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Histamine modulates nitric oxide release by microglia and dopaminergic neuronal survival

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Resumo

As células microgliais, células imunitárias residentes no cérebro, desempenham um papel crítico na etiologia e progressão de várias doenças neurodegenerativas. A doença de Parkinson (DP) é uma doença neurodegenerativa caraterizada por uma grande perda dos neurónios dopaminérgicos na Substantia Nigra (SN), diminuição dos níveis de dopamina no estriado e complicações motoras. Várias evidências clínicas e experimentais sugerem que a neuroinflamação tem um papel crítico na patogénese da DP através da ativação das células microgliais e consequente produção de mediadores inflamatórios, incluindo o óxido nítrico (ON). A Histamina (HIS), uma amina que atua como neurotransmissor e mediador inflamatório, tem sido descrita como tendo um importante papel na patogénese da DP. Alterações nas inervações histaminérgicas no estriado e SN bem como um aumento das concentrações de histamina no sangue, estriado e SN foram observadas em pacientes com DP. Com base nestes dados, o nosso objetivo foi avaliar o efeito da histamina nas células microgliais obtidas da Substantia Nigra de ratos Wistar e seguidamente avaliar de que forma fatores solúveis libertados pela microglia previamente estimulada com histamina podem modular a sobrevivência neuronal dopaminérgica. Inicialmente foram utilizadas culturas de células microgliais para estudar o efeito da histamina e os seus recetores na produção de ON, o qual foi quantificado pelo teste de Griess. Demostramos que a HIS provoca um aumento da produção de ON quando comparado com o controlo, um efeito mediado pela ativação do recetor 4 da histamina (H4R). Contudo, num contexto inflamatório induzido pelo Lipopolissacarídeo (LPS), a HIS inibe a produção de ON induzida pelo LPS não só pelo R4H, mas também possivelmente através da ativação do recetor 1 da histamina (R1H). Em seguida, recolhemos o meio condicionado das células microgliais (MCM) tratado com HIS e/ou LPS para avaliar o seu efeito na viabilidade dos neurónios dopaminérgicos presentes em co-culturas de neurónios e astrócitos isoladas do mesencéfalo. De facto, o meio condicionado obtido das células microgliais expostas ao LPS ou à HIS levaram a uma diminuição do número de neurónios positivos para a Tirosina Hidroxilase; sendo este efeito anulado quando o MCM é obtido das células microglias tratadas com a HIS mais LPS. Curiosamente, o mesmo efeito foi observado quando a HIS e/ou LPS foram adicionados diretamente nas co-culturas de neurónios e astrócitos. Assim, estes resultados sugerem que a HIS por si só atua como um mediador pró-inflamatório, enquanto, num contexto inflamatório, a HIS tem supostamente um efeito anti-inflamatório promovendo desta forma a sobrevivência dos neurónios dopaminérgicos.

Palavras-chave

Neuroinflamação; Histamina; Microglia; Óxido Nítrico; Doença de Parkinson

Abstract

Microglia cells, the resident immune cells in the brain, play a critical role in the development and progression of several neurodegenerative diseases. Parkinson's disease (PD) is a neurodegenerative disorder characterized by a dramatic loss of dopaminergic neurons (DA) in the substantia nigra (SN), striatal dopamine depletion and motor impairments. Accumulating clinical and experimental evidences suggest that neuroinflammation plays a critical role in the pathogenesis of PD through the activation of microglia cells and the subsequent production of a vast array of inflammatory mediators, including nitric oxide (NO). Histamine (HIS), an amine that acts as a neurotransmitter and inflammatory mediator, has been reported to play a role in the pathogenesis of PD. Indeed, alterations in the histaminergic innervations in the striatum and SN and increased histamine concentrations in the blood, striatum and SN were found in PD patients.

Based on these data, our aim was to uncover the effects of histamine on microglia cells derived from the SN of Wistar rats and then evaluate whether soluble factors released by microglia previously stimulated with histamine could modulate dopaminergic neuronal survival. Firstly, microglia cell cultures were used to study the effects of HIS and its receptors on NO production, which was measured by the Griess assay. We demonstrated that HIS triggered an increase of NO production as compared with control, an effect mediated by histamine H4 receptor (H4R) activation. Interestingly, in the presence of an inflammatory context, mimicked by lipopolysaccharide (LPS), HIS inhibited LPS-induced NO production not only by H4R but, possibly through histamine H1 receptor (H1R) activation. Then, conditioned medium derived from microglia cells (MCM) challenged with HIS and/or LPS was collected to evaluate its effects on the viability of DA neurons present in neuron-astrocyte midbrain co-cultures. In fact, conditioned medium derived from microglia cells exposed to LPS or HIS induced a decrease in the number of Tyrosine Hydroxylase positive neurons; whereas this noxious effect was abolished when MCM obtained from microglia challenged with HIS plus LPS was used. Curiously, the same effects were observed when HIS and/or LPS were added directly on neuron-astrocyte midbrain co-cultures. Together, our results suggest that HIS *per se* acts as a pro-inflammatory mediator, whereas, in an inflammatory context, HIS has a putative anti-inflammatory profile that can protect dopaminergic neurons.

Keywords

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Abbreviations

Ag H$_4$R	Agonist of histamine 4 receptor (4-methylhistamine dihydrochloride)
Ant H$_1$R	Antagonist of Histamine 1 receptor (mepyramine maleate)
Ant H$_2$R	Antagonist of Histamine 2 receptor (cimetidine)
Ant H$_3$R	Antagonist of Histamine 3 receptor (carcinine ditrifluoroacetate)
Ant H$_4$R	Antagonist of Histamine 4 receptor (JNJ7777120)
ATP	Adenosine 5'-triphosphate
Bax	Bcl-2–associated X protein
BSA	Bovine Serum Albumin
CD11b	cluster of differentiation molecule 11b
CNS	Central nervous system
COX-2	Cyclo-oxygenase 2
DMEM	Dubecco's modified eagle medium
FBS	Fetal bovine serum
H$_1$R	Histamine 1 receptor
H$_2$R	Histamine 2 receptor
H$_3$R	Histamine 3 receptor
H$_4$R	Histamine 4 receptor
HIS	Histamine
IFN-γ	Interferon-γ
IL-1ß	Interleukin-1 beta
iNOS	Inducible Nitric oxide synthases
LPS	Lipopolysaccharide
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MCM	Conditioned medium derived from microglial cells
MPT	Mitochondrial permeability transition
NBM	Neurobasal medium
NMDA	N-Methyl-D-aspartate
NO	Nitric oxide
PBS	Phosphate buffered saline

PD	Parkinson' disease
PFA	Paraformaldehyde
PGE	Prostaglandin E2
PI	Propidium iodide
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute media
RT	Room temperature
SN	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
TNFR-1	TNF-α receptor 1
TNF-α	Tumor necrosis factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end belling

Chapter 1

<u>INTRODUCTION</u>

1.1. NEUROINFLAMMATION

In the past, the central nervous system (CNS) was considered an immune-privileged site. Nowadays, it is well established that the activation of the immune cells present in the CNS to infection, trauma, toxins, among other stimuli plays a crucial role in the development and progression of neurodegenerative and neuropsychiatric diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), bipolar disorder (BD), schizophrenia (SZ) and depression. The inflammatory responses in the brain, also known as neuroinflammation, are a complex combination of acute and chronic responses of several types of cells, including neurons, microglia, astrocytes and infiltrating leukocytes. The acute inflammatory responses are believed to be beneficial, since it tends to minimize further injury and contributes to repair of damaged tissue. On the other hand, chronic neuroinflammation produces long-lasting and self-perpetuating neuroinflammatory mediators that remain after the initial neuroinflammatory insult has passed (Frank-Cannon et al., 2009; Kraft and Harry, 2011; Rao et al., 2012).

1.1.1 MICROGLIA IN HEALTH AND DISEASE

Microglia are the resident immune-competent cells of the CNS and have a role in monitoring the brain for immune insults and invading pathogens. Ramon and Cajal considered microglia to be part of the 'third element' of the CNS, being neither neuronal nor astrocytic (Long-Smith et al., 2009).

The origin of microglia still remains highly debated. The hypothesis most accepted is the ''myeloid-monocytic hypothesis'', which states that resident microglia, as well as the other tissue resident macrophages, are derived from circulating blood monocytes, during the late embryonic life and post-natally (Flügel et al., 2001; Kaur et al., 2001; Polazzi and Monti, 2010;).

Microglia cells are distributed throughout the CNS, represent around 5-20% of the total adult brain cells, depending on the species, and constitute approximately 20% of the glial cell population. Interestingly, the density and the morphology of microglia are region-

specific. This strongly suggests that these differences might be related to a microglial functional heterogeneity (Lawson et al., 1990; Polazzi and Monti, 2010).

Major features of microglia are their highly ramified morphology and plasticity that allow them to supervise the extracellular CNS parenchyma and to be quickly activated in response to pathological conditions, thus exerting typical macrophagic functions, such as phagocytosis, secretion of proinflammatory cytokines and antigen presentation (Gehrmann et al., 1995; Stence et al., 2001; Ladeby et al., 2005;).

It was presumed for many years that under normal physiological conditions microglial cells are quiescent and in a resting state. But *in vivo* two-photon microscopy studies in living mice showed that microglial processes are substantially motile, and survey their local surroundings thought formation of random filopodia-like protrusions, extensions and withdrawal of bulbus endings. This state of high motility facilitates the microglial processes to perceive the status of their microenvironment, to endocytose nutrients and to clear debris and apoptotic cellular material (Nimmerjahn et al., 2005; Napoli and Neumann, 2009). They are also actively involved in the determination of cell fate (elimination/survival) of developing neurons by enforcing the programmed elimination of neural cells or enhance neuronal survival through the release of trophic and anti-inflammatory factors. In addition, in the mature brain, microglia facilitate brain repair through the guided migration of stem cells to the site of inflammation and injury, and might be involved in neurogenesis (Marín-Teva et al., 2004; Ekdahl, 2012). Microglia are potentially also promoters of the migration, axonal growth, and terminal differentiation of different neuronal subsets, through the release of extracellular matrix components, soluble factors and direct cell–cell contact. Moreover, the cross–talk with neurons is believed to be an important factor in guarding microglia cells in a quiescent state. For example, interaction of the neuronal membrane protein CD200 with the myeloid cell receptor CD200R dampens microglial activation. Mice deficient in CD200 show morphological and molecular signs of microglia activation in the resting CNS, and the microglial response to different forms of experimental brain injury is excessive (Polazzi and Contestabile, 2002; Streit, 2002). The interaction between microglia and other glial cells namely astrocytes is also complicated due the reciprocal interaction, both in health and unhealthy brain, and like microglia cells, astrocytes play diverse functions in the brain, both harmful and beneficial. For example, it is known that activated microglia facilitates astrocytic activation and activated astrocytes in turn regulate microglial activities and also promote microglial activation. Astrocytes play a dual role in CNS inflammatory diseases, not only having the ability to enhance immune responses and postpone restoration, but also limiting CNS inflammation and being neuroprotective (inhibitory effect on activated microglia). Therefore an important question is how these two totally opposite effects coexist because the degree of inflammation is crucial (De Keyser et al., 2008; Liu et al., 2011; Rocha et al., 2012). Clearly, much more remains to be learned about the intricate functional inter-relationships that exist between microglia and astrocytes, as well as their meaning for neuronal regeneration and degeneration.

Apart from these important roles of microglial cells in healthy brain, microglia also plays an important role in unhealthy brain since it is exquisitely sensitive to disturbance of their microenvironment. Microglia detect the changes in its environment through the expression of a great number of cellular surface receptors and nuclear receptors that play a critical role in the initiation and/or modulation of its immunitary responses (Hanisch, 2002; Block, 2007).

