Inês Isabel Pinheiro Paiva

Licenciatura em Bioquímica

Exploring the role of carbon monoxide as a modulator of microglia neurotrophism

Dissertação para obtenção do Grau de Mestre em Bioquímica para a Saúde

Orientadores: Helena Luísa De Araújo Vieira, PhD, Principal Investigador, UCIBIO, FCT-UNL and CEDOC FCM/UNL

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Júri: A definir

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Abril, 2021

Acknowledgments

Em primeiro lugar, quero agradecer à minha orientadora Dra. Helena Vieira por me ter aceitado no seu grupo e permitido trabalhar numa área que tanto gosto. Agradecer à Dra. Helena nunca será suficiente por toda a ajuda, apoio, ensinamentos e conselhos que me deu e me ajudaram a manter-me firme até nas situações mais adversas, sendo para mim uma referência em todos os campos.

Tenho inevitavelmente de agradecer ao meu mentor Dr. Nuno Soares, o meu "pai" de laboratório, por tudo o que me ensinou, por me ter sempre encorajado e por ser um grande amigo e exemplo. Obrigada por tornares todos os serões de trabalho em momentos incríveis e por todos os ataques incontroláveis de riso que me proporcionaste.

Quero também agradecer à Daniela Pedroso, por tudo o que me ensinou, pela imensa paciência e por toda ajuda que me deu. Obrigada por todos os bons momentos e por seres a minha "mãe adotiva".

À minha colega e "gémea siamesa", Cristiana Manuel, obrigada por teres sido a minha companhia dentro e fora do laboratório, pelo companheirismo e amizade.

Aos restantes membros e colegas do meu grupo, Pedro Cipriano, Raquel Domingues, Inês Mollet e Miguel Mira quero agradecer pelos bons momentos e aprendizagens que me proporcionaram.

Aos meus amigos "Covids anónimos" por todos os almoços, cafés, lanches, serões de conversa e de jogos, sempre acompanhados de boa disposição e sorrisos.

Um agradecimento especial à minha amiga Raquel Sequeira por ter sido o melhor presente que ganhei no decorrer desta etapa. Obrigada por tudo o que fizeste e fazes por mim e por nunca me deixares cair.

A toda a minha família, especialmente à minha mãe, à minha madrinha e ao meu irmão um enorme obrigada por serem o meu pilar mesmo nos momentos mais difíceis. Obrigada pelo amor e por serem a minha força e inspiração para continuar. Ao meu pai, tenho a agradecer por todas as memórias e ensinamentos que me deixou, que me tornaram na pessoa que sou e me dão tantas vezes a força que preciso. Ao meu padrinho, que perdi no decorrer desta etapa e a quem dedico este trabalho em forma de homenagem, agradeço por ter sido o exemplo de pessoa que quero ser, por tudo aquilo que me deu e me permitiu chegar até aqui.

Ao meu namorado Nuno Anjos, por todo o companheirismo, amor e compreensão. Obrigada por seres o meu melhor amigo e acreditares tanto em mim, por me animares e seres a minha alegria até nos momentos mais complicados e críticos de cansaço.

Às minhas lindas afilhadas, Rute e Andrea, por me serem as minhas filhas do coração e me fazerem querer ser melhor todos os dias para ser um bom exemplo a seguir.

A todos os meus amigos, em especial aos Hidrolisados, Família Preguiça e Gang do Serrado por estarem sempre lá, presencialmente ou à pequena distância de um clique, por toda a companhia e apoio que me ajudou a manter-me sã e motivada. Não há muito que já não vos diga todos os dias, obrigada por serem uma família e acreditarem tanto em mim.

Obrigada a todos que, direta ou indiretamente, tornaram esta tese de mestrado possível. Numa altura tão atípica e particularmente difícil tenho a sorte de estar rodeada de pessoas magníficas que nunca me deixaram desamparada.

Abstract

Cellular communication is essential for the maintenance of neuronal functional integrity and environmental homeostasis in the Central Nervous System (CNS). Microglia are responsible for various functions in the CNS such as synaptic pruning, patrol of the microenvironments and, most importantly, immune response and inflammation, vital for neuronal survival and tissue homeostasis. Microglia is capable of performing very diverse and distinct roles due to the bi-directional communication with other cell types, including neurons. Microglial activity needs to be under tight regulation since exacerbated actions can lead to uncontrolled inflammation and promote irreversible damage.

Carbon monoxide (CO) is an endogenous gasotransmitter with already proven cytoprotective and anti-inflammatory effects when present at low levels in the CNS, including the limitation of pro-inflammatory microglial output.

Nevertheless, the influence of CO in basal microglial function remains unexplored. Therefore, the main objective of our study was to understand the effect of CO in the microglial neuroprotection modulation, under physiological conditions.

Firstly, in order to achieve this purpose, a conditioned media protocol was established with BV2-microglia and CAD-neuron cell lines. Neurons incubated with microglia medium showed morphological changes and increased viability suggesting that the microglial secretome induces neuroprotection. Secondly, to understand how CO modulates this response, a new CO-releasing molecule (ALF-826) was used to treat microglia and their neuroprotective effect was enhanced.

We have found that CO treatment increased the expression of neurotrophic factor BDNF and Interleukin-10 secretion in microglia. Likewise, CO also enhanced microglial ectonucleotidase CD73 expression, indicating that purinergic signaling, in particular adenosine might be involved.

In conclusion, CO modulates microglial secretome towards neuroprotection. Furthering the knowledge regarding how CO regulates microglia function could be a step forward towards a new potential CO application as a preventive procedure by modulation and improvement of microglia neurotrophism.

Resumo

A comunicação celular é essencial para a manutenção da integridade e homeostase neuronal no Sistema Nervoso Central (SNC). A microglia é responsável por várias funções no SNC, tais como o refinamento sináptico, patrulha dos microambientes e, principalmente, pela resposta imunitária e inflamação, vitais para a sobrevivência e bom funcionamento neuronal.

A microglia desempenha diversas funções devido à comunicação bidirecional com outros tipos de celulares, incluindo os neurónios. A regulação da microglia é crucial, uma vez que ações exacerbadas podem levar a uma inflamação descontrolada e promover danos irreversíveis. O monóxido de carbono (CO) é uma molécula endógena com propriedades citoprotectoras e anti-inflamatórias comprovadas quando presente em níveis baixos no SNC, incluindo a limitação da produção microglial de agente pró-inflamatórios.

No entanto, a influência do CO na função basal da microglia permanece desconhecida. O objetivo principal deste estudo foi compreender a ação do CO na modulação da neuroprotecção microglial, em condições fisiológicas.

Foi estabelecido um protocolo de meio condicionado usando linhas celulares de BV2-microglia e CAD-neurónios. Os neurónios incubados com meio de microglia apresentaram alterações morfológicas e aumento de viabilidade sugerindo que o secretoma da microglia induz neuroprotecção. De forma a compreender a influência do CO nesta resposta migroglial foi realizado um tratamento com uma nova molécula libertadora de CO (ALF-826).

Verificámos que o tratamento com CO aumentou a expressão do factor neurotrófico BDNF e a secreção da Interleucina-10 na microglia. Do mesmo modo, o CO também aumentou a expressão ectonucleotidase microglial CD73, indicando um possível envolvimento da sinalização purinérgica, em particular da adenosina.

Concluímos que o CO reforça a neuroprotecção através da modulação do secretoma microglial. O progresso do conhecimento sobre a função reguladora do CO na microglia pode ser considerado um avanço de uma nova potencial aplicação do CO como procedimento preventivo através da melhoria do neurotrofismo da microglia.

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Abbreviations

A2AR – Adenosine 2A receptor

ACAMP - Apoptotic-cell-associated molecular pattern

ATP – Adenosine triphosphate

ADP – Adenosine diphosphate

BBB – Blood-brain barrier

BDNF – Brain-derived growth factor

BR - Bilirubin

BSA – Bovine serum albumin

BRV - Biliverdin reductase

CD200 – Cluster of differentiation 200

CD200R – Cluster of differentiation 200 receptor

CNS – Central nervous system

CO – Carbon monoxide

COHb – Carboxyhemoglobin

CORM – Carbon monoxide releasing molecules

CX_3CL_1 – Fractalkine

CX_3CR_1 – Fractalkine receptor

DAMP – Damage associated molecular pattern

EDTA – Ethylenediamine tetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FBS – Fetal Bovine Serum

GDNF- Glial-derived neurotrophic factor

GLUR- Glutamatergic receptors

HO – Heme oxygenase

IFN– Interferon

IL – Interleukin

LPS – Lipopolysaccharide

MAPK – Mitogen activated protein kinase

NADPH Oxidase – Nicotinamide adenine dinucleotide phosphate-oxidase

NGF – Nerve growth factor family

NO – Nitric oxide

Nrf-2 – Nuclear respiratory factor 2

NT – Neurotrophin

PAMP - Pathogen-associated molecular patterns

PBS – Phosphate buffer saline

PI – Propidium iodide

PNS – Peripheral Nervous System

PRR - Pattern Recognition Receptor

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

sGC – Solube guanylyl cyclase

SR - Scavenger receptor

***t*-BHP –** *tert*-butyl-hydroperoxide

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor α

TREM - Triggering Receptor Expressed On Myeloid Cells

UDP – Uridine diphosphate

UTP - Uridine triphosphate

1. Introduction

1.1. Nervous system

The nervous system is the most complex network system of the human body and is crucial for its survival and proper functioning. This system presents the capacity of monitorization of external and internal stimuli, as well as their integration.[19] This allows the activity management of various systems and functions including mental activity and control of muscles and glands to reach and maintain homeostasis.[19,24,60] Anatomically, the nervous system is composed of two major components: Central Nervous System (CNS) constituted primarily by the brain and spinal cord and Peripheral Nervous System (PNS) constituted by the cranial and spinal nerves and the intramural nervous system including their receptors and effector organs, such as muscles and endocrine glands.[19,33]

1.2. Neurons as a functional unit of the Nervous System

The nerve cells, neurons, are considered the functional unit of the nervous system. Neurons are post-mitotic, highly specialized and excitable cells that use electrochemical signaling to process and transmit information.[19,33]

