 pmol of G6PD siRNA (Santa Cruz Biotech) in Opti-MEM medium® (Invitrogen). Culture medium was gently mixed at room temperature, allowing the formation of liposomes. Cells were subsequently transfected in the absence of antibiotics. In the next 27 h and 48 h, the efficiency of transfection was assessed by Western Blot (WB) assay.

G6PD activity

G6PD activity was measured by a commercially available kit from Sigma-Aldrich (Glucose-6-phosphate dehydrogenase Activity Assay Kit MAK015), based on colorimetry and manufacturer's instructions were followed.

High Performance Liquid Chromatography (HPLC)

An HPLC with fluorescence detection was used to quantify the *glutathiomic* profile: the fraction of GSH in its free form and bound to proteins; glutathione in the reduced (GSH) and oxidized form (GSSG) in order to obtain GSH/GSSG ratio; as well as the GSH precursor cysteine (Cys) and glutamylcysteine (CysGlu); and the GSH degradation product cysteinylglicine (CysGly). Following 7 days of neuronal differentiation, cell extracts were collected and performed by lysing cells with 250 µL of 0.01% (v/v) Triton X-100 in PBS (1X) followed by centrifugation at 16000 *g* for 5 min, at 4 °C.

Aminothiols in cell extracts and culture media (supernatants) were reduced with tris(2-carboxyethyl)phosphine (TCEP) derivatized with ammonium 7- fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), as previously described by our group[48,49]. Samples were analysed by HPLC system (Shimadzu) with a RF-10AXL fluorescence detector, operating at 385 nm (λ excitation) and 515 nm (λ emission). The analytes Cys, CysGly, GluCys and GSH were separated on a LiChrospher 100 RP-18 (250×4 mm, 5 µm; Merck), with a mobile phase consisting of a mixture of 0.1 M acetate buffer (pH 4.5, adjusted with acetic acid): methanol (99:1 v/v), at a flow rate of 0.8 mL/min, at 29 °C. The run time was 20 min.

Statistical analysis

The data concerning cell culture were carried out at least in three independent preparations. Data are present as mean ± standard deviation (SD), n ≥ 3. Statistical comparisons between multiple groups were performed using one-way ANOVA. Whenever two groups were compared unpaired two-tailored t-test was used. In some specific cases, such as the analysis of WB band intensity, Q-PCR for mRNA expression and Cys, CysGly, GluCys and GSH measurements, paired t-tests were performed. Statistical significance was considered for p-value below 0.05; p-values are described in the figures and the specific used analysis are described in figure's legends. Statistical analysis was performed in GraphPad Prism® Version 6.

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) (Figure 1A). CORM-A1 was used as a supplement of the classical differentiation procedure with RA. The neuronal differentiation process of SH-SY5Y cells occurs during 7 days, followed by 5 days of neuronal enrichment with anti-mitotic agent treatment (Figure 1A).

After 7 days of differentiation, there is a mixed cell population composed of fully differentiated neurons and yet undifferentiated precursor cells[16]. This occurs mainly because cells are not synchronized in the beginning of the process. As expected, this population was composed of neurons and proliferative cells expressing Tuj1 and ki67 proteins, respectively, as assessed by immunocytochemistry (Figure 1B). When SH-SY5Y cells were differentiated in the presence of 25 µM CORM-A1 supplementation, the total cell number of the mixed cell population at day 7 significantly increased, which was assessed by nuclei counting (Figure 1C).

To further control that CORM-A1 effect is due to CO gas, CO-depleted inactive form iCORM-A1 was evaluated during neuronal differentiation. Neuronal differentiation triggered by RA and RA supplemented with iCORM-A1 presented the same amount of mixed cell population (Figure S1). Thus, the observed increased levels of mixed cell population that follow neuronal differentiation with RA supplemented with CORM-A1 is dependent on CO gas.

In order to address 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) for further neuronal cell quantification and morphology characterization by immunocytochemistry. Neuronal morphology is similar whenever differentiation was performed in the presence or absence of CORM-A1 (Figure 1D). For the same number of inoculated cells in the beginning of neuronal differentiation process, the enriched neuronal population increased about 4.5 times in the presence of CORM-A1 (Figure 1E). Thus, CO released by CORM-A1 presents a positive modulatory role in neuronal differentiation of SH-SY5Y cell line. Moreover, it improves the final neuronal production yield, as also previously demonstrated in Almeida and co-authors (2016)[16].

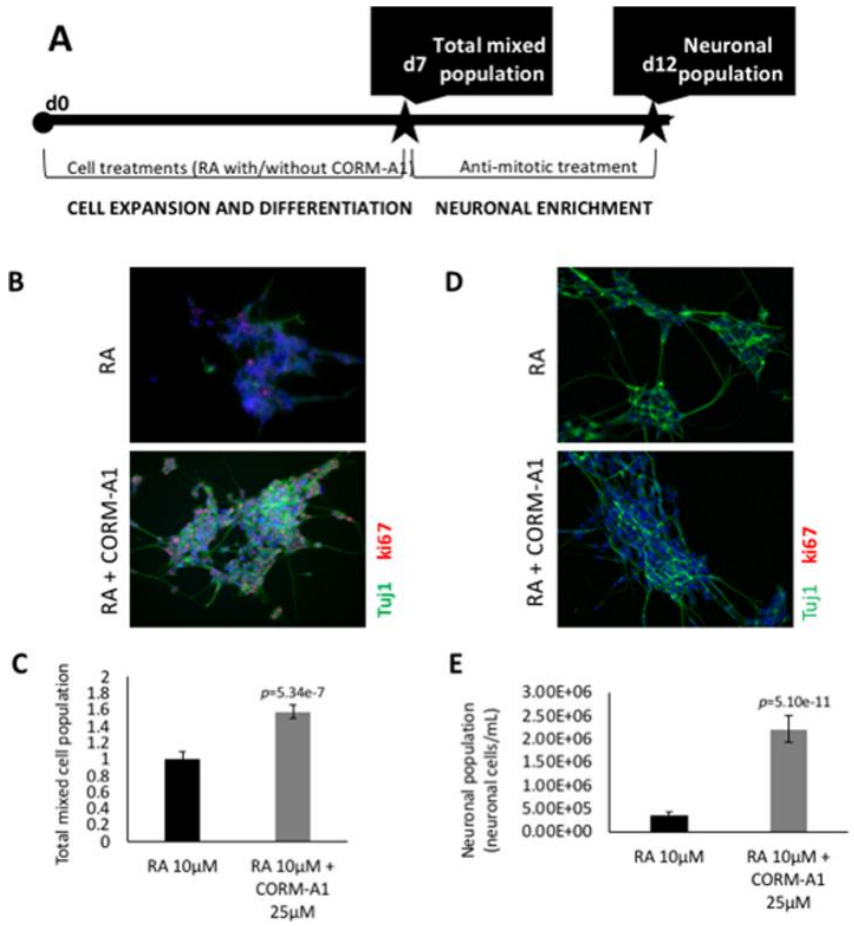

Figure 1: SH-SY5Y neuronal differentiation procedure and CORM-A1 improves neuronal differentiation yield. **(A)** SH-SY5Y cells were induced to differentiate during 7 days (d7) with 10µM of RA treatment with or without 25µM of CORM-A1, subjected to a differentiation medium exchange at day 4. After 7 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). **(B, C)** Characterization of SH-SY5Y cells by immunocytochemistry (green staining: Tuj1; blue staining: DAPI; red staining: ki67; magnification 100x); **(D)** nuclei counting of total mixed cell population, using RA at 10µM as reference and **(E)** nuclei count of neuronal enriched cell population. After 7 days of differentiation **(B, D)** and after anti-mitotic treatment during 5 days **(C, E)**. The used statistical analysis was unpaired two-tailored t-test.

CORM-A1 effect on glycolytic metabolism

Because neuronal differentiation involves modulation of glycolytic metabolism[2,3,50–52] and CO regulates cell metabolism[13,29], the CORM-A1 effect on glycolysis was assessed during neuronal differentiation. Characterization of cell metabolism, particularly the balance between glycolytic and oxidative metabolism, can be indirectly addressed by extracellular quantification of lactate production *per* glucose consumption over time [13]. When lactate production/glucose consumption ratio is 2 there might occur 100% of glycolysis. Also, as lower is this ratio, the higher is the level of oxidative phosphorylation. Herein lactate/glucose ratio remained unchanged with or without CORM-A1 supplementation during neuronal

differentiation process (Figure 2A); indicating that there might be no alteration on glycolysis or oxidative phosphorylation due to CO supplementation during SH-SY5Y neuronal differentiation.

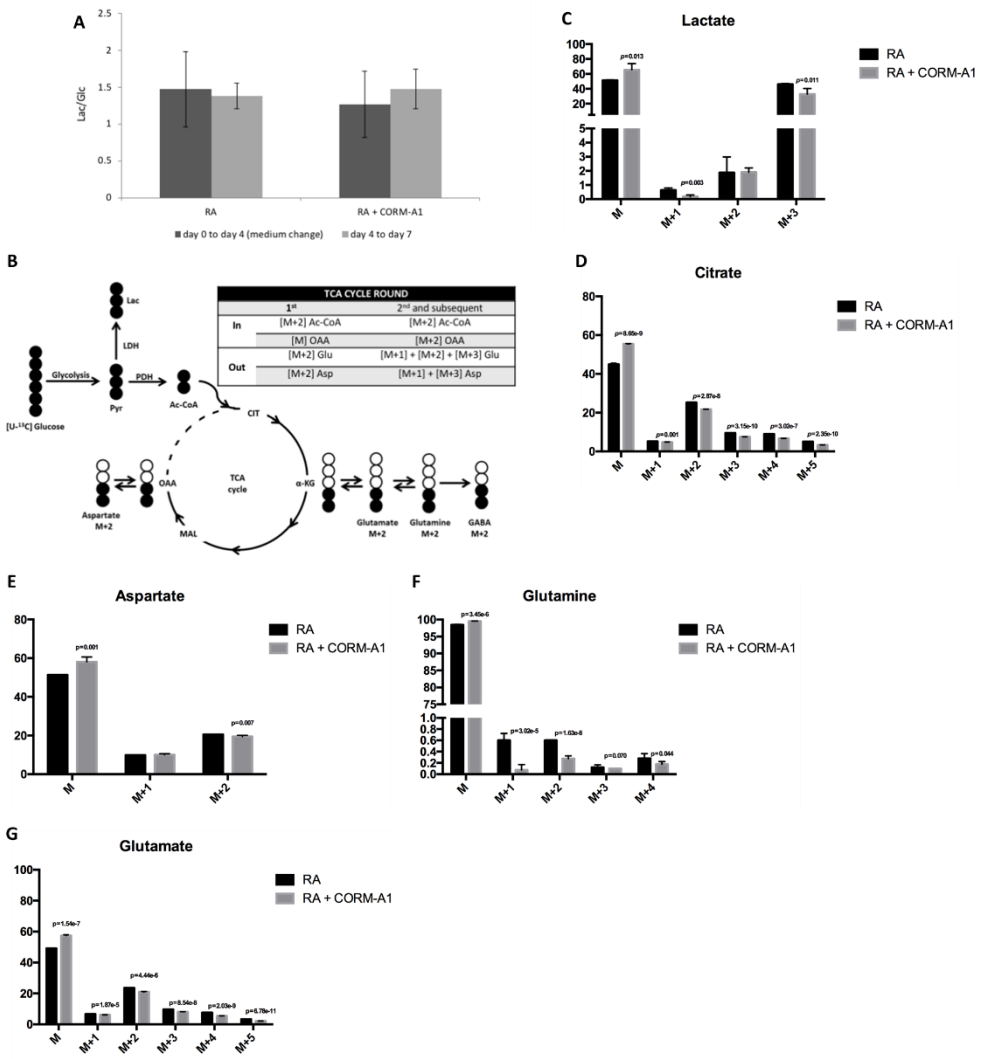

Figure 2: Glycolytic metabolism profile. (A) Lactate production per glucose consumption (qLac/qGlc) ratios calculated between treatments: day 0 to day 4 and day 4 to day 7; **(B)** Labelling patterns derived from [U-^{13}C]glucose metabolism; The % enrichment with ^{13}C leading to the formation of molecules 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 24 hours incubation with medium containing [U-^{13}C]glucose; dark grey corresponds to RA-treated cells and light grey to CORM-A1 supplemented cells, with * p<0.05; **(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. RPL22 was used as house-keeping gene and mRNA expression in non-differentiated cells was used as reference. The used statistical analysis was unpaired two-tailored *t-test*, while for panel H it was used paired *t-test*.

In order to further assess the role of glucose during neuronal differentiation, glycolysis flux progression was evaluated using labelled glucose (^{13}C) and GC-MS analysis. During glycolysis, glucose is converted in pyruvate, which can follow three distinct pathways: it can be converted (i) to lactate by lactate dehydrogenase (LDH) activity, (ii) to acetyl-CoA by pyruvate dehydrogenase (PDH) action followed by

coenzyme A ligation, feeding the tricarboxylic acid cycle (TCA) or (iii) to alanine by reductive alkylation. 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 treated 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, glutamine and glutamate, as well as in the 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 Figure 2C, only about 50% of lactate incorporated ^{13}C, accordingly to the fact that differentiated neurons present low glycolytic levels. Moreover, neuronal metabolism relies mostly on oxidative phosphorylation and PPP is very active in neurons[17,53,54]. Nevertheless, there is a low difference of M+3 lactate levels between control cells and CORM-A1 supplemented ones. These data corroborate the ratios of lactate production *per* glucose consumption presented in Figure 2A, suggesting that there is no increase in 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 (Figure 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 (Figure 2B). GC-MS analysis of SH-SY5Y cell extracts treated with [U-^{13}C] glucose showed that the percentage of ^{13}C labelling of citrate, aspartate, glutamine and glutamate was decreased for M+2 (Figure 2D-G) in the presence of CORM-A1. In summary, CORM-A1 supplementation of SH-SY5Y cells during neuronal differentiation seems not to interfere with glycolysis, but it leads to a slight decrease in mitochondrial metabolism. Likewise, CORM-A1 supplementation promotes a decrease of mRNA expression of PDH and an increase on lactate dehydrogenase (LDH) mRNA expression (Figure 2H). This result is in accordance with the lower mitochondrial metabolism observed in CORM-A1 supplemented cells (Figure 2C-G) and with the unaltered ratio lactate/glucose and the amount of lactate obtained during differentiation in the presence of CORM-A1 (Figure 2A and C, respectively). Consequently, one may hypothesize that, upon CORM-A1 supplementation, glucose can feed other pathways than glycolysis. PPP is a strong candidate pathway since it is important during cellular differentiation and it is parallel and interconnected to glycolysis. Likewise, PPP is a key cellular pathway against oxidative stress by increasing NADPH levels, which are needed for GSH/GSSG recycling. It is worthy of note that GSH is the first antioxidant cellular line of defence[8] and that GSH regeneration from GSSG is catalysed by glutathione oxidases and is dependent on electrons transferred from NADPH[55,56].

CORM-A1 modulates Pentose Phosphate Pathway (PPP)

Our group has recently demonstrated that ROS are key signalling molecules in CO-induced SH-SY5Y neuronal differentiation because: (i) CO promoted anion superoxide and hydrogen peroxide production and (ii) N-acetylcysteine (NAC) pre-treatment reverted CO-induced improvement of neuronal differentiation[16]. Thus, it can be hypothesized that CO-induced ROS production reinforces PPP in order to increase NADPH levels and to boost GSSG/GSH recycling system, which can limit potential oxidative stress. Therefore, PPP was indirectly addressed by GSH/GSSG ratio quantification. The GSH/GSSG ratio in cell extracts of neuronal differentiated SH-SY5Y cells with or without CORM-A1 supplementation was analysed by HPLC at the end of differentiation procedure day 7 (Figure 3A). There is an increase of the intracellular GSH/GSSG ratio with CORM-A1 supplementation, which can be correlated with higher amounts of intracellular NADPH and consequently with PPP flux.

To further evaluate PPP during neuronal differentiation, mRNA expression of two PPP key enzymes was assessed, namely phosphogluconate dehydrogenase (PGDH) and transketolase (TKT), from oxidative and non-oxidative phases of PPP respectively (Figure 3B).

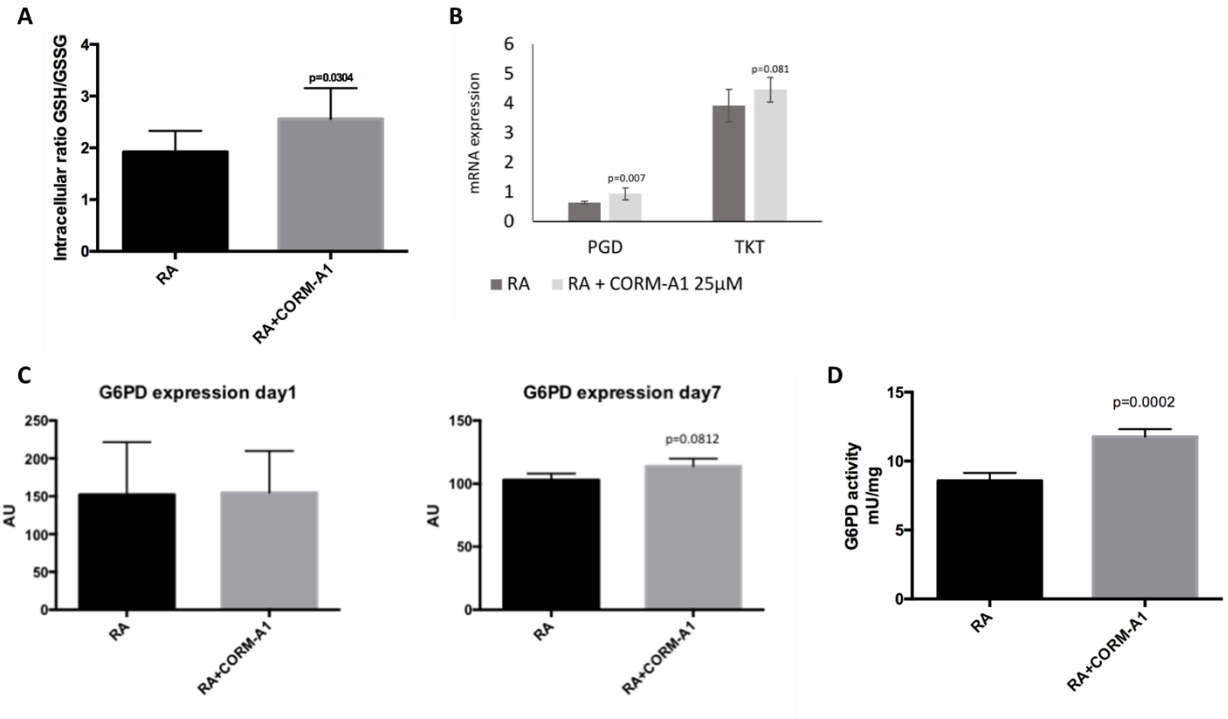

Figure 3: CO modulation of pentose phosphate pathway. (A) GSH/GSSG ratios were obtained 7 days after CORM-A1 supplementation; unpaired *t-test* with * *p<0.05*; **(B)** mRNA expression of specific PPP enzymes (PGDH for phosphogluconate dehydrogenase and TKT for transketolase) in SH-SY5Y mixed cell population after 7 days of neuronal differentiation. RPL22 was used as housekeeping gene and mRNA expression in RA-treated cells was used as reference, with ** *p<0.01* and # *p<0.1*; **(C)** Protein expression of G6PD after 24 hours and 7 days of neuronal differentiation assessed by WB, with # *p<0.1*; **(D)** Quantification of enzymatic activity (mU/mg of protein) of G6PD in the presence of RA or RA+CORM-A1 after 7 days of neuronal differentiation. The used statistical analysis was paired two-tailored *t-test* for panels A, B and C, while for panel D it was used unpaired *t-test*.

Actually, cellular expression of both enzymes increased in the presence of CORM-A1 during neuronal differentiation process (Figure 3B). Likewise, protein expression level of the PPP rate-limiting enzyme Glucose-6-phosphate dehydrogenase (G6PD) was quantified by WB during neuronal differentiation process in the presence of CORM-A1 (Figure 3C). After 24 h of neuronal differentiation, there is no difference in G6PD expression with CORM-A1 supplementation, while after 7 days an increase of G6PD expression is observed, although p-value is 0.0812, being above 0.05, which is usually the confidence interval for considering statistically different. (Figure 3C). Furthermore, G6PD expression duplicates in SH-SY5Y cells treated with RA compared with non-treated cells (data not shown), indicating that PPP is a critical metabolic pathway in neuronal differentiation. Finally, G6PD activity was assessed following 7 days of neuronal differentiation and CORM-A1 supplementation increased G6PD enzymatic activity (Figure 3D). In summary, the higher levels of PGDH and TKT mRNA and G6PD protein expression and activity in the presence of CORM-A1 supplementation, along with an increase in GSH/GSSG ratio, corroborate with the hypothesis of CO-induced PPP stimulation during neuronal differentiation.

Functional role of PPP during neuronal differentiation

In order to further validate the role of PPP during neuronal differentiation, G6PD was knocked down by small interference RNA (siRNA) approaches. By partially limiting PPP, one can validate its importance on neuronal differentiation process. Knocking down G6PD gene expression was confirmed by protein expression analysis via WB assay at 3 h and 24 h of neuronal differentiation (Supplementary Material Fig.S2). Whenever G6PD is knocked down, CO-induced increase on total cell population following 7 days of neuronal differentiation was reverted to similar levels as control cells treated only with RA (Figure 4). Of note, no difference on total cell population was found in RA control with or without knocked down expression of G6PD, indicating that the role of PPP might be important only for the CO-induced improvement of neuronal differentiation. Thus, this data evidences that CO improvement on neuronal differentiation in SH-SY5Y cells is dependent on PPP stimulation.

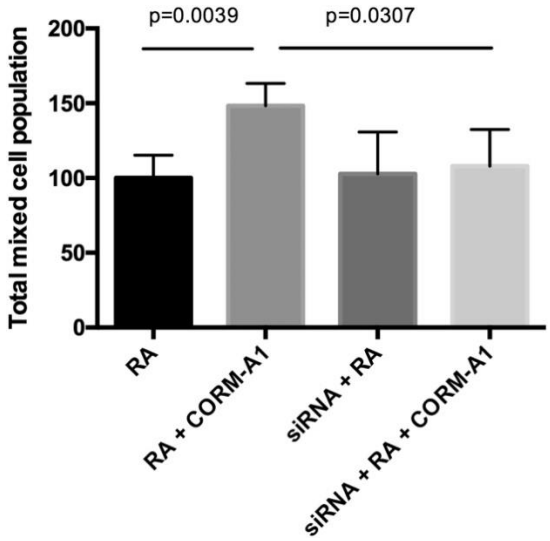

Figure 4: Role of PPP during neuronal differentiation of SH-SY5Y cell line. Total mixed cell population, nuclei count *per* volume of SH-SY5Y cells and normalized by control cells treated with RA, following 7 days of differentiation, in the presence or absence of CORM-A1 supplementation and with down-regulation of G6PD expression. The used statistical analysis was unpaired two-tailored *t-test*.

CORM-A1 modulates glutathiolomic profile

The cell GSH/GSSG ratio is mostly dependent on GSH/GSSG recycling, which is associated with NADPH availability and PPP. Nevertheless, one might consider that the levels of GSH are also dependent on its synthesis and catabolism. Thus, it is also necessary to evaluate the role of CO on glutathiolomic profile during neuronal differentiation. Quantification of intracellular precursors cysteine (Cys) and glutamyl-cysteine (GluCys) was used to address glutathione anabolism, being Cys the rate-limiting factor for GSH synthesis[57,58]. While evaluation of glutathione catabolism was performed by quantification of the ratio cysteinylglicine (CysGly) to glutathione in extracellular environment[58].