Virtually, every neurological disorder leads to inflammation, with activation of resident microglia, accompanied by an increase in number and change in phenotype of glial cells, a phenomenon generally termed ''reactive gliosis''. Acute neurodegenerative diseases, such as stroke, hypoxia, and trauma, compromise neuronal survival and indirectly trigger neuroinflammation, as microglia become activated in response to the insult itself, thus adopting a phagocytic phenotype and releasing inflammatory mediators, mainly cytokines and chemokines. This acute neuroinflammatory response is generally beneficial to the CNS, since it tends to minimize further injury and it contributes to repair of damaged tissues.

In contrast, chronic neurodegenerative diseases, including AD and PD, are known to be associated with chronic neuroinflammation, even if several differences have been identified among these pathologies. Chronic neuroinflammation is a long-standing and often self-perpetuating response that persists long after an initial injury or insult either genetical or environmental in nature. It is generally characterized by a long-standing activation of microglia and subsequent sustained release of inflammatory mediators leading to increased oxidative and nitrosative stress. This, in turn, works to perpetuate the inflammatory cycle, activating additional microglia, promoting their proliferation and resulting in a further release of inflammatory factors (Fig. 1). Besides playing a protective role as acute neuroinflammation does, chronic neuroinflammation is most often considered detrimental and damaging to nervous tissue. Thus, whether neuroinflammation has beneficial or harmful outcomes in the brain may depend critically on the duration of the inflammatory response and on the kind of microglial activation (Frank-Cannon et al., 2009; Polazzi et al., 2010).
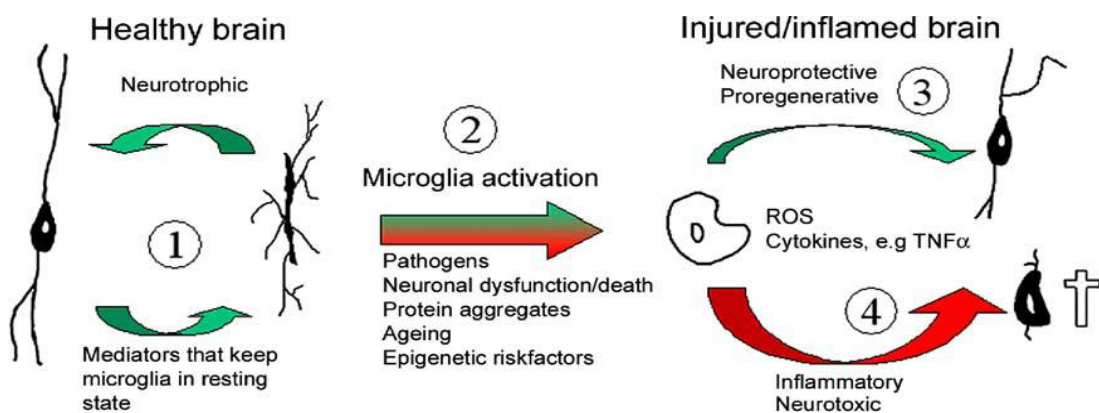

Figure 1: **Microglia activation by endogenous and/or exogenous stimuli**. (1) In the healthy brain microglia support neuronal well-being, and in turn receives cues from neurons and glial cells to remain in the resting state. (2) In response to a wide array of noxious stimuli microglia undergo activation.

Activation may be beneficial to the host (3) when reactive oxygen spices (ROS) and secreted cytokines are kept at low and/or transient levels. In this instance these proinflammatory mediators are neuroprotective. However, when they surpass a certain level of host tolerance (4) these mechanisms become neurotoxic and result in neuronal dysfunction and cell death, which may further contribute to microglial activation (from Vilhardt, 2005).

1.1.2 CONTRIBUTION OF NEUROINFLAMMATION TO EXCITOTOXICITY

Neuroinflammation may play a critical role in the modulation of excitotoxicity that occurs in several neurodegenerative diseases. Excitotoxicity refers to a process of neuronal death caused by excessive or prolonged activation of receptors for the excitatory amino acid neurotransmitter glutamic acid (Zimmer et al., 2000). Glutamate-induced death of neurons can be mediated by: (a) activation of the N-Methyl-D-aspartate (NMDA) subtype of glutamate receptor, resulting in Ca^{2+} and/or Na^+ overload of the neuron; (b) activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) or (c) glutamate inhibition of cystine uptake, resulting in oxidative stress/death of the neuron. Calcium elevation may: (a) stimulate calcineurin causing Bcl-2-associated death promoter (Bad) and Bcl-2–associated X protein (Bax) activation; (b) stimulate mitochondrial oxidant production and mitochondrial permeability transition (MPT); and (c) stimulate neuronal Nitric Oxide Synthases (nNOS) production of NO and oxidants. Activation of Bax pores and/or MPT may result in release of apoptosis inducing factor (AIF) and cytochrome c, and/or cause ATP depletion (Fig. 2) (Brown and Bal-Price, 2003; Ankarcrona et al., 1995; Doble, 1999).

Microglial cells express both ionotropic and metabotropic glutamatergic receptors, that when overactivated in pathological conditions induce microglia activation and subsequent release of pro-inflammatory cytokines (Noda et al., 2000 and Taylor et al., 2005). In addition to glutamate, adenosine triphosphate (ATP) is a co-transmitter also released by injuried or dying neurons following brain insults that can modulate microglial activation, via interaction with both metabotropic (P2YR) and ianotropic (P2XR) receptors (Davalos et al., 2005). All these mechanisms lead to neuronal cell death and therefore, we can't consider a single mechanism involved in chronic neurodegenerative disease, but a set of mechanisms that may act in synergy, where microglial cells play a central role.
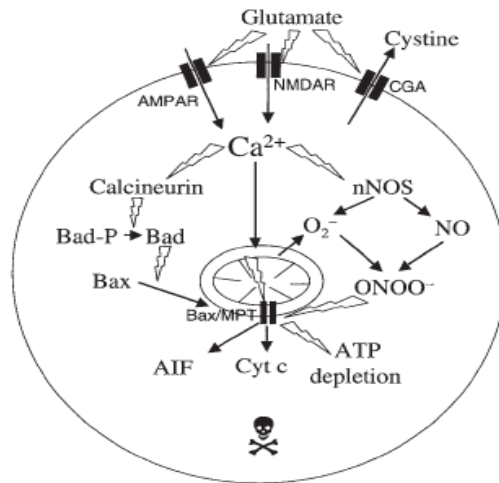
Figure 2: Possible mechanisms of glutamate induced neuronal death. Arrows indicate movement of reaction/production; thunderbolts indicate activation (from Brown et al., 2003).

1.1.3 ROLE OF NO IN NEUROINFLAMMATION

Several mechanisms underlying the activation of the microglia, such as oxidative stress and mitochondrial dysfunction may be involved in neuronal cell death (Doble, 1999; Andersen, 2004; Witte et al., 2010). The NO released from microglial cells, play an important link between microglia activation and these mechanisms that lead to neuronal cell death (Duncan and Heales, 2005). NO is an important second messenger, having a crucial role in intercellular communication and in intracellular signaling in many tissues (Moncada et al., 1989; Kerwin et al., 1995), including the brain (Garthwaite et al. 1988). NO can be produced by three nitric oxide synthase (NOS) genetically different isoforms: the neuronal NOS (nNOS), the endothelial isoform (eNOS) and, the inducible NOS (iNOS). The iNOS is expressed in microglia and cells from the immune system, leading to a production of large amounts of NO that may be cytotoxic. For example, NO itself can causes rapid, selective, potent but reversible inhibition of cytochrome oxidase that leads to mitochondrial respiration inhibition and consequently ATP depletion, ATP depletion causing failure of the sodium pump, resulting in plasma-membrane depolarization and removal of the Mg^{2+} block of the NMDA channel with glutamate release (Brownand Bal-Price, 2003).

Certainly the actions of NO in neuroinflammation are complex and varied, thus future research to cover the regulatory and signalling effects of NO on cell death are necessary.

1.2. ETIOLOGY AND PATHOGENESIS OF PARKINSON'S DISEASE

PD was first described in 1817 by Dr. James Parkinson in his monography entitled "An essay on the Shaking Palsy". The median age of onset is 60 years and the mean duration of the disease from diagnosis to death is 15 years, with a mortality ratio of 2 to 1. Evidence exists that men are about 1.5 times more likely than women to develop PD (Andrew, et al., 2009; Trimmer and Bennett, 2009). PD is characterized by cardinal motor features such tremor, rigidity, slowed body movements (bradykinesia), unstable posture and difficulty in walking (characterized by the patient's shuffling gait). Although non-motor symptoms are also typically observed in patients with PD including neuropsychiatric symptoms, sleep disturbances, autonomic impairments, and sensory dysfunctions (Singh et al., 2007; Kim et al., 2009). Yet, as there are no specific markers to identify the onset of PD or any of the stages of disease progression, the diagnosis is based on clinical signs and symptoms.

The causes of PD are unknown but considerable evidences suggest a multifactorial etiology involving genetic and environmental factors, neuronal injury such as traumatic brain injury or stroke, bacterial or viral infections and age-related factors (Collins et al., 2012). Pathological hallmarks comprise the loss of dopaminergic neurons in the *Substantia Nigra* (SN) that results in the loss of dopaminergic neurotransmission in the striatum and by the presence of insoluble protein inclusions termed Lewy bodies and Lewy neurites, located in either the neuronal cell body or neuronal processes, respectively (Dunning et al., 2012). Therefore, cerebrospinal fluid (CSF) profiles of dopamine and its metabolites are potential neurochemical biomarkers and together with the 6-[18F]fluorodopa positron emission tomographic (PET) scanning, can help in diagnosis of PD (Goldstein et al., 2008; Vernon, 2008; Andrew et al., 2009).

PD is still an incurable progressive disease, but treatment substantially improves quality of life and functional capacity. Dopamine replacement with Levedopa remains the gold standard regarding symptomatic efficacy. However, long-term treatment with Levedopa is often complicated by the development of various types of motor response oscillations over the day. Dopamine agonists as early treatment have been reported to reduce the risk of motor fluctuations. Deprenyl, a monoamina oxidase-B (MAO-B) inhibitor was found to be effective in parkinsonian patients (Caraceni and Musicco, 2001; Poewe et al., 2010). Deep brain stimulation of the subthalamic nucleus (STN-DBS) is an established therapy for advanced PD patients with motor complications (Weaver et al., 2009). Furthermore several neuroprotective agents have been study in order to delay the progression of disease. Antiapoptotic agents, antioxidants, glutamate antagonists, neurotrophic factors and nonaspirin nonsteroidal anti-inflammatory drug (NSADs) are some examples (Bornebroek et al., 2007; Löhle and Reichmann, 2010). Other promising therapeutics is the intrabody technology as a novel tool to modulate the function of intracellular proteins such as alpha-synuclein (α-syn) or the use of stem cells which enables the replacement of dopaminergic

neurons and others systems that degenerate in PD patients (Svendsen, 2008; Zhou and Przedborski, 2009). However, there is still much to learn in order to design a fully effective therapy to cure PD.

1.2.1 ROLE OF MICROGLIAL CELLS IN PARKINSON'S DISEASE

Even if the causes and underlying mechanisms of PD remain uncertain, recent studies suggest that neuroinflammation and microglia activation play important roles in PD pathogenesis (Tansey and Goldberg, 2010; Collins et al., 2012). Activated microglial cells might contribute to dopaminergic cell death by releasing cytotoxic inflammatory compounds such as proinflammatory cytokines (TNF-α, IL-1ß, and interferon γ (IFN-γ)) (Fig. 3). Among these cytokines, TNF-α might have a direct damaging effect on dopaminergic neurons by activating an intracellular death pathway coupled with TNF-α receptor 1 (TNFR-1) expressed on the cell surface of these neurons (Mogi et al., 2000; Long-Smith et al., 2010). Pathways transduced by activation of TNFR-1 are linked to the induced expression of cyclo-oxygenase 2 (COX2) within dopaminergic neurons. COX-2-positive neurons release prostaglandin E2 (PGE_2), which promotes the production of microglial-derived mediators, which, in turn, help in killing neurons (Teismann et al., 2003; Sánchez-Pernaute et al., 2004 Hewet et al., 2006). These cytokines might also stimulate the expression of iNOS within microglial cells (Sheng et al., 2011). This process might lead to the production of toxic amounts of NO free radicals (Hunot et al., 1996). In turn, these free radicals could potentiate the expression and release of TNF-α by adjacent microglial cells, thereby amplifying further the inflammatory reaction (McCoy et al., 2006; Hirsch and Hunot, 2009).