Neurons can be divided in three main types (i) the sensory neurons (afferent) responsible for transmitting impulses from the sensorial receptors to the CNS, (ii) the motor neurons (efferent) that transmit the impulses from the CNS to the viscera, muscles, or glands, and (iii) the interneurons (association neurons) responsible for connecting the sensory and motor neurons.[45]

Despite the different functions, sizes and shapes that neurons can exhibit, these cells are typically composed of the cell body (soma), which contains the nucleus and other organelles, and the neurites (dendrites and axons) and respective terminals.[33] (Figure 1.1) This morphology promotes the communication between highly organized neuronal cells. Dendrites are usually branched neuronal processes that act as sensitive structures to receive the signals from other neurons.[19] The signal is transmitted as an action potential along the axon to another cell. Axons can present a large extension with many ramifications facilitating the communication with the target cells - other neurons, muscles, or glands.[19] In order to accelerate action potential propagation axons are surrounded by myelin sheaths, which are extensions of plasmatic membranes of oligodendrocytes.[16]

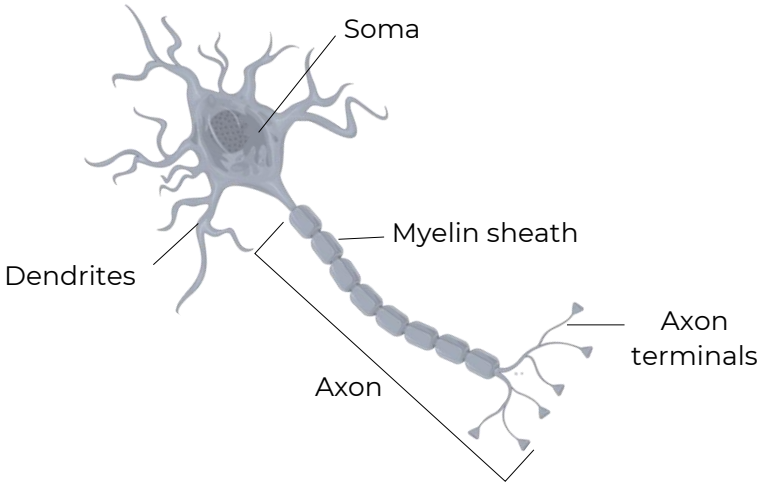

Figure 1.1 – Illustration of neuron's morphology. Morphological composition of neurons constituted of the cell body (soma) and neurites composed by the dendrites and the axon surrounded by myelin sheaths to accelerate the transmission and propagation of the impulses.

The communication between neurons occurs at specialized junctions– the synapses, where the signal is transmitted from a neuron (presynaptic neuron) to the next (postsynaptic neuron) through neurotransmitters (chemical synapses) or electrochemical signaling (electrical synapses).[19]

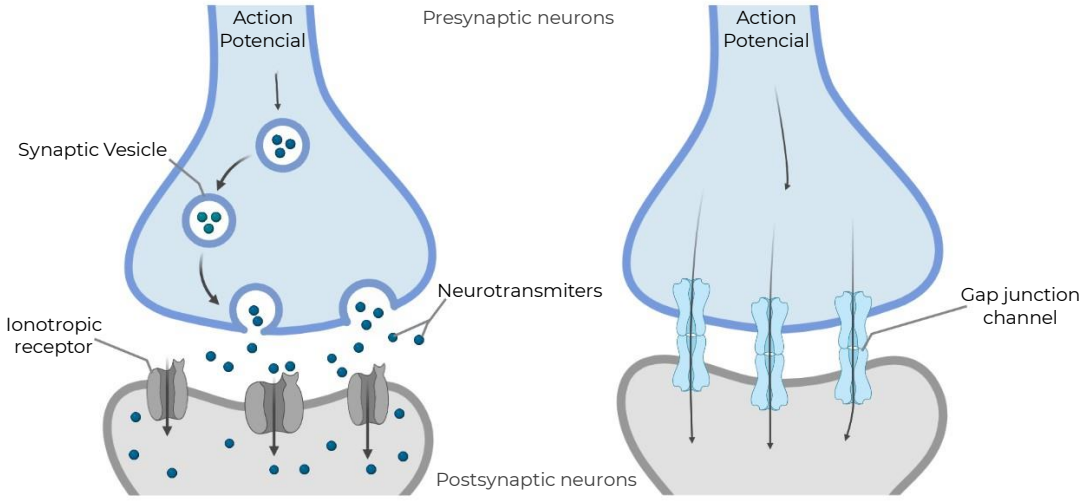

Figure 1.2 – Representation of chemical and electrical synapses. (A) Chemical Synapse. (B) Electrical synapse. Illustration made on biorender.com website.

These two classes of synapses are very distinct, chemical transmission that requires neurotransmitters release is regulated by a complex presynaptic molecular machinery.[76] When an action potential invades the synaptic terminal, it leads to depolarization and activation of voltage-gated calcium channels, which in turn

increases intracellular Ca^{2+} concentration and promotes the release of neurotransmitters. In the postsynaptic membrane, ionotropic and metabotropic receptors are required to detect and translate the information (neurotransmitters) into postsynaptic events, thus amplifying the presynaptic signal.[76] (Figure 1.2 A)

The electrical transmission implies a physical connection between adjacent cells and the signal is propagated by the bidirectional passage of electrical currents (ions) and small molecules through specialized pores (gap junction channels)[76] (Figure 1.2 B). In this type of synapses, the transmission of information occurs more rapidly (< 0.3 msec) than chemical transmission, which is crucial for synchronization of cells in *flight* or *fight* responses, synchronized development and uniform responses for essential functions, such as contraction of the heart muscle.[19,76]

1.3. Glial Cells

The survival and maintenance of neurons including their aptitude to communicate are dependent on another group of cells named glial cells. The term "glia," derived from the Greek word for "glue" suggests the neuronal supportive role of glial cells.[87] Glia are the most abundant cells in CNS and are indispensable to numerous functions including maintenance of extracellular homeostasis, pH, information processing and metabolic support for neurons.[33] In the human brain, glia present distinct morphologies, functions and locations [4]. In mammals, the three main types of glial cells in the CNS are astrocytes, oligodendrocytes, and microglia.[40] (Figure 1.3)

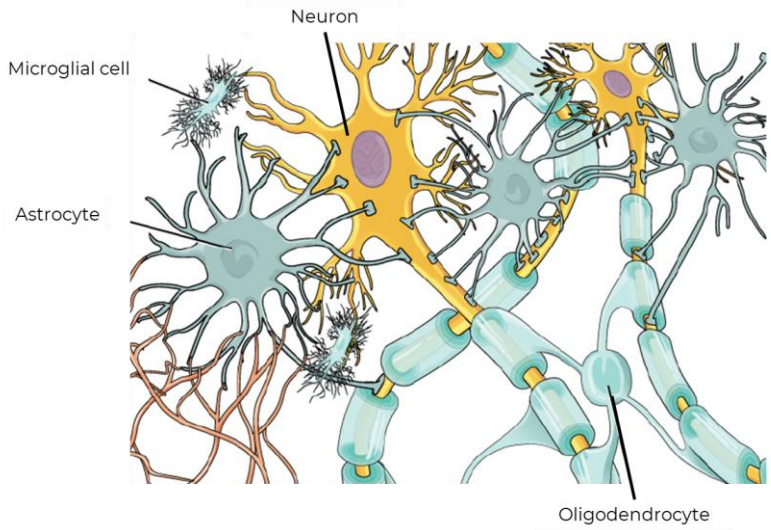

Figure 1.3. – Glial Cells in the Central Nervous System. Astrocytes, microglia and oligodendrocytes are the three main glial cells present in the CNS, essential for survival and maintenance of neurons. Adapted from https://opentextbc.ca/biology/wp-content/uploads/sites/96/2015/03/Figure_35_01_06.jpg.

Their function is essential for the maintenance of neuronal homeostasis and the functional neuronal network. These include, among other functions, neurotransmission support, ionic balance maintenance in the extracellular space, and axons insulation to speed up electrical communication.[4]

Astrocytes are the most abundant glial cells in the central nervous system (CNS)[36] and provide metabolic and trophic support, perform a crucial role in synapse formation and function, adult neurogenesis, and brain vascular tone[28,111,119]. These cells contain numerous processes and present functional heterogeneity, many of which also brain region-specific, different populations can exhibit heterogeneous morphology.[79,107] Some examples of astrocytic roles are (i) participation in the control of blood brain barrier; (ii) uptaking excessive glutamate after synaptic signaling and recycling it into glutamine which is then released into the intercellular space and (iii) supply of lactate to be metabolically used by neurons.[3]

Oligodendrocytes are highly specialized cells responsible for the formation of layers of myelin in the CNS. This substance allows a faster and more efficient flow of the electrical signal through neuronal axons. In this way, oligodendrocytes are essential for rapid communication between neurons and their targets.[4] Besides myelination, among other functions oligodendrocytes are responsible for regulating their microenvironment[16] and also provide metabolic support to neurons.[77]

Microglia represents only 5% of the glial cells in the cortex[75] and are mainly recognized for their role as part of the innate immune system. These cells are derived from a monocytic-macrophage lineage and present evident differences from macrophages due to the unique regulation by the CNS environment. For this reason, microglial cells are known as the resident macrophage population of the central nervous system[35,44,106,112]. Microglia express a variety of receptors, such as Pattern Recognition Receptors (PRRs), scavenger receptors, adhesion molecules, cytokine receptors, that sense molecular patterns such as PAMPs associated with pathogens, DAMPs associated with damage during tissue injury or ACAMPs associated with apoptotic cells.[54,58,116]. Nevertheless, microglial cells play several essential roles in the brain, namely: (i) cerebral development, (ii) preservation of the neural environment, (iii) tissue repair and (iv) in some cases propagation of tissue injury.[35,44,53,79] The role of microglia and its crosstalk with neurons will be described in the next sub-section.