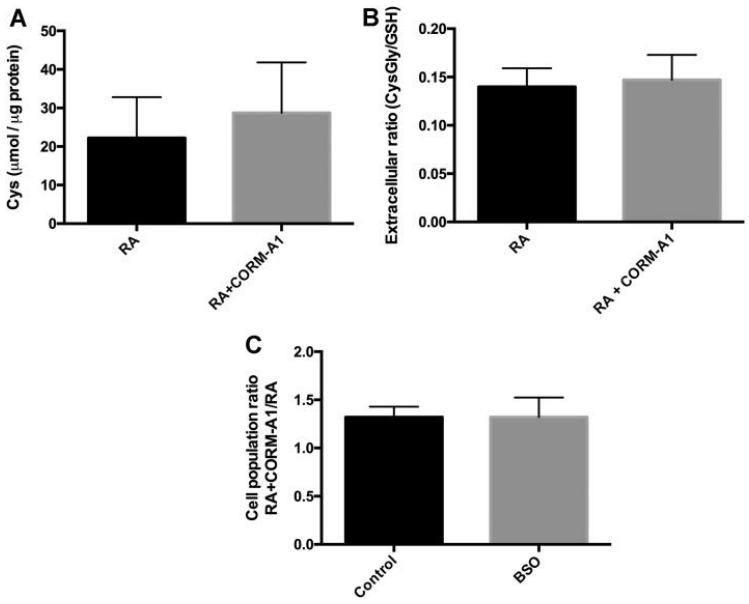

Figure 5: CO modulation of glutathione metabolism. Following 7 days of neuronal differentiation in the presence and absence of CORM-A1 supplementation, it was quantified **(A)** intracellular total cysteine; **(B)** extracellular ratio CysGly/GSH; **(C)** Total mixed cell population, nuclei count *per* volume of SH-SY5Y cells and normalized by control cells treated with RA, following 7 days of differentiation, in the presence of BSO for inhibiting GSH synthesis. Results are represented as the increase on total cell population due to CORM-A1 supplementation under control and BSO conditions.

Cellular synthesis of glutathione is through the condensation of three aminoacids: cysteine, glutamate and glycine. CORM-A1 supplementation seems not to change glutathione anabolism, since intracellular levels of total cysteine are similar between control cells and cells treated with CORM-A1 during neuronal differentiation (Figure 5A). Likewise, the levels of GluCys were below the detection limit of HPLC (0.625 µM) indicating that GSH synthesis occurs at residual levels or does not occur. Taken all together, these data might suggest that CO does not modulate GSH synthesis. Furthermore, no alteration in GSH catabolism was found, since the extracellular ratio of CysGly/GSH was not altered in the presence of CORM-A1 (Figure 5B).

To further confirm that GSH synthesis does not play any role on the improvement of neuronal differentiation and the increase on GSH/GSSG ratio is due to PPP reinforcement, the role of CORM-A1 on neuronal differentiation was assessed in the presence of buthionine sulfoximine (BSO), which is a pharmacological inhibitor of GSH synthesis. Total mixed cell population at day 7 was counted and the growth on cell population was similar in the presence or absence of BSO (Figure 5C). In conclusion, CORM-A1-induced improvement of neuronal differentiation is caused by an increase on PPP, promoting reduced GSH regeneration and is independent on new GSH synthesis or impaired catabolism.

DISCUSSION

Our team has recently demonstrated that CO improves neuronal differentiation in a ROS dependent manner, using the human SH-SY5Y neuroblastoma cell line as a model[16]. Indeed, CO triggered ROS generation during neuronal differentiation, while NAC prevention of ROS production reverted the CO stimulation of neuronal differentiation[16]. The present work focuses on the role of CO in cell metabolism modulation, in particular the pentose phosphate pathway, since its metabolism is a key cellular process during neuronal differentiation[3].

CO reinforces oxidative phosphorylation and decreases glycolysis in different models, namely astrocytes[13], carcinoma cells[59] or NT2 cells during neuronal differentiation[17]. Nevertheless, in the SH-SY5Y cell model of neuronal differentiation, CO supplementation did not change the balance between glycolysis and oxidative phosphorylation of SH-SY5Y cells during neuronal differentiation (Figure 2). Therefore, the PPP was targeted as a potential metabolic pathway involved in CO's modulation of neuronal differentiation in the SH-SY5Y cell model. Although the PPP is a minor contributor to total glucose oxidation, it is essential for cellular function due to the importance of the products: (i) ribose-5-phosphate, which are building blocks for biosynthetic processes during neurogenesis and (ii) the electron donor NADPH that is needed for GSH recycling and antioxidant defence[60–65]. It is worthy of note that the GSH system is a key in antioxidant cell defence, by directly reacting with radicals and by being an electron donor in reactions catalysed by glutathione peroxidases, which in turn generates glutathione disulphide (GSSG)[8]. Thus, the CO-induced higher levels of ROS production during neuronal differentiation might promote the PPP stimulation in order to enhance the antioxidant defence through NADPH production and GSH recycling. In fact, CORM-A1 supplementation during SH-SY5Y cell line neuronal differentiation increased: (i) mRNA expression of 6-phosphogluconate dehydrogenase (PGDH) and transketolase (TKT) enzymes for oxidative and non-oxidative phases of PPP, respectively; (ii) protein expression and activity of Glucose-6-phosphate dehydrogenase (G6PD) the rate-limiting enzyme of PPP and (iii) the intracellular ratio of GSH/GSSG (Figure 3). Thus, one can conclude that CO does stimulate the PPP during neuronal differentiation in the SH-SY5Y cell line model. Likewise, whenever G6PD was knocked down using the siRNA approach and the PPP was partially inhibited, CO-induced improvement of neuronal differentiation reverted to levels similar to control, which is the standard RA treatment (Figure 4). Of note, modulation of PPP promoted endodermal differentiation of embryonic stem cells[66]. In conclusion, CO stimulates the PPP, which is already a well-established important metabolic pathway for neuronal differentiation.

In addition, PGDH and TKT are expressed in various cell types in the brain[67–69], but PGDH activity decreases after birth[70], while TKT activity increases during postnatal development[71,72]. Taking into account that there is low mRNA expression of PGDH and high mRNA expression of TKT, it can be speculated that SH-

SY5Y cell model mimics adult neurogenesis. 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)[73,74].

The cellular availability of reduced GSH is dependent on: GSSG reduction, GSH synthesis and GSH catabolism. The levels of total and free cysteine are similar in the presence or absence of CO, which indirectly indicates that CO does not modulate GSH synthesis. Likewise, the extracellular levels of CysGly/GSH ratio were found similar in the presence of CORM-A1, indicating no alteration on GSH catabolism. Finally, whenever GSH synthesis is pharmacologically inhibited by BSO, no CO-induced improvement was found on neuronal differentiation (Figure 5). In conclusion, CO's improvement of neuronal differentiation is dependent on the increase of reduced GSH levels via metabolic regulation of PPP and enhanced generation of NADPH. Interestingly, Kaczara and colleagues have recently demonstrated that CORM-401 (a new CORM containing manganese as metal centre) enhances PPP in human endothelial cells[75].

Finally, in the present experiments CO did not affect glycolysis but slightly decreased mitochondrial metabolism (Figure 3). These results are not in accordance with data previously published by our group[17], showing that CORM-A1 promotes neuronal differentiation of NT2 cells by reinforcing mitochondrial metabolism. In fact, the discrepancy between these results can be due to the different cell models used. NT2 are teratocarcinoma-derived cells that present a pluripotent phenotype, while SH-SY5Y cells already present a neuronal predisposition and have the ability to differentiate along the neuronal lineage. Thus, one can speculate that the NT2 cell line differentiation represents an early stage of neurogenesis process, while the SH-SY5Y cell line is a model more appropriate to characterize end phases of the neuronal differentiation process. Accordingly, at early stages, the switch from glycolytic to oxidative metabolism can be more relevant for the change between cell proliferation and the differentiation processes, which could explain the different CO effects in the NT2 and SH-SY5Y cell models.

In summary, our results (i) corroborate the importance of antioxidant defences during neuronal differentiation, (ii) present the PPP as relevant metabolic pathway for the modulation of neurogenesis and (iii) validate CO for improvement of neuronal yield by modulating the PPP and glutathione metabolism. Altogether, this study is a step forward in clarifying the role of cell metabolism involved in adult neurogenesis process.

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SUPPLEMENTARY FIGURES

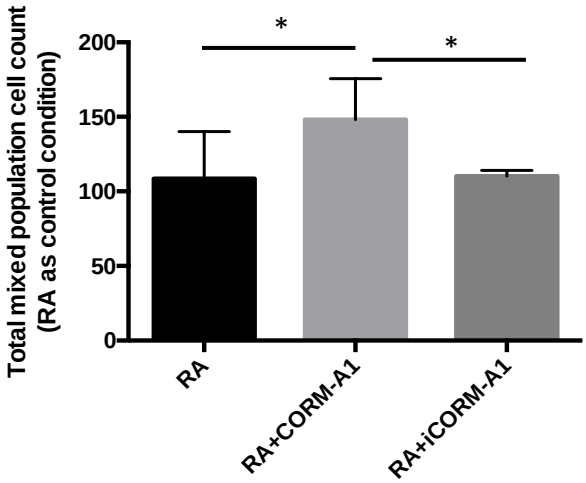

Figure S1: iCORM-A1 does not improve neuronal differentiation yield. Nuclei count *per* volume of SH-SY5Y cells and normalized by control cells treated with RA, following 7 days of differentiation. p <0.05.

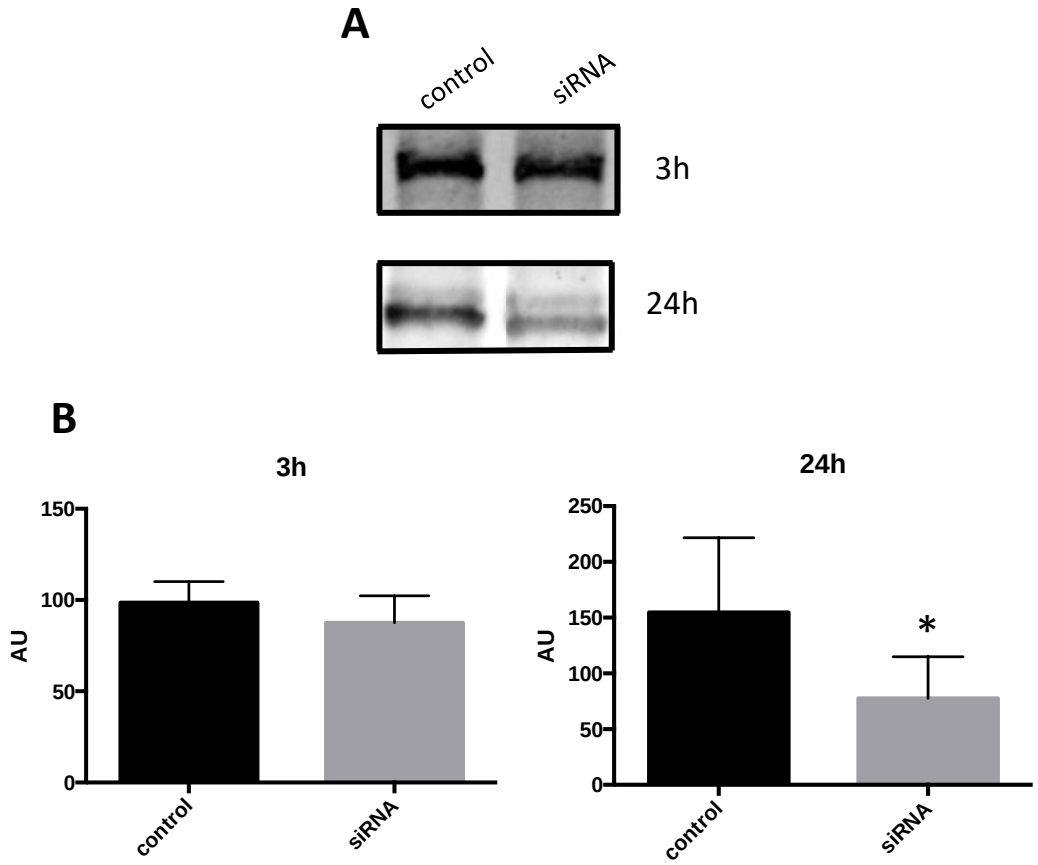

Figure S2: Quantification of G6PD protein expression following siRNA silencing approach. Protein quantification was assessed at 3 hours and 24 hours of neuronal differentiation, which corresponds to 27 hours and 48 hours of siRNA silencing, respectively. (A) Representative WB membrane for each time point; (B) WB band quantification. * p-value<0.05.

III

CARBON MONOXIDE MODULATION OF MICROGLIA-NEURON COMMUNICATION: ANTI-NEUROINFLAMMATORY AND NEUROTROPHIC ROLE

This chapter is based on data to be published as:

'Carbon monoxide modulation of microglia-neuron communication: anti-neuroinflammatory and neurotrophic role'

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Manuscript under final preparation

Nuno L. Soares 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.

ABSTRACT

Microglia, the 'resident immunocompetent cells' of the central nervous system (CNS), are key players in innate immunity, synaptic refinement and homeostasis. Dysfunctional microglia contribute heavily to the creation of a toxic inflammatory milieu, a driving factor in the pathophysiology of several CNS disorders. Strategies for modulation of microglial function are required to tackle exacerbated tissue inflammation. Carbon monoxide (CO) is an endogenous gaseous molecule, produced by the degradation of haem, which presents several biological functions, namely anti-inflammatory, anti-apoptotic, pro-homeostatic and cytoprotective. For this study the novel molybdenum based CO-releasing molecule, ALF826 was used. ALF826 was shown to modulate neuron-microglia remote communication. Using a BV2 microglia-CAD neuron conditioned media approach, CO provided indirect neuroprotection by limiting lipopolysaccharide-activated microglia expression of reactivity markers (ROS, CD11b) and secretion of inflammatory factors (TNF-α, nitrites). This consequently prevented neuronal cell death and morphological alterations induced. In the absence of inflammation, conditioned media from CO-treated microglia improved neuronal morphological complexity. Likewise, it also prevented neuronal cell death induced by pro-oxidant *tert*-Butyl hydroperoxide (*t*-BHP). ALF826 treatment reinforced microglia secretion of Interleukin-10 (IL-10), a key anti-inflammatory cytokine, which may be involved in protection against *t*-BHP stress in this remote communication model. Our findings indicate that CO has a modulatory role on microglia-to-neuron communication, promoting neuroprotection in a non-cell autonomous way. In conclusion, CO-induced neuroprotection is afforded by (i) blockade of exacerbated microglial inflammation and (ii) the microglial release of neurotropic factors. For the first time it is described CO improvement of microglial neurotrophism under non-inflammatory conditions.

INDEX

BACKGROUND

Microglia are the main immune cell population in the Central Nervous System (CNS)[1]. Originating from a primitive myeloid precursor[2], microglia populate the brain and spinal cord, where they account for 5-10% of total glial population with crucial biological functions[1,3]. As the brain first line of defence, microglia are sentinels that constantly screen the parenchyma and initiate an inflammatory response when encountering potentially pathogenic or toxic agents that endanger the CNS integrity[1,3]. These homeostatic imbalances trigger major functional and morphological changes in microglia-altered expression of surface receptors, intracellular enzymes and secreted molecules[1]. Release of inflammatory mediators, such as reactive oxygen species (ROS)[4,5] and nitric oxide (NO) derivatives[3,6,7], cytokines[3,6,7], metabolites and extracellular vesicles[8] induce cell death on compromised cellular populations and/or potentiate the microglial phagocytosis of bacteria, viruses and debris[1,9,10]. Additionally, microglia recruit other cells and help set up the adaptive immune response[11]. Conversely, microglia also have a relevant role in neurotrophic support[12], as well as brain development, removing immature and apoptotic populations[13].Microglia also participate in the regulation of synaptic plasticity[14–16], and stimulate synaptogenesis and synaptic maturation[17,18]. Several of these functions are reliant on microglia paracrine mechanisms, through secretion of specific factors like thrombospondin, Insulin growth factor 1 (IGF-1)[12,19–23], brain-derived neurotrophic factor (BDNF), ATP, Interleukin-10 (IL-10) as well as low levels of inflammatory cytokines TNF-α (Tumour necrosis factor α) and IL-1β[24–27]. Being both the hub of neuroinflammation, and crucial for neuronal development, survival and activity, microglia function is under tight regulation. Deficient microglial function causes imbalances in brain tissue homeostasis, mostly triggered by exacerbated inflammation, which is a common feature of both chronic and acute CNS disorders[28,29]. Altered microglial also affect synaptic plasticity and overall neuronal function [1].

Known for its toxicity, carbon monoxide (CO) is a gaseous molecule, endogenously produced by the degradation of haem catalysed by haem oxygenase (HO)[30]. This reaction yields CO, along with free iron (Fe^{2+}) and biliverdin[30,31]. HO has two known isoforms: the constitutive form HO-2 and HO-1, an inducible isozyme, whose expression is activated by a wide array of stress signals[31]. CO is a signalling molecule and the CO/HO-1 axis is protective in several biological contexts, modulating inflammation, apoptosis, cell differentiation and metabolism[32–34] CO-derived ROS production primes the anti-inflammatory player PPAR-γ (Peroxisome proliferator-activated receptor gamma co-activator 1 α) in macrophages[35] and in lung[36], decreasing downstream pro-inflammatory mediators. CO also regulates the activities of mitogen activated protein kinase, resulting in lower secretion of key cytokines, which limits pro-inflammatory stimulation[37–39]. In CNS, CO has been described as neuroprotective by preventing neuronal cell death in vitro[40,41] and in ischaemia and reperfusion in vivo models[42,43]. In glial cells, CO is cytoprotective for astrocytes, namely CO limited

apoptosis by regulating mitochondrial membrane permeabilization[44], by improving oxidative metabolism[45] and modulating P2X7 receptors[46]. Likewise, vasodilation is promoted by CO via astrocytic glutamate receptors[47]. In microglia, CO reduces LPS (lipopolysaccharide), thrombin or Interferon γ induced (IFN-γ) inflammatory response[37,48]. This anti-inflammatory effect was amplified by chemically inhibiting PI3K (Phosphoinositide 3-kinase) an ERK (Extracellular-signal-regulated kinase)[37,48]. CO also decreases neuroinflammation in microglia *via* modulation of cell metabolism, namely by enhancing mitochondrial respiration[49]. *In vivo* models of CNS disorders have highlighted the potential of CO as a regulator of local brain immunity. In fact, CO treatment ameliorates tissue inflammation and limits cell loss and disease progression in models for autoimmune encephalomyelitis[50] and haemorrhagic stroke[51].

Herein, we aimed to assess how CO alters microglia function and paracrine communication with the surrounding cellular milieu, particularly with neurons. In fact, CO's role on microglia-neuron communication has not been an object of study. Thus, the present work targets the underlying molecular mechanisms of this inter-cellular communication, primarily the secretome of microglia and how it affects neuronal survival, morphology and function. Hence, it focuses on how CO regulates two main aspects: (i) the impact on microglial neuroinflammation in neurons and (ii) microglia's role on neurotrophism. Because CO is a promising therapeutic molecule with an increasing number of applications[31], CO-releasing molecules (CORMs) have been under development to circumvent several limitations regarding CO's pharmacological delivery, namely safety and tissue specificity[52–54]. Herein, a novel CORM, ALF826, is used to assess the impact of CO on microglia neuroinflammation and microglia-neuron communication. ALF826 is a molybdenum carbonyl complex which spontaneously releases CO in biological media with a half-life of 37 min in Hepes 7.4 buffer. Its cytotoxicity is negligeable at 100µM (98% survival of RAW246.7 macrophages at 24h) and its total CO effective load is >3 equivalents of CO per mol (Information provided by Proterris (Portugal) Lda.). Molybdenum is a trace element that is essential for the function of at least three human enzymes: xanthine oxidase, sulfite oxidase and aldehyde oxidase. Thus Mo-based CORM presents the advantage of having an endogenous metal that is not toxic and does not accumulate, since the human body presents biological machinery to eliminate Molybdenum from the organism.

METHODS

Materials

All cell culture plastics were purchased from Corning (Corning, NY) and Sarstedt (Sarstedt, Germany). Chemicals of analytical grade were obtained from Sigma-Aldrich (St Louis, MI), unless stated otherwise.

Cell line cultures

BV2 murine microglia cell line was kindly supplied by Dr Ana Raquel Santiago (Faculdade de Medicina da Universidade de Coimbra). Cells were grown in RPMI-1640 medium (Sigma-Aldrich), supplemented with 10% foetal bovine serum (FBS, Thermo-Fisher Scientific), 4 mM L-glutamine (Thermo-Fisher Scientific), penicillin (100 units/mL) and streptomycin (100 µg/mL) (Thermo-Fisher Scientific). CAD mouse catecholaminergic neuronal cell line was gently provided by Dr Federico Herrera (Faculdade de Ciências da Universidade de Lisboa). DMEM/F12 was used as basal medium (Thermo-Fisher Scientific) and supplemented with 8% FBS (Thermo-Fisher Scientific), penicillin (200 units/mL) and streptomycin (200 µg/mL) (Thermo-Fisher Scientific). For differentiation, growing medium was replaced by a serum free DMEM/F12 supplemented solution. Both cell lines were maintained in a humified incubator at 37°C and 5% CO_2.

Primary cell cultures

Animals were housed in standard laboratory conditions with free access to water and standard rodent chow. All animal experiments were performed with the approval of the i3S Animal Ethics Committee and in accordance with European Union Directive on the protection of animals used for scientific purposes (2010/63/EU) and the Portuguese Law that transposes it (Decreto-Lei n.º 113/2013). All efforts were made to minimize animal suffering. Neuron hippocampal cell cultures were isolated from embryonic day 18-19 Wistar rats. Pregnant females were euthanized in a CO_2 chamber, their abdominal region was pulverized with 96% ethanol and opened using surgical scissor and tweezers. Embryos were retrieved, decapitated and the brains were placed in a Petri dish containing Hank's Balanced Salt Solution (HBSS, Thermo-Fisher Scientific) supplemented with 100 µg/mL Gentamicin (Lonza). The brains were dissected under a magnifying glass and the hippocampi were collected. Hippocampal tissue was incubated for 15 minutes at 37°C with 1.5 mg/mL of trypsin (Thermo-Fisher Scientific) and after allowing the hippocampi to sediment, the supernatant was removed and a 10 mL HBSS + 10% FBS (Thermo-Fisher Scientific) solution was added. Following gentle agitation, the supernatant was discarded, and a washing step was performed with fresh HBSS. The

supernatant was again removed and 5mL of Neurobasal medium (Thermo-Fisher Scientific) supplemented with 25 µM Glutamate (Sigma-Aldrich), 0.5 mM L-glutamine (Thermo-Fisher Scientific), 100 µg/mL Gentamicin (Lonza) and 1% B27 supplement (Thermo-Fisher Scientific) was added, and the tissue was dissociated by thorough pipetting. The suspension was then put through a cell strainer, collected and cells were counted with a Neubauer counting chamber. Neurons were plated on 24 multi-well plates (100 000 cells/well), on top of Poly-D-Lysine (PDL, 1 mg/mL, Sigma-Aldrich) coated glass coverslips. Cells grew *in vitro* for 10 days before any analysis was carried forward, with fresh medium being added every two days.

Mixed glial cell (MGC) cultures were obtained from cortices of 7-day old Wistar pups. Animals were quickly sacrificed, with brains being removed and dissected consequently in HBSS (Thermo-Fisher Scientific) with penicillin (100 units/mL) and streptomycin (100 µg/mL) (Thermo-Fisher Scientific). White matter and meninges were discarded, and the remaining tissue was collected and mechanically homogenized. The cell suspension was passed through a gauge syringe and incubated with 0.1 U/mL DNAse I (Zymo) and 1.5 mg/mL trypsin (Thermo-Fisher Scientific) for 15 minutes at 37°C. After incubating, trypsin was inactivated by adding DMEM GlutaMAX (Thermo-Fisher Scientific) supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL) (Thermo-Fisher Scientific), 10% FBS (Thermo-Fisher Scientific) and the suspension was centrifuged afterwards (550 g, 15 minutes). The resulting supernatant was carefully discarded, and the pellet was resuspended in 10mL of the same medium and put through a 100 µm cell strainer. The obtained suspension was distributed equally into 75cm^2 t-flasks (approximately 2 brain per flask) to make a total media of 10mL per flask. Prior to adding the cells, the t-flasks were coated with 1 mg/mL PDL (Sigma-Aldrich) for 1 hour. The cell medium was changed every other day.

Primary microglia cells were obtained from 10-day-old MGC cultures, which were placed on an orbital shaker incubator for 2h at 200 rpm. The cell suspension was collected, centrifuged (260 rcf, 10 minutes) and the supernatant was discarded. The pellet was resuspended in DMEM/F12 (Thermo-Fisher Scientific) and cells were counted using a Neubauer counting chamber. Microglia cells were always obtained from either 10 (shake 1) or 17 days *in vitro* (DIV, shake 2) MGC cultures. Older cell cultures were not considered for this. Microglial cells were plated on 24 multi-well plates, with DMEM/F-12 GlutaMAX (Thermo-Fisher Scientific) and 4% L-glutamine (Sigma-Aldrich), penicillin (10 units/mL) and streptomycin (10 µg/mL) (Thermo-Fisher Scientific) and 10% FBS (Thermo-Fisher Scientific) supplement.