Besides these proinflammatory factors, others cytotoxic factors, such glutamate, eicosanoids, reactive oxygen species (ROS) and others reactive nitrogen species (RNS) are released by activated microglia (Smith et al., 2012). In addition, several cytokines released from microglia can increase the blood–brain-barrier (BBB) permeability and enhanced movement of leukocytes into the CNS by increasing expression of cell adhesion molecules essential for extravasation (e.g., intracellular adhesion molecule 1 [ICAM-1], vascular cell adhesion molecule 1 [VCAM-1]) and trafficking. For example, activated CD4+ T cells might express and release several inflammatory factors, such as TNF-α, IFN-γ, and Fas ligand. In fact, Fas ligand-derived CD4+ T cells might have a deleterious effect on dopaminergic neurons directly (by activating an intracellular death pathway coupled with Fas receptor expressed on the cell surface of dopaminergic neurons) or indirectly (by activating Fas receptor expressed on activated microglial and reactive astrocytic glial cells), thereby stimulating their activation and the release of additional inflammatory factors (Hirsch and Hunot, 2009).
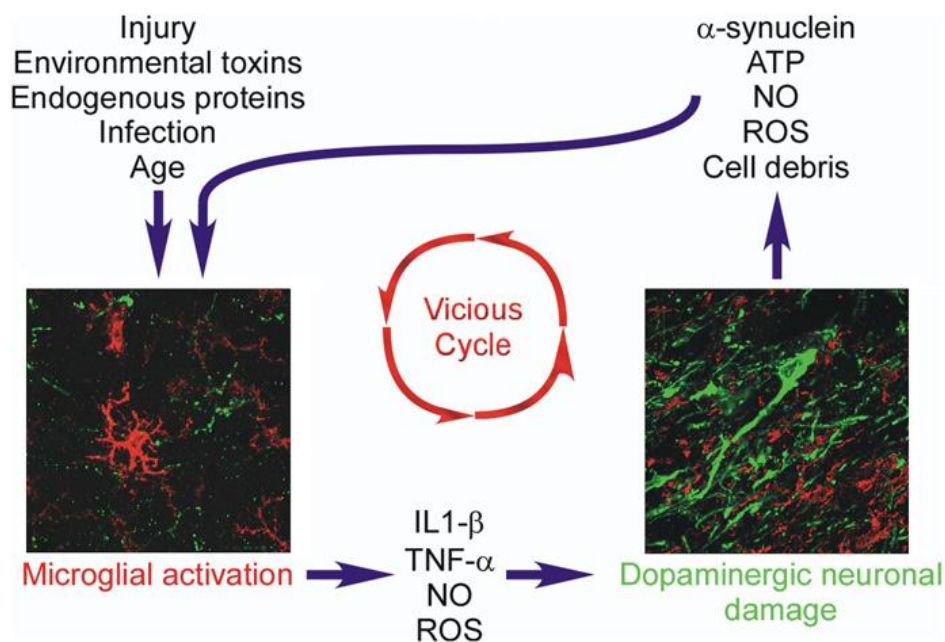
Figure 3: Relationship between microglia activation and dopaminergic neuronal damage. Schematic representation of the impact of microglial activation on dopaminergic neuronal survival, and the consequent effects of substances released from dying dopaminergic neurons on microglial activation (from Collins et al., 2012).

1.2.2 LIPOPOLYSACCHARIDE-INDUCED PARKINSON'S DISEASE ANIMAL MODEL

As stated above, PD is characterized by a selective and gradual loss of dopaminergic innervations from the SN to the striatum of the basal ganglia. Findings from epidemiological studies and analysis of postmortem PD brains and animal PD models have provided increasing evidence to support a role for inflammation and microglia activation in the pathogenesis of PD. Due to the role of inflammation in PD, the need for purely inflammation-driven animal models has emerged. An animal model widely used is the lipopolysaccharide PD animal model (Gao et al., 2002).

LPS, an endotoxin found in the Gram-negative bacteria cell wall, is a potent inducer of inflammation, a powerful activator of microglia cells. LPS associates with the soluble LPS binding protein (LBP) and CD14 which is anchored in the outer leaflet of the plasma membrane. Signal transduction across the plasma membrane is made possible through the interaction of the LPS CD14 complex with the transmembrane Toll-like receptor-4 (TLR-4) and the extracellular accessory protein MD-2 (Dutta et al., 2008). Activation of this receptor triggers major intracellular signaling pathways such as the mitogen-activated protein kinase

(MAPK) pathway. MAPK are serine-theronine kinases that mediate intracellular signaling and leads to a variety of cellular responses including cell proliferation, differentiation, survival and cell death. There are three main members of the MAPK family, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, each member exerts different biological functions. For instance, activated ERK1/2 pathway is involved in proliferation and survival whereas JNK and p38 MAPK are associated with apoptosis (Svensson et al., 2011). Downstream the activation of MAPK leads to activation of transcription factors, such as, nuclear factor (NF)-kB and activator protein 1 (AP-1). These transcription factors may be thus involved in the expression of genes involved in pro-inflammatory processes (Kim and Kim, 2005).

Besides these signaling pathways activated by LPS that leads to inflammation, various brain regions are differentially susceptible to LPS-induced degeneration. Neurons in the *Substantia Nigra* (SN) are the most sensitive region to bacterial endotoxin LPS-induced neurotoxicity, whereas neurons in hippocampus or cortex remain insensitive to this treatment, even with higher concentrations of LPS. In the SN, LPS induce a rapid activation of microglia followed by a delayed, progressive and selective destruction of nigral dopaminergic neurons (Gao et al., 2002). The region-specific susceptibility to LPS-induced degeneration is most likely attributable to the abundance of microglia in that region and consequently a high concentration of the inflammation-related factors produced by these cells, such as TNF-α and NO (Pintado et al., 2001; Kim et al., 2000). Selective degeneration of nigral dopaminergic neurons can be also related with the particular vulnerability of these neurons to oxidative stress as they operate under high oxidant conditions due to reduced levels of the anti-oxidant glutathione and increased nigral iron content. Moreover dopamine can generates redox metabolites including semiquinone, quinone, zwitterionic 5,6-hydroxyindoles, and possibly oxygen free radicals that increases the susceptibility of dopaminergic neurons to oxidative stress and consequently contributes to LPS-induced degeneration (De Pablos et al., 2005; Machado et al., 2011).
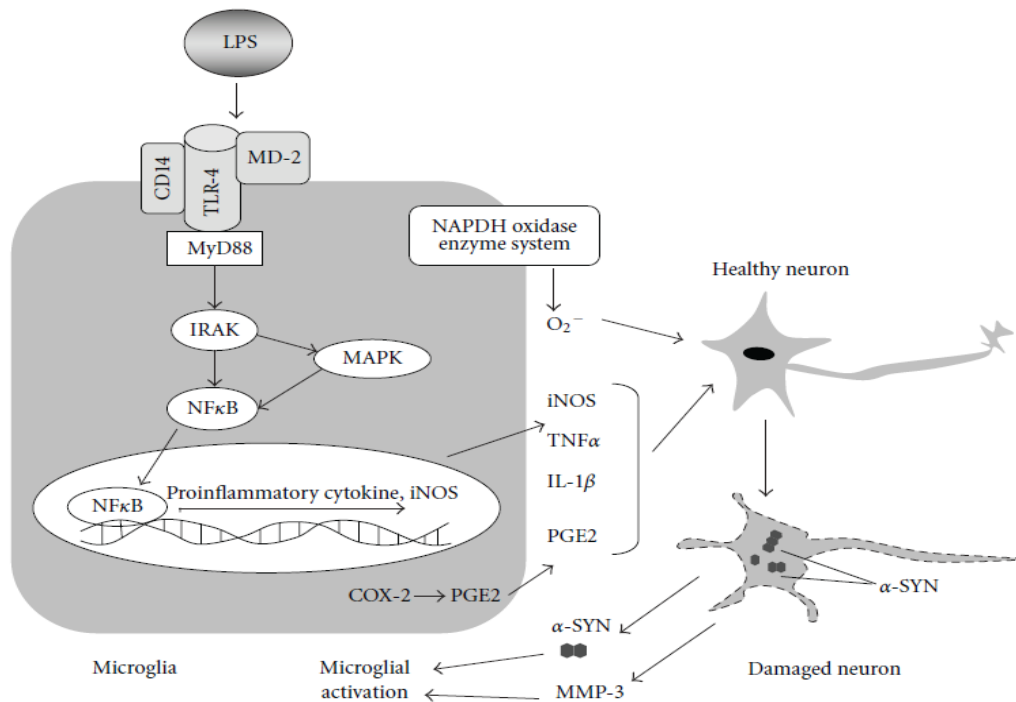
Figure 4: Simplified schematic representation of the link between LPS-induced microglial activation and dopaminergic neurodegeneration. LPS activates microglia cells by binding to its intermediate receptor CD14, in concert with TLR4 and the accessory adaptor protein MD2. This complex triggers the activation of the MyD88-dependent cascade which initiates NFκB activation, leading to the upregulated expression of pro-inflammatory cytokines (TNFα, IL-1ß) and increased production of other inflammatory mediators (NO and PGE2, synthesized by iNOS and COX-2, respectively). These soluble mediators collectively damage nigral dopaminergic neurons. Conversely, MMP-3 and alpha-Synuclein (α-SYN) released by stressed neurons may aggravate microglial activation and, ultimately, exacerbate dopaminergic degeneration (from Tufekci et al., 2011).

1.3 HISTAMINE

Histamine (4-imidazolyl-2-ethylamine) is a biogenic amine present as a normal constituent of the body with multiple effects in several organs (Fernández-Novoa and Cacabelos, 2001).

Histamine is mostly stored in the granules of mast cells and basophils. Other sources of histamine include T cells, dendritic cells, platelets and gastric enterochromaffin like cells, to name a few (Schneider et al., 2002). In the CNS, there are three main types of histamine-producing cells: neurons (where it acts as a neurotransmitter), mast cells and microglial cells (Katoh et al., 2002). Histamine exerts its effects by activating four types of receptors, namely: H1R, H2R, H3R, and H4R. All of these histamine receptors belong to the G protein-

coupled receptor family (Marson, 2011). Depending on the type of receptor, histamine plays multiple functions (Table 1).

Table 1: Expression, Function, and Signaling of Histamine Receptors and the G Proteins Involved
(Adapted from: Jadidi-Niaragh and Mirshafiey, 2010; Marson, 2011).

Histamine receptor	Expression	Function	Intracellular signaling pathway	G protein involved
H_1	nerve cells, smooth muscle, neutrophils, eosinophils, monocytes, dendritic cells, T and B cells, etc.	Wakefulness, inflammatory responses, decreasing blood pressure	Ca^{2+}, cGMP, phospholipaseD phospholipase A, NF-κB	GR_q
H_2	nerve cells, airway and vascular smooth muscle, hepatocytes, epithelial and endothelial cells, neutrophils, monocytes, dendritic cells, T and B cells	Regulation of gastric acid secretion, relaxationof airway and vascular smooth muscle	adenylate cyclase, cAMP, c-FOS,-Jun, PKC	GR_s
H_3	histaminergic neurons, eosinophils, dendritic cells, monocytes; low expression in peripheral tissues	neurotransmitter modulation: decreases release of histamine, acetylcholine, serotonin, and norepinephrine	enhanced Ca2+, MAPK, inhibition of cAMP	$G_{i/o}$
H_4	Hematopoietic cells, Cerebellum, hippocampus	immunomodulation	Inhibition of PKA, activation of PLC, MAPK	$G_{i/o}$

The H4R was the last receptor discovered, and its expression appears to be mostly in cells of the immune system, especially in mast cells, lymphocytes, and dendritic cells. This receptor is linked to a chemotactic effect on mast cells and eosinophils (Marson, 2011). However, the expression of H_4R in the brain has remained controversial. Several groups could not detect any H4R mRNA in the brain, while a few labs reported their expression by RT-PCR in various parts of the CNS, including amygdala, cerebellum, hippocampus, caudate nucleus, substantia nigra, thalamus and hypothalamus (Strakhova et al., 2009). Interestingly, a recent report demonstrated that all known HRs are expressed in a N9 microglia cell line. Moreover,

was also demonstrated the expression of H4R in microglia from the cortex. The same authors show that histamine *per se* stimulates microglia motility and most interestingly they saw that in a LPS-induced inflammatory context histamine plays an inhibitory action on microglia migration and in the release of IL-1ß (Ferreira et al., 2012).These findings could provide a new approach for the treatment of CNS pathologies or neurodegenerative disorders which are commonly accompanied by inflammation.