1.4. Microglia-neuron interactions under physiological conditions

Microglia are plastic and strongly influenced by the local CNS microenvironment including neuronal function.[52,90,109] Microglia differ in number, morphology, abilities and functions. Under physiological conditions, microglia present several crucial roles in the brain, such as brain development, elimination of apoptotic cells and debris and synaptic pruning.[35,40,49]

Microglia have acquired multiple functional roles associated with various phenotypes. Microglial cells in the absence of harmful stimuli are named “surveillant”, playing an essential role in neuronal plasticity and patrol function of the nervous system.[35,49] Surveillant microglia present a phenotype with highly branched and motile cell processes that allow them to continuously sense and patrol the microenvironment of the CNS where they are located. Surveying microglia secrete a diversity of factors such as brain and glial-derived neurotrophic factors (BDNF, GDNF) and nerve growth factors (NGF).[43] These factors are involved in synapse elimination, sprouting and cellular differentiation needed to increase the efficiency of the neural network. They also secrete other molecules such as anti-inflammatory cytokines, such as interleukin 10 (IL-10)) in order to communicate with other microglial cells and preserve the surveillant state.[43] Specific microglial processes and their interaction with neurons are described in the next sub-sections.

1.4.1. Brain development

During brain development, the construction of neuronal circuits occurs in various steps. The main steps are stem/progenitor cell proliferation, differentiation, migration to specific areas where occurs axonal elongation and synaptogenesis with the establishment of the circuits.[103] However, these events occur in excessive numbers, with numerous inadequate or inappropriate branches and synapses; thus reshaping/ reformulation of this neuronal network is necessary.[34] This reformulation occurs through elimination events, such as regulation of cell death, axon pruning and/or elimination of synapses.[97] In addition to the functions described previously, microglia can also contribute with constructive events to synapse remodeling including proliferation, differentiation, axonal elongation, and synaptogenesis.[34] These processes are dynamic and closely dependent on microglia activity via phagocytosis of neuronal death cells and synapses.[34,97]

1.4.2. Phagocytosis

Microglia are important for the clearance of apoptotic cells and debris, by phagocytosis, which can become neurotoxic. In fact, microglia are attracted to apoptotic neurons by diverse "find-me" signals promoting microglial migration, and by "eat-me" signals on the membranes of the apoptotic cell that trigger their engulfment and phagocytosis.[34,96] The process initiated by the apoptotic cells to start microglial migration is the release of extracellular nucleotides, such as ATP and UTP.[96] In microglia, P2Y6 receptors have been discovered to function as sensors for phagocytosis. Injured neurons release uridine-5'-triphosphate (UTP) that is hydrolyzed into uridine diphosphate (UDP) which is a P2Y6-receptor agonist leading to its activation, which facilitates the clearance of dying cells or debris in the CNS.[42] Another signaling pathway of apoptotic cells is the expression of fractalkine/CX_3CL_1 since microglia express the fractalkine receptor (CX_3CR_1) that by establishing a direct receptor-binding contact on the cell membrane promotes phagocytosis.[69,104]

After microglial migration, there are complementary pathways, which are involved in the function of target recognition and apoptotic cell engulfment. For this purpose, phagocytes have a diversity of receptors that allow them to recognize their targets (eat-me markers) and distinguish them from the remaining cells. [86,91,96]. The *receptors differ* depending on the nature of their targets, in presence of infectious pathogens, highly conserved motifs termed Pathogen-associated molecular patterns (PAMPs) are detected by Scavenger receptors (SRs) and pattern recognition receptors (PRRs) including Toll-like receptors (TLR) such as the CD14/TLR4 complex, or receptors of the immunoglobulin superfamily.[96] In the same way, cellular patterns associated with apoptotic cells (ACAMPs) are detected by several receptors, including brain-specific angiogenesis inhibitor 1 (BAI-1)[11] and Triggering Receptor Expressed On Myeloid Cells 2 (TREM2).[37,96,100] Besides direct target recognition, phagocytosis can also be induced by soluble opsonins like some antibodies and complement system proteins that bind to receptors in target cells, facilitating the recognition and make them more susceptible to internalization into microglia.[96,105,113]

1.4.3. Neuroprotection

Microglia are also involved in neuroprotective and neuroregenerative events, namely by expressing a variety of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), fibroblast growth factor 2 (FGF-2), macrophage colony-stimulating factor, osteopontin, and insulin growth factor 1 (IGF-1).[34] These factors are recognized as neurotrophic due to the demonstrated support of neuronal proliferation and survival.[9,34]

Overall, in addition to the developmental stage, microglia are important for the control of neuronal activity and essential for the preservation and function of neural circuits.[93] This means that they also contribute to several brain functions such as learning and memory. [34,99] The bidirectional communication between microglia and neurons is fundamental for the construction of neuronal circuits mentioned above and for the proper functioning and surveillance of the CNS.[59,99]

1.5. Bi-directional crosstalk neuron-microglia

As mentioned in the previous sub-sections, besides microglial action as the brain's immune guards, these cells are crucial for preserving neuronal homeostasis. This requires efficient bi-directional communication between neuronal and microglial cells.[93] Neurons and microglia express various receptors and ligands that can be activated through a direct connection between cells or numerous secreted soluble molecules. [99]The crucial bi-directional communication is impaired in brain diseases due to certain stimuli that interfere with specific communication pathways. These alterations affect synaptic activity and plasticity or neuronal survival, which can potentially cause profound changes in nerve circuits and associated functions. [55,99] In the next sub-section, several different signaling pathways for neuron-microglia communication are described.

1.5.1. Cytokines

A category of small soluble molecules, named cytokines, is recognized to modulate communication, cell growth, survival, differentiation and functions. Cytokines released by microglia are involved in modulating neuronal functions and may present neuroprotective and detrimental effects. For example, IL-1, IL-10 and IL-19 are linked to a positive contribution to synaptic plasticity and memory. Conversely, a high concentration of cytokines, such as IL-1β, IL-18, IFN and TNFα produced by activation of microglia under inflammatory conditions, exert detrimental effects on neurons, impairing synaptic plasticity.[55,114]

Also, neurotrophins are associated with neuron-microglia communication, these molecules are produced by microglia and their receptors are expressed on both neurons and microglia. In particular, neurotrophins like BDNF and NGF have important roles in neuronal activity and plasticity in the CNS.[55,114]

1.5.2. Neurotransmitters

Several neurotransmitters-based pathways are recognized as crucial for communication. Microglia express ionotropic and metabotropic glutamatergic receptors (GLURs) capable to recognize certain neurotransmitters.[65,78] Neurotransmitters can modulate microglial phenotype, motility, channels and functions. In addition, similarly to neurons, microglia release several substances capable of activating neuronal GLURs and in this way are able to control neuronal function in a feedback loop.[55] For these reasons, neurotransmitters are considered a pathway used for mutual regulation.[55]

1.5.3. Purinergic signaling

The purinergic signaling involves mechanisms of ATP release, of extracellular ATP metabolism by ectonucleotidases, and purinergic receptors.[88] Purinergic receptors are composed of two main classes P1R and P2R receptors. P1R are G protein-coupled receptors selective for adenosine composed of four subtypes (A1, A2A, A2B and A3 receptor). P2R, activated by purine and by pyrimidine nucleotides, are divided into P2X (ligand-gated ion channels) and P2Y (G protein-coupled) receptors.[20,41]

Microglia express functional ionotropic P2X and metabotropic P2Y purinergic receptors. Purinergic receptors are activated by extracellular nucleotides (ATP, ADP, UTP), typically associated with tissue damage or released by neurons.[1,88] In particular, the metabotropic P2Y12 R is expressed on microglia in the CNS and is activated by

ADP deriving from enzymatic degradation of ATP released from neurons, astrocytes and oligodendroglia during their physiological activity or following tissue damage [1,120] and mediate various aspects of intercellular signaling targeting microglia [81,107]. On the other hand, other receptors are relevant for this communication. For example, ATP activation of P2X7 is associated with cytokine release and microglial death and P2X4 expression, which is present at low levels in the brain under physiological conditions, under inflammation P2X4 becomes highly upregulated in microglia, controlling the feeding circuits.[38,55]

Adenosine, a product of ATP catabolism, can also activate microglial receptors such as of A1R and A2AR. In particular, stimulation of A2AR leads to the retraction of microglial processes associated with the activated phenotype of microglia, release and secretion of inflammatory factors and neuroprotection through Nerve growth factor (NGF) release.[72] Microglia are able to release ATP or adenosine depending on the environment sensed and at the same time express ectonucleotidases such as CD73 and CD39, which catalyze the reaction of ATP conversion into adenosine. [55–57]

Extracellular adenosine plays multiple physiological roles in the brain parenchyma as a modulator of synapses, astrocytes, oligodendrocyte, endothelial and microglia. Specifically in microglia, adenosine can control the proliferation, reactivity and communication with neurons.[29,78]

In this way, purinergic signaling is considered one of the key systems of neuron-microglia communication and microglia function.[55]

1.5.4. CX_3CL_1/CX_3CR_1

A widely studied pathway in neuron-microglia communication is the fractalkine axis: CX_3CL_1/CX_3CR_1. Fractalkine (CX_3CL_1) is a chemokine, that are molecules of the cytokine family, with important roles in cell migration in response to stimulus, in physiological or pathophysiological conditions.[95] CX_3CL_1 is produced by neurons and can be found as (i) a membrane-bound form, which promotes cell adhesion and as (ii) a cleaved soluble form, inducing chemo-attraction.[95] These two functions require interaction with microglia through its receptor (CX_3CR_1), a G-protein coupled receptor, whose activation triggers PI3K, AKT and NF-kb pathways.[15] CX_3CL_1/CX_3CR_1 signaling is associated with an anti-inflammatory and homeostatic function of microglia and its reduction leads to the loss of these functions, affecting physiological processes and may lead to neurodegeneration. [55]

1.5.5. Complement system

The complement system is also involved in the bi-direction communication and is composed of diverse proteins associated with membranes or in circulation (c1q, C1, C3, C4, C5) and their respective receptors, expressed in neurons and glia.[55] Microglia express high levels of C1q protein C3 and C5 receptors, however, neurons' expression of complement factors is mainly associated with disease or early states of development. These pathways are responsible to the control of distinct microglial functions, such as motility, phagocytosis and cytokine release.[98] These functions are altered in neurodegenerative disorders where a massive activation and overexpression of complement factors are observed. As expected, a reduction of complement factors reverts detrimental events, namely: synapse loss, impaired cognitive function, aberrant microglial phagocytosis and molecular alterations that precede neurodegeneration.[92] Microglia-mediated neurotoxic effects in neurodegenerative disorders may also involve the induction of a reactive astrocytic phenotype by cytokines and C1q release.[55]