Cell culture treatments

For neuroinflammatory assay, BV2 cells were seeded onto multi-well plates (10×10^4 cells per well), treated with ALF826 (50µM) for 24h before LPS (Lipopolysaccharide, 500 ng/mL, Sigma-Aldrich) was added for another 24h. Subsequently, the microglia conditioned media was collected and centrifuged for 5 minutes, 500g (Figure 1A). Simultaneously, CAD neurons were seeded (6×10^4 cells per well) and 24h later differentiating medium was added. After 48h of differentiation, neuronal supernatant was removed, and CAD were challenged for one day with microglia conditioned media.

For neurotrophic assay, BV2 cells (10×10^4 cells per well) were treated for 48h with ALF826 (50µM), then medium was collected and centrifuged (5 minutes, 500g). The conditioned medium (neuronal culture supernatant) was added into 48h-differentiated CAD neuronal culture. Pro-oxidant *tert*-Butyl hydroperoxide at 7.5 and 10 µM (*t*-BHP, Sigma-Aldrich) was added into the neuronal culture to promote cell death for 24h, with or without the presence of microglia conditioned medium (Figure 7A).

Primary culture of microglia derived from 10 or 17 DIV MGC cultures was collected and plated on 24 multi-wells ($7x10^5$ cells/well). 24h after seeding, cells were treated with 50 µM of ALF826 for 24h then with 10 ng/mL of LPS for another 24h. Microglia medium was collected, centrifuged at 500 g for 5 min and its supernatant was used as conditioned medium. 50% volume of neuronal medium of 10 DIV hippocampal neurons cultured in 24 multi-well plates was replaced by microglia conditioned media. Neuronal cells were analysed following 24h.

Reagents and solution preparation

ALF826 was provided by Proterris (Portugal) Lda. Stock solutions of 2.5mM were prepared by dissolving the compound in Dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluting it 1/10 in a $NaHCO_3$ solution (0.1mM, pH 8.3). ALF826 was then filtered, aliquoted and stored at -80°C at a final concentration of 2.5 mM.

Flow Cytometry

Neuronal cell viability assay

CAD neurons were collected by trypsinization and stained with 1ng/mL of Propidium Iodide (PI, Thermo-Fisher Scientific) for 15 minutes at 37°C. Cell viability was analysed by flow cytometry using the FACS Canto II (BD Biosciences). 488nm laser line was used for excitation and PI was read in the FL-3 channel in the

linear scale. Appropriate controls, such as positive staining controls and unstained samples were always carried out. Data analysis was performed with the FlowJo software (BD Biosciences).

Microglia reactivity assay

BV2 cells were collected by scrapping and stained with 500 ng/mL of PE/Cy7 (Phycoerythrin/Cyanine 7) anti-mouse CD11b antibody (BioLegend) for 15 minutes at 37°C. Microglia reactivity was analysed by flow cytometry using FACS Canto II (BD Biosciences). 488nm laser line was used for excitation and reactivity levels were determined by reading sample's PE/Cy7 Median Fluorescence Intensity in the FL-4 channel in the linear scale. Appropriate controls, such as positive staining controls and unstained samples were always carried out. Subsequent data analysis was performed using FlowJo software (BD Biosciences).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to measure Tumour necrosis factor α (TNF-α), Interleukin-1 β (IL-1β) and IL-10 microglia supernatant levels, using the respective Standard ABTS ELISA Development Kits (PeproTech). For Glial cell-derived neurotrophic factor (GDNF) and Brain-derived neurotrophic factor (BDNF), different kits were used (GDNF ELISA Kit (Abcam), Human/Mouse BDNF DuoSet ELISA (R&D)).Microglia culture media was collected and centrifuged to remove cellular debris, and the pellet was discarded. The resulting supernatant was kept at -80°C until analysis. All experiments were performed in accordance with the respective manufacturer's instructions. Absorbance values were measured at 415nm, with wavelength correction set at 560nm, using an Infinite F200 PRO microplate reader (Tecan).

Griess reaction assay

Nitrite of microglia supernatant was quantified by Griess reaction colorimetric test. Microglia culture media was collected and centrifuged to remove cellular debris and subsequently incubated with Griess reagent (1:1 ratio, Sigma-Aldrich) for 10 minutes at room temperature (RT), protected from light. Absorbance was measured at 540 nm using an Infinite F200 PRO microplate reader (Tecan). Nitrite concentration was calculated with reference to a standard curve generated with known concentrations of sodium nitrite ($NaNO_2$, Sigma-Aldrich).

Immunoblotting

Cell extracts were washed several times with ice-cold PBS and lysed with RIPA (Radioimmunoprecipitation assay buffer) buffer (50 mM Tris-HCl, pH 6.8, 50 mM NaCl (w/v), 0.1% SDS (w/v), 1% sodium deoxycholate (w/v), 1% Triton X-100 (v/v), 10% Glycerol (v/v), 1% protease inhibitor cocktail (v/v). Protein concentration was determined with a Pierce BCA Protein Assay Kit (Thermo-Fisher Scientific), following manufacturer's instructions. Absorbance values were registered at 560 nm using an Infinite F200 PRO microplate reader (Tecan). A Bovine Serum Albumin (BSA, Merck) standard curve was constructed to determine protein concentration.

Equal amounts of protein were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on a polyacrylamide gel (10% gel unless stated otherwise), with a NZYColour Protein Marker II (NZYtech) being used as band size reference. Proteins were then electrically transferred onto an Amersham Protran 0.45 NC nitrocellulose membrane (GE LifeSciences) and blocked with 5% (m/v) BSA in T-TBS for 1h. Membranes were labelled consecutively with primary and secondary antibodies as indicated in Supplementary Table 1. Immunoblots were exposed to ECL Clarity Western Detection Reagent (Bio-Rad) 5 minutes and the reactive bands were detected after the membranes were exposed to X-ray film (Chemidoc Touch Imaging System, Bio-Rad). The resulting area and intensity of the bands were quantified with ImageLab software (Bio-Rad). β-actin was used as internal loading control unless otherwise is stated.

ROS generation assay

ROS, in particular hydrogen peroxide generation, were measured following the conversion of 2',7'-dichlorofluorescein diacetate (H2DCFDA) (Invitrogen) to fluorescent 2',7'-dichlorofluorescein (DCF). BV2 microglia cells were seeded (9×10^3 cells/well) on 96-well black flat bottom plates and treated with ALF826 and/or LPS, as described previously. After treatment, microglia supernatant was removed, and cells were washed twice with ice-cold PBS and treated for 15 minutes with 5 µM of H2DCFDA. Cells extracts were subsequently washed, and fluorescence intensity was measured using a Tecan Infinite F200 PRO microplate reader (λ_{ex} 485 nm/λ_{em} 530 nm). ROS generation was calculated as increase over baseline levels, determined for untreated cells and normalized to total protein quantification for each condition.

Immunofluorescence microscopy

Cells growing on glass coverslips were fixated with 4% (v/v) PFA (paraformaldehyde) and 4% (w/v) sucrose solution (20 minutes at RT), permeabilized with 0.3% (v/v) Triton X-100 solution (15 minutes, RT)

and then blocked with BSA 1% (w/v) and Triton X-100 0.1% (w/v) for half hour at RT. Later, cells were probed with primary (2h incubation) and secondary (1h) antibodies, as described in Supplementary Table 1. Coverslips were mounted onto glass slides with Prolong mounting medium (with DAPI 1:1000 (4',6-diamidino-2-phenylindole, Thermo-Fisher Scientific). Washing with ice-cold PBS was always performed between steps. All antibodies used were diluted in 1%(v/v) BSA and 0,1%(v/v) Triton X-100 solution. Fixating, permeabilizing and blocking solutions were prepared in PBS. Images were captured with a Zeiss Z2 microscope, with at least 5 random micrographs being acquired, unless otherwise stated.

Neuronal morphology analysis

Neurite tracing was done using the NeuronJ Fiji plug-in (Erik Meijering) and following instructions present in an online manual provided by the developer (https://imagescience.org/meijering/software/neuronj/manual/). Neurites were labelled and clustered as primary (originating from cell soma), secondary (from a primary neurite) and tertiary (from a secondary neurite) processes. Software calculation determined total number of neurites per field as well as individual neurite length. Similarly, neuron skeleton reconstruction and Sholl analysis (quantification of intersections at concentric spheres originating from cell soma to 150µm, with radius every 5 µm) were performed using the Simple Neurite Tracer Fiji plug-in, in accordance to instructions present in online tutorials (https://imagej.net/Simple_Neurite_Tracer). For each individual experiments, four to six images were analysed for each glass slide.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Data normality was tested using the Shapiro-Wilk normality test. The Kruskal-Wallis test, followed by Dunn's multiple comparison test, were utilized to compare different conditions and a 95% confidence interval was considered. All statistical analysis was performed using the Prism 6.0 software (GraphPad).

Supplementary Table 1 - List of primary and secondary antibodies used in this work.

Primary Antibody	Dilution	Host	Manufacturer	Technique
Cleaved caspase 3	1:1000	Rabbit	Cell Signaling Technologies (#9664)	Immunoblotting
TNF-α	1:1000	Mouse	Santa Cruz Biotech (sc-52746)	Immunoblotting
IL-6	1:1000	Mouse	Santa Cruz Biotech (sc-57315)	Immunoblotting
BDNF	1:1000	Mouse	Santa Cruz Biotech (sc-546)	Immunoblotting
β-actin	1:5000	Mouse	Sigma-Aldrich (A5441)	Immunoblotting
βIII-tubulin	1:1000	Mouse	Sigma-Aldrich (T8660)	Fluorescence Microscopy
NF-κB p65	1:1000	Mouse	Santa Cruz Biotech (sc-8008)	Fluorescence Microscopy
Iba1	1:500	Rabbit	Wako (019-19741)	Fluorescence Microscopy
PE/Cy7 CD11b (M1/70)	1:200	Rat	BioLegend (101216)	Flow Cytometry

Secondary Antibody	Dilution	Host	Manufacturer	Technique
ECL Anti-mouse IgG	1:5000	Sheep	GE LifeSciences (NA931V)	Immunoblotting
ECL Anti-rabbit IgG	1:5000	Donkey	GE LifeSciences (NA934V)	Immunoblotting
Alexa 488 anti-mouse IgG	1:1000	Goat	Thermo Fisher (A11001)	Fluorescence Microscopy
Alexa 594 anti-rabbit IgG	1:1000	Goat	Thermo Fisher (A11012)	Fluorescence Microscopy

RESULTS

Conditioned medium from CO-treated BV2 microglia provides neuroprotection to CAD neuronal culture

In order to assess the role of carbon monoxide (CO) on remote neuron-microglia communication, a conditioned medium approach was used, which allows to evaluate the impact of microglia secretome on neuronal function and survival. BV2 microglia were pre-treated with 50µM ALF826 for 24h and then incubated or not with 500 ng/mL of LPS (Lipopolysaccharide), a TLR-4 (Toll-like receptor) agonist, classically used to elicit an inflammatory-like response. After one day, the microglia media was collected and added to the catecholaminergic neuronal cell line CAD culture for 24h, challenging cell survival (Figure 1A). Neuronal cell survival assessment was based on plasmic membrane integrity (Propidium iodide internalization) by flow cytometry (Figure 1B). Viability of CAD neuronal cells exposed to conditioned media derived from inflammatory microglia decreased, and this effect was partially reverted whenever BV2 microglia were pre-treated with ALF826 (Figure 1B). In addition, expression of neuronal cleaved caspase-3 (Figure 1C and D), which is a classical biomarker of apoptosis, was quantified by Western Blot. Accordingly, supernatant derived from CO-treated microglia also decreased the levels of cleaved caspase-3. These data indicated that CO decreases microglia release of neurotoxic factors, limiting neuronal cell death.

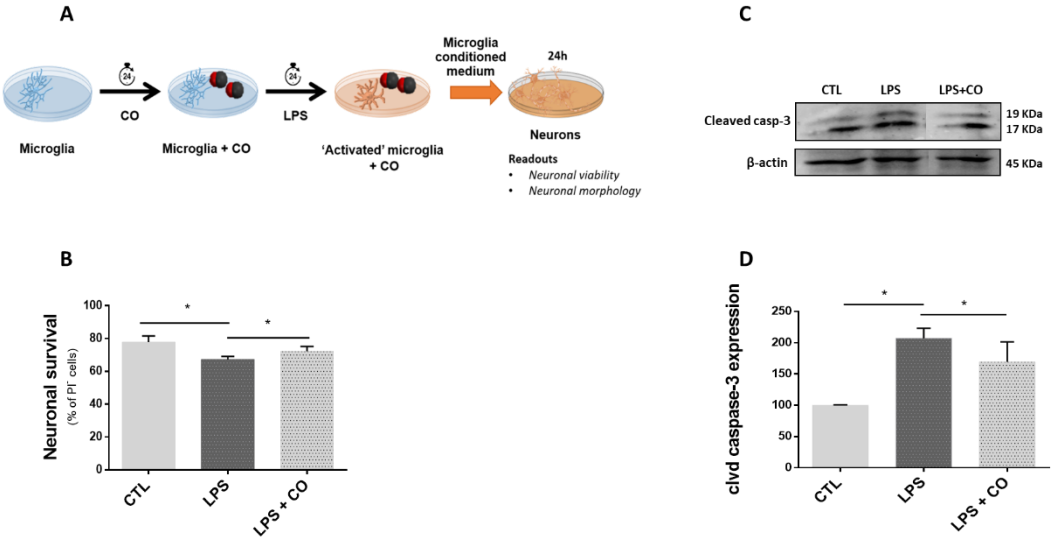

Figure 1 – Carbon monoxide (CO) treatment in microglia attenuates CAD neuronal cell death induced by conditioned medium from LPS-activated microglia. BV2 microglia were treated with ALF826 (50 µM) for 24 hours and with LPS (500 ng/mL) for another 24 hours. CAD neuronal culture was subsequently incubated with microglia conditioned medium (A). Cell viability was assessed by (B) propidium iodide staining quantification in flow cytometry or by (C, D) cleaved caspase 3 protein expression by Western Blotting. The densitometry of cleaved caspase-3 expression was normalized for β-actin and is presented as percentage relative to the control. Representative membranes for the experiments are displayed (B). *n=4*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

Neuronal morphology of differentiated CAD cell line was analysed for indirect evaluation of neuronal function (Figure 2). Neurons exposed to inflammatory microglia media presented a decrease in cellular complexity parameters, such as average neurite length (Figure 2A) and number of neurites *per* cell (Figure 2B). Additionally, data from Sholl analysis, which is a quantitative method used for the analysis of neuronal arborization, further indicated the existence of a decrease of the overall neuronal complexity whenever neurons were challenged with inflammatory conditioned media (Figure 2C and D). Conditioned medium derived from CO-treated microglia prevented the decrease of neurite length and the number of neurites *per* cell and maintained neurite arborization at similar levels as the non-treated control neurons.

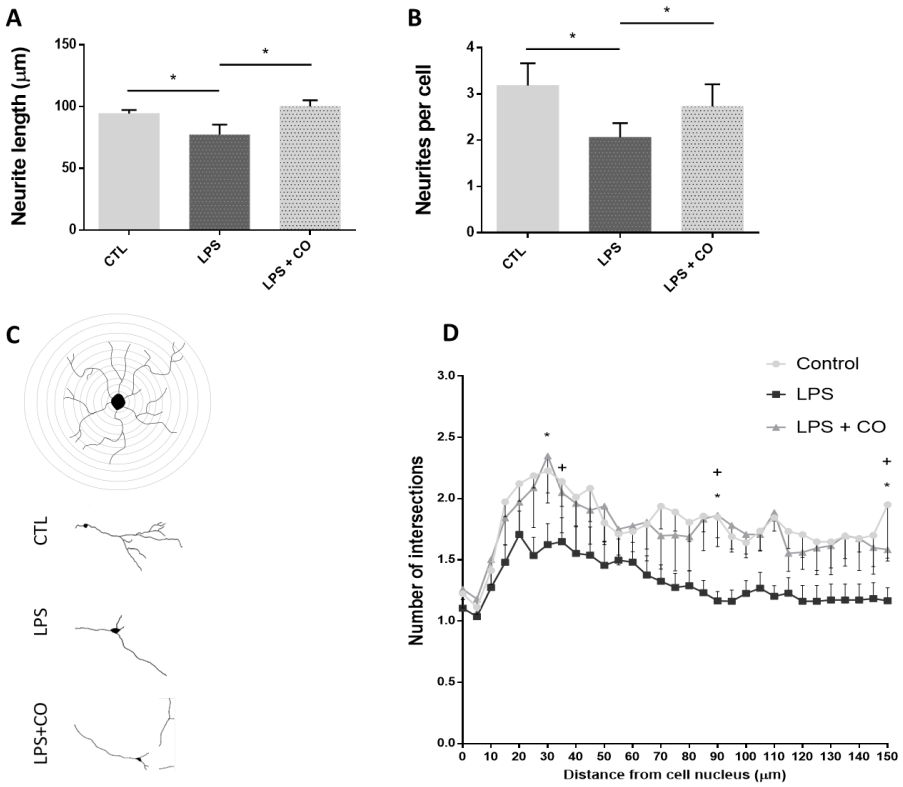

Figure 2 – Administration of CO in microglia limits loss of morphological complexity in neurons exposed to LPS-activated microglia conditioned medium. CAD neurons treated with BV2 microglia conditioned media for 24h were immunostained with a βIII-tubulin antibody. Nuclei were stained with DAPI. Following image acquisition, morphological features, such as (A) neurite length, (B) number of neurites per cell and (D) Sholl analysis were assessed from six independent experiments (10-15 cells per condition). Representative tracings of 2D acquired images were also performed (C). *n=5-6*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

Thus, microglial ALF826 treatment has a paracrine neuroprotective effect on neuronal cells, potentially achieved through modulation of microglial release of neurotoxic pro-inflammatory factors.

CO has an anti-inflammatory role on LPS-challenged BV2 microglia

Since CO appears to provide neuroprotection by regulating neuron-microglia remote communication, we next quantified extra-cellular levels of the pro-inflammatory factors nitric oxide (NO), Tumour necrosis factor α (TNF-α) and Interleukin-1β (IL-1β) in microglial supernatant, all of which promote inflammation. Griess colorimetric assay, which allows for the quantification of nitrites, an indirect measure of NO, indicated an increase in nitrites concentration following LPS exposure (Figure 3A). Thus, ALF826 treatment partially inhibited LPS-induced nitrite release. The decrease of nitrite secretion was dependent on ALF826 concentration (Data not shown). TNF-α was quantified by ELISA, CO also reverted the secretion of TNF-α pro-inflammatory cytokine in LPS BV2 (Figure 3B). For IL-1β, no significant differences were identified in the presence of LPS nor CO (Figure 3C).

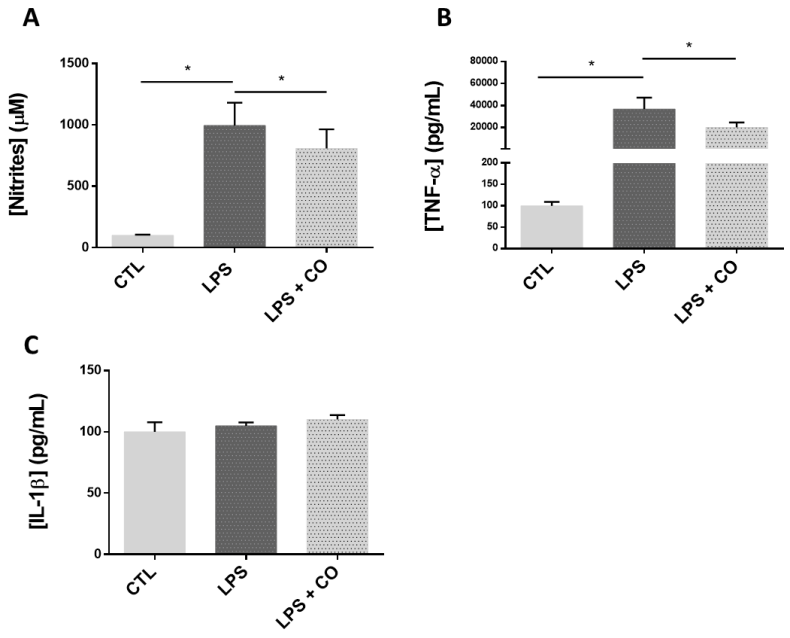

Figure 3 – CO attenuates the inflammatory profile of microglia's secretome. BV2 microglia supernatant was quantified for nitrite levels (B) by Griess colorimetric assay. TNF-α (B) and IL-1ß (C) pro-inflammatory cytokines were assessed *via* ELISA. All results are presented as percentage relative to the negative control. *n=5-6*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA.

To further confirm the anti-inflammatory effect of ALF826 treatment on BV2 function, we quantified the expression of specific microglial reactivity markers (inflammatory cytokines TNF-α and IL-6, intracellular ROS production and surface receptor CD11b). Similarly, ALF826 treatment limited the expression of TNF-α and IL-6 in LPS-treated microglia (Figure S1 A and B). The role of CO on intracellular ROS production was evaluated by H(2)DCF-DA staining that increases its fluorescence in the presence of hydrogen peroxide. Likewise, CO partially inhibited LPS-induced microglia-derived oxidative stress (Figure 4A). Finally, expression

of surface receptor CD11b was quantified *via* flow cytometry. CD11b immunoreactivity increased in LPS-administered microglia and this was partially reverted, in ALF826 pre-treated cells (Figure 4B). Overall, these data strongly suggest that CO suppresses BV2 microglial reactivity. The impact of LPS and CO on microglial morphology was also assessed by performing immunofluorescence microscopy with anti-Iba-1 (Ionized calcium-binding adapter molecule 1) staining (Figure 4C). Morphological complexity was measured by circularity index and cell area (Figure 4D and E). Inflammatory stimulus led to a 1.55-fold increase in microglia size and caused a decrease in cell circularity, which may indicate microglial activation. However, CO did not revert these morphological parameters.

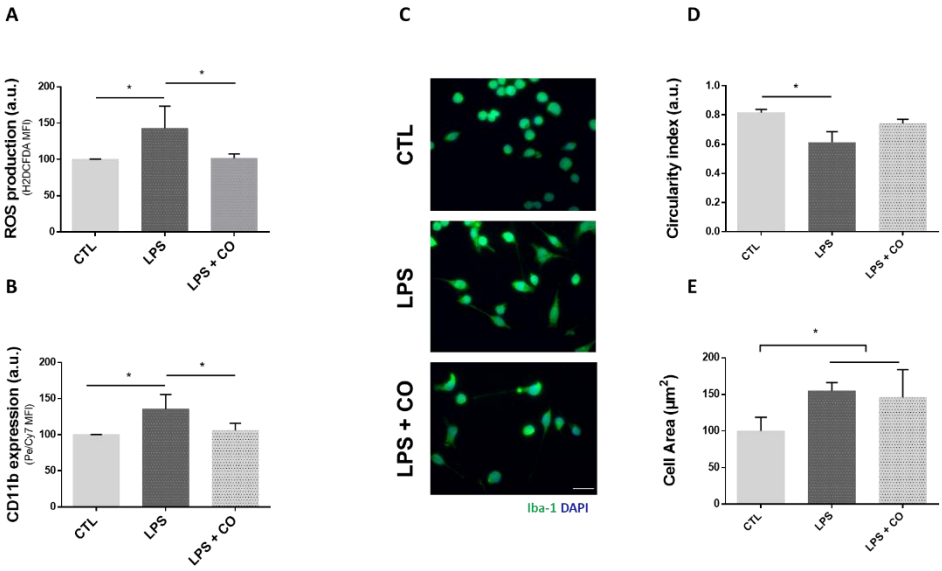

Figure 4 – CO decreases microglial expression of inflammatory reactivity markers. BV2 microglia cells were collected, and expression of inflammatory markers was assessed. (A) Intracellular H_2O_2 levels were measured by fluorescence using H(2)DCF-DA and normalized to total protein levels. (B) CD11b surface expression was quantified by Pe/Cy7 immunofluorescence median fluorescence intensity via flow cytometry. BV2 microglia were immunostained with Iba-1 antibody (green) and DAPI (nuclei, blue) and photographed under a fluorescence microscope (C). Scale bar = 25 µm. Microglia morphological parameters' circularity index (D) and cell size (E) were evaluated. Results in (A), (B) and (E) are presented as percentage relative to the negative control. *n=3-5*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

Overall, the results suggest that ALF826 is, in fact, a strong pharmacological anti-inflammatory agent, limiting the expression and secretion of several pro-inflammatory agents. Thus, CO-promoted non-cell autonomous neuroprotective effect might result from limiting the inflammatory response of microglial cells.