1.3.1 ROLE OF THE HISTAMINERGIC SYSTEM IN PARKINSON'S DISEASE

Histaminergic neurons are located exclusively in the tuberomammillary nucleus (TM) of the hypothalamus, from where they project to practically all brain regions including SN (Lee et al., 2008). The dopaminergic and histaminergic systems interact extensively, but little is known about the role of the histaminergic system in diseases affecting the dopaminergic neurons (Anichtchik et al., 2000). However, it is known that several functions regulated by the histaminergic system including the sleep-wake cycle, sensory and motor adjustment, cognition, attention, learning and memory are altered in PD (Shan et al., 2012).

In post-morten brain from PD patients, has been reported a dramatic increase of histaminergic innervations and histamine concentration in the SN (Anichtchik et al., 2000; Rinne et al., 2002). Moreover, a Thr105Ile polymorphism of histamine methyltransferase (HMT), the main enzyme breaking down histamine, was observed to be associated with PD, suggesting that a changed histamine homeostasis in the CNS is associated with the risk for PD (Palada et al., 2012). Besides, histamine is able to produce a specific degeneration of dopaminergic neurons in SN along with a highly inflammatory process (Vizuete et al., 2000).

Based on these studies, it has been proposed that histamine may play a role in the pathogenesis of PD. However, it is still unclear the exact role of histamine in the degeneration of mesencephalic dopaminergic neurons in the context of PD.

Chapter 2

<u>OBJECTIVES</u>

Recently, Ferreira and collaborators showed that microglial cells from the cortex express the H4R and that histamine may trigger dual effects (in the presence or absence of LPS) in microglia migration and cytokines release (Ferreira et al., 2012). However, there is no information regarding the effects of histamine in microglia derived from the SN, a brain region with a high density of these cells and highly susceptible to dopaminergic neuronal loss present in Parkinson's disease. With this in mind, we proposed to:

- Analyze the expression of histamine H4 receptor in primary microglia cell cultures from SN;

- Study the effects of histamine on the production of NO by microglial cells derived from SN, in the presence or absence of an inflammatory context mimicked by LPS;

- Spell out which histamine receptors are involved on the production of NO, both in the presence or absence of an inflammatory stimulus;

- Evaluate the effects of conditioned medium derived from microglial cells previously treated with LPS and/or histamine in cellular viability of dopaminergic neurons;

- Evaluate the effects of histamine and/or LPS *per se* in cellular viability of dopaminergic neurons.

Chapter 3

MATERIALS AND METHODS

3.1. *IN VITRO* CELL CULTURES

3.1.1. N9 MICROGLIA CELL LINE CULTURE

Murine N9 microglia cell line (kind gift from Prof. Claudia Verderio, CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy) was grown in RPMI medium supplemented with 30 mM glucose (Sigma, St. Louis, MO, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO, Invitrogen, Barcelona, Spain). Cells were kept at 37ºC in a 95% atmospheric air and 5% CO_2 humidified atmosphere. When cells reached an approximately 70% confluence was carried out the passage of the cells with a trypsin solution (Sigma, St. Louis, USA). Number of viable cells was evaluated counting trypan blue-excluding cells which were then plated at a density of 2×10^4 cells *per well* in 24-well trays. Cell treatments included the following incubation setup: histamine dihydrochloride (1-100 µM, Sigma) or LPS (100 ng/ml, Sigma) for 24h.

3.1.2. PRIMARY MICROGLIA CELL CULTURES FROM SUBSTANTIA NIGRA

All animals were handled in accordance with the national ethical requirements for animal research, and with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (2010/63/EU).

Briefly, the ventral midbrain of postnatal day 2 or 3 Wistar rat pups was dissected, carefully stripped of the meninges, and put in iced phosphate buffer saline (PBS: *NaCl 140 mM, KCl 2.7 mM, $KHPO_4$ 1.5 mM and Na_2HPO_4 8.1 mM, pH 7.4*). The tissue was then digested in cysteine solution (1.9 mM $CaCl_2$, 1.3 mM cysteine) and H&B solution (116 mM NaCl, 5.4 mM KCl, 26 mM $NaHCO_3$, 12 mM $NaH_2PO_4.H_2O$, 1 mM $MgSO_4.7H_2O$, 0.5 mM EDTA, 25 mM glicose, pH 7.3) supplemented with 20 U/ml papain and 0.001% phenol red. The average time for digestion was 4 min at 37ºC. After the digestion, the tissue was removed to a sterile tube and washed 3 times with 5 mL of warmed Dulbecco's modified Eagle's medium (*DMEM, Life*

Technologies) with 10% Fetal Bovine Serum (*FBS, Biochrom AG*), and 100 U/ml penicillin plus 100 µg/ml streptomycin (*Sigma*). The tissue was then mechanically dissociated with a 5 mL pipette, followed by further 5-10 sequential passes with techtips. Finally, the tissue was pelleted by centrifugation (*3K18C Bioblock Scientific; Sigma Laboratory Centrifuges*) for 3 min at 405 g and then resuspended in DMEM. Number of viable cells was evaluated counting trypan blue-excluding cells which were then plated at a density of 0.233 x 10^6 cells *per well* in 48-well trays (NO release) and 0.402 x 10^6 cells *per well* in 24-well trays in slides coated with poly-D-lysine (Sigma-Aldrich, St. Louis, USA) (immunocytochemistry). The cultures were kept at 37^oC under a 5% CO_2 and 95% air atmosphere. The medium was changed every 7 days. After 20-21 days *in vitro*, the microglia were obtained by trypsinization of astrocytes with a trypsin solution (*Sigma*) diluted 1:3 in DMEM (without FBS, penicillin and streptomycin) for 40 min. Microglia were kept in DMEM with 10% FBS, and 100 U/ml penicillin plus 100 µg/ml streptomycin at 37 °C in a 5% CO2, 95% air atmosphere for further 5 days.

3.1.3 NEURON-ASTROCYTE MIDBRAIN CO-CULTURES

The embryos of Wistar pregnant females with 15 or 16 days of gestation were removed and the ventral midbrain was dissected, carefully stripped of the meninges, and put in phosphate buffer saline (PBS: *NaCl 140 mM, KCl 2.7 mM, KH$_2$PO$_4$ 1.5 mM and Na$_2$HPO$_4$ 8.1 mM, pH 7.4*). The tissue was then dissociated by enzymatic digestion (*Tripsin 4.5 mg/ml and DNAse 2.5 mg/ml diluted in PBS*) and incubated at 37° C for 5 min. The cells were pelleted by centrifugation (*3K18C Bioblock Scientific; Sigma Laboratory Centrifuges*) for 1 minute at 88 g. A solution containing PBS with 10% Fetal Bovine Serum (FBS) heat-inactivated (*Biochrom, Holliston, USA*) was used to stop the enzymatic digestion and the pellet was then centrifugated for 1 min at 88 g. After discarding the supernatant, cells were rinsed with the PBS solution and then mechanically dissociated with a 5 mL pipette, followed by further 5-10 sequential passes with techtips. Cell suspension was then collected by centrifugation for 3 minutes at 405 g and then resuspended in Neurobasal Medium (*Gibco, Paisley, Scotland, UK*) supplemented with *B27 2%, glutamate 25 μM/mL, glutamine 0.5 mM/mL* and *gentamicine 120 μg/mL*. Viable cells were counted by the trypan blue exclusion method and were plated at a density of 0.8x10^6 cells *per well* in 24-well trays in slides coated with poly-D-lysine (*Sigma-Aldrich, St. Louis, USA)*. The cultures were kept at 37° C in a 5% CO_2 and 95% air atmosphere during 5-6 days. After cells reach confluence, a 5- fluorodeoxyuridine solution (FDU: *uridine 16.5 µg/mL and 5-Fluoro-5'-deoxyuridine 6.7 µg/mL)* (Sigma-Aldrich*, St. Louis, USA*) was added to inhibit further cell culture growth.

3.2. CELL TREATMENTS

Cell treatments include the following incubation setup: histamine dihydrochloride (100 µM, Sigma) and/or LPS (100 ng/ml, Sigma) for 24h (cell death assays); histamine dihydrochloride (1-100 µM, Sigma), LPS (100 ng/ml, Sigma), H1 receptor antagonist, 2-((2-(dimethylamino)ethyl)(p-methoxybenzyl)amino)-pyridine maleate (mepyramine maleate, 1 µM), H2 receptor antagonist, N-cyano-N'-methyl-N''-[2-[(5-methyl-1Himidazol- 4-yl)methyl]thio]ethyl]guanidine (cimetidine, 5 µM), H3 receptor antagonist 3-amino-N-[2-(1H-imidazol-4-yl)ethyl]propanamide ditrifluoroacetate (carcinine ditrifluoroacetate, 5 µM), H4 receptor antagonist, 1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine (JNJ7777120, 1 µM) and H4 receptor agonist, 5-(2-aminoethyl)-4-8methylimidazole dihydrochloride (4-methylhistamine dihydrochloride, 20 µM), (all from Tocris, MO, USA) for 24h (NO release). All histamine receptor antagonists were added 40 min prior to cell treatments.

To assess the effects of soluble mediators released by microglia on dopaminergic neuronal viability, primary microglia cell cultures were exposed for 24h with HIS 100 µM and/or LPS 100 ng/ml and the resulting conditioned medium (MCM) was collected and stored at -80 ºC. The MCM was then added to neuron-astrocyte midbrain co-cultures for further 24h and the viability of TH-neurons was evaluated. In another set of experiments, HIS 100 µM and/or LPS 100 ng/ml were added directly to neuron-astrocyte midbrain co-cultures for 24h and the viability of dopaminergic neurons was then evaluated.

3.3. EVALUATION OF CELL DEATH ASSAYS

3.3.1. PROPIDIUM IODIDE UPTAKE

The propidium iodide (PI; 3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenyl phenanthridinium diiodide) is a stable fluorescent dye absorbing blue-green light (493 nm) and emitting red fluorescence (630 nm). As a polar substance it only enters dead or dying cells with a damaged or leaky cell membrane, interacting with DNA to yield a bright red fluorescence. PI is non-toxic to cells and has been used as an indicator for cellular membrane integrity and cell damage. After cell exposure to histamine and/or LPS for 24h, 3 µg/ml of PI was added for further 40 min at 37º C in a 5% CO_2 and 95% air atmosphere. Then, the cells were fixed for 30 min in PFA 4% at room temperature (RT). For nuclear labeling, cell preparations were counterstained with Hoechst (2 µg/ml) (Molecular Probes) in PBS, for 5 min at RT and mounted with a fluorescent mounting medium (*DAKO, Glostrup, Denmark*). The cellular uptake of PI was recorder by fluorescence microscopy (Zeiss Axio imaging Microscope (*Axiobserver Z1, Zeiss*) using a 63x lens).