1.5.6. TREM signaling

TREM signaling is also a key process in the bidirectional communication between cells, promoting neuroprotection. TREM2 is an innate immune receptor that increases its expression during microglial activation.[99] TREM2 expression increases microglial migration and phagocytic activity, which in turn promotes the elimination of apoptotic cells.[58] Perturbation in this pathway leads to the loss of microglial capacity to maintain homeostasis, associated with neurodegeneration.[55]

1.5.7. CD200/CD200R

Communication also occurs by the formation of protein complexes that are implicated in the control of brain inflammation under pathology. The complex between neuronal CD200 glycoprotein and the microglial receptor CD200R is involved in the regulation of microglial phagocytosis.[44] Moreover, inhibition of microglial CD200R interaction with neuronal CD200 activates microglia, leading to deleterious neuroinflammation activation and neurodegeneration.[44,55]

1.6. Microglia-neuron interactions under pathological conditions

When exposed to a damaging stimulus, such as physical injury, ischemia, inflammation, infection, or neurodegeneration, microglia are rapidly activated (change in morphology and phenotype) along with an increased number of microglia in the affected area – microgliosis and reactivity.[58]

In response to stress, microglia adopt amoeboid morphological features through an enlargement of the soma and highly retracted processes. These microglia are termed 'activated' and present an increased expression of myeloid cell markers and improved phagocytic ability.[43] Moreover, it has been demonstrated that morphologically similar microglia in different models of CNS diseases present widely divergent patterns of gene expression, showing that different stimuli polarize their activation states to achieve appropriated effector responses.[85] Activated microglia recruit other microglial cells and can respond to foreign or injury cells by changing their expression and secretion of molecules.[44] Microglia in the activated state secrete high levels of reactive oxygen/nitrogen species (ROS/RNS), such as nitric oxide (NO), growth factors, chemokines, neurotrophins, and both anti-inflammatory and pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin 1 beta (IL-1β).[43,44] Molecules expressed by microglia are also associated with an ability to stimulate T cells with antigen, inciting a host response.[43,85] Microglia are also responsible for phagocytosis myelin debris, damaged cells and synapses to protect neurons from excessive neuronal excitation.[34] Moreover, microglia are also beneficial for the efficient reconstruction of neural circuits, similarly to what happens during brain development and the formation of new neural circuits.[85]

Activation of microglia in the CNS promotes an event called neuroinflammation, which is a vital process to overcome harmful consequences caused by any source of danger.[44] However, inflammation needs to be under very tight control, cannot be exacerbated or chronic.[43] In fact, chronic or hyper-activation of microglia can promote irreversible damage, causing further tissue degradation, debilitating the BBB, decline of neuronal activity that may be associated with acute and/or chronic neurodevelopmental pathologies [35,44,112]

The plasticity of microglia in density, morphology, gene expression and function is important for the responses of specific brain regions to environmental stimuli.[55] This plasticity makes these cells passive targets for neuroprotective strategies or treatment.

1.7. Carbon monoxide

Cells and tissues have developed strategies to respond and restore homeostasis when submitted to different injury conditions, such as oxidative stress, hyperthermia, hypothermia, ischemia, hypoxia, hyperoxia, inflammation, misfolded protein response or exposure to UV light.[84] Affected cells produce mediators such as cytokines, chemokines and prostaglandins associated with inflammation. The immune cells are mainly the firsts to be activated and recruited to the site of injury leading to the release of ROS and other molecules.[2] When inflammation is persistent the released molecules can be excessive and consequently detrimental.[58] In order to fight against inflammation and oxidative stress, endogenous pathways are activated. Previous studies have revealed that transcription nuclear factor-erythroid 2 (NF-E2) p45-related factor-2) (Nrf2) regulates the expression of phase II detoxifying enzymes including NADPH, NAD(P)H quinone oxidoreductase 1, glutathione peroxidase, ferritin and Heme oxygenase-1 (HO-1) and antioxidant genes with anti-inflammatory actions that protect cells from injuries.[2,10,18,22]

Heme oxygenase (HO) is an enzyme with antioxidant activity and cytoprotective effects, namely anti-inflammatory, anti-apoptotic and anti-proliferative actions, involved in the prevention of disease development and re-establishment of tissue homeostasis.[12] HO can be found in two main isoforms: HO-1, an inducible isoform, found in many tissues and at low levels in the brain under basal conditions; and HO-2, a constitutive isoform present mainly in neurons. [21,84] A third isoform (HO-3), not found in the human, was discovered in rat, however, its function and specific distribution are still controversial.[73]

In the CNS, HO act as a stress-response enzyme, therefore, by increasing its expression or activity in neuronal, glial, and endothelial cells following stress stimulation, namely oxidative stress, inflammation (pro-inflammatory cytokines), hypoxia, ischemia, among others.[17]

HO is responsible for the catabolism of the heme group using NADH and NADPH as electron donors to produce carbon monoxide (CO), free iron (Fe^{2+}) and biliverdin, a tetrapyrrolic bile pigment, which is rapidly reduced to bilirubin (BR), an antioxidant molecule by biliverdin reductase (BVR).[46] (Figure 1.4.)

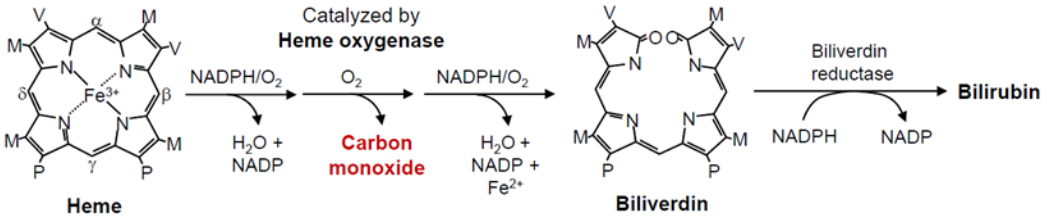

Figure 1.4. –Heme catabolism by Heme-oxygenase (HO). Heme-oxygenase catalyzes the reaction of the heme groups leading to the formation of free iron, carbon monoxide and biliverdin that is rapidly converted to bilirubin by biliverdin reductase. Adapted from [26].

HO activity maintains homeostasis and promotes cytoprotection by the removal of free and toxic heme groups that are released during protein turnover. Furthermore, HO is also cytoprotective due to their products: biliverdin, Fe and CO. Biliverdin and bilirubin have potent antioxidant action and are involved in the modulation of the inflammatory response and insulin signaling [17]. Free Iron (II) induces the expression of ferritin, which prevents free radical damages to cells by binding and storage free metals.[117]

For decades Carbon Monoxide (CO) has been viewed as an environmental pollutant and mostly known as toxic and silent-killer gas since high levels can compromise systemic oxygen delivery and mitochondrial function. This occurs due to its high affinity for heme-proteins in their reduced state (Fe^{2+}), which is present in myoglobin, soluble guanylyl cyclase (sGC), inducible nitric oxide synthase, NADPH oxidase, cytochrome c oxidase and hemoglobin. The interaction of CO with hemoglobin leads to the formation of carboxyhemoglobin (COHb) which consequently decreases its capability to carry oxygen through the body, resulting in hypoxia. At the cellular level, high CO concentrations inhibit cytochrome c oxidase and oxidative phosphorylation, leading to excessive mitochondrial production of ROS and decreasing generation of ATP.[31]

Nevertheless, endogenous CO or low levels or exogenous CO act as a signaling molecule involved in several pathways (for instance sGC and MAPK pathways). Low concentrations of CO are involved in different biological roles including anti-inflammation and anti-apoptosis.[63] During inflammation, CO acts on these pathways leading to decreased expression of inflammatory cytokines (TNF-α, IL-6, IL-1β) and increased expression of anti-inflammatory compounds, like IL-10 and other factors that are related to inhibition of inflammatory signaling.[73,84]

The mechanisms of CO cytoprotection are still under discussion. However, CO is associated with targeting and inhibition of cellular functions and generation of low

levels of reactive oxygen species (ROS) and other signaling molecules (superoxide, H_2O_2 and hydroxyl radicals).[7,17,83] There is evidence showing a partial and reversible inhibition of mitochondrial respiration by CO binding to cytochrome c oxidase, leading to the production of small amounts of ROS at complex III level, due to electron accumulation whenever complex IV is inhibited.[84] Although excessive ROS production is generally considered toxic, evidence suggests that nondetrimental amounts of ROS can act as important regulators of biological processes.[17,70] These nondetrimental levels of ROS can be produced in response to exogenous low doses of CO and act as signaling molecules.[64,82] In fact, ROS promote the cell to undergo oxidative pre-conditioning-like effect that leads to cytoprotective gene expression. CO also leads to the generation of reactive nitrogen species (RNS) derived from NO.[17]

In CNS, CO also presents several different protective roles, for revision.[84] CO has proven anti-apoptotic action in neurons[94,110], as well as the capability of improving neuronal differentiation.[6,8] In astrocytes, CO prevents oxidative stress-induced cell death by reinforcing mitochondrial metabolism [5], by limiting mitochondrial depolarization and membrane permeabilization [82] and by activation of P2X7 receptor.[71] In microglia, CO also presents anti-inflammatory role.[13,50] Nevertheless, the potential effect of CO on generation of neurotrophic factors in microglia has never been explored before.