Validation of neuron-microglia communication in primary cultures

The data obtained in BV2 microglia-CAD neuron conditioned media protocol were then validated in a more physiologically relevant model: hippocampal neuronal and microglia rat primary cultures. Primary culture of microglia was treated with ALF826 (50 µM, 24h) and LPS (10 ng/mL, 24h) and the supernatant was collected, centrifuged and subsequently incubated 1:1 (volume) with neuronal media to challenge 10 days *in vitro* (DIV) hippocampal neurons.

To assess neuronal cell death in primary cultures, neurons were immunolabeled with an antibody against βIII-tubulin (green), the nuclei were stained with DAPI (blue) and cells were imaged using a fluorescence microscope (Figure 5A). Apoptosis was quantified by counting chromatin condensed nuclei (Figure 5B). In accordance with data from Figure 1B, neurons cultured with inflammatory conditioned media presented a significant decrease in cell survival, which was reverted whenever microglia are pre-treated with ALF826.

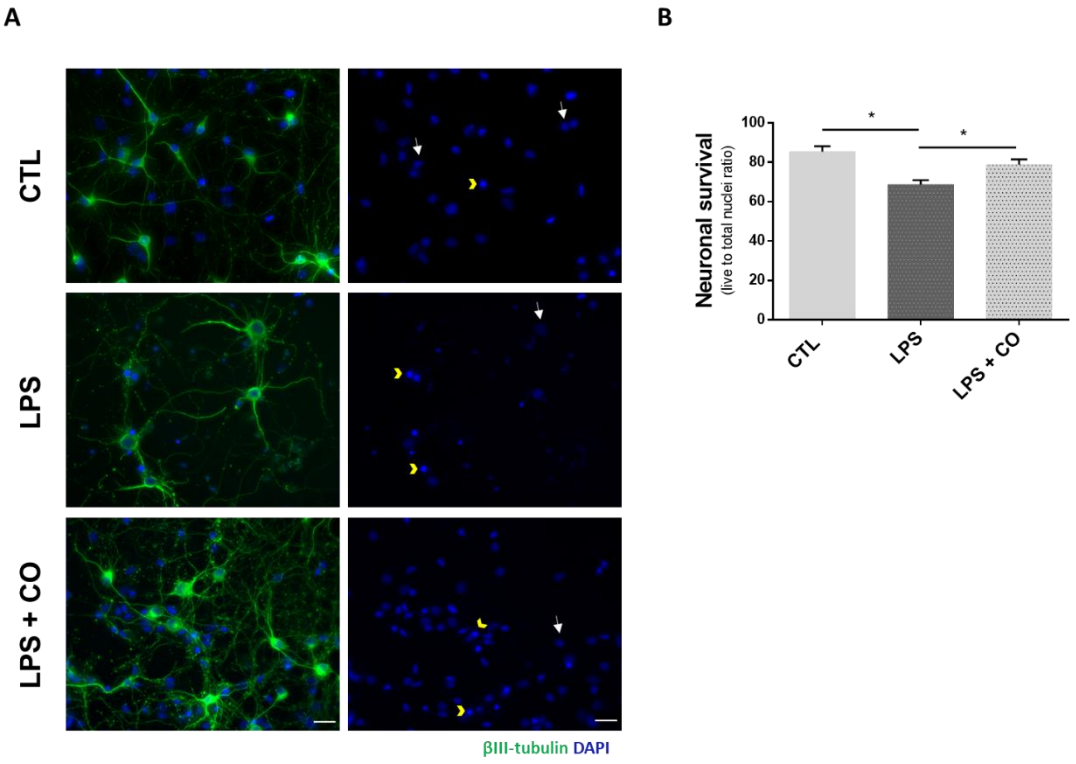

Figure 5 – CO provides neuroprotection in primary hippocampal neurons *via* modulation of primary microglia. Rat hippocampal neurons were challenged with conditioned medium from primary microglia cells for 24 hours and immunostained with βIII-tubulin primary antibody (green) and DAPI (nuclei in blue) (A, scale bar = 25 µm). Cell viability was assessed by counting total and apoptotic neurons. White arrows highlight the nucleus of healthy neurons and yellow arrowheads highlight the nucleus of dead neurons. The live/total nuclei ratio was registered and is presented in (B). *n=3*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

It was next addressed whether CO anti-neuroinflammatory function is present in primary cultures of microglia. Microglial medium was collected for indirect quantification of NO levels by Griess colorimetric

assay and of TNF-α and IL-1β levels by ELISA. For all three secreted factors, LPS triggered a significant increase, which were all partially prevented in the presence of ALF826 pre-treatment (Figure 6A, B and C). Contrary to BV2 cell line, in microglial primary cultures LPS elicited elevated levels of IL-1β, suggesting a strong NLRP3 (NLR Family Pyrin Domain Containing 3) inflammasome reaction.

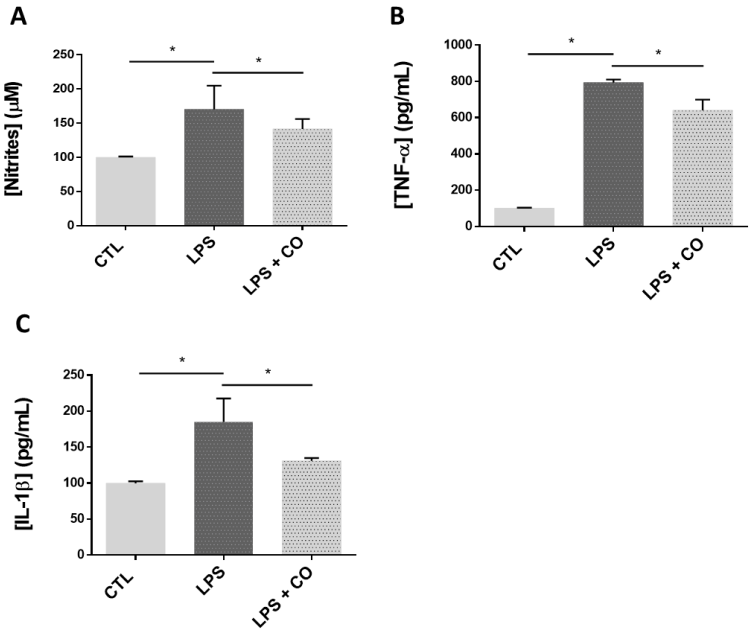

Figure 6 – CO treatment inhibits the inflammatory secretome of primary microglial cells. Supernatant from rat primary microglia was collected and secretome was analysed. (A) Nitrite quantification was performed via Griess colorimetric assay and ELISA assays were utilized for the quantification of TNF-α (B) and IL-1ß (C) levels. All data is presented as percentage relative to the negative control. *n=3*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA.

LPS-treated primary microglia displayed increased NF-κB (Nuclear factor κ-light-chain-enhancer of activated B cells) nuclear translocation (Figure S2 A and B), but CO did not inhibit this effect, which indicates that modulation of primary microglia secretome was dependent on alternative effector pathways.

Altogether, these results further highlight and validate that CO has a modulatory role in the remote neuron-microglia communication, by limiting exacerbated inflammation and consequently promoting neuroprotection.

Medium-derived from 'resting' microglia limits apoptosis in CAD neuronal cells exposed to pro-oxidant stimulus and improves neuronal morphological complexity

Other than being the CNS immune guardians, microglia have a crucial role in neuronal basal function, that range from modulation of proliferation/differentiation balance during development, and trophic support, morphological and synaptic plasticity[18,55,56]. Moreover, up to our knowledge there are no data showing CO's role in the modulation of microglia neurotrophic functions. To test microglia-induced neurotrophic effect and neuroprotection, neuron-microglia conditioned media approach was used. BV2 microglial cells were treated or not with 50 µM ALF826 for 48h, then, microglial supernatant was collected for treating differentiated CAD neurons, which were simultaneously challenged with a pro-oxidant agent (*tert*-Butyl hydroperoxide, *t*-BHP) to induce neuronal cell death for 24h (Figure 7A). Conditioned medium from microglia and from CO-treated microglia, both reduced levels of neuronal death at 7.5 and 10 µM of *t*-BHP. These results indicate that microglia conditioned medium, by itself, has a neuroprotective effect against oxidative stress. While CO does not improve microglia-induced neuroprotection.

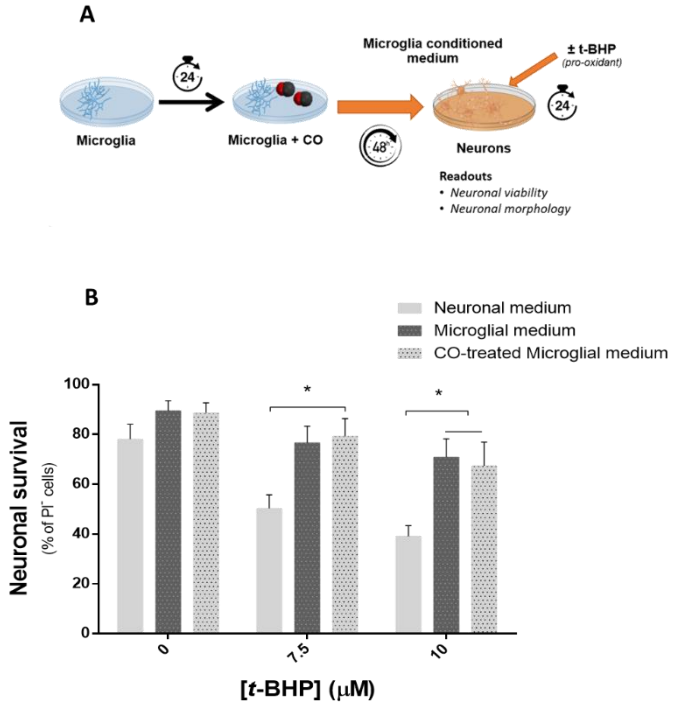

Figure 7 –Microglia conditioned media provides a protective effect in neurons exposed to oxidative stress. BV2 microglia were treated with ALF826 (50 µM) for 48 hours. CAD neuronal culture was simultaneously incubated with microglia conditioned medium and with pro-oxidant agent *t*-BHP (A). Cell viability was assessed by (B) propidium iodide staining quantification in flow cytometry. *n=6*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

As microglia have been described to modulate neuronal network dynamics[13], it was next assessed whether CO affects neuronal morphology under basal conditions. CAD neurons were incubated with conditioned media from control and CO-treated microglia and stained with anti-βIII-tubulin (green) and with

DAPI (nuclei staining) for morphological analysis. Microglial CO treatment modulates neuronal morphology, increasing the number of neurites *per* cell (Figure 8A), and neurite length *per* cell (Figure 8C), but not average neurite length (Figure 8B).

Overall, CO appears to stimulate microglial neurotrophic function, by enhancing neuronal survival under oxidative stress conditions, as well as by improving neuronal morphology under basal conditions.

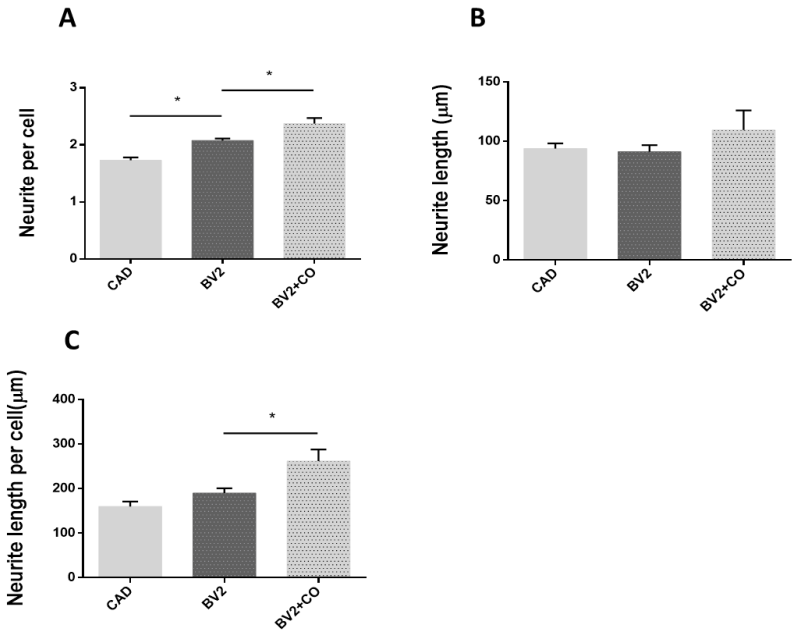

Figure 8 – Administration of CO in 'resting' microglia increases neuronal morphological complexity after exposure to conditioned medium. CAD neurons treated with BV2 microglia conditioned media for 24h were labelled with a βIII-tubulin antibody and DAPI, and fluorescence microscopy was performed. Micrographs were analysed and morphological features (A) number of neurites per cell, (B) neurite length and (C) neurite length per cell were assessed from five independent experiments (10-15 cells per condition). *n=5*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

CO stimulates microglial neurotrophic response

Lastly, the microglia secretome was analysed to disclose which molecular players are involved in CO-mediated microglia neuroprotection and neuronal morphological modulation. Several candidates described as key players in neurotrophism and neuronal support were assessed: BDNF (Brain-derived neurotrophic factor), GDNF (Glial cell-derived neurotrophic factor) and the anti-inflammatory cytokine IL-10. In fact, CO stimulated microglial IL-10 secretion demonstrated by ELISA (Figure 9). However, CO administration did not increase BDNF or GDNF secretion (Data not shown).

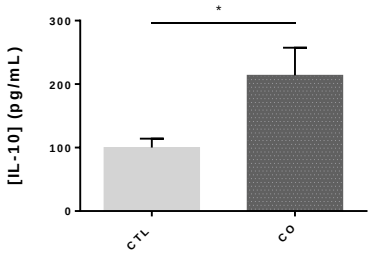

Figure 9 – CO stimulates 'resting' microglial secretion of anti-inflammatory factor IL-10. BV2 microglia supernatant was collected, and secretion levels of IL-10 was quantified by ELISA. All results are presented as percentage relative to the negative control. *n=5*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA.

Altogether, there are evidence pointing towards a neurotrophic role of CO. In fact, IL-10 overexpression is not only relevant for resolution of inflammation, but also for regulation of cell survival, neurite dynamics, development and synaptogenesis [25,57,58].

DISCUSSION

In this study, we have shown that carbon monoxide modulates microglia function and impacts on microglia-to-neuron remote communication. Using a conditioned media protocol, it was demonstrated that carbon monoxide (CO) ultimately promotes indirect neuroprotection: (i) by limiting microglial pro-inflammatory reactivity and (ii) by enhancing basal microglial neurotrophism. The non-cell autonomous effect of CO acting on microglia and promoting neuroprotection has never been described before. Furthermore, a novel Molybdenum-based CO-releasing molecule: ALF826, was used herein. This molecule presents low cytotoxicity in microglia and macrophage cell lines (Proterris (Portugal) Lda.) and delivers a large CO load.

The anti-inflammatory feature of CO has been extensively studied in macrophages[39,59]. Microglia treated with CORM-3 (CO-releasing molecule 3) also secreted lower levels of tumour necrosis factor α (TNF-α), Interleukin-1β (IL-1β) and nitrites after being triggered with different stimuli (Lipopolysaccharide, thrombin and Interferon γ)[37, 38]. Although there are data demonstrating the anti-neuroinflammatory role of CO in microglia, not much is known regarding direct CO modulation of neuron-microglia communication in an inflammatory setting, and how it affects neuronal survival and function.

Herein, we demonstrated that conditioned media derived from microglia treated with a pro-inflammatory stimulus (LPS) promoted neuronal cell death. Neurons challenged with inflammatory microglial supernatant partially lost morphological complexity. Moreover, ALF826 microglial pre-treatment rescued changes in neuronal morphology and partially reverted neuronal death. Neuronal structural degradation is a marker of cell senescence and cell death in general and can be a consequence of exacerbated inflammation[60]. Alterations in neuronal network complexity are a common phenotype of neurodegenerative disorders and play a role in defective synaptic connectivity[61–64]. Moreover, it has been shown that exposure of primary embryonic neurons to high levels of exogenous TNF and IL-1β alters cell morphology, affecting total arborization, number and length of dendrites, growth cone dynamics, as well as dendritic spine density and maturity[65]. Thus, loss of neuronal morphological complexity can also be partially a consequence of pro-inflammatory pathways disruption cytoskeleton signalling mechanisms, which CO could alleviate by inhibiting microglial inflammatory output. These results indicate that LPS-treated microglia drive a noxious response that causes severe neuronal damage, which can be prevented by ALF826 microglial pre-treatment.

To test this hypothesis, we assessed microglial production of soluble inflammatory factors in the presence and absence of CO administration. LPS increased secretion of soluble inflammatory factors TNF-α and nitrites, which were attenuated by CO administration in microglia. In contrast, LPS did not promote IL-1β release in BV2 microglia cell line. IL-1β maturation and secretion is controlled by the NLRP3 (NLR Family Pyrin Domain Containing 3) inflammasome activation[66], which requires both a priming (activated by

cytokines, endotoxins) and an activation step (viral RNA, ATP)[66]. Hence, it is possible that LPS stimulus was not sufficient to elicit an inflammasome response. Alternatively, this result could be due to limitations of the cell line model, since in primary culture of microglia, LPS alone was enough to increase secretion of IL-1β, and ALF826 pre-treatment abrogated this effect. This is in accordance with a previous publication showing that CO blocks IL-1β and IL-18 secretion in macrophages, by suppressing NLRP3 inflammasome activation via prevention of mitochondrial dysfunction[67].

CO did not affect p65 nuclear translocation in primary microglia, suggesting the involvement of other pathways for inflammatory regulation in microglia. In RAW 264.7 macrophages, CO limits inflammatory cytokine output by modulating activity of mitogen activated protein kinase activation (MAPKs) [46,81]. CORM-3 attenuates BV2 inflammation by modulation of MAPK/ERK (Extracellular-signal-regulated kinase) pathway[37]. CO has been shown to regulate NF-κB (Nuclear factor κ-light-chain-enhancer of activated B cells) in cultured endothelial cells[70] and vascular tissue[71], but not yet in immune populations.

Also, ALF826 treatment limited microglial reactivity in LPS-activated BV2 cells, namely by decreasing hydrogen peroxide levels, as well as TNF-α, IL-6 and CD11b protein levels. CD11b is a surface β-integrin expressed on microglia in the brain[72,73]. It has been shown in LPS challenged BV2 microglia that CD11b overexpression was dependent on NO production[74]. Accordingly, CO regulates the expression of inducible nitric oxide synthase (iNOS)[31]. Here, we showed that ALF826 treatment inhibited NO production, which could suggest that downregulation of CD11b is dependent on this effect. LPS's treatment also affected microglial morphology, increasing ramified pattern and size. Nevertheless, CO did not revert microglial morphological changes. Importantly, these data do not recapitulate the classical amoeboid-ramified dichotomy, which characterizes microglial populations in *in vivo* settings.

Despite microglia modulation of inflammation and CNS immunity, these myeloid cells also regulate brain development, synaptogenesis, neuronal turnover and provide strong neurotrophic support to neuronal cells[12,18–23,55,56,75,76]. Interestingly, CO modulation of microglia neurotrophism has never been explored. Herein, we showed that conditioned media derived from non-activated microglia protected neurons from oxidative damage by partially preventing neuronal cell death.

Microglia neuroprotection can result from secretion of neurotrophins (BDNF, Neurotrophin-3, Nerve growth factor)[19,77], inflammatory cytokines[23] or survival growth factors (IGF-1)[12], which impact cell fate. In the present work, CO did not increase microglial production and secretion of BDNF or GDNF, while IL-10 release was stimulated by CO. Microglial IL-10 is a key anti-inflammatory regulator, anti-apoptotic and an important synaptogenic mediator *in vivo*[25,78–80]. Accordingly, using *in vivo* acute inflammation model and *in vitro* LPS-stimulated macrophages, CO promoted IL-10 production[39]. Under physiological conditions without inflammatory stimulation, this is the first work showing CO-induced production of IL-10 in microglia. Furthermore, IL-10 promotes neurite outgrowth and synapse formation in OGD (Oxygen-glucose deprivation)-challenged primary cortical neurons[58]. Herein, conditioned medium from CO-treated microglia

increased number of neurites per cell and neurite length. Because IL-10 has a neuromorphogenic role[58], one may speculate that IL-10 is involved in neuronal morphology improvement by reinforcing neurotrophism. Still, further experiments are needed to confirm this link. It can also be hypothesized that neuronal protection against oxidative stress could indicate that microglia, under basal conditions, secrete specific anti-oxidant or scavenging agents. In fact, microglia release extracellular vesicles, which contain enzymes, chaperones, nucleic acids, and metabolites, which can provide a cytoprotective effect on target cells[8].

In summary, neurotrophic factors, anti-oxidant molecules or even anti-apoptotic players can be involved in microglia-induced neuroprotection under physiological conditions.

Taken together, ALF826 is a promising new CO delivery molecule that limits microglial reactivity and secretion of inflammatory mediators and improves the release of neurotrophic factors. Both effects protect neurons against cell death and improve neuronal morphology. In fact and for the first time, it was shown that CO affects basal microglia function, stimulating neurotrophism and remotely protecting neuronal cells against an oxidative stress-induced cell death. Hence, CO regulates neuron-microglia remote communication and promotes neuroprotection in a two-fold non-cell autonomous manner. The novel ALF826 has also proven to be a useful CORM for the reproducible and non-toxic delivery of CO enabling the study of delicate intercellular processes.

ACKNOWLEDGMENTS

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SUPPLEMENTARY FIGURES

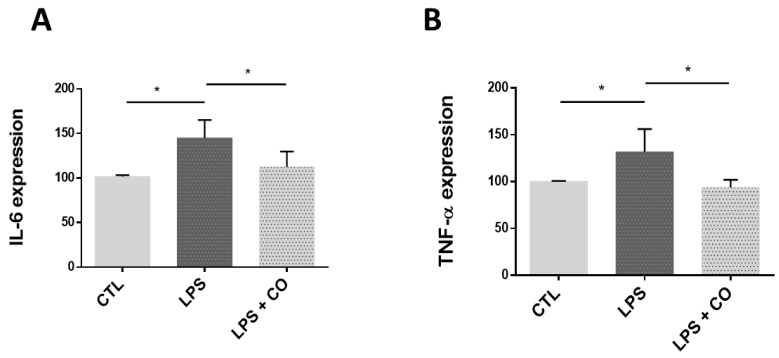

Figure S1 – CO decreases microglial expression of inflammatory cytokines. BV2 microglia expression of inflammatory markers on protein extracts was performed after incubation with CO. Intracellular IL-6 (A) and TNF-α (B) protein levels were quantified via Western Blotting. Cytokine band densitometry was performed and normalized to βIII-tubulin expression, as explained previously. Results are presented as percentage relative to the negative control. *n=3-5*, error bars represent mean ± SEM, *p<0,05 by one-way ANOVA test.

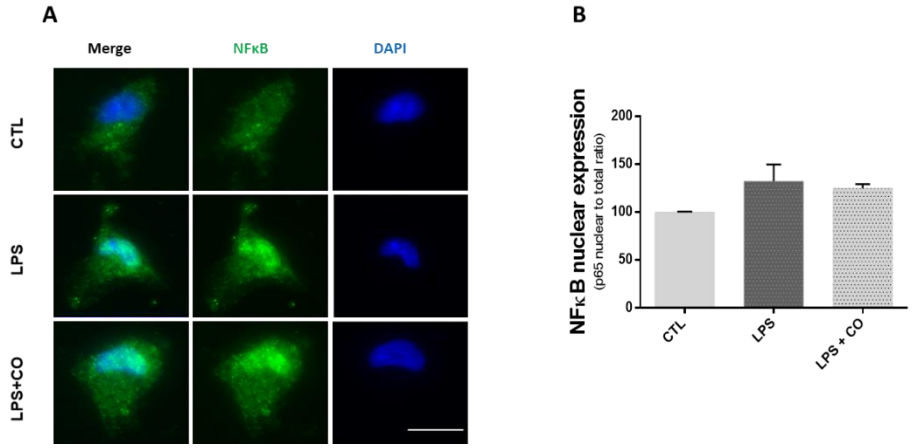

Figure S2 – CO inhibits NF-κB nuclear translocation in primary microglia. Primary microglia were immunostained with NFκB p65 antibody (green) and DAPI (blue) (A). Scale bar = 10µm. p65 nuclear to total ratio was measured following micrograph acquisition (B). Results are expressed as percentage relative to the negative control. *n=3*, error bars represent mean ± SEM, *p<0,05 by one-way ANOVA.