3.3.2. TUNEL LABELING

Cell apoptosis in microglial cells was evaluated by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL). This method in based on the specific binding of TdT, which attaches nucleotides (dUTP), to 3'-OH ends of the DNA generated during apoptotic-induced DNA fragmentation. Incorporation of biotinylated dUTPs allows the detection of cell apoptosis by immunocytochemistry procedures. At the end of each Histamine and/or LPS incubation (24h), microglia cells were fixed for 30 min in PFA 4% at RT and rinsed in PBS. Then, cells were incubated in a humidified atmosphere with a TUNEL reaction mix (Roche kit, REF: 11684795910) for 60 min at 37ºC. For nuclear labeling, cell preparations were counterstained with Hoechst (2 µg/ml) (Molecular Probes) in PBS, for 5 min at RT and mounted with a fluorescent mounting medium (*DAKO, Glostrup, Denmark*). TUNEL labeling were assessed using fluorescence microscopy (Zeiss Axio imaging Microscope (*Axiobserver Z1, Zeiss*) using a 63x lens).

3.4. MEASUREMENT OF NITRIC OXIDE (NO) RELEASE

Nitric oxide concentration was determined by measuring the total amount of nitrite ($NaNO_2$, including nitrate that is converted to nitrite by the Griess reagent), one end product of NO oxidation that is released to the culture medium. This assay relies on a diazotization reaction that was originally described by Griess in 1879, and is based on the chemical reaction which uses sulfanilamide and NED (*N*-1-napthylethylenediamine dihydrochloride) under acidic conditions. The amount of NO formed was determined from the accumulation of the stable NO metabolite (nitrite) in the supernatant after 24h of stimulation. Supernatants (50 µl) were collected, transferred to a 96-well plate, and mixed with an equal volume of the Griess reagent (sulfanilamide plus NED). The mixture was incubated in the dark for 10 min at RT, and the absorbance was read at 540 nm. To ensure accuracy of the nitrite quantification, a reference curve was prepared using as a matrix DMEM. The concentration of nitrite in the samples was determined from a sodium nitrite ($NaNO_2$) standard curve.

3.5. IMMUNOCYTOCHEMISTRY

Cells were fixed in 4% PFA for 20 min at RT. After washing with PBS, unspecific binding was prevented by incubating cells in a PBS solution with 3% Bovine Serum Albumin (BSA) and 0.5% Triton X-100 for 30 min, at RT. The cells were then incubated overnight at 4°C with the primary antibodies diluted in a PBS solution with 0.3% BSA and 0.1% Triton X-100, then washed with PBS the following day, and incubated for 1h at RT with the corresponding

secondary antibodies diluted in PBS. Antibodies were used as listed on Table 1. For nuclear labeling, cell preparation were stained with Hoechst (2 µg/ml) (Molecular Probes) for 5 min at RT and mounted with a fluorescent mounting medium (*DAKO, Glostrup, Denmark*). Fluorescent images were acquired using a fluorescence microscopy (Zeiss Axio imaging Microscope (Axiobserver Z1, Zeiss) using a 63x lens).

Table 2. Primary and secondary antibodies used for immunocytochemistry.

Primary antibody	Target	Dilution	Company	Secondary antibody	Dilution	Company
Mouse anti CD11b	Microglia	1:600	Chemicon	Goat anti mouse 488	1:200	Invitrogene
Mouse anti TH	Dopaminergic neurons	1:1000	Abcam	Goat anti mouse 488	1:1000	Invitrogene
Rabbit anti MAP2	Neurons	1:200	Chemicon	Goat anti rabbit 594	1:1000	Invitrogene
Goat anti H_4R	Histamine H_4 receptor	1:100	Santa Cruz Biotechonology	Rat anti Goat 488	1:200	Molecular probes

Legend: CD11b, cluster of differentiation molecule 11B; TH, tyrosin hydroxylase; MAP2, Microtubule-associated protein 2; H4R, histamine H4 receptor.

3.6 DATA ANALYSIS

Data are expressed as percentages of values obtained in control conditions or as percentages of the total number of cells, and are presented as mean ± S.E.M. of at least three independent experiments, performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by the Dunnett's test. Values of $P<0.05$ were considered significant. All statistical procedures were performed using GraphPad Prism 5 Demo (GraphPad Sotware Inc., San Diego, CA).

Chapter 4

<u>RESULTS</u>

4.1 Characterization of primary microglia cell cultures derived from the SN.

The cell culture methodology for purification of microglia from the SN was an indirect method starting from a mixed primary co-culture of astrocytes and microglial cells which grow for 21 days. The microglia cells were then purified by the removal of astrocytes using a mild trypsinization protocol (see section 3.1.2). To assess the purity of these primary microglia cell cultures, we performed an immunocytochemical staining for the alpha chain of αMβ2-integrin, CD11b, a well known surface marker for microglia, whose over-expression is associated to microglial activation (Fig. 5). We found that about 95% of cells were CD11b positive microglial cells (n=3 independent cell cultures). However some residual astrocytic cells could be also found on our culture, especially at the border of the glass slides (Fig. 5B).
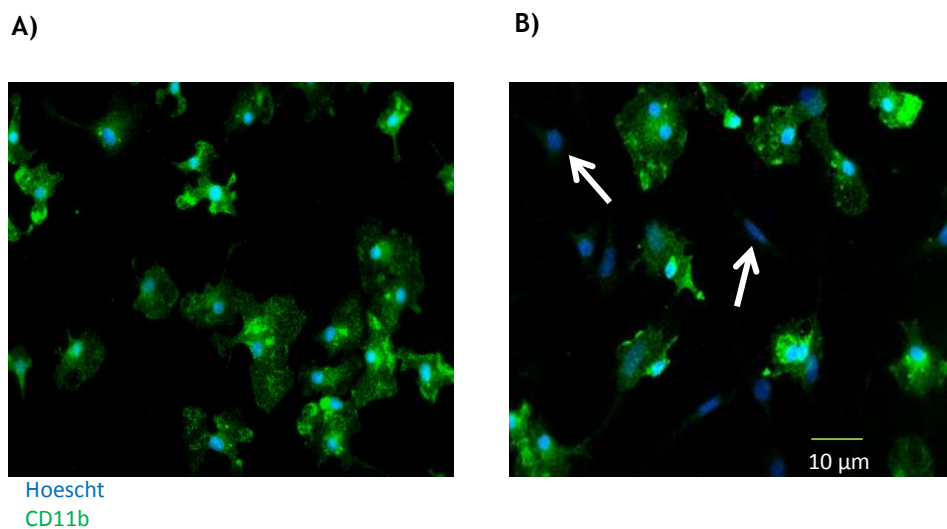

Figure 5: Immunocytochemical stainings of a primary microglia cell culture derived from SN of the Wistar neonatal rats. Representative photomicrographs taken at the center of slide (A) and in periphery (B). The microglia cells were stained with an anti-CD11b antibody (green) and for nuclear labeling, cell preparations were counterstained with Hoechst 3342 (blue). White arrows depict some cells that do not stain for CD11b.

4.2 Expression of histamine H4 receptor in microglial cells derived from SN.

Recently, it was reported by Ferreira and collaborators (Ferreira et al. 2012) that both the N9 microglia cell line as well as primary microglia cells isolated from the cortex of rats express the H4R. In this project, we analyzed the expression of this receptor in primary microglia cell cultures derived from SN, a region with a density of microglia 4-5 times higher than in other brain regions. The results show that SN-derived microglial cells indeed express H_4R, as detected by immunocytochemistry (Fig. 6A) and western blot (Fig. 6B). Furthermore, to determine whether differences regarding the pattern of receptor expression existed in an inflammatory context, we stimulated the microglial cells with LPS (100 ng/ml) for 24h and then immunocytochemistry against H4R was also performed. As shown in Fig.6A, no differences in H_4R protein expression were found between the control and the LPS-treated cells. Negative controls were performed to confirm the specificity of the primary antibody used for the detection of H4 receptors.
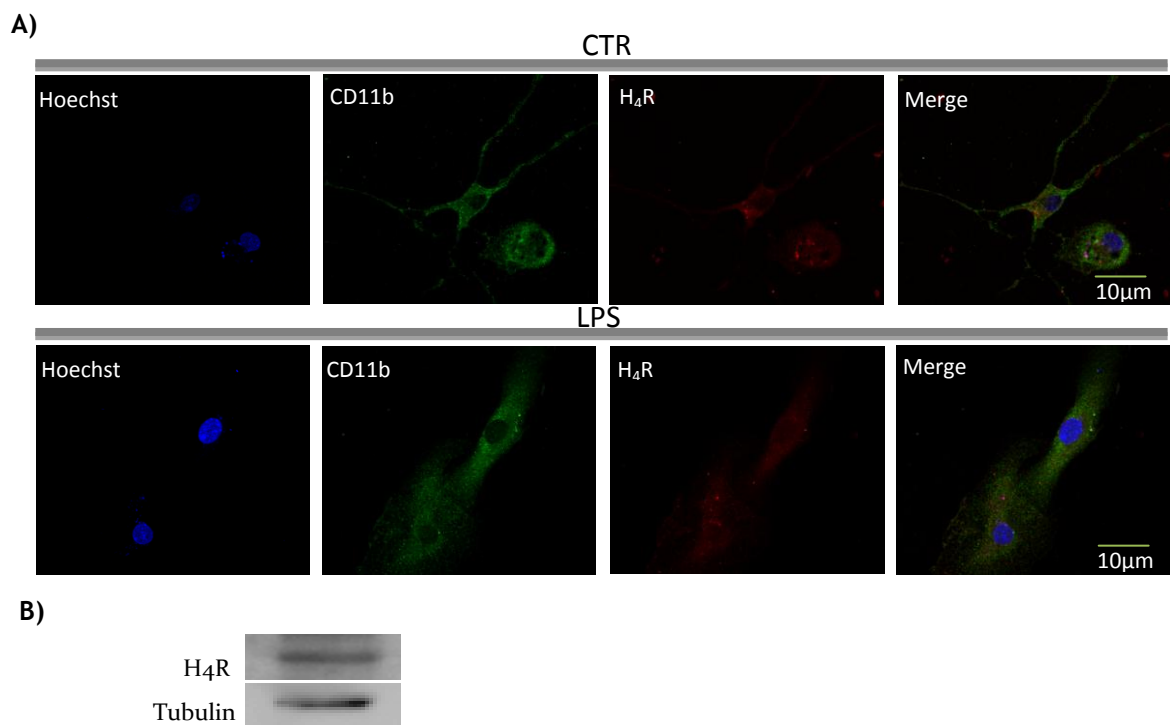
A)

B)

Figure 6: Microglia cells from SN express the histamine H4 receptor. A) Immunocytochemical analysis of histamine H4 receptor expression on untreated microglial cells (CTR) and treated with 100 ng/mL LPS for 24h. Photomicrographs depict staining for nuclei (Hoechst, blue), microglial cells (CD11b, green) and histamine H4 receptor (H4R, red). **B)** Histamine receptor expression analysis by western blot showed that microglial cells express the histamine H4 receptor (H4R: 44 KDa; Tubulin: 55 KDa).