The potential of CO as a therapeutic agent led to the development of strategies to deliver it in an efficient, safe and controlled manner. The application of exogenous CO gas by inhalation presents several disadvantages, mainly the lack of specificity, which can cause partial systemic hypoxia and toxicity.[27,89] To overcome these problems, pro-drugs named CO-releasing molecules (CORM) were developed to deliver CO in a more physiologically appropriate and specific manner. [61]

1.8. CORMS

CO-releasing molecules (CORM) are complex carbonyl compounds (M(CO)y), containing generally a transition metal in the center – such as molybdenum, cobalt, iron, manganese, or ruthenium - and carbonyl groups as coordinated ligands. [61] These small organometallic molecules improve the administration of CO by partially overcome several issues associated with CO inhalation as toxicity, low specificity, and meticulous controlled administration.[61]

CORM administration can be more practical using alternative routes like oral or parenteral. [64,89] These releasing molecules present different characteristics to

overcome issues as toxicity associated with transition metals, insolubility, or instability through the nature of the ligands.[61,89]

Another advantage of CORMS is the potential increased tissue specificity, which can be achieved by specific alterations of the molecule. For instance, CORMs can be altered in (i) the central metal ion, (ii) the number and the spatial arrangement of the CO, and (iii) the type, number, and coordination of ligands [121], according to the target and respective microenvironment. [64,89] CORM specificity leads to increased efficiency of CO action since, to achieve the same biological response, lower doses of CORMs are required.[89]

CORMS can be classified into two groups depending on CO release. The first class comprises the spontaneous and initiated release by hydrolysis, redox reactions at the metal core or other factors like temperature, pH and ligand substitution.[121] The second main type of release encompasses CORMs that require external stimuli to promote the liberation of CO. Examples of the previous category are "Enzymatically triggered CORMs" and "Photo-CORMs" that release CO as a result of an enzymatic reaction and light exposure, respectively.[121]

The first two commercially available CORMs, CORM-1 (Mn_2CO_{10}) and CORM-2 ($Ru(CO)_3Cl_2$) were lipid-soluble and stable under aerobic conditions. Furthermore, the CO release processes are distinct: CORM-1 (Figure 1.5 A) presents a photo-induced CO release and CORM-2 (Figure 1.5 B) releases CO molecules in a spontaneous manner presenting a half-life of approximately 1 minute.[61] Thus, these features do not facilitate their use in biological systems. Then the water-soluble CORM-3 [$Ru(CO)_3Cl(glycinate)$] (Figure 1.5 C) was developed and presents a half-life of CO release at approximately 1 minute.[27] While CORM-A1 [H_3BCO_2]Na_2 (Figure 1.4 D) is a nontransition-metal molecule complex, water-soluble and releases CO slowly under physiological conditions (pH and temperature).[62] Besides the referred CORMs, numerous other molecules with different properties have been developed and have already proven to be effective in biological systems. [39,121]

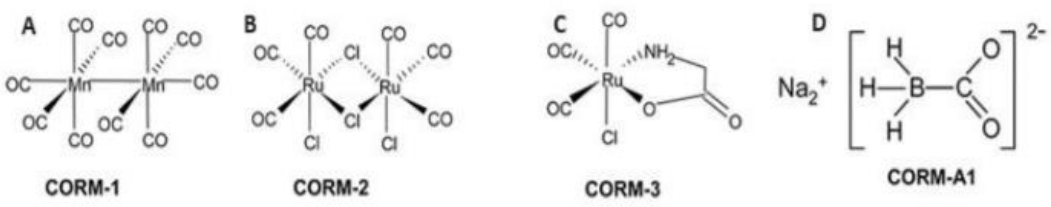

Figure 1.5.- Chemical structures of CO-releasing molecules. (A) CORM-1, (B) CORM-2, (C) CORM-3 and (D) CORM-A1. Adapted from www.fasebj.org/content/19/2/284/F3.large.jpg

The described beneficial effects of CORMs range to various models of disease [27,32,48,65,66,74,108] and cellular processes. [14,13,115] This supports that CO with the appropriate delivery is a potential therapeutic molecule.

ALF-826 is a new developed molecule containing molybdenum as central metal. Preliminary and confidential data have demonstrated the anti-inflammatory role of ALF-826 using *in vivo* models.

1.9. Final remarks and objectives

In the CNS, the interaction between microglia and neurons is crucial for homeostasis maintenance, function preservation and adaptative behavior under physiological or injury situations. Considering the relevance of this communication, targeting microglia is an approach with great potential to promote neuroprotection. [99] In fact, as long term impact, one can propose that modulation of microglial function can become a new strategy to improve the outcome in neurological and neurodegeneration disorders. Carbon monoxide appears as a promising therapeutic molecule with already proven anti-inflammatory, anti-apoptotic and cytoprotective potential in several cells and disease models. [14,13,17,115] Still, CO can also act as an anti-neuroinflammatory agent in microglia.[14,13,50]

Therefore, the main goal of this thesis is to study CO as a modulator of microglia neurotrophism – whether and how CO modulates microglia to promote neuroprotection and improve neuronal function under physiological conditions.

In order to fulfill this main goal, three specific questions are addressed:

(i) "Does CO promote neurotrophic and/or neuroprotective response in neurons by targeting microglia?"

(ii) "How does CO modulate the microglial expression and release of neurotrophic factors?"

(iii) "How these neurotrophic factors participate in the communication microglia-neuron?".

2. Materials and Methods

2.1. Cell culture

CAD (Cath.-a-differentiated) cell line, a variant of a CNS catecholaminergic cell line established from a brain tumor in a transgenic mouse was used to mimic neurons. This catecholaminergic cell line was maintained in T-flasks at 37°C, 5% CO_2, in a DMEM:HAMS F12 medium (Thermo Fisher) supplemented with 8% (v/v) FBS (Thermo Fisher), 2% (v/v) Pen/Strep (Thermo Fisher) and 1% (v/v) L-glutamine (Thermo Fisher). The culture was passed 2 times per week with a dilution of 1:4.

The immortalized murine microglial cell line BV2 was used to mimic microglia. This microglial cell line was maintained in T-flasks at 37°C, 5% CO_2, in a RPMI-1640 medium (Thermo Fisher) supplemented with 10% FBS (Thermo Fisher), 1% Pen/Strep (Thermo Fisher) and 2% L-Glutamine (Thermo Fisher). The culture was passed 3 times per week with a dilution of 1:4.

2.2. Optimization of the viability model

Neuronal CAD cells were seeded onto 24-well plates with 8×10^4 cells per well and differentiated after 24 hours in a DMEM:HAMS F12 medium (Thermo Fisher) supplemented with 2% (v/v) Pen/Strep (Thermo Fisher) and 1% (v/v) L-glutamine (Thermo Fisher). One day after differentiation, CAD cells were incubated with different concentrations (0, 5, 7.5, 10 and 15 µM) of *t*-BHP (Sigma-Aldrich). Cell viability was performed after 24h by flow cytometry.

2.3. Conditioned Media Protocol

CAD cells were seeded onto 24-well plates, previously coated with a 5mg/100ml concentration of poly-D-lysine (Sigma-Aldrich), with 8×10^4 cells per well and BV2 cells were seeded onto 24-well plates with 10×10^4 cell per well. One day after seeding, differentiation of CAD cells was induced in free-serum conditions (DMEM:HAMS F12 medium (Thermo Fisher) supplemented with 2% (v/v) Pen/Strep (Thermo Fisher) and 1% (v/v) L-glutamine (Thermo Fisher)) and microglia were pre-treated or not with ALF-826 (50 µM). 48 hours after treatment, microglial media were collected and centrifuged for 5 min at 500g to ensure that only soluble factors were present in the media. Neuronal media were then removed and substituted by microglial media collected previously. In viability experiments, after media substitution neuronal cells were incubated with 7.5, 10 µM of *t*-BHP (Sigma-Aldrich).

2.4. Neuronal Viability by Flow Cytometry

CAD cells, after 24 hours of incubation with *t*-BHP, were detached and collected by trypsinization using trypsin (Thermo Fisher) at 37°C for 5 minutes, after thoroughly washing the wells with PBS. Subsequently, collected cells were incubated for 15 min at 37°C with 1 ng/mL of Propidium iodide (PI), a fluorescent agent that intercalates with DNA in cells with compromised plasmatic membrane integrity, allowing the discrimination between dead and alive cells. Cell viability was performed using a flow cytometer (FACS Canto II, BD Biosciences) with a 488nm laser 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 and a threshold of acquisition of 5000 cells was maintained. Data obtained were analyzed by Flowjo software.

2.5. Protein extract collection

Collection of protein extracts was done by washing repeatedly with ice-cold PBS to Eppendorf tubes, 48 hours after CO treatment in BV2 cells and 24h after condition media protocol in CAD cells, using a lysis buffer (20 mm Tris HCL pH 8, 137 mm NaCl, 10% glycerol, 1% Igepal, 2mm EDTA) supplemented with 10% of protease inhibitor (Merck) and stored at -20°C.

2.6. Total protein quantification protocol

Total protein concentration was quantified using a BCA protein calibration curve (Pierce BCA protein assay kit, Thermo Scientific). Cells extracts were diluted (1:10) and 25 µL of each was added onto 96-well plates as well as the BSA standards. The working reagent (200 µL) was then added to the wells and the plate was covered and incubated at 37°c for 30 minutes.

To access total protein concentrations, absorbance was measured at 592 nm, using a Tecan Infinite F200 PRO microplate reader.

2.7. Western blot

Protein extracts of microglial BV2 cells were collected and quantified to access BDNF precursor (32 kDa) and mature form (14 kDa) and CD73 (63 kDa) protein concentrations after treatment with ALF-826 (50 µM).

In the same way, protein extracts of neuronal CAD cells were collected and quantified to access A2A receptor (45 kDa) after conditioned media protocol (2.3). If necessary, samples were concentrated in ethanol overnight.

The samples, containing the same amount of protein, were centrifuged at 20238 rcm for 25 minutes and ethanol was completely removed. The samples were resuspended in 25 µL of Loading Buffer diluted 5x at RIPA 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)) and incubated at 97°c for 10 minutes. After the previous procedure, samples and molecular marker (Nzycolour Protein Marker II, nzytech) were added to a polyacrylamide gel and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 135 - 150V (fixed) for 1hmin-1h30min. The separated target proteins were transferred onto nitrocellulose (Sigma Aldrich) or PVDF membrane (Merck) for 1h15min at 500 ma (fixed) and then blocked for 1 hour with 5% BSA solution (Merck). After blocking, membranes were incubated with the respective primary antibody (Table 2.1) overnight at 4°c. On the next day, the membrane remained 2 hours at R.T. with the primary antibody and then was incubated for 1h with the secondary antibody (Table 2.1) with 3 T-TBS washing steps after incubations. The membrane was then incubated with ECL for 5 minutes and images of the membranes were acquired using Chemidoc Touch equipment (Bio-Rad). β-Actin (42 kDa) was used as a loading control due to its consistent expression across eukaryotic cells that do not vary drastically due to cellular treatment. Acquired data were analyzed by Image Lab software.