IV

CARBON MONOXIDE AND NEURON-MICROGLIA DIRECT COMMUNICATION: ROLE OF PHAGOCYTOSIS AND INFLAMMATION

This chapter is based on data to be published as:

'Carbon monoxide and neuron-microglia direct communication: role of phagocytosis and inflammation'

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Unpublished data

Nuno L. Soares 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.

ABSTRACT

Microglia are the resident macrophage population in the Central nervous system (CNS). These cells are the engine of innate immunity in the brain, but are also involved in synaptic network development, tissue clearance and support neuronal survival. Microglia inflammatory activity is tightly regulated by the microenvironment and interactions with other glial cells and neurons. Loss of specific cell-to-cell communication pathways can result in exacerbated inflammation, dysfunctional engulfment of live cells and lead to a pathogenic response. Carbon monoxide (CO) is an endogenous gas produced by haem oxygenase that is a stress sensitive enzyme. It is well established that CO presents anti-neuroinflammatory and neuroprotective properties, but CO modulation of the direct microglia-neuron crosstalk is still not explored. Herein, a microfluidic neuron-microglia co-culture system was used to assess CO regulation of cell-to-cell interaction under normal and inflammatory conditions. CO limited microglial secretion of inflammatory mediators (TNF-α, nitrites, IL-1β) and consequently blocked neuronal morphological atrophy and apoptosis. Additionally, LPS inhibited microglia phagocytosis of neuronal post-synaptic content (PSD-95), but not pre-synaptic (VGLUT1). CO reverted this effect, improving microglial synaptic pruning. In contrast, experiments with fluorescent latex beads showed that CO limited the increase of beads internalization by LPS-treated microglia. To further understand whether CO directly modulates phagocytic machinery in a cell autonomous manner or if CO effect on phagocytosis is dependent on other cell signaling, BV2 microglia were incubated with either viable or apoptotic CAD neurons. In fact, CO had a dual role on phagocytosis by: (i) suppressing inflammatory-induced microglia engulfment of viable neurons and (ii) enhancing the clearance of apoptotic neurons. Moreover, CO treatment enhanced expression of neuron-microglia communication axis CD200-CD200R, suggesting a reinforcement of cellular cross-talk. In summary, CO regulates neuron-microglia crosstalk under inflammatory context and promotes a homeostatic cue in microglial phagocytosis. The mechanisms that mediate this CO-driven homeostasis are currently under investigation.

INDEX

BACKGROUND

Neuroinflammation is an inflammatory response in the central nervous system (CNS), which is a tightly controlled process, crucial for pathogen clearance and overall brain homeostasis[1–3]. However, depending on the intensity and duration, neuroinflammation can contribute heavily to the creation of a toxic milieu, which has physiological consequences in the tissue and acts as a major driving factor in ageing and in the pathophysiology of several CNS disorders[4–6]. As life expectancy continues to increase, there is an increasing global burden of neurodegenerative and other chronic disorders.

Microglia are the main orchestrators of inflammation in the CNS[1]. As the 'resident brain immune cells', microglia patrol the parenchyma, secreting nourishing factors and responding to homeostatic imbalances[1]. Upon a damage cue, microglia undergo transcriptional and morphological alterations, producing cytokines, glutamate, reactive oxygen species (ROS), proteases, degrading and clearing compromised tissue and pathogenic agents[1,2,7]. Microglia are also highly phagocytic, removing pathogens and eliminating supernumerary and dysfunctional synapses, which is crucial for neuronal network wiring[1,8,9]. During development, microglia phagocyte apoptotic cells and can induce cell death, either by releasing soluble factors or by directly engulfing immature neuronal precursor cells: phagoptosis[10,11]. Despite being reparative in nature, exacerbated microglial TNF-α (Tumour necrosis factor α) production can activate apoptotic pathways, ROS can attack susceptible cellular constituents and accumulation of glutamate leads to excitotoxicity[12,13]. Moreover, inflammation can disrupt phagocytic mechanisms ('find me', 'eat me' and 'don't eat me' signals), leading to microglia removing viable neurons[14]. This ultimately has lasting consequences in brain cells[1–3,15,16].

Neuroinflammatory homeostasis is largely dependent on microglia exposition to microenvironmental cues, namely to their interaction with other glial cells and neurons[17–23]. Neuron-microglia interactome: a collection of signalling molecules, receptors and ligands, is paramount for the fine tuning of microglia function, with several neuroimmune regulatory pathways acting as immune 'breaks'[18]. Examples of neuron-microglia communication pairs are CD200-CD200R, CX3CL1-CX3CR1 (fractalkine-fractalkine receptor) and CD45-CD45L[24–27]. The CD200-CD200R axis is a pair of transmembrane glycoproteins through which neurons modulate microglia inflammation[28]. Disruption of CD200-CD200R cooperation in rodent models of Experimental Autoimmune Encephalomyelitis (EAE) augments inflammation, which can be partially rescued by overexpressing the neuronal CD200[29]. Interestingly, brains of Alzheimer Disease (AD) patients also display lower expression of the CD200-CD200R pair, with CD200 suppression being more pronounced in regions most affected by AD pathology[30]. These interactome pathways are also crucial for phagocytosis, namely: primary microglia from CD200 deficient mice are more phagocytically proficient[31] whereas knockdown of microglial receptor TREM-2 (Triggering receptor expressed on myeloid cells 2) blocks

phagocytosis of apoptotic neurons and increases secretion of inflammatory cytokines[32]. Likewise, in CX3CR1 knockout mice synaptic pruning in the hippocampus was hampered, which caused alterations in network excitatory functions[9]. Altogether, understanding the underlying molecular mechanisms of neuron-microglia communication opens new doors to novel anti-neuroinflammatory and neuroprotective therapeutic approaches targeting non-cell autonomous processes.

Carbon monoxide (CO) is a gaseous molecule, endogenously produced by free haem recycling, in a reaction catalysed by haem oxygenase (HO)[33]. CO has a key role in cellular homeostasis by regulating inflammation, apoptosis, cell metabolism and differentiation processes[34–38]. Due to its pharmacological potential, delivery strategies have been developed to safely deliver CO in biological contexts. CO-releasing molecules (CORMs) are organic or organometallic pro-drugs which release CO under specific physiological conditions. Herein, ALF826, which is a newly developed molecule based on molybdenum, is used to study CO modulation of microglia-neurons communication. ALF826 is non-toxic to RAW264.7 macrophage cells at 100µM at 24h (98% survival). It spontaneously releases CO with a half-life of 37 min in Hepes 7.4 buffer, defining half-life as the time needed to release 0.5 equivalents of CO out of a load of 4 equivalents per mole. Moreover, its available CO load enables slow, steady, delivery of > 3 equivalents of CO per mole (Information provided by Proterris (Portugal) Lda.). Molybdenum reacts rapidly with oxygen forming molybdate ion ($MoO4^{2-}$), which is very soluble in water and can be rapidly eliminated from the body, a key feature for safety issues.

The anti-inflammatory role of CO was first described in macrophages *in vitro*, modulating cytokine secretion[39], TLR-4 (Toll-like receptor 4) signalling[40] and mechanisms of host-bacteria interaction[41]. In microglia, *in vitro* CORM-3 administration suppresses secretion of inflammatory mediators, such as NO (nitric oxide) and TNF-α[42–44]. Moreover, CO impacts overall neuroinflammatory homeostatic tissue balance, disease progression and outcome in *in vivo* models such as haemorrhagic stroke[45] and EAE[46]. Previous work of our group has demonstrated that CO presents a non-cell autonomous effect. In fact, CO protected neurons against cell death by targeting astrocytic metabolism and modulating neuron-astrocyte communication *via* reinforcement of purinergic pathways[47]. Recently, we have also described that CO can impact neuron-microglia remote communication: CO modulates microglia secretome, which provides neuronal protection. This occurred by limiting microglial exacerbated inflammatory output (TNF-α, nitrites) and by reinforcing neurotrophic signalling (Interleukin-10) in basal conditions (manuscript under final preparation – Chapter III). Herein, we focused on understanding if CO regulates neuron-microglia direct communication, disclosing which are the CO-regulated interactome pathways, and their consequences on phagocytic homeostasis and overall inflammatory regulation.

METHODS

Materials

All cell culture plastics were purchased by Corning (Corning, NY) and Sarstedt (Sarstedt, Germany). Chemicals of analytical grade were bought from Sigma-Aldrich (St Louis, MI) unless stated otherwise.

Primary cell cultures

Animals were housed in standard laboratory conditions with free access to water and standard rodent chow. All animal experiments were performed with the approval of the i3S Animal Ethics Committee and in accordance with European Union Directive on the protection of animals used for scientific purposes (2010/63/EU) and the Portuguese Law that transposes it (Decreto-Lei n.º 113/2013). All efforts were made to minimize animal suffering.

Neuron hippocampal cell cultures were obtained from embryonic day 18-19 Wistar rats. Pregnant females were euthanized in a CO_2 chamber, their abdominal region was pulverized with 96% ethanol and opened using surgical scissor and tweezers. Embryos were retrieved, decapitated and the brains were placed in a petri dish containing Hank's Balanced Salt Solution (HBSS, Thermo-Fisher Scientific) supplemented with 100 µg/mL Gentamicin (Lonza). The brains were dissected under a magnifying glass and the hippocampi were collected. Hippocampal tissue was incubated for 15 minutes at 37°C with 1.5 mg/mL of trypsin (Thermo-Fisher Scientific) and after allowing the hippocampi to sediment, the supernatant was removed and a 10 mL HBSS + 10% foetal bovine serum (FBS, Thermo-Fisher Scientific) was added. Following gentle agitation, the supernatant was again discarded, and a washing step was performed with fresh HBSS. The supernatant was removed and 5mL of Neurobasal medium (Thermo-Fisher Scientific) supplemented with 25 µM Glutamate (Sigma-Aldrich), 0.5 mM L-glutamine (Thermo-Fisher Scientific), 100 µg/mL Gentamicin (Lonza) and 1% B27 supplement (Thermo-Fisher Scientific) was added, and the tissue was dissociated by thorough pipetting. The suspension was then put through a cell strainer, collected and cells were counted with a Neubauer counting chamber.

Mixed glial cell (MGC) cultures were obtained from cortices of 7-day old Wistar pups. Animals were quickly sacrificed, with brains being removed and dissected consequently in HBSS with penicillin (100 units/mL) and streptomycin (100 µg/mL). White matter and meninges were discarded, and the remaining tissue was collected and mechanically homogenized. The cell suspension was passed through a gauge syringe and incubated with 0.1 U/mL DNAse I (Zymo) and 1.5 mg/mL trypsin (Thermo-Fisher Scientific) for 15 minutes at 37°C. After incubating, trypsin was inactivated by adding DMEM GlutaMAX (Thermo-Fisher Scientific)

supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL) (Thermo-Fisher Scientific), 10% FBS (Thermo-Fisher Scientific) and the suspension was centrifuged afterwards (550g, 15 minutes). The resulting supernatant was carefully discarded, and the pellet was resuspended in 10mL of the same medium and put through a 100 µm cell strainer. The resulting suspension was distributed equally into 75cm^2 t-flasks (approximately 2 brain per flask) to make a total media of 10mL per flask. Prior to adding the cells, the t-flasks were coated with 1 mg/mL PDL (Poly-D-Lysine, , Sigma-Aldrich) for 1 hour. The cell medium was changed every other day.

Primary culture of microglia was obtained from 10-day-old MGC cultures, which were placed on an orbital shaker incubator for 2h at 200 rpm. The cell suspension was collected, centrifuged (269 rcf, 10 minutes) and the supernatant was discarded. The pellet was resuspended in DMEM/F12 (Thermo-Fisher Scientific) and cells were counted using a Neubauer counting chamber. Microglia cells were always obtained from either 10 (shake 1) or 17 DIV (days *in vitro*) (shake 2) MGC cultures. Older cell cultures were not considered for this.

Microfluidic cell culture chambers

AXIS™ Axon Isolation microfluidic cell culture devices were purchased from Merck (AX15005PB). The devices are fabricated from a Polydimethylsiloxane polymer (PDMS) and have a design that consists of four separate wells, two main reservoirs and a set of microgrooves. The wells are paired, connected by the reservoirs that are opposite of each other and connected by the microgrooves. The grooves, centrally located, have a 150 µm length x 5 µm x 10 µm width. Each device has approximately 120 individual grooves.

Prior to use, microfluidic devices were sterilized and mounted on top of PDL-coated glass coverslips. One day after, hippocampal neurons were seeded on one of the central reservoirs at a concentration of 100 000 cells/microfluidic, whereas the opposite side was filled with fresh culture media. Cells grew for 10 days in microfluidic chambers, allowing for neural network formation and expansion. Neurobasal medium (Thermo-Fisher Scientific) supplemented with 0.5 mM L-glutamine (Thermo-Fisher Scientific), penicillin (100 units/mL) and streptomycin (100 µg/mL) (Thermo-Fisher Scientific) and 1% B27 supplement (Thermo-Fisher Scientific) was added to microfluidic culture every three days on the neuronal side. After 10 DIV, the opposite chamber was emptied, and MGC-derived microglial cells were plated at the same seeding concentration, using DMEM/F-12 GlutaMAX (Thermo-Fisher Scientific) with 4% L-glutamine (Sigma-Aldrich), penicillin (10 units/mL), streptomycin (10 µg/mL) (Thermo-Fisher Scientific) and 10% FBS supplement (Thermo-Fisher Scientific) as growing medium. Microglia adhered for 24 hours before any treatment was performed. Cells were subsequently treated with ALF826 (50µM) for 24h and the microglia reservoir was then incubated with LPS (Lipopolysaccharides, 10 ng/mL, Sigma-Aldrich) for an additional 24h. One day later, supernatant from microglia reservoir and coverslip adhered cells were collected for further analysis.

Cell line cultures

BV2 murine microglia cell line was kindly provided by Dr Ana Raquel Santiago (Faculdade de Medicina da Universidade de Coimbra). Cells were grown in RPMI-1640 medium (Sigma-Aldrich), supplemented with 10% FBS, (Thermo-Fisher Scientific), 4 mM L-glutamine (Thermo-Fisher Scientific), penicillin (100 units/mL) and streptomycin (100 µg/mL) (Thermo-Fisher Scientific). CAD mouse catecholaminergic neuronal cells were gently provided by Dr Federico Herrera (Faculdade de Ciências da Universidade de Lisboa). DMEM/F12 was used as basal medium (Thermo-Fisher Scientific) and supplemented with 8% FBS (Thermo-Fisher Scientific), penicillin (200 units/mL) and streptomycin (200 µg/mL) (Thermo-Fisher Scientific). For differentiation, the growing medium was removed and substituted by a serum free DMEM/F12 supplemented solution. Both cell lines were maintained in a humidified incubator at 37°C and 5% CO_2.

Reagents and solution preparation

ALF826 was provided by Proterris (Portugal) Lda. Stock solutions of 2.5mM were prepared by dissolving the compound in Dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluting it 1/10 in a $NaHCO_3$ solution (0.1mM, pH 8.3). ALF826 was then filtered, aliquoted and stored at -80°C at a final concentration of 2.5 mM.

Immunofluorescence microscopy

Cells growing on glass coverslips were fixated with 4% (v/v) PFA (paraformaldehyde) and 4% (w/v) sucrose solution (20 minutes at room temperature - RT), permeabilized with 0.3% (v/v) Triton X-100 solution (15 minutes, RT) and then blocked with Bovine Serum Albumin (BSA, Merck) 1% (w/v) and Triton X-100 0.1% (w/v) for half hour at RT. Later, cells were probed with primary (2h incubation) and secondary (1h) antibodies, as described in Supplementary Table 1. Nuclei were stained with Hoechst 33342 (1:2000, Thermo-Fisher Scientific) and coverslips were mounted onto glass slides with Glycergel mounting medium (Dako). Washing with ice-cold PBS was always performed between steps. All antibodies used were diluted in 1%(v/v) BSA and 0,1%(v/v) Triton X-100 solution. Fixating, permeabilizing and blocking solutions were prepared in PBS. Images were captured with Z2 Axio Imager microscope (Zeiss), with at least 5 random micrographs being acquired, unless otherwise stated. Some samples were analysed under an LSM 710 confocal microscope (Zeiss).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to measure TNF-α (Tumour necrosis factor α) and IL-1β (Interleukin-1β) microglia supernatant levels, using Murine TNF-α and IL-1β Standard ABTS ELISA Development Kits (PeproTech).

Microglia culture media was collected and centrifuged to remove cellular debris, and the pellet was discarded. The resulting supernatant was kept at -80°C until analysis. All experiments were performed in accordance with the respective manufacturer's instructions. Absorbance values were measured at 405nm, with wavelength correction set at 650nm, using an Infinite F200 PRO microplate reader (Tecan) for 50 minutes, with readings every 5 minutes.

Griess reaction assay

Nitrite microglia supernatant levels were assessed by performing the Griess reaction colorimetric test. Microglia culture media was collected and centrifuged to remove cellular debris and subsequently incubated with Griess reagent (1:1 ratio, Sigma-Aldrich) for 10 minutes at RT, protected from light. Absorbance of the resulting solution was measured at 540 nm using an Infinite F200 PRO microplate reader (Tecan). Nitrite concentration was calculated with reference to a standard curve constructed with known concentrations of sodium nitrite ($NaNO_2$, Sigma-Aldrich).

Phagocytosis assays

Fluorescent latex beads assay

Primary culture of microglial cells derived from 10 or 17 DIV MGC was plated on 24 multi-well plates (200.000 cells/well), adhering to Poly-D-Lysine (1 mg/mL, Sigma-Aldrich) coated glass coverslips. After one day of adhesion, cells were treated with ALF826 (50µM, 24h) and subsequently with LPS (10 ng/mL, 24h). Microglia were afterward incubated with 1% 0.5 µm carboxylate-modified polystyrene latex fluorescent beads for 30 minutes at 37°C. Afterwards, medium was removed, and cells were washed twice with ice-cold PBS. Microglia were then labelled with Iba-1 (Ionized calcium-binding adapter molecule 1) and the nucleus was stained with Hoechst 33342 as described in 'Immunofluorescence microscopy'. For calculation of Phagocytic efficiency, the following formula, described in [48] was considered, with *xn* representing the number of cells containing *n* beads. Cell size and circularity were also calculated. All image analysis was done using the Fiji software (FlowJo).

$$\text{Phago Eff.}\,(\%) = \frac{(1 \times x1 + 2 \times x2 + 3 \times x3 \ldots + n \times xn)}{\text{Total number of cells}} \times 100\%$$

Neuronal engulfment assay

Cell treatment

BV2 cells were seeded onto multi-well plates (6×10^4 cells per well), treated with ALF826 (50µM) for 24h and with LPS (500 ng/mL) for a subsequent 24h. Following 48h of differentiation, CAD neurons were treated or not with 20 µM of *t*-BHP (tert-Butyl hydroperoxide, Sigma-Aldrich) for 24h, before staining with 5-(and-6)-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA; Thermo-Fisher Scientific) 25 µM, rinsed three times with warm PBS, resuspended and seeded on top BV2 culture (2:1 microglia to neuron ratio), which were stained with microglia specific Isolectin B4 – Alexa488, prior to this step.

Immunofluorescence microscopy

After 3h, cultures were rinsed with PBS twice to remove excess cells and live cells were imaged under an Axiovert 40 microscope (Zeiss). For each individual experience, six images were obtained for each condition, with the ratio of 5-TAMRA$^+$ BV2 microglia to total BV2 cells being quantified. All image analysis was performed using the Fiji software (ImageJ).

Flow cytometry

Cells were collected from individual wells, washed twice with PBS and incubated at 37°C for 10 minutes. Using a FACS Canto II (BD Biosciences) cytometer, sample fluorescence was measured in the FL-1 and FL-3 channels. 488nm laser line was used for excitation. To exclude CAD cells, debris and other events, FL-1 negative events were gated out of the analysis. Phagocytosis levels were analysed by quantifying FL-3 mean fluorescence intensity of FL-1 positive samples (microglia). Appropriate controls, such as positive staining controls and unstained samples were always carried out, allowing adjusting auto-fluorescence levels and compensation parameters. Post-acquisition data analysis was performed with the FlowJo software (BD Biosciences).

To assess CAD neuronal viability after *t*-BHP treatment, cells were stained with 5-TAMRA at 25 µM. After 24h of *t*-BHP incubation, neurons were collected by trypsinization and stained with 1ng/mL of propidium iodide (PI, Thermo-Fisher Scientific) for 15 minutes at 37°C. PI was analyzed in the FL-3 channel in the linear scale to assess cell viability.

Immunoblotting

Cell extracts were washed several times with ice-cold PBS and lysed with RIPA buffer (Radioimmunoprecipitation assay buffer, 50 mM Tris-HCl, pH 6.8, 50 mM NaCl (w/v), 0.1% SDS (w/v), 1% sodium deoxycholate (w/v), 1% Triton X-100 (v/v), 10% Glycerol (v/v), 1% protease inhibitor cocktail (v/v). Protein concentration was determined with a Pierce BCA Protein Assay Kit (Thermo-Fisher Scientific), following manufacturer's instructions. Absorbance values were registered at 560 nm using an Infinite F200 PRO microplate reader (Tecan). A BSA (Merck) standard curve was constructed to determine protein concentration.

Equal amounts of protein were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a polyacrylamide gel (10% gel unless stated otherwise), with a NZYColour Protein Marker II (NZYtech) being used as band size reference. Proteins were then electrically transferred onto an Amersham Protran 0.45 NC nitrocellulose membrane (GE LifeSciences) and blocked with 5% (m/v) BSA in T-TBS for 1h. Membranes were labelled consecutively with primary and secondary antibodies as indicated in Supplementary Table 1. Immunoblots were exposed to ECL Clarity Western Detection Reagent (Bio-Rad) 5 minutes and the reactive bands were detected after the membranes were exposed to X-ray film (Chemidoc Touch Imaging System, Bio-Rad). The resulting area and intensity of the bands were quantified with ImageLab software (Bio-Rad). β-actin was used as internal loading control unless stated otherwise.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Data normality was tested using the Shapiro-Wilk normality test. The Kruskal-Wallis test, followed by Dunn's multiple comparison test, were utilized to compare different conditions and a 95% confidence interval was considered. All statistical analysis was performed using the Prism 6.0 software (GraphPad).

Supplementary Table 1 - List of primary and secondary antibodies used in this work.

Primary Antibody	Dilution	Host	Manufacturer	Technique
CD200	1:1000	Goat	R&D Systems (AF2724)	Immunoblotting
CD200R	1:1000	Goat	Santa Cruz Biotech (sc-14392)	Immunoblotting
β-actin	1:5000	Mouse	Sigma-Aldrich (A5441)	Immunoblotting
βIII-tubulin	1:1000	Mouse	Sigma-Aldrich (T8660)	Fluorescence Microscopy
Iba-1	1:500	Rabbit	Wako (019-19741)	Fluorescence Microscopy
PSD-95	1:2000	Mouse	Thermo Fisher (MA1-045)	Fluorescence Microscopy
VGLUT1	1:1000	Rabbit	Synaptic Systems (135302)	Fluorescence Microscopy
NF-κB p65	1:1000	Mouse	Santa Cruz Biotech (sc-8008)	Fluorescence Microscopy

Secondary Antibody	Dilution	Host	Manufacturer	Technique
ECL Anti-mouse IgG	1:5000	Sheep	GE LifeSciences (NA931V)	Immunoblotting
ECL Anti-rabbit IgG	1:5000	Donkey	GE LifeSciences (NA934V)	Immunoblotting
HRP Anti-goat IgG	1:5000	Rabbit	Abcam (ab6741)	Immunoblotting
Alexa 488 anti-rabbit IgG	1:1000	Goat	Thermo Fisher (A11008)	Fluorescence Microscopy
Alexa 488 anti-mouse IgG	1:1000	Goat	Thermo Fisher (A11001)	Fluorescence Microscopy
Alexa 594 anti-rabbit IgG	1:1000	Goat	Thermo Fisher (A11012)	Fluorescence Microscopy
DyLight 650 anti-goat IgG	1:1000	Donkey	Abcam (ab98517)	Fluorescence Microscopy

RESULTS

Carbon monoxide provides neuroprotection in neuron-microglia communication model by targeting inflammation

To study the role of carbon monoxide (CO) on neuron-microglia cell-to-cell communication, a microfluidic cell culture system (Figure 1A) was used. This device features four different wells, which are connected in pairs by two main cellular reservoirs, which in turn are connected to each other by a central microgroove system. Microfluidic cell culture allows greater experimental flexibility in a very controlled microenvironment, both physically and chemically[49]. This type of system is more tailored to single cell analysis than macroscopic cell culture, making it more robust in studying cell-to-cell interactions.