4.3 Effect of histamine on the production of NO.

A feature of brain inflammation is the release of inflammatory mediators, such as NO, by activated microglial cells (Stence et al., 2001; Gibbons and Dragunow, 2006). It is known that histamine can induce microglia mobility and IL-1ß release (Ferreira et al., 2012) but their effect on NO production is unknown. To study this effect, we measured the amount of nitrite (a stable metabolite of NO) released by an N9 microglia cell line culture after 24h of treatment with different histamine concentrations (1 µM; 10 µM and 100 µM). The results showed that histamine stimulation significantly increased the NO release, and this increase is directly proportional with the histamine concentration (mean$_{HIS1\mu M}$ = 144.3±6.3; mean$_{HIS10\mu M}$ = 150.6±8.3; mean$_{HIS100\mu M}$ = 174.3±12.4, n=7-11) (Fig 7A). As we expected, LPS stimulation (100 ng/ml; positive control) leads to an increase of NO production (mean$_{LPS100ng/ml}$= 218.6±19.1, n=11) (Fig 7A). The same experimental conditions were also applied to a primary microglia cell culture from SN, since it mimics the physiological condition better than a N9 microglia cell line. Similarly to the *in vitro* cell line model, both the LPS and the histamine treated cells showed higher levels of NO release as compared to the control condition (mean$_{HIS1\mu M}$ = 143.7±9.8; mean$_{HIS10\mu M}$ = 125.0±9.4; mean$_{HIS100\mu M}$ = 149.0±5.6; mean$_{LPS100ng/ml}$ = 161.3±6.7, n=4-9) (Fig 7B). Based on these results, we then decided to use primary microglia cell cultures to disclose the receptor involved in histamine-induced NO release.
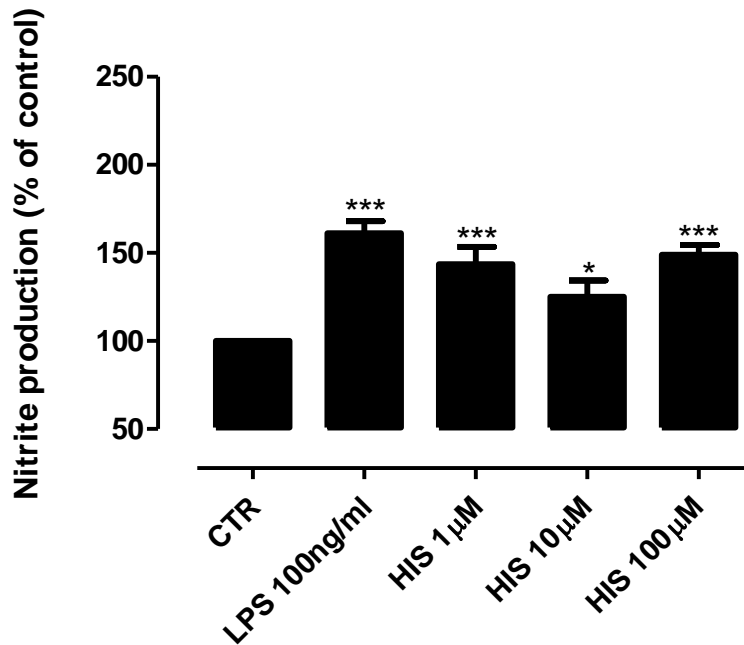
A)
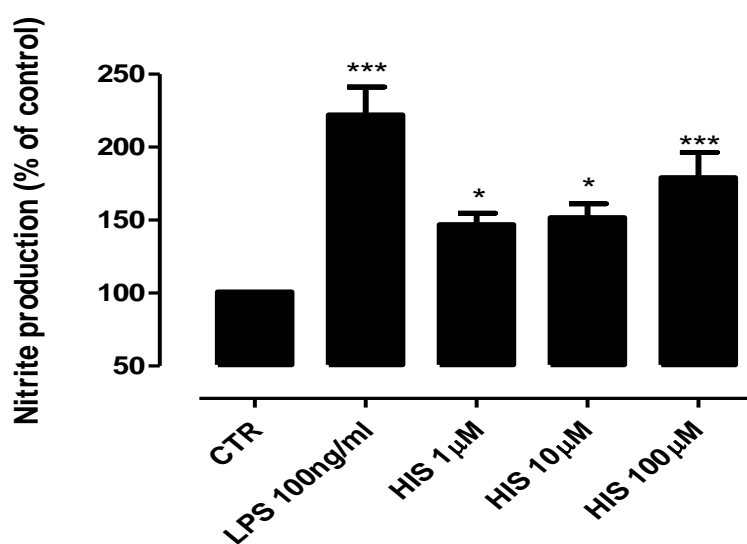
B)

Figure 7: Histamine-induced NO release from microglia cells. Histamine at 1 μM, 10 μM and 100 μM triggered an increase of NO release in (A) N9 microglia cell line culture and (B) primary microglia cell culture derived from the SN of neonatal rats. LPS (100 ng/ml) was used as a positive control and also increased significantly the production of NO in both cell cultures. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's correction (*P<0.05; ***P<0.001 as compared with control). The control was set to 100%.

4.4 H4R activation mediates the production of NO triggered by histamine

To uncover which histamine receptor was involved in the modulation of NO production by microglial cells, we then pretreated primary microglia cells culture for 40 min with all histamine receptors antagonists individually (Ant H_1R, 1 μM; Ant H_2R, 2.5 μM; Ant H_3R, 5 μM; Ant H_4R, 1μM) followed by an co-incubation for 24 h with histamine (100 μM). As shown in Fig. 8, histamine induces NO production via H4R activation, since in the presence of a H_4R antagonist (JNJ7777120, 5 μM), the stimulatory effect on NO production is reversed to values near to control, and the same is not observed for all others antagonists (mean$_{HIS100μM}$ = 149.0±5.6; mean$_{HIS+H1RAnt}$ = 149.7±11.2; mean$_{HIS+H2RAnt}$ = 140.0±10.4; mean$_{HIS+H3RAnt}$ = 139.0±16.5;

mean$_{HIS+H4RAnt}$ = 96.4±13.9, n=4) (Fig. 8). Furthermore, when microglial cells were treated with a H$_4$R agonist (4-methylhistamine dihydrochloride, 20 µM) for 24h, the NO production was similar to the levels induced by histamine *per se* (mean$_{HIS100µM}$ = 149.0±5.6; mean$_{H4RAg}$ = 134.7±5.0)(Fig. 8). These data suggest that histamine *per se* induced NO release by microglia *via* H4R activation.
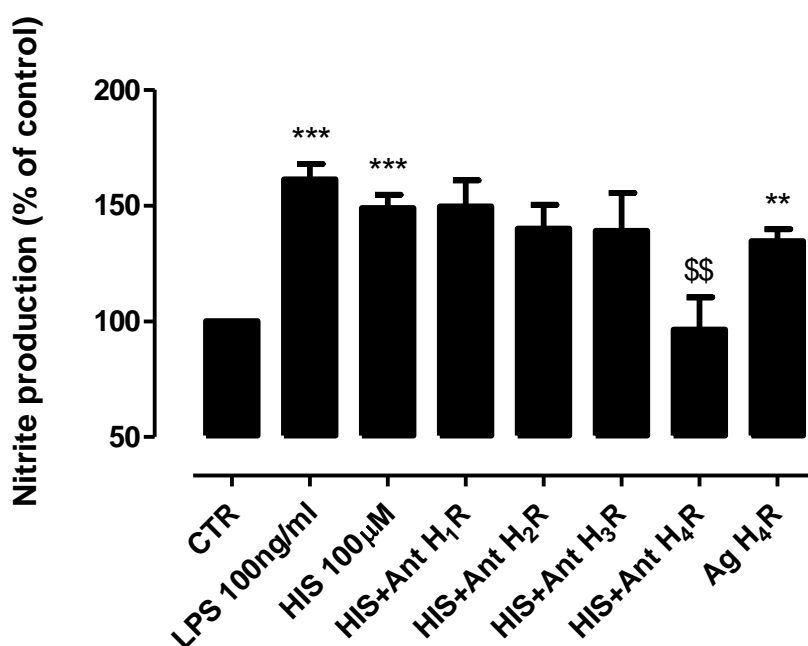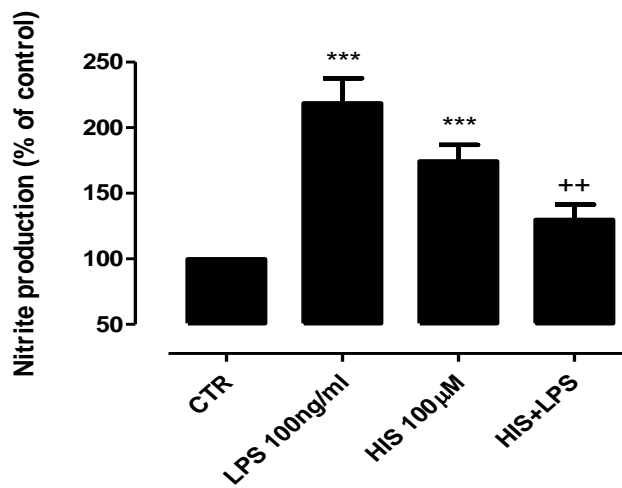

Figure 8: Histamine induces NO release by microglia cells *via* H4 receptor activation. NO production by microglia cells derived from the SN was increased when cells were treated with 100 ng/mL LPS, 100 µM histamine or 20 µM of an H4R agonist (4-methylhistamine dihydrochloride). Furthermore, histamine induced NO production is abolished in the presence of an H4R antagonist (JNJ7777120; 5 µM). Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's correction (**P<0.01; ***P<0.001 as compared with control and ^{SS}P<0.01 as compared with histamine). The control was set to 100%.

4.5 Effect of Histamine on NO production in an inflammatory context induced by LPS

Since histamine *per se* can induce NO release by microglial cells (Fig. 7), next we evaluated the role of histamine in an inflammatory context induced by LPS. For this, we treated N9 microglia cell line cultures with histamine (100 µM), concentration at which there was a higher increase of NO production (Fig. 7) together with LPS (100 ng/ml) for 24h. Surprisingly, upon LPS and histamine co-administration, NO release induced by LPS alone was significantly inhibited to levels similar to control cultures (mean$_{LPS100ng/ml}$ = 218.6±19.1;

mean$_{HIS+LPS}$ = 129.8±11.6, n=7-11) (Fig. 9A). We later explored the role of histamine upon an inflammatory challenge triggered by LPS (100 ng/mL) in a more complex biological model by using a primary microglia cell culture derived from the SN of neonatal rats. Similarly to the *in vitro* cell line model, we observed that the co-administration of LPS and histamine prevented NO release triggered by LPS *per se* (mean$_{LPS100ng/ml}$ = 161.3±6.7; mean$_{HIS+LPS}$ = 105.7±9.9, n=7-8) (Fig. 9B).

A)

B)
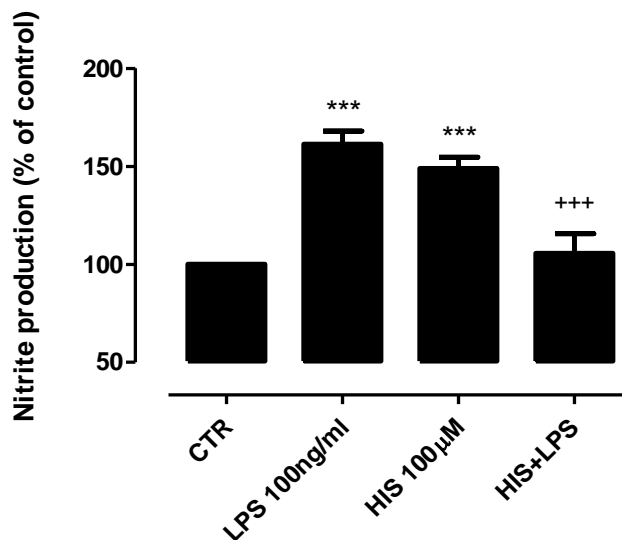
Figure 9: LPS-induced NO release is inhibited by Histamine. LPS and histamine individually increased NO release while co-administration abolished this effect, both in a (A) N9 microglia cell line culture and in (B) primary microglia cell cultures derived from the SN. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's correction (***P<0.001 as compared with control; ++P<0.01 and +++P<0.001 as compared with LPS).