2.8. Immunofluorescence Microscopy

The immunofluorescence experiment in neuronal cells was performed using 3 conditions - CAD cells, CAD cells in contact with BV2 media and CAD cells in contact with BV2 cells media pre-treated with 50 µM of ALF-826.

After the conditioned media protocol (2.3), CAD cells adhered to the coverslip were fixed with 4% PFA (+ 4% Sucrose) in PBS (20 min at R.T.), permeabilized with 0.3% TX-100 in PBS (15 min at R.T.) and blocked with bovine serum albumin (BSA) 1% (w/v) and Triton X-100 0.1% (w/v) in PBS (30 min at R.T.), with PBS washing before each step.

After the previous procedure, cells were incubated with primary antibody to neurite (β-tubulin) staining (Table 2.1) for 2h at R.T. and incubated with secondary antibody (Table 2.1), with PBS washing after each step. Then Prolong mounting media (Invitrogen) with DAPI to stain the nucleus of cells was added to the coverslip. After the drying of the mounting media cells were prepared for observation using Zeiss Z2 microscope. Morphological parameters were determined using neuronj plugin in Imagej software.

2.9. ELISA

BV2 cells were plated in 24-well plates with a concentration of 10×10^4 cells/well. 24 hours after seeding, BV2 were treated with 50 μM of ALF-826 and one day after the media were collected and stored at -20°c until used.

To measure the concentration of IL-10 secreted by BV2 cells, a Murine IL-10 Standard ABTS ELISA Development kit (Peprotech) was used following the manufacturer's instructions. The concentration was determined by monitorization of color development at 415 nm with wavelength correction set at 560 nm using a Tecan infinite F200 PRO microplate reader.

2.10. Statistical analysis

The statistical analysis between different condition groups was done by One-way ANOVA Dunnett's Multiple Comparison. The statistical analysis between the same condition groups was done by t-test. Significant differences were considered whit p-values lower than 0.05 when compared with respective controls.

Table 1 – List of antibodies used for immunofluorescence microscopy and western blot experiments.

Primary Antibodies	**Species**	**Type**	**Technique**	**Dilution**	**Supplier**
BDNF	Rabbit	Polyclonal	WB	1:200	Thermofisher
CD73	Mouse	Monoclonal	WB	1:1000	Abcam
A2AR	Mouse	Monoclonal	WB	1:200	Sta. Cruz
B-Actin	Mouse	Monoclonal	WB	1:5000	Sigma-Aldrich
B-Tubulin	Mouse	Monoclonal	IF	1:500	Sigma-Aldrich
Secundary Antibodies		**Type**	**Technique**		**Supplier**
Anti-Rabbit		Polyclonal	WB	1:5000	GE LifeSciences
Anti-Mouse		Polyclonal	WB	1:5000	GE LifeSciences
Anti-Mouse (Alexa Fluor 488)		Polyclonal	IF	1:1000	Thermofisher

3. Results

3.1. Establishment of experimental model

Neuronal survival is an important parameter to assess how particular components influence neurotrophism by promoting neuroprotection. In order to establish our viability experimental model, neuronal cells were challenged *tert*-butyl hydroperoxide (*t*-BHP) at different concentrations for inducing cell death. *t*-BHP is a pro-oxidant organic agent that induces oxidative stress and consequent apoptosis.[118]

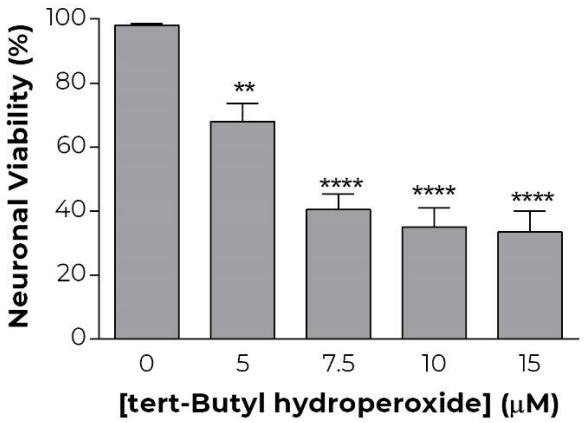

Figure 3.1 – *t*-BHP induces a cell death dose-response to cultured neuronal CAD cells. Neuronal CAD cells were incubated 24h after differentiation with different concentrations of tert-Butyl hydroperoxide (*t*-BHP). Cell viability was performed after 24h by flow cytometry (PI internalization). Statistical significance was analyzed by One-way ANOVA Dunnett's Multiple Comparison: **p≤0.01; ****p≤0.0001 compared with negative control, n=4 biological replicates.

Viability was determined by flow cytometry using Propidium Iodide (PI), a fluorescent DNA intercalating agent, which stains dead cells since it only crosses the plasmatic membrane when their integrity is compromised. *t*-BHP agent induces a consistent cell-death dose-response curve in CAD cells, showing that increased concentrations of *t*-BHP decrease neuronal viability. (Figure 3.1.). This result confirms that this agent can be used as an inducer of cell death in CAD cells in the following experiments.

3.2. Microglia-neuron communication

3.2.1. Neuron viability and carbon monoxide effect

After the establishment of the viability model, a microglia-derived conditioned media protocol was performed (Figure 3.2 A), by treating CAD neuronal cells with microglial secretome for 24 hours. This procedure allowed the assessment of the neuronal viability in the presence of microglial conditioned medium in order to disclose the role of microglia on neuronal viability and response to stress. Moreover, this conditioned medium protocol mimics conditions that are more similar to physiological events, since neurons are not isolated cells and communication is crucial for their maintenance and function. Regardless of the simplification of the model, the performance of this experiment allowed the study of the role of microglia-neuron communication and its importance for neuroprotection. Carbon monoxide was used in the experiment to study its potential as a neuroprotective molecule and stimulator of microglial neurotrophism.

CO was delivered by using the molybdenum base CORM called ALF-826. The optimal concentration for microglia is 50 μM, which was previously established (data not shown). To understand how microglia-neuron communication and CO presence affect neuronal viability in response to oxidative stress (*t*-BHP treatment), 3 conditions were used: (1) control neurons that did not contact with microglial media (black, Fig 3.2. B), (2) neurons incubated with microglial media without any treatment (grey, Fig 3.2. B) and (3) neurons incubated with CO-treated microglial media (black and white stripes, Fig 3.2. B).

The data showed that in the absence of the cell-death-inducing agent, *t*-BHP, no major difference in the 3 conditions was found, presenting high viability. However, in presence of *t*-BHP, microglial media (treated and untreated with CO) rescue neuronal viability, which has substantially decreased in monoculture of neurons (Figure 3.2. B). Nevertheless, the neuronal viability following *t*-BHP challenge in the presence of untreated microglial media or CO-treated microglial media were not statistically significant. In conclusion, microglial conditioned medium promoted neuroprotection against oxidative stress-induced cell death.

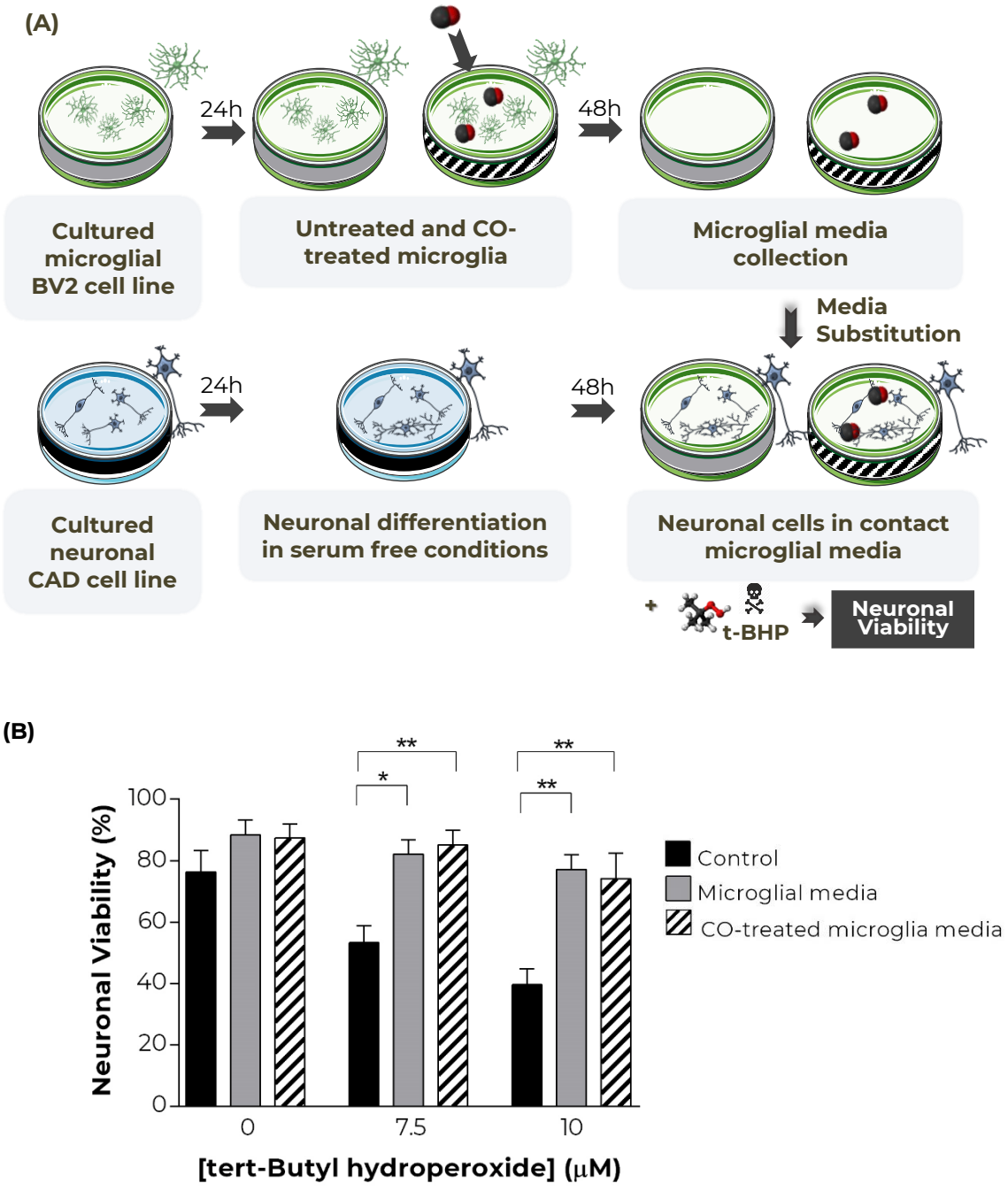

Figure 3.2 – Microglial media, untreated and treated with CO, in contact with neuronal cells increase their viability in presence of cell death-inducing agent. Neuronal CAD cells were plated, 48h after differentiation, with either microglial BV2 media or microglial BV2 media treated with 50 μM of ALF-826, followed by administration of different concentrations (7.5 and 10 μM) of tert-Butyl hydroperoxide (*t*-BHP), a cell death-inducing agent (A).). Cell viability was performed after 24h by flow cytometry (PI internalization). (B). Statistical significance was analyzed by One-way ANOVA Dunnett's Multiple Comparison: *p≤0.05; **p≤0.01 compared with respective controls, n=5 biological replicates.