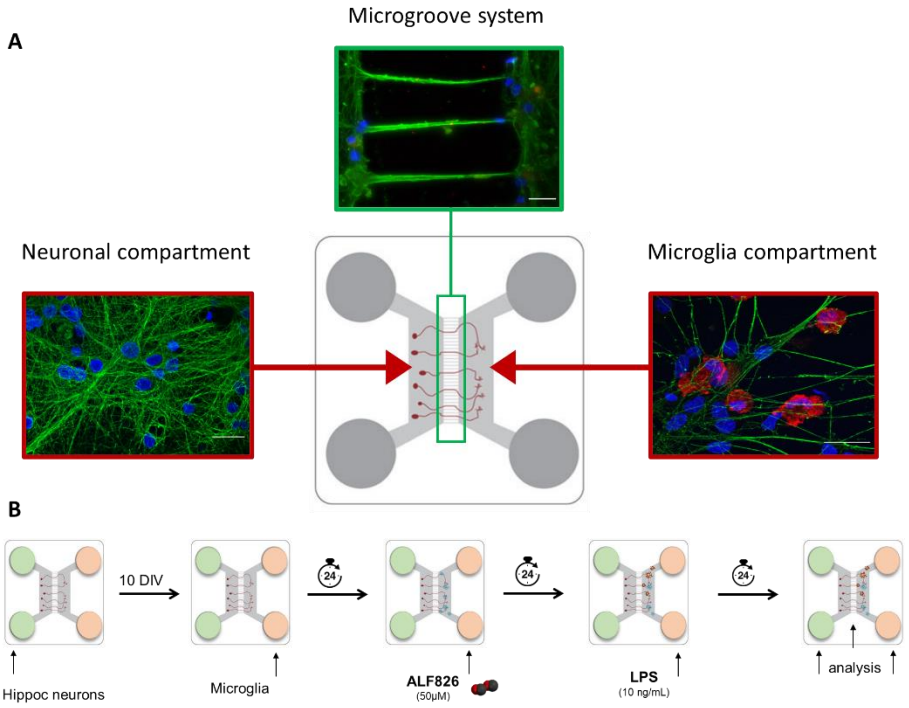

Figure 1 – Neuron-microglia microfluidic co-culture system. Schematic representation of the microfluidic device (A, Scale Bars = 25 µm), highlighting the two opposing compartments, where cell somas are localized and the 150 µm long microgroove region, through which neuronal processes span across and into the microglia reservoir. (B). Neurons were immunostained with βIII-tubulin antibody (green) and microglia were stained with Iba-1 antibody (red). Nuclei are shown in blue (Hoechst 33342). Experimental protocol: neurons were cultured in Poly-D-Lysine (PDL)-coated coverslips attached the individual microfluidic devices (left compartments); after 10 days microglia were added into the opposite compartment (right one), and allowed to adhere for 24h. Microglia were subsequently treated with ALF826 (50 µM) for 24 hours and with LPS (Lipopolysaccharide, 10 ng/mL) for another 24 hours.

Primary culture of hippocampal neurons was plated on the microfluidic devices, on one of the reservoirs. Growing neurons for 10 DIV (Days *in vitro*) allowed neuronal maturation and formation of a

neuronal network and neurite expansion through the microgrooves. Then primary culture of microglia was plated on the opposite reservoir and allowed to adhere for 24h. ALF826 at 50 µM was administered for 24h at microglial compartment, followed by the addition of LPS at a final concentration of 10 ng/mL for another 24h (Figure 1B). Then, coverslips were stained with Hoechst 33342 (nuclei, in blue), and with antibodies against βIII-tubulin (green, neurons) and Iba-1 (red, microglia) for characterization of cell culture and for the assessment of cell death. Neuronal apoptosis was quantified by counting chromatin condensed nuclei at the neuronal reservoir. Whenever microglia were incubated with LPS, there was a decrease in neuronal cell survival (Figure 2A and D), which is partially reverted if microglia are treated with ALF826 24h prior to the inflammatory stimulus. Likewise, in order to indirectly assess neuronal function, neuronal network integrity was analysed in both the main neuronal reservoir and the grooves (closer to neuron-microglia interaction). While the differences at the neuronal chamber are inconclusive, the neuronal processes at the microgrooves in LPS-treated condition displayed a partial atrophy, which was absent in all other conditions (Figure 2B). Since inflammation can present a strong neurotoxic effect, we subsequently quantified microglial inflammatory activity within the microfluidic co-culture.

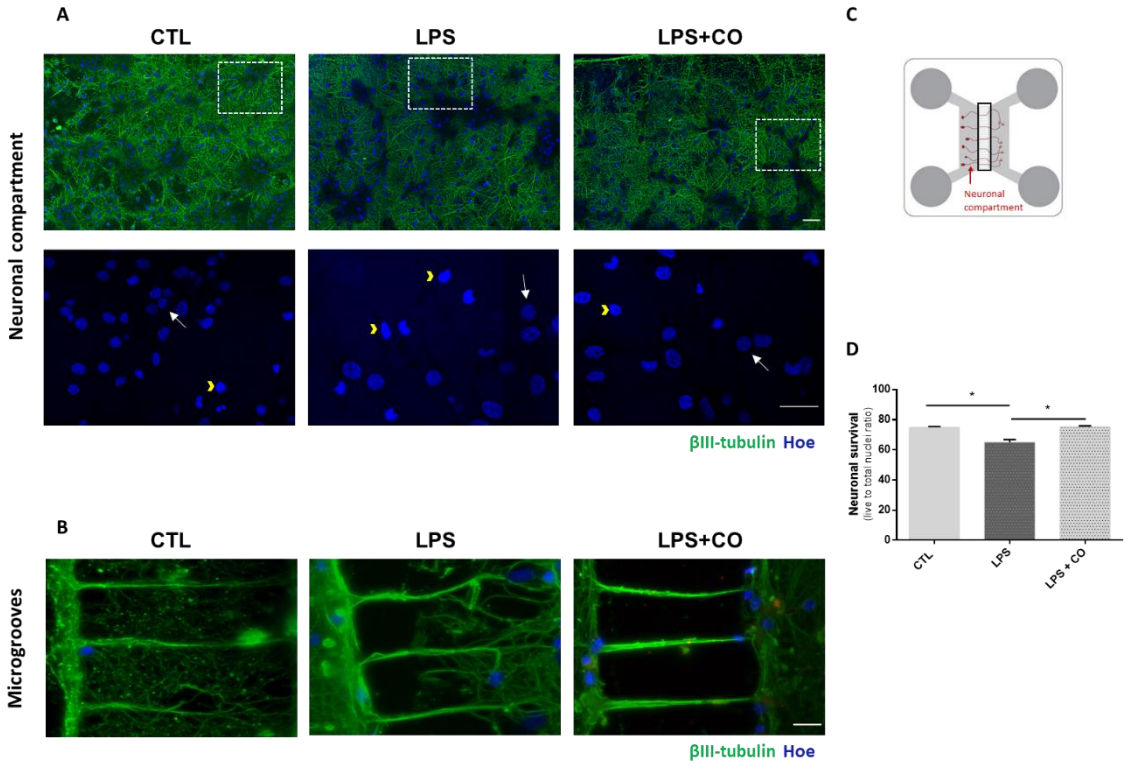

Figure 2 – Carbon monoxide (CO) treatment in a neuron-microglia microfluidic culture system limits neuronal death triggered by LPS-induced inflammation. Primary rat hippocampal neurons and microglia were cultured in microfluidic devices and immunostained with βIII-tubulin (green) and Iba-1 antibodies (red, not shown). Nuclei are shown in blue (Hoechst 33342). Micrographs of hippocampal neuronal network in the main neuronal reservoir (A, Scale Bar = 50 µm). White arrows highlight the nucleus of viable neurons and yellow arrowheads highlight the nucleus of dead neurons. Neurite outgrowth in the microgroove region (B, Scale Bar = 25 µm). Schematic representation of the microfluidic device (C), highlighting the neuronal compartment regions. Apoptotic and total number of nuclei in the neuronal reservoir were counted and the ratio was calculated and shown in (D). *n=3*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

Previously, we have shown that CO can impact remote neuron-microglia communication and cell survival by modulation of inflammatory secretome (manuscript under final preparation – Chapter III). Thus, we next collected supernatant from microglia microfluidic compartment to measure nitrites (an indirect indicator of nitric oxide) and pro-inflammatory cytokines IL-1β (Interleukin-1β) and TNF-α (Tumour necrosis factor α). LPS treatment elicited an increase in nitrites, IL-1β and TNF-α (Figure 3A, B and C), while ALF826 limited the secretion of these three soluble pro-inflammatory factors by microglia, although the levels of IL-1β are not significantly different.

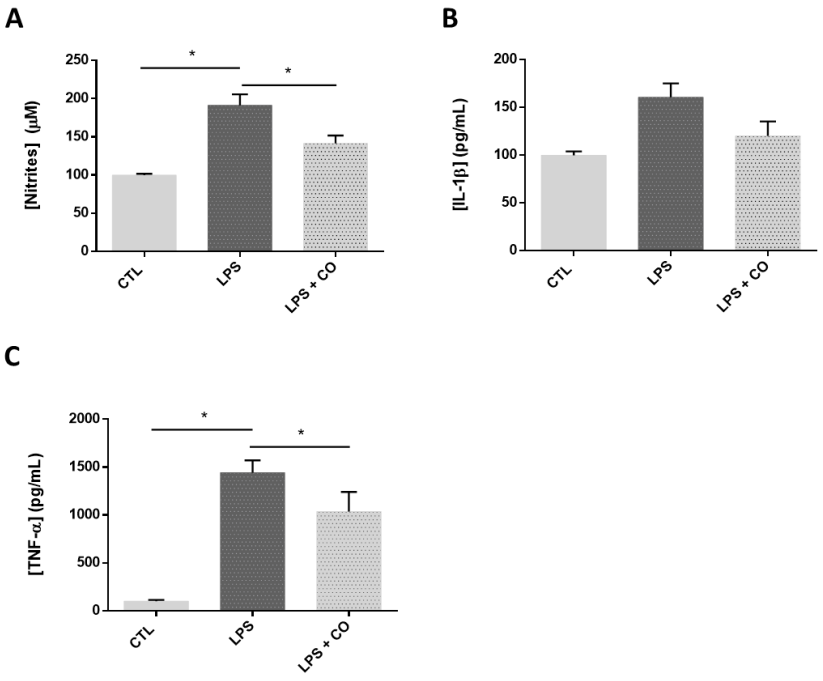

Figure 3 – CO attenuates the inflammatory profile of microglial secretome in a neuron-microglia microfluidic setting. Supernatant from primary microglia growing in a microfluidic device was collected for assessment of soluble inflammatory factors. Nitrite levels were quantified via Griess reagent colorimetric assay (A) and secretion of TNF-α (B) and IL-1ß (C) was done by ELISA. All results are expressed as percentage relative to the negative control. *n=3-4*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA.

To further assess if CO limits inflammation through the activity of the NF-κB (Nuclear factor κ-light-chain-enhancer of activated B cells) signalling pathway, we quantified NF-κB nuclear translocation (Figure S1). However, the ratio between nuclear and total intensity was unaltered upon LPS treatment. These results are inconclusive and further experiments are needed.

In summary, CO limits microglia-mediated inflammation in a co-culture system featuring direct cell-to-cell contact, preventing neuronal apoptosis and morphological breakdown. Further experiments need to be performed to assess which neuroimmune specific pathways are regulated by CO.

CO directly modulates microglia synaptic pruning in a neuron-microglia communication model

Some neurons and neurites can grow from neuronal compartment into the microgrooves and sparsely populate the microglial reservoir, which allows assesing more intimate neuron-microglia communication. In the microglia reservoir, cells were stained with Iba-1 (Ionized calcium-binding adapter molecule 1) antibody for labelling microglia and neurons were stained with either synaptic proteins PSD-95 (Postsynaptic density protein 95), VGLUT1 (pre-synaptic marker Vesicular glutamate transporter 1) or βIII-tubulin for the assessment of neuron-microglia interaction.

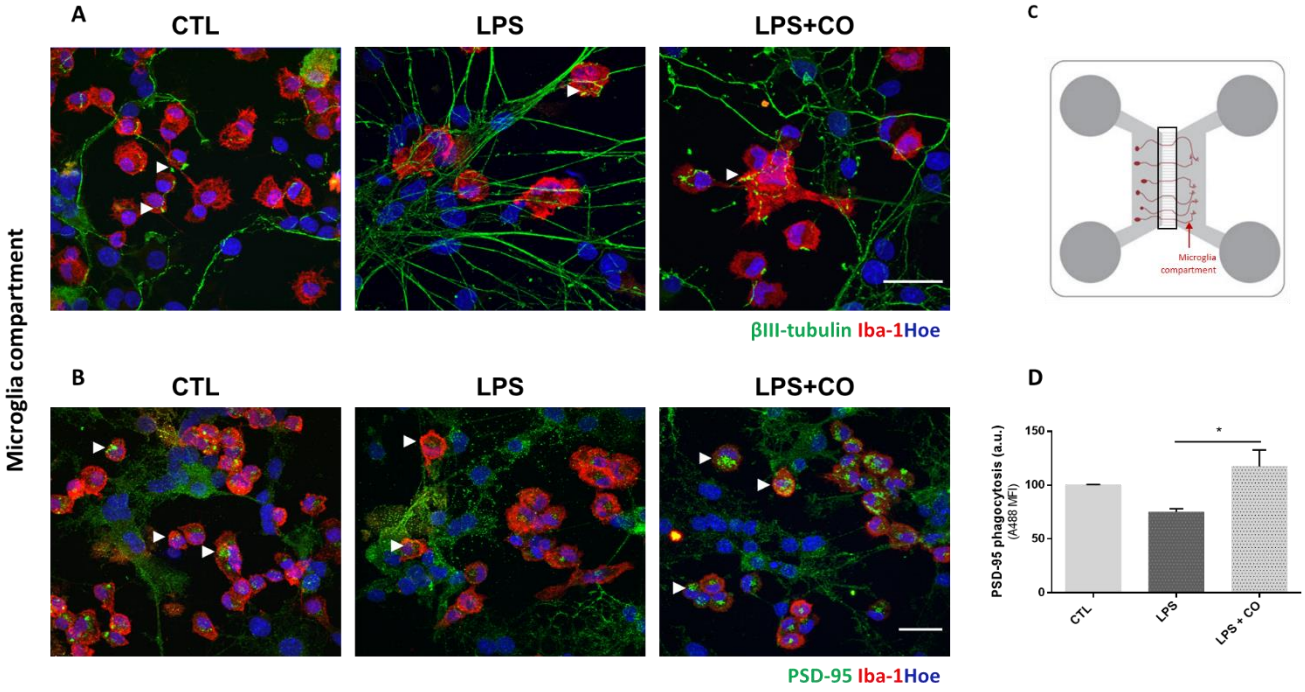

Figure 4 – CO stimulates microglia phagocytosis of post-synaptic material in a neuron-microglia microfluidic culture system. Primary rat hippocampal neurons and microglia were cultured inside microfluidic devices. Microglia were labeled with Iba-1 (red) and neurons were stained with either βIII-tubulin (A) or PSD-95 (B) antibodies, both in green. Hoechst 33342 (blue) was used as nuclear staining. The microglia reservoir (neuronal axonal side) was photographed under a fluorescence microscope. Scale bars = 25 µm. In (A), arrowheads point to microglia close nibbling/contact with neuronal processes. Arrowheads in (B) highlight microglia phagocytosis of synaptic content. Phagocytosis of PSD-95 was quantified by calculating microglia Alexa Fluor-488 mean fluorescence intensity (D). Schematic representation of the microfluidic device (C), highlighting the microglial compartment regions. Data is presented in (D) as percentage relative to the untreated condition. n=3, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA.

Arrowheads in Figure 4A show microglia in close contact with βIII-tubulin stained neurites, appearing to partially engulf some neurites. At the same region, microglia in contact with neuronal processes engulfed post-synaptic content (PSD-95) in Figure 4B. This is not unexpected, since these immune cells have an active role in synaptogenesis, synaptic pruning and overall plasticity[8,9]. Furthermore, LPS treatment caused a

decrease in the capacity of microglia engulfment of synaptic material (Figure 4B), showing that inflammation may perturb synaptic pruning. This effect was reverted when microglia were pre-treated with ALF826 (Figure 4B and D). The results indicate that CO can have an active role on governing microglia communication with neurons, regulating engulfment of synaptic material. Interestingly, microglial populations were shown to not engulf any vGlut-1 (Figure S2), regardless of the experimental condition. This could indicate that CO regulation of neuron-microglia communication and pruning is specific to the nature of the synapse. This effect needs to be clarified by performing additional experiments with other synaptic markers, to confirm the robustness of these data.

Nevertheless, one lingering question here is whether CO modulates cell autonomous microglial phagocytosis machinery or CO regulates neuron-microglia intracellular signalling.

CO has a dual, quality control effect on microglia phagocytosis

In order to assess whether CO directly modulates microglial phagocytosis independent on neuronal presence, 10 or 17 DIV primary culture of microglia were cultured in 24-well plates, treated with ALF826 and LPS, as previously described and then treated with fluorescent latex beads for the direct assessment of phagocytosis (Figure 5A). LPS stimulus enhanced primary microglia phagocytic ratio comparatively to control, while ALF826 treatment reduced this effect (Figure 5B). Analysis of morphometric parameters also showed differences: LPS stimulation increases microglia cell size, which is limited by CO presence (Figure 5C). Moreover, LPS lowered cell circularity index, indicating a higher morphological complexity, but CO did not revert the decrease on circularity index (Figure 5D). Contrary to LPS effect on microglial engulfment of synaptic material, in this case, pro-inflammatory stimulation increased the phagocytic level of beads, while ALF826 treatment reverted the LPS-induced phagocytosis (Figure 5A and B).

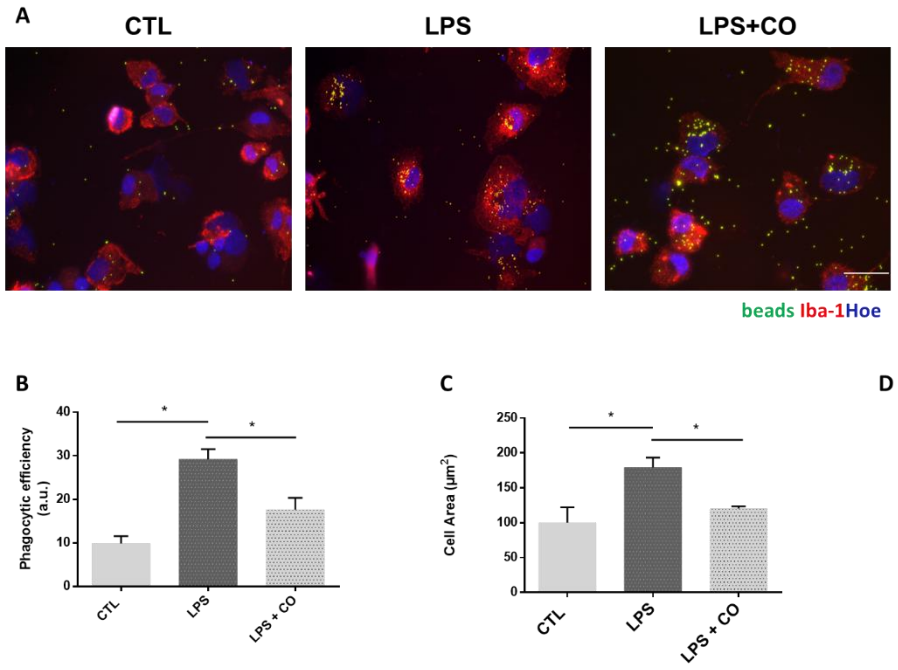

Figure 5 – Microglia phagocytic capacity is limited by CO treatment. Primary microglial cells were cultured and incubated 30 minutes with fluorescence latex beads before being washed, stained and photographed under a fluorescence microscope. Microglia are stained in red (Iba-1) with blue nuclei (Hoechst 33342) and incorporated green fluorescence latex beads (A). Scale bar = 25 µm. Phagocytic efficiency was calculated using a formula already described (B). Additionally, microglia area (C) and circularity index (D) were also calculated for all experiments. Data in (C) is shown as a percentage relative to the negative control. *n=5*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA.

Taken together, CO modulation of phagocytosis seems to be much context dependent, particularly tied to the nature of target and target recognition system. These results could indicate that CO, *per se*, does not improve microglia phagocytic function, and that there might be regulating microglia-inherent pathways that controls cell and environmental interactions and phagocytosis.

Another phagocytosis model with BV2 microglia and CAD neuronal cell lines was used to understand whether CO's effect on microglia engulfment is context-dependent (Figure 6A). Monocultures of BV2 cells were treated with ALF826 for 24h, followed by treatment with LPS for more 24h. Simultaneously, CAD neurons were grown in monoculture for 48h and subsequently treated or not with *t*-BHP (*tert*-Butyl hydroperoxide), a pro-oxidant molecule that promotes cell death, for 24h and then stained with 5-TAMRA. After washing steps, both cell types were co-cultured for 3h in order to analyse microglial phagocytosis of live neurons or t-BHP-challenged neurons, which contained a mixture of viable neurons (25.6±5.9%), dead neurons (88.1±2.3%) and neuronal debris (Figure S3).

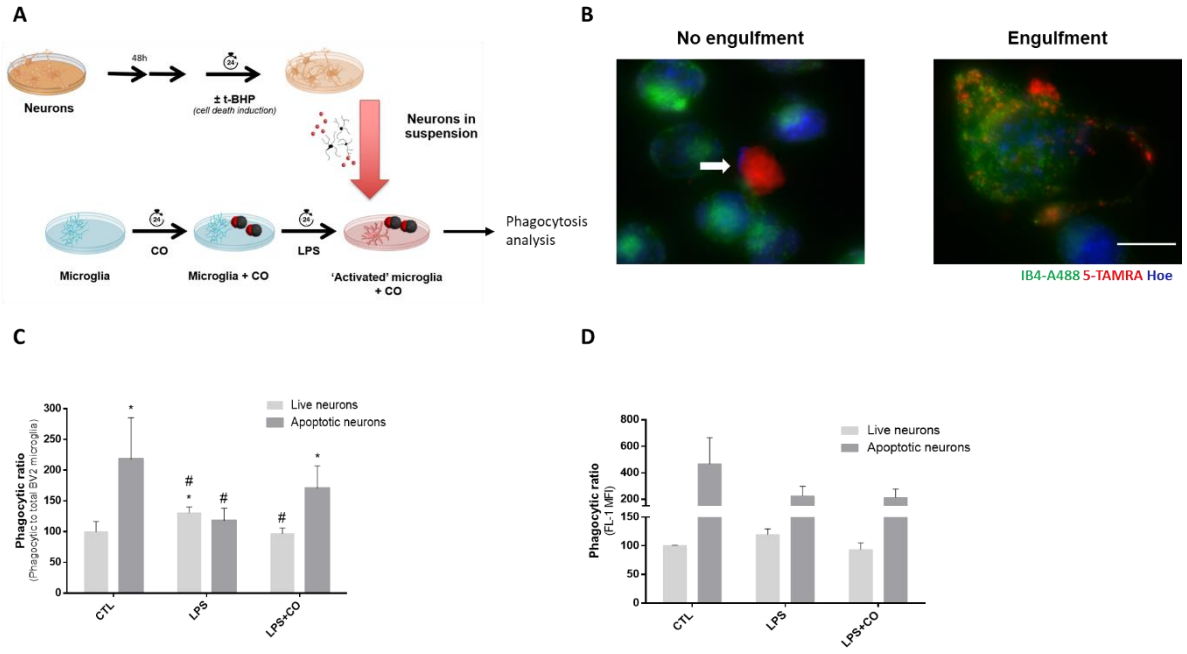

Figure 6 – CO administration has a dual, context dependent modulatory effect on microglia engulfment of live and apoptotic neuronal cells. BV2 microglia were treated with ALF826 (50 µM) and LPS (500 ng/mL) for 24 hours each, and subsequently co-cultured with a cell suspension of differentiated CAD neurons that were previously treated with a cell death inducer (*t*-BHP) (A). After 2h of incubation, cells were photographed using a fluorescence microscope (B), with microglia stained in green (Alexa Fluor-488 conjugated Isolectin B4), neurons in red (5-TAMRA) and nuclei in blue (Hoechst 33342). Microglia phagocytic ratio was calculated *via* both fluorescence microscopy analysis (C) and flow cytometry (D). For microscopy, engulfment of neuronal content was considered only when observing colocalization of red, puncta-like events with microglia, as exemplified by the arrowhead in (B). For flow cytometry, this quantification was performed by registering FL-3 median channel shifts for FL-1^{+} events. All data is presented as percentage using the negative control as reference. *n=5-6*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA.