4.6 Effect of Histamine on cellular viability

Above, we showed that histamine reduces NO production in an LPS-induced inflammatory context (Fig. 9). Nevertheless, in order to confirm that this decrease in NO production is not due to cell death, we then evaluated whether histamine and/or LPS could modulate microglia viability. To address this issue, primary microglia cells were exposed to histamine (100 μM) and/or LPS (100 ng/ml), for 24h, and cell necrosis or apoptosis were evaluated by propidium iodide uptake and the terminal deoxynucleotidyl transterase-mediated dUTP nick end labeling (TUNEL) assay, respectively. No significant effects on cell death by necrosis or apoptosis were observed after cell exposure to histamine alone or in co-administration with LPS (Fig. 10). Based on these results, we may suggest that 100 μM histamine in presence or absence of LPS is not toxic to microglia cells, and the reduction of the NO production shown in Fig. 9 is due in fact to a dual role of histamine in NO production.
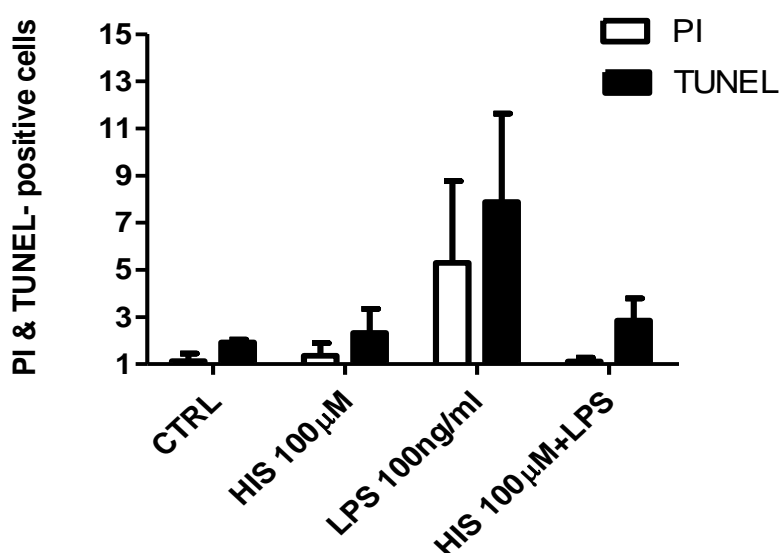

Figure 10: Microglia cell viability after histamine and/or LPS exposure. Percentage of PI-positive and TUNEL-positive cells in control cultures and in cultures exposed to 100μM histamine and/or 100 ng/mL LPS for 24h. Data are expressed as mean ± SEM of three independent experiments performed in duplicate.

4.7 Histamine H4 and H1 receptors activation modulates LPS-induced NO production

As show in Fig. 9B, co-administration of histamine together with LPS inhibits significantly the NO production induced by LPS *per se*. In order to investigate which histamine receptor is involved in this inhibitory effect, primary microglial cells were pre-treated with all histamine receptors antagonists individually (Ant H_1R, 1 µM; Ant H_2R, 2.5 µM; Ant H_3R, 5 µM; Ant H_4R, 1 µM) for 40 min, followed by a co-incubation for further for 24h with 100 ng/ml LPS and/or 100 µM histamine. As shown in the Fig. 11, both the histamine H_1R receptor as well as the H_4R receptor are involved in the inhibitory effect driven histamine on NO production induced by LPS alone. In fact, in the presence of the antagonists of these two receptors, the inhibitory effect of histamine upon LPS was prevented ($mean_{HIS+LPS}$ = 105.7±9.9; $mean_{HIS+LPS+H1RAnt}$ = 163.3±5.3; $mean_{HIS+LPS+H4RAnt}$ = 147.4 7±7.5, n=4-7) (Fig. 11). Moreover, to confirm that histamine H_4R receptor is involved in this modulatory effect, we treated microglial cells with LPS together a histamine H_4R receptor agonist. As show in Fig. 11 this receptor is in fact involved in this effect, since this agonist in presence of LPS can also inhibit the effect induced by LPS ($mean_{LPS100ng/ml}$ = 161.3±6.7; $mean_{LPS+H4RAg}$ = 105,7±10.8, n=4). Future experiments using the H1R agonist will be also performed.
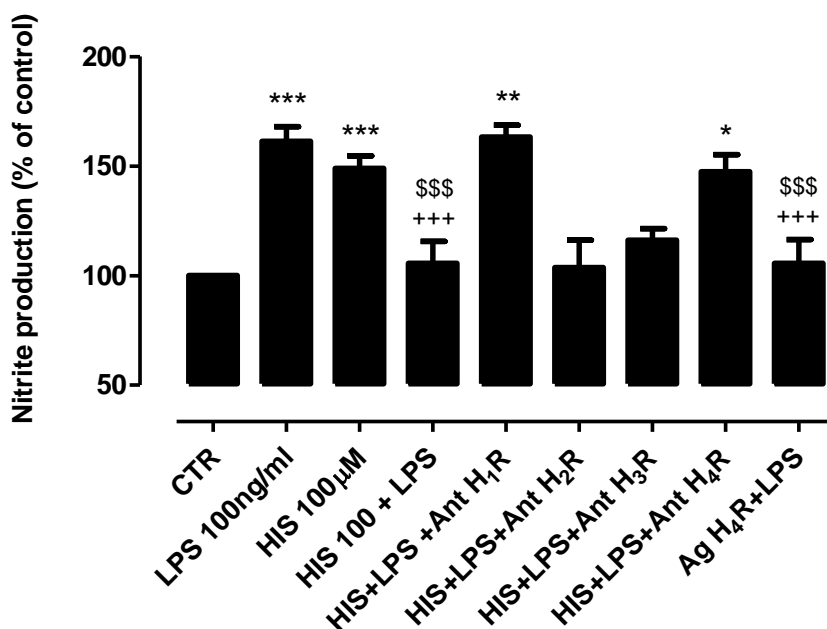
Figure 11: LPS-induced NO release is inhibited by histamine, *via* H1 and H4 receptors activation. LPS and histamine individually increased NO release while co-administration abolished this effect. Likewise, in the presence of an H4R agonist, LPS-induced NO release was also decrease. Application of the H1R and H4R antagonists restored LPS-induced NO release in the presence of histamine while H2R and H3R antagonists have no effect. Data are expressed as mean ± SEM. Statistical analysis was

performed using one-way ANOVA with Dunnett's correction (*P<0.05; **P<0.01; ***P<0.001 as compared with control; [+++]P<0.001 as compared with LPS and [$$$]P<0.001 as compared with histamine).

4.8 Effect of conditioned medium derived from microglial cells treated with LPS and/or Histamine in the viability of dopaminergic neurons

To evaluate the effects of the soluble factors released by microglia upon histamine and/or LPS stimulation on viability of dopaminergic neurons, we then treated primary microglia cells with histamine 100 µM and/or LPS 100 ng/ml for 24h, the conditioned media was retrieved and used to treat neuron-astrocyte midbrain co-cultures. In the control condition we used the conditioned medium derived from untreated microglia cells. Dopaminergic neuronal viability on neuron-astrocyte midbrain co-cultures was assessed by counting TH-positive cells as a percentage of total cells (Fig. 12A). As shown in figure 12B, the media derived from histamine or LPS-treated microglia induced a reduction concerning 30% in the number of TH positive cells ($mean_{HIS}$ = 69.2±4.3; $mean_{LPS}$ = 68.5±1.5, n=4) . However, MCM derived from histamine and LPS treated cultures restored dopaminergic neuronal survival to levels similar to control ($mean_{HIS+LPS}$ = 100.1± 2.1, n=4). Finally, since the MCM was applied to neuron-astrocyte midbrain co-cultures in a proportion of 1:1 (MCM: Neurobasal Medium), we then evaluated whether the Dulbecco's modified Eagle's medium (DMEM) *per se* could also modulated dopaminergic neuronal survival. Therefore, neuron-astrocyte midbrain co-cultures were treated with DMEM plus Neurobasal Medium (NBM), but no differences were seen in the percentage TH-positive neurons as compared with the control ($mean_{DMEM+NBM}$ = 97.2±2.5, n=4) (Fig. 12B).

A)
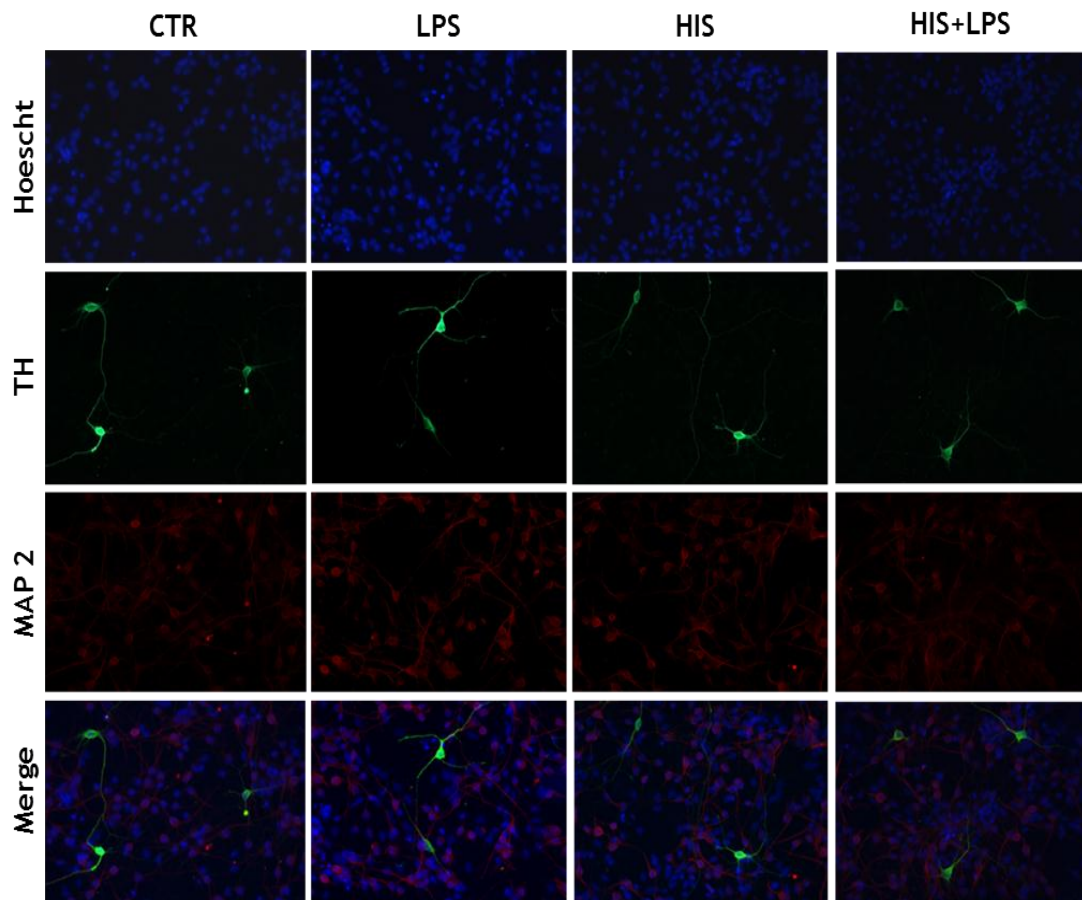
B)
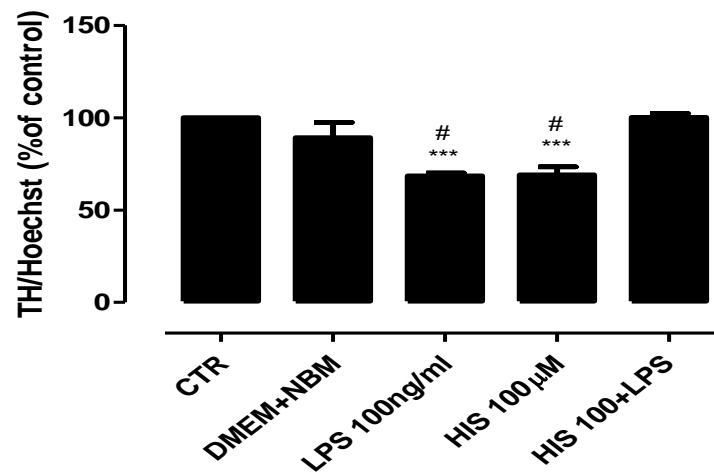
Figure 12: Microglia protects dopaminergic neurons from LPS injury when co-stimulated with histamine. The conditioned medium derived from microglial cells treated with 100 ng/mL LPS or 100 µM histamine, individually, decreased TH-positive neurons while co-administration abolished this effect. Results are expressed as the mean value of TH positive cells in relation to all nuclei stained with Hoescht ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's correction (***$P<0.001$ as compared with control and #$P<0.05$ as compared with DMEM plus NBM).