3.2.2. Neuron morphology and carbon monoxide effect

Neuronal viability is not the only parameter that can be accessed to study the effects of CO-treated microglia (and their released soluble factors) on CAD neuronal function. Neuronal morphology is also an important feature to understand the potential protective effect of microglial secretome when treated with CO. Thus, neuronal morphological changes were assessed under the 3 conditions: (1) control neurons that did not contact with microglial media, (2) neurons incubated with microglial media without any treatment and (3) neurons incubated with CO-treated microglial media, which were previously presented. Neuronal morphology was followed by staining neurons with β-Tubulin III antibody coupled with a fluorescent secondary antibody and images were taken using a fluorescence microscope.

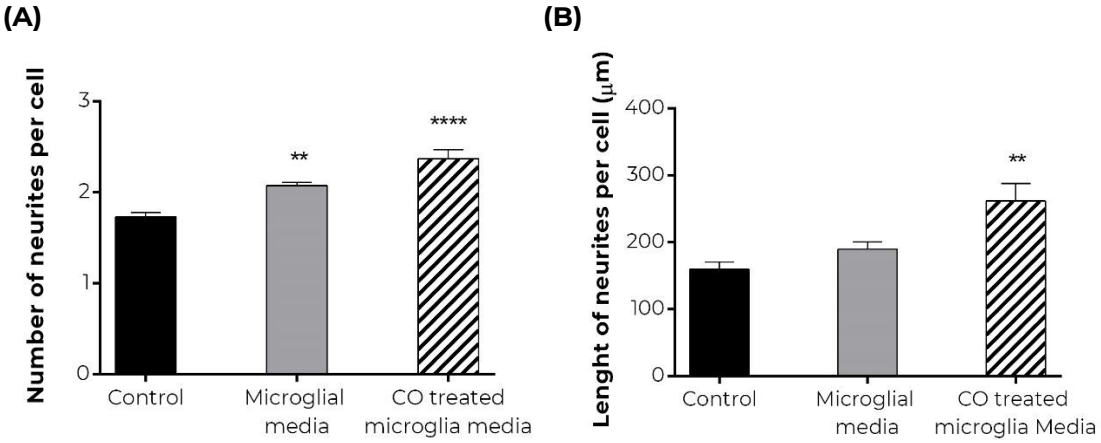

Figure 3.3 – Effect of microglial media treated and untreated with ALF-826 in neuronal morphology. Neuronal CAD cells were incubated 48h after differentiation, with either microglial BV2 media or microglial BV2 media treated with 50 μM of ALF-826. After 24h, neuronal cells were prepared to perform fluorescent microscopy using Zeiss Z2 microscope with nucleus (DAPI) and neurite (β-Tubulin III) staining. **(A-B)** Number of neurites per cell and Lenght of neurites per cell parameters were obtained using imagej plugin neuronj. Statistical significance was analyzed by One-way ANOVA Dunnett's Multiple Comparison: **p≤0.01; ****p≤0.0001, n=5 biological replicates.

The parameters presented in Figure 3.3., (A) number and (B) length of neurites were obtained using a specific program (Image J) and software (Neuronj plugin) that allowed to define the neurites for each cell in the acquired images. As it can be observed in Figure 3.3 A, microglial media increase the number of neurites *per* cell with significantly higher levels when microglial media were pre-treated with CO.

When looking at neurite's length (Figure 3.3 B) the difference between the effect of untreated and CO-treated media became more evident. The absence of alteration in the length of neurons in untreated microglial media compared with control contrasted with the significant increase in neurites' length in neurons incubated with CO-treated microglial media. In summary, CO appears to improve neuronal morphology by acting on microglial secretome.

3.3. Effect of carbon monoxide in microglial soluble factors

After the previous finding that neuronal viability and morphology are altered by microglial secretome and some alterations are potentiated by carbon monoxide microglia pre-treatment; the next step of this study was to identify which key soluble factors are secreted by microglia; in particular which ones are more secreted in the presence of CO. In order to find which factors are involved in this process, some potential candidate molecules were investigated.

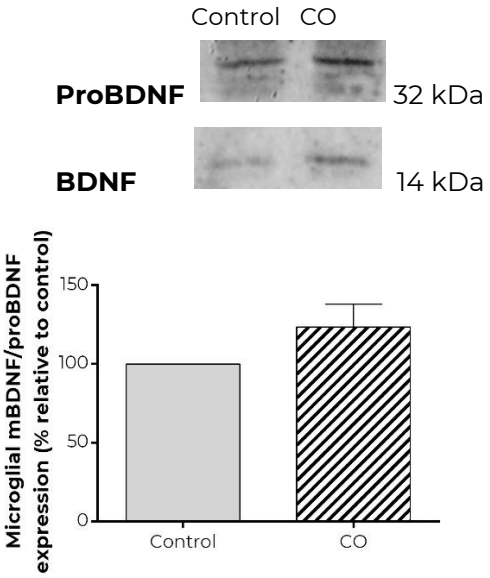

Figure 3.4 – Effect of Carbon monoxide in mBDNF/porBDNF expression in microglia. Microglial BV2 cells were treated with 50 µM of ALF-826. Total protein extracts of microglial cells were collected 24h after CO treatment and analyzed by Western blot. Statistical significance analyzed by t-test: ns: p>0.05. N=4 biological replicates.

Brain-Derived Neurotrophic Factor (BDNF), which is a neurotrophin with well-established protective roles in brain development, physiology and pathology of neurological disorders [15] was accessed. Neurotrophins are synthesized as precursors (proneurotrophins)[51], BDNF precursor – proBDNF (32 kDa) is synthesized in the endoplasmic reticulum (ER) and is converted into the mature form - mBDNF (14 kDa)

in an activity-dependent manner.[102] Increasing evidence shows that mature and pro-BDNF present different roles in the CNS, the mature form is associated with the promotion of neuronal survival, differentiation and synaptic plasticity, in contrast to its precursor associated with apoptosis and growth cone retraction.[51,101] The conversion between BDNF forms is a dynamic mechanism dependent on molecular signals sensed by cells to improve synaptic efficacy.[102] Therefore, the presence of proBDNF and mBDNF were analyzed in microglia medium treated or not with CO by Western Blot techniques. As it can be observed in Figure 3.4., the ratio of mature and precursor isoforms in presence of CO tends to increase, indicating a tendency to the cleavage of proBDNF to mBDNF.

Another potential participant in this microglial-neuronal communication is adenosine that is involved in purinergic signaling.[38] In order to indirectly access adenosine, we determined the expression of CD73, an ectonucleotidase expressed in microglia, responsible for the final limitation step of extracellular ATP conversion into adenosine.[20,57] CD73 expression in microglia when treated or not with CO was quantified in Figure 3.5. There was a significant increase of CD-73 expression in CO-treated microglia, which may indicate an improvement of adenosine signaling.

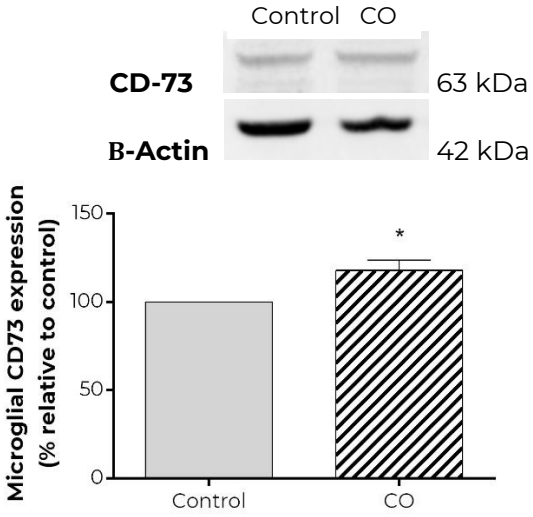

Figure 3.5 – Effect of Carbon monoxide in CD73 expression in microglia. Microglial BV2 cells were treated with 50 µM of ALF-826. Total protein extracts of microglial cells were collected 24h after CO treatment and analyzed by Western blot. Statistical significance analyzed by t-test: ns: p>0.05. N=5 biological replicates.

The neuronal adenosine A2A receptor (A2AR) is the main receptor involved in adenosine-mediated signaling[72]. For that reason, neuronal A2AR expression in neurons was also determined by Western Blot. (Figure 3.6.)

Besides the absence of a statistically significant difference between control (monoculture of neurons) and microglia conditioned medium (with or without CO) treated neurons, there is a tendency for increased expression of this receptor in neurons incubated with microglial media.