In fluorescence microscopy-based analysis, phagocytic ratio was calculated as the number of engulfing microglia (red labelled with puncta-like co-localization) divided by total BV2 number (Figure 6B and C). Microscopy analysis indicated that LPS administration increases microglial engulfment of viable neurons, which is inhibited by ALF826 pre-treatment (Figure 6C), reverting phagocytic ratio back to control levels. For microglial phagocytosis of neurons under apoptotic conditions, there was an expected increase of engulfment of dying neurons by microglia (Figure 6D). Starkly, LPS treatment limited clearance of apoptotic neurons while ALF826 pre-treatment, reverted this effect, increasing phagocytic ratio of apoptotic neuronal content closer to control levels. In flow cytometry analysis, results corroborated the data obtained via microscopy (Figure 6D).

Hence, depending on the nature of the substrate, CO presents a distinct and opposite dual effect on microglia engulfment of neuronal content. In fact, CO suppresses the engulfment of live neurons, but enhances the phagocytosis of apoptotic cells. The opposing results suggest that CO has a homeostatic role for the control of phagocytosis. Likewise, it is potentially a consequence of a CO-induced fine tuning of specific neuron-to-microglia communication pathways and recognition of environmental cues.

CO modulates neuron-microglia cell-to-cell direct communication

CD200R-CD200 axis is a specific pathway for neuron-microglia signalling, which limits neuroinflammation. Thus, the microglial receptor CD200R and its neuronal ligand CD200 were quantified by immunoblotting in monocultures of BV2 microglia and CAD neurons. ALF826 administration seems to reinforce this pathway, since CO increased expression of neuronal CD200 (Figure 7A) and partially reverted the LPS-induced suppression of microglial CD200R (Figure 7B, data not statistically significant).

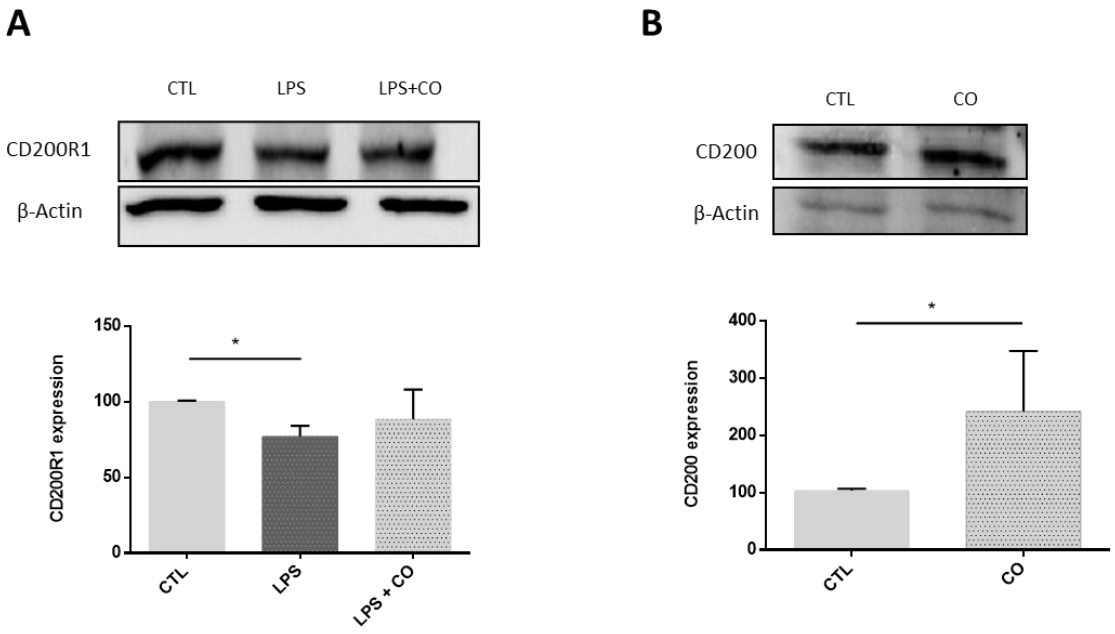

Figure 7 – CO administration improves CD200-CD200R communication pathway. BV2 microglia expression of surface receptor CD200R (A) and CAD neuronal expression of surface ligand CD200 (B) were quantified via Western Blotting. Protein band densitometry was performed and normalized to β-actin and is presented as percentage relative to the control. n=6, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

The CD200-CD200R axis is a key direct pathway of the neuron-microglia interactome, involved in phagocytosis control[50]. Thus, subsequent functional studies will be performed in order to assess the effect of this pathway on CO-promoted phagocytic homeostasis. Moreover, proteomic experiments are underway to identify other potential targets of CO neuron-microglia regulation.

DISCUSSION

In the present study, a neuron-microglia microfluidic cell culture system was used to target microglia-neuron interactome. We have shown that carbon monoxide (CO) affords neuroprotection by limiting microglia inflammation. ALF826 treatment partially reverted LPS-induced microglia reactiveness and limited hippocampal neuron apoptosis and loss of neuronal morphological integrity at the microgrooves. Also, CO regulated synaptic pruning under inflammatory conditions. Furthermore, we demonstrated that CO has a distinct dual quality control response concerning microglia phagocytosis: improves phagocytic clearance of apoptotic neurons and limits removal of viable neurons. Such biological response had not been described until now and indicates CO might play a role in governing neuron-microglia interaction pathways towards homeostasis. For this study we used a novel molybdenum based CO-releasing molecule, ALF826, which presents negligible cytotoxicity at 100µM, has a 37 min half-life in Hepes buffer 7.4 and delivers a large load of CO. CO has been largely known to be an anti-inflammatory endogenous compound, which regulates reactivity of immune cell populations[42,45,46,51–53]. Herein the focus is to understand whether CO regulates the neuron-microglia direct communication system in the context of inflammation. This communication system is ultimately key for regulating microglia migration, activation, proliferation and phagocytosis during the recognition of environmental cues[18,24,26,27,54–59]. Neuroimmune bidirectional pathways (between neurons and microglia) provide essential inflammatory 'calming cues', acting as natural breaks for exacerbated inflammation[32,60–64].

In our microfluidic co-culture system, whenever microglia were challenged with LPS (Lipopolysaccharides), hippocampal neurons presented decreased cell viability. Under the same pro-inflammatory conditions, neuronal network was mostly unaltered in the main neuronal compartment, but some regions revealed partial disruption of network integrity. Further experiments are required to confirm the level of structural damage at the neuronal compartment. However, neurites at the microgroove region were shortened and amorphous. We attributed this effect to elevated levels of pro-inflammatory mediators produced by microglia, as exacerbated inflammatory output is a major driving force for neurotoxicity[65,66]. In fact, microglia did increase secretion of TNF-α (Tumour necrosis factor α), IL-1β (Interleukin-1β) and nitrites. A recent report using the same *in vitro* model showed similar results, as TNF-α-treated microglial reactivity led to neuronal apoptosis and partial morphological disruption[67]. Microglia pre-treatment with ALF826 partially limited neuronal apoptosis and rescued neuronal process morphology at the microgrooves. Concurrently, CO-treated microglia secreted lower levels of inflammatory mediators, which further indicates the connection between neuronal loss and an exacerbated microglia response. The present results point to a neuroprotective, anti-neuroinflammatory role in a model of direct neuron-microglia cross-talk.

To deeper assess neuron-microglia direct communication, we looked at the microglia reservoir region adjacent to the microgrooves, where microglia were in contact with a smaller neuronal population and processes extending from the neuronal reservoir. We assessed microglial communication with βIII tubulin-stained processes (cytoskeleton), PSD-95 and vGlut-1 puncta (post and pre-synapses). Microglia close contact with neuronal processes is crucial for synaptic modulation and axonal growth[9,68–70]. Indeed, microglia can locally alter and remodel neuronal cytoskeletal structures[71,72]. Herein, microglia contacted directly with, and partially engulfed, βIII tubulin-stained neuronal extensions, however no major differences were seen between different conditions. Next, we looked at interaction between microglia and PSD-95 (Postsynaptic density protein 95) or vGlut-1 (Vesicular glutamate transporter 1) stained neurons. Microglia cells adjacent to neurons phagocyted and accumulated considerable amounts of post-synaptic material (PSD-95), but not pre-synaptic (vGlut-1). Likewise, in LPS-treated conditions, microglia decreased neuronal PSD-95 engulfment, while CO reverted this effect. Microglia synapse modulation is a widely described function, as these cells actively remove 'weak' or superfluous synapses[9,73,74]. vGlut-1 results are inconclusive, as no engulfment was observed regardless of conditions, despite microglia being known to engulf this type of synaptic markers[75]. Neuron-microglia communication pathways like the CX3CL1-CX3CR1 axis and P2Y12R purinergic signalling have been described as crucial for synaptic fine-tuning[74,76,77]. Hence, lower levels of phagocytosis of the post-synaptic marker PSD-95 could be attributed to inflammatory disruption of cell-to-cell crosstalk, which was partially rescued by CO. Still, to fully confirm that microglia is enhancing phagocytosis of synaptic material under CO conditions, lysosomal function needs to be addressed. For this purpose, microfluidic co-cultures could be stained with lysosomal marker CD68 (colocalization) or a lysosomal pH indicator, to track cargo degradation. Also, we cannot exclude, that CO could also cause an over-engulfment of synaptic material, deleterious for network connectivity. Future neuron functional studies, such as electrophysiology experiments, would be helpful in determining whether CO's effect in promoting synaptic pruning is ultimately beneficial. Installing microchip technology within the microfluidic system could be a potential solution for this question. Spine morphology and density could also be quantified, as an additional readout of synaptic plasticity. Phagocytosis of other pre-synaptic markers could be assessed, to understand if CO-induced microglia synaptic pruning is specific to post-synapses or not.

It can be hypothesised that inflammatory-induced changes in synaptic engulfment by microglia is not a consequence of neuron-microglia cross-talk regulation but related to changes in microglial phagocytic machinery, especially since CO has been shown to act on RhoGTPases, which regulate F-Actin dynamics[78]. To answer this question, latex fluorescent beads were used as substrate of microglial phagocytosis. LPS stimulus greatly increased phagocytosis of latex beads, and this was inhibited by CO. Accordingly, inflammatory stimulation enhances secretion of opsonins, expression of NADPH Oxidase, expression of Fc receptors and multiple other cell surface recognition receptors[79,80]. Likewise, LPS-treated microglia also presented an increase in cell size, which ultimately could affect bead recognition and engulfment. One could argue that

ALF826 treatment primed microglial cell autonomous anti-inflammatory machinery, which subsequently caused a suppression in phagocytosis efficiency, while the regulation of synaptic protein removal could be dependent on neuron specific signalling that trigger synaptic clearance by microglial phagocytosis.

In order to evaluate whether and how CO modulates the bi-directional communication between neurons and microglia for the control of phagocytosis, it was also crucial to follow how CO regulates microglial phagocytosis in the presence of viable neurons, apoptotic neurons, and cell debris. Indeed, data demonstrated the apparent dual nature of CO in the regulation of microglia engulfment of biological entities. LPS-activated microglia were more proficient engulfing material from live neurons, while CO suppressed this effect. Phagoptosis, which is a cell death process by phagocytosis of target viable cells, can occur under an inflammatory setting and is potentially pathogenic[81]. Exacerbated microglia reactivity can lead to neurons transiently exposing 'eat me signals' like phosphatidylserine, leading to their engulfment and neuronal cell death[82,83]. On the other side of the same coin, LPS decreased the capacity of microglia for engulfing apoptotic neurons, while pre-treatment with ALF826 pushed back the levels of microglial phagocytosis for clearance of neuronal apoptotic cells and debris. The decrease on LPS-microglia phagocytic activity could indicate that inflammation leads to a loss of function in microglial recognition of target cells. Interestingly, Schallner and colleagues have shown that stimulating the CO/HO-1 axis improved microglial erythrophagocytosis and prevented neuronal damage in a Subarachnoid haemorrhage (SAH) model[84]. They subsequently showed that CO enhanced erythrophagocytosis and suppressed inflammation by increasing the expression of surface scavenger receptor CD36[85]. These data can provide a blueprint for understanding the mechanisms by which CO provides phagocytic homeostasis.

We hypothesize that CO might modulate (i) specific 'eat me'/'don't eat me' machinery or/and (ii) direct neuron-microglia interactome. Pathways such as CD200-CD200R axis[86,87], CX3CL1-CX3CR1[9,74], and the microglial receptor TREM-2 (Triggering receptor expressed on myeloid cells 2)[32,60,88], are involved in microglial synaptic pruning, phagocytic recognition, and engulfment. Here, preliminary data with monocultures of BV2 microglia and CAD neurons suggest that CO could regulate CD200-CD200R. This effect will be confirmed and the functional consequences of CO on CD200-CD200R regulation on inflammation and phagocytosis will be assessed. Lastly, we postulate that CO can also be targeting cargo trafficking, degradation, and overall lysosomal function, as these downstream mechanisms are also crucial for phagocytic efficiency, cell metabolism and survival[89–91]. Impaired removal of synaptic content and apoptotic neurons can be a consequence of hindered lysosomal function. To assess these hypotheses, we will undertake a mass spectrometry study using primary culture of microglia cells, for the identification of CO-induced differentially expressed proteins, for understanding the underlying biological effects of CO in the modulation of phagocytosis and/or inflammation.

Altogether, we have used a novel Mo-based CORM molecule with great efficacy in the modulation of neuroinflammation and in the control of microglia-neuron communication, in particular by promoting a homeostatic state of phagocytosis. Indeed, CO can act as a mediator of microglia-neuron bidirectional direct communication. Herein, CO administration impacted microglial removal of neuronal synaptic material and regulates phagocytosis of neuronal cells, improving clearance of apoptotic cells and blocking engulfment of viable neurons. We are currently performing further experiments to identify the underlying CO-regulated communication pathways and further understand its biological consequences in the Central nervous system (CNS). These results are a steppingstone for CO as both a tool to disclose the physiological mechanisms of the neuron-microglia interactome system, as well as potentially opening a novel pharmacological avenue in brain pathology.

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SUPPLEMENTARY FIGURES

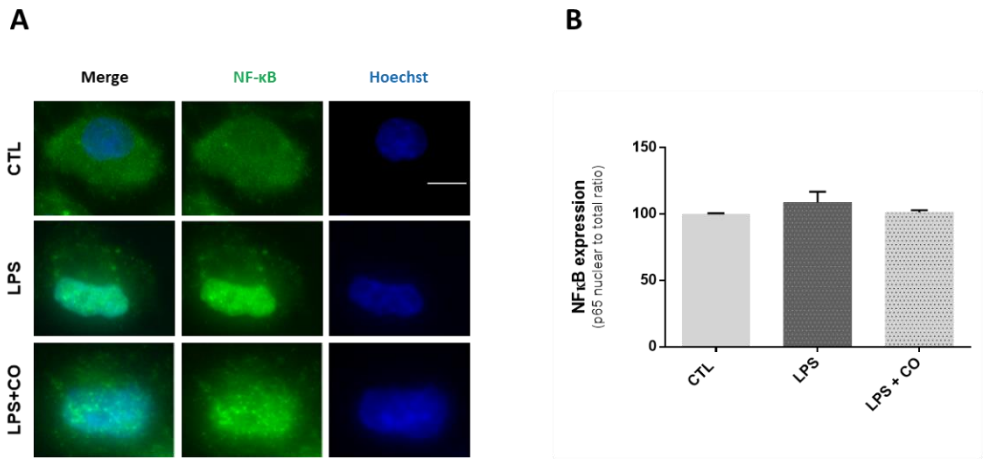

Figure S1 – Primary microglia NF-κB nuclear translocation in a neuron-microglia microfluidic setting. Primary microglia were immunostained with NFκB p65 antibody (green) and Hoechst 33342 (blue) (A). Scale bar = 10 µm. p65 nuclear to total ratio was quantified following micrograph acquisition (B). Results are expressed as percentage relative to the negative control. *n=3*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA.

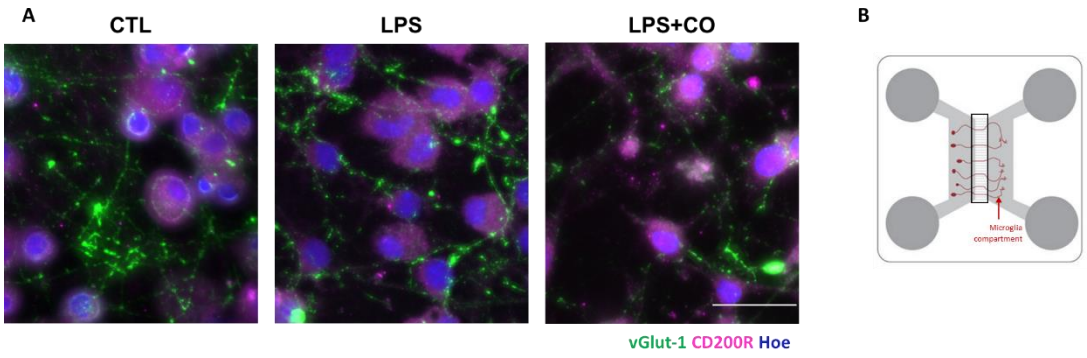

Figure S2 – Microglia phagocytosis of pre-synaptic material in a neuron-microglia microfluidic culture system. Primary rat hippocampal neurons and microglia were cultured inside microfluidic devices. Microglia was labeled with CD200R (magenta) and neurons were stained with a vGlut-1 antibody (green). Hoechst 33342 (blue) was used as nuclear staining. The microglia reservoir (neuronal axonal side) was photographed under a fluorescence microscope (A). Scale bars = 25 µm. Schematic representation of the microfluidic device (B), highlighting the microglial compartment regions.

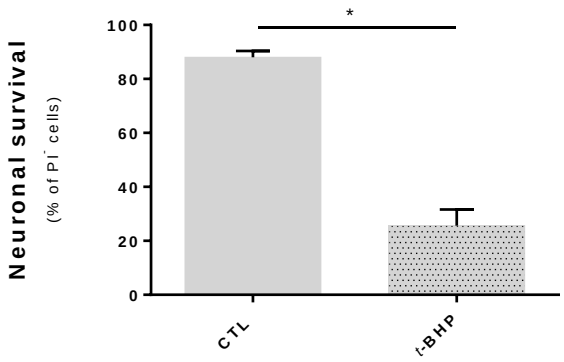

Figure S3 – CAD neuronal survival in the presence of a pro-oxidant cell death inducer (*t*-BHP). Differentiated CAD neurons viability was assessed via propidium iodide staining quantification in flow cytometry after treatment with a cell death inducer for 24 hours (*t*-BHP). *n=6*, error bars represent mean ± SEM, *p<0.05 by one-way ANOVA test.

V

DISCUSSION AND FUTURE WORK

INDEX

Nuno L. Soares has written the whole chapter based on the referred bibliography

1. Introduction

The brain is a very complex organ that is the main functional hub of the Central Nervous System (CNS)[1]. Brain tissue is very vulnerable to homeostatic imbalances, which can cause alterations in regular activity[1]. Neurological disorders are diverse in symptoms, severity, and treatment. According to World Health Organization (WHO), the mortality and morbidity numbers, as well as the costs associated with healthcare, will continue to increase in the forthcoming decades[2]. Neurodegenerative pathologies, such as Alzheimer's (AD) and Parkinson's Disease (PD) have very complex aetiology and diagnosis still is not accurate[3]. In stroke, therapeutic options are limited and there is an ongoing effort to discover predictive circulating biomarkers[4]. Hence, it is necessary to disclose molecular mechanisms of disease in the CNS and develop new therapeutical approaches and molecules, with the intent of improving long-term neurological status.

1.1. Adult neurogenesis

For a long time, it was widely agreed that the brain was unable to produce new neuronal cell populations after very early post-natal development, despite increasing evidences suggesting particular regions of the brain were capable of sustaining production of new neurons[5]. It was only at the turn of this century that adult neurogenesis became widely accepted[6].

Understanding the molecular mechanisms of adult neurogenesis is important for several reasons. Altered neurogenesis has been observed in different neurologic disorders[7]. PD animal models have revealed impairments in proliferation and survival of newly generated neurons[8]. In fact, impairment in hippocampal neurogenesis has been related with PD pre-motor symptoms[9]. Decrease in progenitor cells in the hippocampus was registered in Huntington's disease rodent models as well[10]. Moreover, neurogenesis is a very complex and integrated process. Understanding the pathways of cell differentiation can be a step forward towards comprehending the aetiology of neurodegeneration. Studying adult neurogenesis can also be seen as a potential therapeutic strategy, by stimulating endogenous mechanisms which will contribute to partially restore brain function after injury. Alternatively, modulation of neurogenesis can be applied for cell therapy *in vitro* production of neuronal cells, which can be eventually used for transplantation and integrated in neuronal networks[11]. For both strategies it is crucial to deeper understand the underlying cellular and molecular processes of neuronal differentiation.

1.2. Microglia modulation of neuronal function

Microglia are the resident immune CNS cells[12]. Originating from a myeloid progenitor population in the yolk sac, which migrates to and colonizes the brain[13], microglia have several homeostatic immune and non-immune functions in the CNS[12,14–16]. However, microglial dysfunction can cause serious consequences in the brain[17]. Exacerbated inflammation is a common feature in numerous neurological disorders and during ageing, creating a potentially neurotoxic microenvironment which is a pathological driving force[14,18,19]. Microglial activity is regulated by complex interactions between the immune cell and the involving milieu[20–22]. Neuron-microglia communication in particular is fundamental for the fine regulation of microglial function, as paracrine communication and direct neuron-microglia pathways act as modulatory mechanisms[20–22]. Breakdown of this bidirectional system can result in damage by inflammatory overload, defective synaptic pruning and phagocytic dysfunction, and consequent engulfment of otherwise viable cells[23,24]. Considering the central role microglia play in CNS pathology, it is essential to disclose both (i) the soluble factors and (ii) direct cell-to-cell physical contact that make up the neuron-microglia interactome, and how modulation of these pathways can maintain brain homeostasis.

1.3. Aims

Carbon monoxide (CO) is an endogenous molecule, produced *via* haem degradation[25]. CO has proven to be anti-inflammatory and anti-apoptotic, emerging as a potential therapeutical tool in the recent past[26–29]. In the CNS, CO improves neurogenesis by limiting cell death of precursor cells[30] and by reinforcing mitochondrial metabolism[27]. Furthermore, CO decreases microglia pro-inflammatory functions[31,32]. This thesis aims to explore CO's potential as modulator of neurogenesis and a potent anti-neuroinflammatory compound. In **Chapter II**, further assessments were made regarding how CO metabolic programming affects neuronal differentiation, in particular *via* pentose phosphate pathway (PPP). While, in **Chapters III** and **IV** the molecular mechanisms of microglia-modulation in the CNS, and how CO regulation of microglia affects neuronal function were focused. Ultimately, each chapter aimed at increasing knowledge regarding protective endogenous CNS pathways and unveiling novel roles for CO-induced protection. The main achievements of this thesis are presented in Figure 1.