4.9 Effect of histamine and/or LPS *per se* on cellular viability of dopaminergic neurons

Since cellular viability of dopaminergic neurons was affected when incubated with MCM derived from microglia treated with histamine (100 µM) or LPS (100 ng/mL), but not when treated with these stimuli together, we decided to study the effects of histamine and/or LPS *per se* on cellular viability of dopaminergic neurons. For this, neuron-astrocyte midbrain co-cultures were treated with 100 µM histamine and/or 100 ng/ml LPS for 24h. Accordingly, a slight reduction of the percentage of TH-positive neurons was observed when neuron-astrocyte midbrain co-cultures were treated with histamine or LPS individually, while when co-treated with histamine and LPS no differences were seen (mean$_{HIS}$ = 69.2±2.6; mean$_{LPS}$ = 77.3±13.1; mean$_{HIS+LPS}$ = 101.0±13.5, n=3) (Figure 13). However, these differences were not statistically significant using one-way ANOVA test with Dunnett's correction. Additional experiments will be performed in order to disclose whether histamine or LPS may have a detrimental effect of dopaminergic neuronal survival.
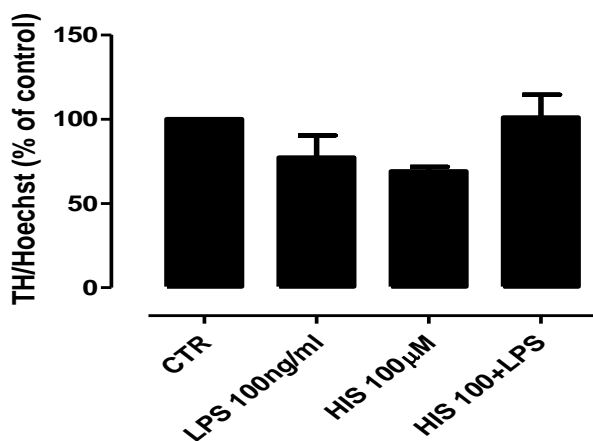

Figure 13: Effects of Histamine and/or LPS *per se* on dopaminergic neuronal survival. LPS and histamine individually decreased the percentage of TH-positive neurons while co-administration abolished this effect. Results are expressed as the mean value of TH-positive cells in relation to all nucleus stained with Hoechst ± SEM (n=3). These results were not statistically significant.

Chapter 5

<u>DISCUSSION</u>

Accumulating evidences indicate that inflammatory responses, involving the activation of microglia and astrocytes, can be a risk factor for the onset and progression of neurodegenerative diseases (Christopher et al., 2010; Rappold and Tieu, 2010). A major unsolved question is whether the inhibition of these responses will be a safe and effective means of reversing or slowing the course of disease. In accordance, several studies showed that a variety of compounds such as resveratrol, catechol compounds and aspirin to name a few, present a therapeutic potential for the treatment of neurodegenerative diseases, such as Parkinson's disease (Elisabetta et al., 2001; Long et al., 2008; Wang et al., 2012).

In response to an injury or infection, microglia cells, the immune resident cells in the brain, became activated and release a cocktail of inflammatory molecules, migrate to the injury site and became phagocytic. Histamine, an amine released by microglia recently gained attention due to its role in the modulation of microglia migration and cytokines release. In fact, Ferreira and collaborators showed that histamine can trigger microglia motility *per se*, whereas, in a presence of an inflammatory stimulus mimicked by LPS, histamine H4 receptor agonists were able to counteract LPS mediated inflammatory actions, namely migration and IL-1ß release (Ferreira et al., 2012). Based on this data, wherein we investigated the modulatory effects of histamine and its receptors on NO release by microglia cells. First, we showed for the first time the expression of the histamine H4 receptor on microglia derived from SN, a region with an density of microglia 4-5 times higher than in other regions and with a particularly high number of activated microglia in the brain of post-mortem PD patients (Lawson et al., 1999; Long-Smith et al., 2009). Moreover, we found that the expression of this receptor was not altered when microglial cells were stimulated with LPS. These results are in accordance with the ones reported by Ferreira et al., 2012, in which they showed that the H4R is expressed in both N9 microglia cell lines and in primary microglial cultures derived from cortex and its expression does not change upon LPS stimulation.

Then, to evaluate the effects of histamine on the release of NO, a molecule highly involved in several neuroinflammatory processes, we adopted two experimental models, with different degrees of complexity: N9 microglia cell line culture and primary microglia cell culture derived from the SN. We first observed that histamine *per se* stimulated NO production in both these two experimental models. This effect was mediated through H_4R activation. This suggests that histamine alone acts similarly to LPS with pro-inflammatory properties, and like LPS, histamine could be one of NO-regulating factors, by inducing iNOS expression. In fact, Tanimoto and colleagues found that histamine upregulated the iNOS

expression through activation of H_1R in intimal smooth muscle cells (Tanimoto et al., 2007). Furthermore, dithiaden (an antagonist of H_1R) can inhibit NO production by a murine macrophage cell line RAW 264.7, an effect caused by a decrease in iNOS expression (Králová et al., 2008). In our work, we showed that histamine leads to NO release by microglial cells, an effect mediated through H_4R activation, which is mimicked by a H_4R agonist (4-methylhistamine dihydrochloride). The mechanism that leads to NO production is unknown, but the involvement of iNOS enzyme is a possibility. The evaluation of iNOS protein expression and iNOS mRNA levels can be helpful to uncover its involvement.

Curiously, given an inflammatory context mimicked by LPS, histamine inhibited NO production. Furthermore, by using all four antagonists of histamine receptors, we showed that this anti-inflammatory effect occured not only by H_4R, but also, possibly through H_1R activation. Moreover, when microglial cells were incubated with histamine H_4R agonist plus LPS, the NO production induced by LPS was also inhibited. This is in agreement with the results obtained when microglia were incubated with histamine and LPS, which suggests the involvement of H_4R in this dual effect triggered by histamine on NO production. Regarding the involvement of H_1R, future experiments are needed and the use of an agonist of this receptor can be helpful for decipher if the H_1R is in fact involved in this anti-inflammatory effect. Again, these results are in agreement with the ones reported by Ferreira and collaborators, in which they showed that in the presence of an inflammatory agent (LPS), histamine inhibited IL-1ß production and microglia migration however, histamine alone induces microglia migration but not IL-1ß production (Ferreira et al., 2012). There are also some studies showing the anti-inflammatory properties of H4R. Two H_4R agonists (6,7-dichloro-3-(4-methylpiperazin-1-yl)quinoxalin-2(1H)-one and 2-benzyl-3-(4-methyl-piperazin-1-yl)quinoxaline) shown in vivo to have an anti-inflammatory effect in a carrageenan-induced paw edema model (Hague and Jones, 2008). However, the molecular mechanisms involved on the modulatory effect of histamine on NO production are unknown. Nevertheless, is known that activation of ERK, JNK and p38 MAPK leads to activation of transcription factors, such as, nuclear factor (NF)-kB and activator protein 1 (AP-1), that are involved in expression of iNOS gene, which is followed by the sustained production of NO by active microglia (Kim and Kim, 2005). Since LPS-induced NO production by microglia involves these intracellular signalling pathways, we hypothesize that the activation of H4R and/or H1R can modulate these pathways. The use of selective inhibitors of these pathways as well as the evaluation of their expression and activation may be helpful to disclose the downstream signalling pathways involved in NO-mediated histamine effects.

Even though microglia have often been indicated as dangerous effector cells on neuronal survival, this perspective was opposed by beneficial microglial features such as neuroprotection mediated by a plethora of neurotrophic factors (NTF) such nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Harada et al., 2002; Mizuno et al., 2004). Furthermore, glutamate receptors and transporters in microglia are involved in induction of NTF expression,

which suggest that microglia play a neuroprotective role during the excitotoxic state in neurodegenerative diseases (Schwab and Schluesener, 2004). In fact, when dopaminergic neurons are exposed to MCM, this can promote their survival and development, an effect that may be due to a NTF released from microglia (Engele et al., 1991; Nagata et al., 1993; Engele et al., 1996).

In this work, using neuron-astrocyte midbrain co-cultures, we showed that the exposure with conditioned media derived from microglia previously challenged with histamine and/or LPS had different effects on dopaminergic neuronal viability. A decrease in the percentage of tyrosine hydroxylase-immunoreactive (TH-IR) neurons was observed when neuron-astrocyte midbrain co-cultures were exposed to MCM derived from microglia challenged with histamine. On the other hand, a similar percentage of TH-IR neurons was found between neuron-astrocyte midbrain co-culture exposed with MCM derived from control microglia cultures (untreated) or cultures exposed with histamine plus LPS. These results suggest that histamine *per se* may trigger the release of several pro-inflammatory molecules by microglia cells, including NO, that may lead to dopaminergic neuronal death. However, conditioned media derived from microglia previously co-treated with histamine and LPS had no effect on dopaminergic neuron survival. Regarding these results, raises a question if this neuroprotective effect of histamine on the inflammatory context mediated by LPS, is due through release of anti-inflammatory factors by microglia or by inhibiting the intracellular mechanism activated by LPS, for instance the release of NO and IL-1β (Ferreira et al., 2012). Future experiments will be done to answer this question.

Concerning the effect of histamine and/or LPS *per se* on cellular viability of dopaminergic neurons in neuron-astrocyte midbrain co-cultures, we found that LPS had a slight but not significant toxicity effect. This effect was likely mediated through the activation of astrocytes. In fact, both astrocytes and microglial cells have been shown to respond to LPS in the induction of iNOS as well as other inflammatory factors such as IL-1β and TNF-α (Wilms et al., 2010; Sheng et al., 2011; Harms et al., 2011). These cytokines, as well as, up-regulation of iNOS, may be involved in the LPS-induced degeneration of dopaminergic neurons (Li et al., 2006; Long-Smith et al., 2010). Like LPS, stimulation of neuron-astrocyte midbrain co-cultures with histamine had a slight but not significant toxicity effect on viability of dopaminergic neurons. In accordance, Vizuete and colleagues (2000) showed that histamine infusion in SN induces a selective dopaminergic neuronal death, with an increase of astrocyte and microglia activity. Once our neuron-astrocyte midbrain co-cultures have no microglial cells, this effect can be due to the presence of astrocytes that response to histamine with increases in intracellular free Ca^{2+} concentration that can trigger glutamate release which possibly leads to excitotoxicity (Shelton and McCarthy 2000; Nakamura et al., 2003). Moreover, histamine can enhance glutamate receptor activation, suggesting that histamine may contribute significantly to NMDA-mediated excitotoxic neuronal death (Langlais et al., 1994; Doble, 1999). Nevertheless, treatment of neuron-astrocyte midbrain co-cultures with histamine in an inflammatory context induced by LPS, do

not affect the viability of dopaminergic neurons. However, how histamine has this dual effect on viability of dopaminergic neurons is unknown, and more studies are necessary to clarify this issue.

Altogether, our results suggest that histamine *per se* may have a pro-inflammatory effect either by triggering NO release and promoting dopaminergic neuronal death (both by the direct incubation or the collection of the conditioned medium released by microglia). While, in an inflammatory context mimicked by LPS, histamine has a putative anti-inflammatory profile that can protect dopaminergic neurons from an inflammatory stimulus.

Chapter 6

<u>CONCLUSIONS</u>

Apart from the neurotransmitter function, histamine is also involved in inflammatory processes in CNS. With this work, we showed that histamine *per se* can trigger NO release from microglial cells, an effect mediated by H_4R activation. However and most importantly, we showed that in an inflammatory context, histamine acting via H_4R and possibly through H_1R, plays an inhibitory action on NO release. Moreover, this dual effect on microglia-induced NO release, seems to be linked to dopaminergic neuronal survival.

Since, to the best of our knowledge, there are no reports showing an involvement of histamine on NO release by microglial cells and the subsequent dopaminergic neuronal survival, with this work we open a new perspective for the therapeutic use of histamine and histamine receptor agonists to treat or ameliorate inflammation-associated processes, like those seen in pathogenesis of PD.

Chapter 7

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