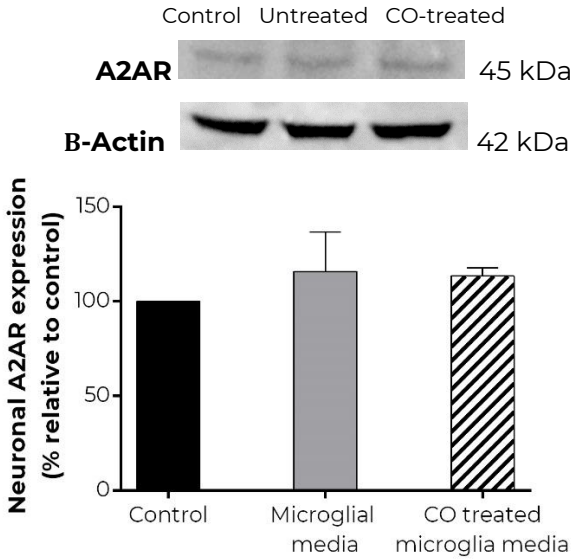

Figure 3.6 – Effect of Carbon monoxide in A2AR expression in neuronal cells. Neuronal CAD cells were incubated 48h after differentiation, with either microglial BV2 media or microglial BV2 media treated with 50 µM of ALF-826. Total protein extracts of neuronal cells were collected 24h after incubation and analyzed by Western blot. Statistical significance was analyzed by t-test: ns: $p>0.05$. (A) n=4 biological replicates, (B) n=3 biological replicates.

Because microglia can also release anti-inflammatory factors and promote neuroprotection, in the following step of this study, Interleukin-10 was quantified in the supernatant on microglial cells by ELISA. IL-10 is a 17 kDa homodimeric anti-inflammatory type II cytokine produced by a variety of cell types, including microglia. [30,80] IL-10 is recognized to have multiple immunoregulatory effects including inflammatory cytokines and ROS inhibition, enhanced B cell proliferation and antibody production.[80] In addition, IL-10 is also known as a promoter for the expression of heme oxygenase-1 (HO)-1, enzyme responsible for the production of endogenous CO by catabolism of heme.[80] CO is capable of inhibiting some pro-inflammatory cytokines and increasing, at the same time, IL-10 expression in different cell lines after inflammatory stimuli such as LPS. [23,47,80]

To understand the influence of CO in IL-10 under physiological conditions, concentration of secreted IL-10 in microglia media was determined. The result is

represented in Figure 3.7. and shows a significant increase in the concentration of extracellular IL-10 when microglia were treated with CO.

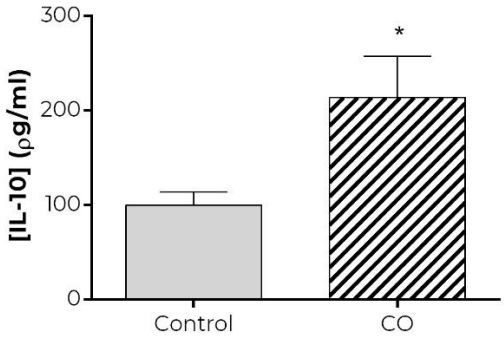

Figure 3.7. – Carbon monoxide increase IL-10 secretion in microglial cells. Microglial BV2 cells were treated with 50 μM of ALF-826. The supernatant of microglial cells was collected 24h after CO treatment and the concentration of IL-10 was determined using an ELISA protocol. Statistical significance was analyzed by t-test: *p≤0.05, n=5 biological replicates.

The increase of this cytokine in the microglial secretome suggests that CO act as a neuroprotective signaling molecule that will be beneficial for neurons not only to overcome inflammation as reported in previous studies but also to promote mechanisms to ensure tissue integrity and homeostasis.

4. Discussion and conclusion

Microglia have been long recognized as the motors and key cells for inflammatory responses in the CNS. Neuroinflammation in the CNS induces a short lived, beneficial protective immune action. Nevertheless, exacerbated microglial activity can lead to uncontrolled inflammation and promote irreversible damage. Carbon-monoxide is an endogenous molecule with already proven anti-inflammatory potential [64], including limitation of pro-inflammatory output of microglia.[14,13,25]

The final aim of this thesis is to study the unexplored microglia-neuron communication under physiological conditions mimicking the healthy brain and to understand if CO, in the absence of inflammation, promotes neurotrophic and/or neuroprotective effects in neurons by indirectly targeting microglia.

For that purpose, a model of BV2 microglia and CAD neuronal cell lines was used, as well as neuronal culture treated with microglial conditioned medium. In fact, a microglia-neuron conditioned media system was applied to create a more *in vivo* like protocol to mimic communication between these cells (Figure 3.2. A). In the performed experiments, neurons were incubated with microglia media previously treated or not with CO using a CO-releasing molecule, ALF-826.

This simplistic model does not include information regarding the diversity of other cell types and molecules that will affect several signaling pathways in vivo nor cell-to-cell communication, giving only information about communication through soluble factors. Nevertheless, this simple model allows the study of microglia to neuron communication via soluble factors.

In order to access viability, after neuronal contact with microglial secretome, neuronal cells were challenge with *t*-BHP, a pro-oxidant organic agent, known to produce free radicals and reactive oxygen species, components involved in oxidative stress and apoptosis.[118] The results in Figure 3.2. B suggests that in the absence of the oxidative stress challenge, the microglial secretome was not essential for neuronal viability, however, once the cell death-inducing agent (*t*-BHP) was introduced in the system, the microglial secretome was crucial for neuronal survival. This result suggests that, under physiological conditions, microglia output indirectly triggers a neuroprotective effect essential for survival after a challenging event. The results of microglia media pre-treated with CO did not present a significant difference from no treated microglia media. Nonetheless, it is important to take into consideration that access viability using a cell death agent can be a too drastic experiment, where discrete protective functions may be undetectable, such as neuroprotective and

neurotrophic potential effects of soluble molecules released by microglia in the presence of CO. Interestingly microglia conditioned media did prevent neuronal cell death in a CO independent manner.

Morphology is a feature intimately related to neuronal function and sense of the environment, and for that reason, neuronal morphological changes between conditions were also determined, in accordance with the viability experiment (Figure 3.2. B). The morphology data also showed differences between control neurons and neurons incubated with microglial secretome (Figure 3.3). However, in contrast to the viability experiment where no significant difference was observed when CO treatment was applied, the impact of CO on neuronal morphology was undeniably positive, improving neuronal morphology (number and length of neurites. This suggests that CO modulates the secretion of microglial soluble factors, being a promising candidate as a neuroprotective and neurotrophic molecule by indirectly acting on microglia.

In order to understand how microglia and CO promote morphological changes and survival improvement in neurons, a class of molecules named neurotrophins seem to be promising candidates because of their crucial roles in numerous important CNS processes including differentiation, neuronal survival, synaptogenesis, plasticity and neuronal function. The neurotrophin, Brain-Derived Neurotrophic Factor (BDNF) expression, was chosen to be a cytoprotective molecule with already important proven roles in brain development, physiology and pathology of neurological disorders, such as Parkinson's disease, Alzheimer's disease, multiple sclerosis and Huntington's disease. [15]

According to diverse evidence, contrary to the precursor form of BNDF, the mature BDNF is related to neurotrophic functions.[51] The conversion between mBDND and proBDNF is a dynamic mechanism dependent on molecular signals sensed by cells to improve synaptic efficacy.[51,102] Our results showed a tendency to the cleavage of proBDNF to mBDNF (Figure 3.4) suggesting that BDNF is one of the microglial neuroprotective mechanisms enhanced by CO.

Adenosine involved in purinergic signaling is a key molecule in processes like cellular viability, able to shape microglia-neuron communication and microglial behavior.[57,72] These capacities turned it into a candidate molecule involved in the CO neuroprotective signaling. Adenosine signaling was indirectly assessed by: (1) microglial expression of CD73 (ectonucleotidase responsible for the last step of conversion of ATP into adenosine)[57]. The increase of microglial CD73 expression (Figure 3.5) and a tendency for increased neuronal expression of A2AR (Figure 3.6)

indicate an increase of adenosine in the microglial output, suggesting that this molecule and this pathway are involved in the neuroprotection potentialized by CO.

Interleukin 10 (IL-10) is an anti-inflammatory cytokine known for its multiple immunoregulatory effects including the capacity to inhibit the production of several inflammatory cytokines and antigen presentation in inflammatory scenarios.[80] IL-10 is involved in the conversion of heme to CO among other products by heme oxygenase-1 (HO)-1, a stress-inducible protein with anti-inflammatory action. Some evidence also suggests that IL-10 not only attenuates neuronal inflammation but also apoptosis and promotes neurite outgrowth and synapse formation via the JAK1/STAT3 signaling pathway in cultured primary cortical neurons after (oxygen-glucose deprivation) OGD injury.[67,68] In our study, in the absence of an inflammatory stimulus or injury, IL-10 secretion by microglia also increases by the influence of CO (Figure 3.7). This suggests that IL-10 is also neuroprotective in physiological conditions.

For many years, CO has been extensively described as a crucial molecule to overcome exacerbated inflammation, limiting neuronal damage in several neuronal diseases [64,84] In this study a new role of CO in microglia was demonstrated for the first time. The increase of microglial mBDNF/proBDNF ratio, CD73 expression and IL-10 secretion after treatment of microglia media with CO suggests that this molecule can also act as a neuroprotective signaling molecule. These findings can indicate that CO biological actions promote mechanisms to ensure tissue integrity and homeostasis.

As future work, in order to overcome some of the current system limitations, results should be reproduced in primary cultures of microglia and neurons that better mimic the in vivo cellular behavior. The establishment of mixed primary cell culture (co-culture systems) or systems in which cells are able to exchange soluble factors and physical contacts, even if cells are located in separated compartments through channels (such as microfluidic chambers) would resolve the lack of information about cell-cell physical communication.

Other molecules involved in microglia and neurons cross-talk, such as interleukins (e.g. IL-1β, IL-6), prostaglandin E2 (PGE2), interferons, and receptors involved signaling pathways like the purinergic (e.g. P2X7, P2X4 and A1R), complement (e.g. C3R and C5R), TREM, TLR and CD200R and respective molecular players should be investigated to support our results.

To further prove that adenosine and other molecules acting as neurotransmitters are participating in this effect, levels of adenosine should be quantified in the conditioned medium. This quantification can be addressed using other techniques like high-performance liquid chromatography (HPLC).

Overall, our findings reveal CO as a neuroprotective molecule by targeting microglia. The knowledge of the role of CO in modulation of microglial neurotrophism can be the beginning of new potential applications of CO. CO is a molecule that can be administered as a preventive procedure previous to any injury or disorder in order to attenuate or prevent future serious brain damage. Moreover, a new CORM ALF-826 has proven to be effective in cellular model for the assessment of microglia-neuron communication.

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