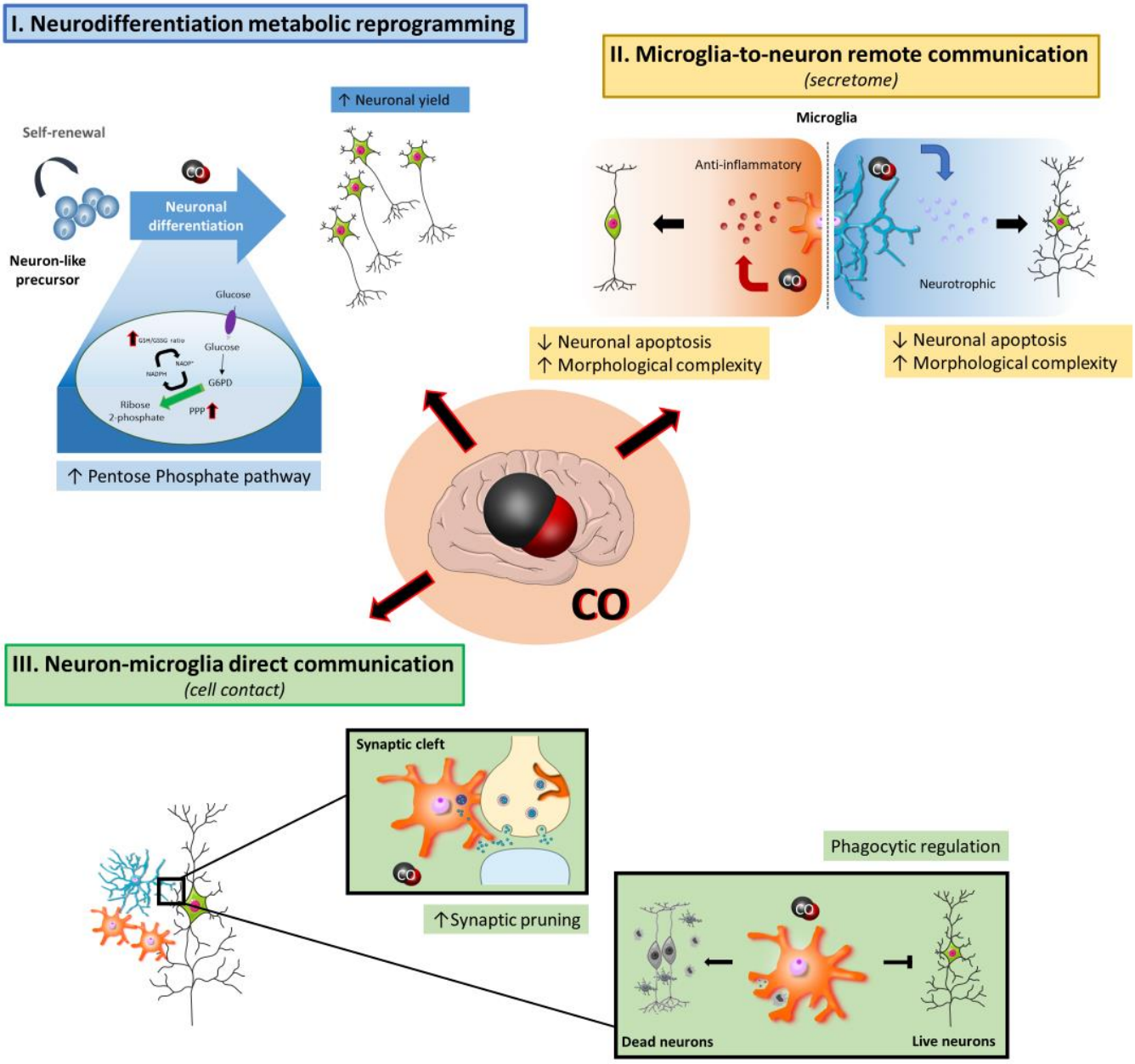

Figure 1 - Main achievements of this PhD thesis. CO improves neuronal differentiation by enhancing the PPP (Chapter II). CO modulates neuron-microglia remote communication (via soluble factors) and improves neuronal survival, both by limiting microglial inflammation and by enhancing microglial neurotrophism (Chapter III). Concerning neuron-microglia direct physical contact communication, CO regulates microglial synaptic pruning and CO maintains homeostasis on microglial phagocytosis of neuronal cells (Chapter IV).

2. Cellular mechanisms of neurogenesis

Adult neurogenesis is a very complex, multi-step process, and the mechanisms of cell differentiation are still poorly understood[11]. Disclosing such processes could improve the prospects of therapeutic application.

Metabolic reprogramming is crucial for neurogenesis: Neural Stem/Progenitor Cells (NSPCs) are mostly glycolytic, but following activation become increasingly energy consuming and oxidative and require *de novo* lipid synthesis[33,34]. Our lab has published that CO reprogrammes NT2 cell line metabolism, shifting it towards an oxidative profile and improving neuronal production in an *in vitro* differentiation protocol[27]. Cell differentiation is also characterized by extensive morphological alterations and organelle synthesis[35]. Moreover, as a consequence of oxidative phosphorylation, cells on later stages produce mitochondria-derived reactive oxygen species (ROS), which requires production of reducing agents to limit oxidative damage at the cell level[36]. Considering the energetic and biomass requirements of differentiating populations, we focused on the effect of CO on the PPP, a crucial pathway for production of nucleotides and electron donors. In fact, PPP supports glutathione (GSH) recycling, which is important for balancing ROS generated during differentiation process.

In **Chapter II**, we showed that CO improves neuronal yield in an SH-SY5Y differentiation protocol, via improvement of the PPP flux. Interestingly, Kaczara and colleagues have shown with a different CO-releasing molecules (CORM), that CO activates the PPP in an endothelial cell line (non-differentiation model)[37]. The effect herein observed was dependent on improvement of Glucose-6-phosphate dehydrogenase (G6PD) expression, the rate-limiting enzyme of this pathway, as silencing reverted final neuronal yield improvement. The effect on G6PD had been undescribed. However, CO is known to produce residual ROS, which act as a downstream signalling mechanism[38]. Since G6PD expression is responsive to redox imbalances, it could be hypothesized that CO-derived ROS prime transcriptional alterations in neurons and induce G6PD expression. Several molecular players have been identified as possible regulators of G6PD gene transcription[39]. One of them is the transcription factor Nrf2 (Nuclear factor erythroid 2-related factor 2), which is involved in anti-oxidant signalling and is a major effector of the CO/HO-1 route[40]. However, differences in G6PD expression were only registered at day 7, and not day 1, which would be expected if G6PD CO-modulation was dependent on ROS specific signalling. Further experiments with ROS scavengers at different timepoints would be necessary to support this hypothesis.

The fact that CO requires PPP flux increase to enhance SH-SY5Y neuronal yield further highlights the importance of metabolism, and this pathway in particular, in neurogenesis. PPP is fundamental for production of electron donors[41,42] (NADPH), which sustain anti-oxidant defences. This is crucial in differentiation cells, that are increasingly more dependent on oxidative metabolism. Here, CO

supplementation does not alter *de novo* synthesis and breakdown of GSH, but increases GSH/GSSG ratio, an indirect indicator of increased PPP flux.

Interestingly, CO supplementation did not increase SH-SY5Y mitochondrial metabolism. Previously, our lab had shown that CO increased NT2 differentiation via enhancement of oxidative phosphorylation[27]. This variance could be explained by the differences in cell line characteristics. NT2 cells represent a more pluripotent population in early activation, where metabolic reprogramming is required, whereas SH-SY5Y cells display an immature neuron-like phenotype. Thus, one can speculate that SH-SY5Y metabolic shift has already occurred, and glucose oxidation is more important for strengthening anti-oxidant defences.

Here, data supported the notion that CO does have a very active, yet context dependent, role in regulating neuronal metabolism during neurogenesis. Still, several questions need to be addresses, such as: Is CO-derived ROS production essential for anti-oxidant defence strengthening? How does CO promote G6PD expression and activity?

3. Neuron-microglia communication

Neuron-microglia bidirectional communication is mediated by several soluble factors (cytokines, neurotrophins, 'find me' signals, purine nucleotides, neurotransmitters), extracellular vesicles and ligand-receptor pairs (CD200-CD200R, CD172a-CD47)[20,21]. This complex interactome is crucial for microglia inflammatory activity, motility, viability, and phagocytosis[20–22]. Loss of cell specific interactions results in unrestrained microglia reactivity and overall dysfunction[24,43–45].

CO is an anti-inflammatory endogenous compound (**Chapter I**)[38], but its effect on neuroimmune communication is undescribed. To assess the role of CO on neuron-microglia cross-talk, two strategies were utilized: (i) targeting neuron-microglia remote communication, focusing on CO's non-cell autonomous role on neurons by acting on microglia secreted soluble factors (**Chapter III**) and (ii) targeting direct cell-to-cell pathways (**Chapter IV**).

3.1. Neuron-microglia remote communication

In **Chapter III**, CO provided indirect neuroprotection in a neuron-microglia conditioned media protocol with cells lines, *via* suppression of microglial inflammatory soluble mediators TNF-α (tumour necrosis factor α) and nitrites. This data were subsequently validated using primary cultures of neuron and microglia. It was the first time that ALF826, a novel molybdenum-based molecule, was applied for assessing neuroinflammation and neuroprotection. Existing literature corroborates our results, as CORM-3 attenuates

secretion of TNF-α and NO in LPS-treated BV2 microglia[31,32]. Some authors have also shown that CO reinforces anti-inflammatory machinery by production of signalling mitochondrial ROS[46–48].

CO modulation of LPS-activated microglia secretome also affected neuronal morphology, limiting loss of arborization and overall complexity. This is possibly a consequence of CO regulation of remote neuron-microglia communication by inhibiting secretion of specific inflammatory secretome. Microglial inflammatory cytokines have neuroactive properties, such as synapse regulation[49] and overall circuit excitability[50], but can also disrupt function. TNF-α can activate neuronal small GTPase RhoA (Ras homolog family member A), which inhibits neurite growth and branching[51].

In a neuron-microglia microfluidic co-culture model (**Chapter IV**), CO also suppressed LPS-microglia inflammatory secretome, consequently reverting neuronal apoptosis in the neuronal reservoir. Moreover, neuronal processes inside the microgrooves were unstructured under inflammatory conditions, and this was reverted by ALF826 treatment. These data further validated that CO has a central role in regulating microglia-neuron paracrine communication. Since the microfluidic *in vitro* model featured physical contact between cell types, it could be argued whether CO anti-inflammatory effect is by simply acting on microglia cell autonomous mechanisms, or by reinforcing specific neuroimmune pathways, which can control the profile of microglial secretome[21].

In the absence of inflammation, microglia have important homeostatic functions, namely during early brain development, recycling superfluous and deficient cells, acting at the synapse level, and providing survival and migration cues to specific populations[52–54]. Still, no data has been published regarding the role of CO on microglia neurotrophism. Herein, we showed that conditioned media from non-activated BV2 microglia provided protection to CAD neurons challenged with pro-oxidant *t*-BHP (*tert*-Butyl hydroperoxide). Cytoprotection was further enhanced whenever neurons were incubated with CO-treated microglia conditioned media, although not in a significative manner and only at higher concentrations of the pro-oxidant *t*-BHP. This indicated both microglia media and ALF826 supplementation provided a cytoprotective stimulus. The results were not unexpected, as microglia conditioned medium increased survival, neurite extension and dopamine uptake in a primary culture of mesencephalic neurons[55]. In an *in vivo* model of chronic pain and depression, microglia depletion disrupted BDNF (Brain-derived neurotrophic factor) signalling and worsened animal behaviour[56]. Herein, we also observed that, in the absence of *t*-BHP incubation, conditioned media from CO-treated microglia, but not for control, improved neuronal morphology. While the conditions here are unrelated to the data presented before, this could strengthen the idea that CO affect neuronal cytoskeleton pathways inducing morphological alterations, by acting on microglia's secretome. Thus, CO might promote the release of microglial factors that improves neuronal morphology, and possibly function.

Looking at CO's effect on microglial secretome, there was an increase in the levels of anti-inflammatory cytokine IL-10 following CO treatment. Previously, it has been published that CO increased IL-10 production in LPS-activated macrophages [57]. Nevertheless, under basal conditions, it is for the first time demonstrated that CO increases IL-10 release from microglia. Previous authors have shown that exogenous IL-10 promotes neurite growth and synaptogenesis in primary cortical neurons in an *in vitro* OGD (oxygen-glucose deprivation) model, by activating the JAK1/STAT3 (Janus kinase 1/ Signal transducer and activator of transcription 3) pathway[58]. Similarly, increasing concentrations of exogenous IL-10 protect cortical neurons from OGD-induced apoptosis[59].

In summary, we showed that ALF826 modulates microglia secretome, limiting inflammation and enhancing neurotrophism, which in turn and indirectly prevents neuronal cell death. CO-derived microglial media improve neuronal morphometric parameters, but the underlying molecular mechanisms need further scrutiny.

3.2. Neuron-microglia direct communication

When assessing direct neuron-microglia contact in the microglia compartment of a microfluidic co-culture system (**Chapter IV**), we registered that microglia phagocyted post-synaptic material PSD-95 (Postsynaptic density protein 95). It is known that microglia play an active role at synapse stripping, modulation, and induction[53,60–62]. However, herein, LPS-treated microglia phagocyte less PSD-95, and ALF826 treatment promotes a great increase in its capacity to engulf post-synaptic content. This could indicate that CO favours pruning of synapses under inflammatory stress conditions, potentially by reinforcing direct cell cross-talk. In fact, specific communication mechanisms are essential for microglia regulation at the synapse level: Microglia 'sense' neuronal activity by expressing surface neurotransmitter receptors and P2Y12 Receptor[63,64].CX3CR1-CX3CL1 (fractalkine receptor – fractalkine) is a crucial for synapse removal. In a *Cx3cr1*$^{-/-}$ mouse model, reduced synaptic pruning, functional connectivity and altered animal behaviour is observed [61]. The classical complement pathway is also important for microglia and astrocyte synapse removal[65].

Interestingly, microglia did not engulf any pre-synaptic marker VGLUT1 (Vesicular glutamate transporter 1), regardless of the experimental condition, which could indicate that these cells are specifically targeting post-synaptic terminals. However, it is known that microglia interact and can prune both pre- and post-synaptic terminals[62,66]. These results need to be further explored by assessing other markers of pre-synapse.

CO had a dual effect on microglia phagocytosis in a co-culture model where BV2 microglia were incubated with either live or apoptotic CAD neurons. CO blocked LPS-activated microglia engulfment of live neurons but enhanced activated microglia phagocytosis of apoptotic cells. This has never been described before and indicates that CO provides a homeostatic balance, avoiding deficient phagoptosis but increasing clearance of compromised material. One can speculate that CO potentially acts *via* reinforcement of specific neuron-microglia direct interactome. In fact, preliminary data suggested CO enhances the CD200-CD200R communication in monocultures of BV2 microglia and CAD neurons. While further functional validation is required, CD200R agonism inhibited reactive macrophage phagocytosis of oligodendrocyte progenitors[67]. Importantly, in **Chapter III**, CO decreased BV2 microglial expression of CD11b, which is part of the Complement Receptor 3 (CR3)[68]. Microglial CR3 is important for synaptic pruning[60], neuronal removal during development and phagocytosis of apoptotic cells[69].

This chapter of the thesis is an ongoing project and there are some questions that remain unanswered. It is currently under investigation which are the CO effector molecules on microglia-neuron communication, and how CO modulation alters microglia inflammation and phagocytosis, as well as neuron survival and function. We postulate that CO regulates microglial phagocytosis by acting at different levels: (i) on neuron-microglia direct communication, by improving specific neuroimmune signalling, such as CD200-CD200R; (ii) by regulating the phagocytic 'eat-me' pathways (Phosphatidylserine-BAI1; Phosphatidylserine-MFGE8/MerTK) or 'don't eat-me' pathways (CD47-SIRPα; Sialic acid-Siglecs) and (iii) by directly controlling microglia cell-autonomous phagocytic process, such as cytoskeletal rearrangements, cargo trafficking and lysosomal activity for cargo degradation.

Mass spectrometry experiments are being undertaken to identify candidate proteins and clarify the mechanisms through which CO actively regulates several aspects of the work, such as neuron-microglia direct communication and microglia phagocytosis.

4. Carbon monoxide as a therapeutical molecule

We demonstrated, in **Chapters II-IV**, that CO is a cytoprotective molecule, capable of enhancing neurogenesis via metabolic reprogramming, as well as providing an anti-inflammatory and neuroprotective effect, regulating remote and direct neuron-microglia communication and phagocytosis. CO also regulates apoptosis, proliferation and has bactericidal properties[26,70,71]. The broad biological effect observed herein highlights the current belief that there is no single CO molecular target, and multiple molecular targets exist.

Much of the current literature supports the notion that CO acts as a primer of protective endogenous mechanisms. We, along with other authors, have shown that CO creates a mild oxidative burst, which primes cellular anti-oxidant, cytoprotective and anti-inflammatory defences[26,47,72–74]. This and the fact that CO can

either be applied exogenously or stimulated endogenously, greatly increases its therapeutical value. Nonetheless, application of CO in biological systems, in particular for human use, has great limitations.

CO-releasing molecules (CORMs) are synthetic molecules which, unlike inhaled CO, provide a safer and more targeted delivery[75,76]. CORMs still present delivery problems, such as molecule traceability, capacity to cross biological membranes, the level of tissue specificity and chemical structure and metabolization[77]. Moreover, CORMs are very chemically diverse, with CO release kinetics being a major variable, as CO can be release in one burst (similar to gaseous solutions), or in a prolonged period of time. This can ultimately affect the physiological response[77]. Here, we utilized two different CORMs. CORM-A1 in **Chapter II** and ALF826 in **Chapters III and IV**. ALF826 is a newly synthesized Molybdenum-based molecule that delivers a large CO load while presenting low cytotoxicity at 100 µM in both RAW246.7 macrophages (Proterris (Portugal) Lda.) and BV2 microglia (Data not shown), at 24 hours. Moreover, Molybdenum is an endogenous metal which does not accumulate in the human body.

5. Future work

We demonstrated in this PhD thesis that CO provides protection in two distinct *in vitro* setting: Enhancing PPP flux and overall neurogenesis in a neuron differentiation model; and modulating remote and direct neuron-microglia communication. Still, to better understand the impact of these findings, and further contribute to understanding the biological functions of CO, several questions need to be addressed, particularly at a subcellular level. Future work and hypothesis are listed and discussed below:

5.1. In vitro approaches:

- In **Chapters III and IV**, important negative controls need to be added, as experiments were not carried out using the depleted form of ALF826 (iALF826). ALF826 cytotoxicity tests were performed prior to use, but metallic centres can still interfere with redox balance of the cell. Although the inactive form of ALF826 is a great mixture of different compounds, it can help to validate the results, excluding the possibility that the obtained biological function was not a non-specific consequence of the CORM skeleton, and not of CO itself. In fact, this has been observed by some authors in very diverse studies[78,79]. Experiments with iALF826 controls will be added prior to submission for publishing. Using alternative sources of CO could also be considered in the future, , such as CO gas that is the cleanest way to supply CO. Genetically or chemically promoting expression of HO-1, as a source of endogenous CO production, would help in corroborating results obtained with CORM administration.

- In **Chapter II**, CO improved neuronal yield in an SH-SY5Y differentiation protocol, by increasing PPP flux *via* rate-limiting enzyme G6PD. CO is known to promote mild ROS production, which acts as a downstream activator of cytoprotective and anti-oxidant machinery[38]. We hypothesize that the increase in PPP flux and GSH/GSSG ratio are a consequence of CO supplementation transiently increasing ROS production. We will test this and, to further clarify the involvement of ROS signalling, we could use a ROS scavenger prior to CORM-A1 supplementation in our SH-SY5Y differentiation model. Also, we are interested in disclosing the molecular mechanisms which regulate CO-driven G6PD overexpression. Potential candidates include transcription factors Nrf2 and HIF-1α (Hypoxia-inducible factor 1 α), target molecular players of CO[80,81], which are involved in redox balance and have been shown to regulate G6PD expression[39,82].

- ALF826 application modulated microglia-to-neuron remote communication, providing indirect neuroprotection (**Chapter III**). To clarify the causality between inflammation and neuronal survival, we propose to incubate neurons with conditioned medium containing anti-TNF-α (and/or anti-IL-1β) antibodies, to neutralize the action of inflammatory cytokines. Similarly, using anti-IL-10 antibodies would allow to also clarify the importance of this cytokine for CO modulation of neuronal morphological complexity.

- Since microglia conditioned media provided neuroprotection against oxidative damage (**Chapter III**), we hypothesized that microglia could secrete anti-oxidant or scavenging agents that limit cell death. In fact, microglia produce anti-oxidant enzymes such as Peroxiredoxin-5, Haem Oxygenase-1, Thioredoxins and Glutathione peroxidase 1[83]. Additionally, microglia release of other paracrine neuroactive factors (Transforming growth factor β, Insulin growth factor 1, Nerve growth factor, Neurotrophin-3, nucleotides, and nucleosides) could also be assessed. Since microglia secrete exosomes containing enzymes, chaperones, metabolites and microRNAs[84], extracellular vesicle signalling could also be analysed.

- The conditioned media protocol utilized in **Chapter III** only allows to assess microglia-to-neuron unidirectional communication, and neuron-microglia is a synchronous, two-way dialogue[20]. Neurons secrete multiple soluble factors, both basally and under duress, which can either suppress or increase microglial inflammatory function, motility, and proliferation and neurotrophic support[21]. Using a co-culture system with a permeable divider or a simple trans-well insert would allow for a more robust and comprehensive reconstruction of neuron-microglia soluble factor exchange, and ultimately of the effect of CO on this remote cross-talk.

- In **Chapter IV**, we observed that CO limits neuroinflammation and provides neuroprotection in a microfluidic co-culture system. While this *in vitro* setting allowed for a closer recapitulation of neuron-microglia direct cross-talk, it is not possible to confirm if CO provided an anti-inflammatory effect by solely acting on microglia cell autonomous machinery, or also by modulating neuron-microglia specific direct communication pathways, which are known to provide an immune restraint cues in the brain[21]. To do so, we will assess the expression of cell-to-cell communication pairs (CD200-CD200R, CX3CL1-CX3CR1) in the presence and absence of ALF826 treatment. The functional consequences of CO modulation of these pathways can then partially be assessed by blocking or silencing specific key molecules. Alternatively, a microglia monoculture could be used, targeting a candidate pathway by treating cells with a soluble recombinant form of the neuronal agonist of interest. As an example, recombinant CD200 Fc has been used both *in vivo* and *in vitro* to treat glia and macrophage populations under different experimental conditions, in order to manipulate CD200-CD200R signalling and better understand the functional consequences this has on inflammation and phagocytosis[67,85,86]. This rational can be replicated to other pairs of proteins in microglia-neuron communication.

- Herein, we have shown that, in an inflammatory context, CO-treated microglia in proximity with hippocampal neurons phagocyte increased levels of post-synaptic content (**Chapter IV**). Microglia are active synapse-strippers, and this function is very relevant for network connectivity in the brain[53]. Since we have greater accumulation of PSD-95 in CO-treated microglia, we argue that CO is enhancing microglial removal of deficient or stressed synapses. However, it is possible that intracellular accumulation is due to inhibited cargo degradation, and not because of enhanced engulfment. To fully exclude this possibility, we need to perform functional studies for vesicle trafficking and lysosomal digestion. If we confirm that CO does enhance microglia synaptic pruning, experiments are required to understand the functional consequences of this. To address it, microchips can be installed within the microfluidic system, which would allow to assess the electrophysiological properties of synapses at different conditions. Furthermore, since spine turnover is a marker of synaptic structural plasticity, spine density and morphology could be tracked in this system. Other biochemical markers could also be assessed, such as density of additional synaptic proteins, NMDA receptor (N-methyl-D-aspartate) subunits and adhesion molecules. Since pre-synaptic marker was not phagocyted here, pruning of other pre-synaptic proteins could be assessed (Synaptosomal-Associated Protein, 25kDa or synapsin I), to further clarify this result.

- Our work indicates that CO afforded a homeostatic effect on microglial phagocytosis, blocking engulfment of live neurons during inflammation, but facilitating the phagocytosis of apoptotic cells and debris (**Chapter IV**). This data indicate that CO can be regulating neuron-microglia specific pathways, or phagocytic sensing and recognition molecules ('find me' and 'eat me' recognition pathways). We are undergoing a comprehensive mass spectrometry of primary mouse microglia, to identify differentially expressed proteins in CO treated cells, and perform subsequent functional studies. This proteomic profiling will help to integrate all data herein obtained by clarifying the role of CO as a homeostatic regulator of neuron-microglia interactome and phagocytosis.

5.2. Ex vivo/In vivo approach

- To further validate results from **Chapters III and IV**, a more physiological approach could be employed. A possible alternative would be the usage of organotypic brain slice cultures (OBSCs). OBSCs feature intact tissue, neural circuitry and other cellular populations that were not considered here (astrocytes, oligodendrocytes). Moreover, it is a flexible experimental setup that would allow integrating CO's effect on microglial function and cross-talk with neurons.

6. Conclusion

This PhD thesis has further proven that CO is an endogenous molecule with therapeutical potential. CO has no described unitary pathway for cytoprotection, indirectly acting on a plethora of molecular targets, most of which are still poorly described.

Here, we showed that CO is a modulator of neuronal differentiation, enhancing PPP metabolism. Moreover, CO provides a strong anti-inflammatory effect on neuron-microglia communication, limiting cell death and acting as a homeostatic modulator of phagocytosis by regulation of neuron-microglia cross-talk. For the first time, it was shown that CO promotes neuroprotection via microglia modulation without pro-inflammatory conditions, probably by improving neurotrophic pathways.

In conclusion, the knowledge gathered will contribute to better understand the diverse molecular targets of CO, the biological consequences of its modulation, and ultimately contribute to advance CO into therapeutical usage.

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