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Impact of Peripheral Inflammation on the Susceptibility to Neurodegenerative Diseases

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Resumo

O lipopolissacarídeo (LPS) é um agente inflamatório amplamente utilizado para induzir respostas inflamatórias periféricas em modelos animais. Quando administrado a baixas concentrações e durante um curto intervalo temporal, tem a capacidade de induzir neuroproteção em modelos animais da Doença de Alzheimer e de Acidente Vascular Cerebral. Este efeito neuroprotetor deve-se à ativação do sistema imunitário periférico e, subsequentemente, ativação e indução da memória imunitária inata nas células da microglia. Assim, o principal objetivo deste trabalho foi compreender os efeitos do LPS e da histamina, uma amina biogénica também envolvida em reações inflamatórias periféricas, na capacidade de induzir memória imunitária inata e, consequentemente, promover neuroproteção dos neurónios dopaminérgicos num modelo animal da Doença de Parkinson (DP). Para tal, murganhos C57BL/6J foram sujeitos à administração intraperitoneal com LPS ou histamina e, após 3 semanas, foram expostos à lesão intraestriatal com 6-hidroxidopamina (6-OHDA) para mimetizar a DP. A resposta inflamatória foi avaliada através da expressão de citocinas pro-inflamatórias (TNF-α e IL-1β) e anti-inflamatórias (IL-10) no plasma sanguíneo, e da expressão da molécula adaptadora de ligação ao cálcio ionizado 1 (Iba-1), marcador celular de células de microglia e macrófagos, no cérebro. Para avaliar a sobrevivência dopaminérgica e o comportamento motor, recorreu-se às técnicas de imunohistoquímica para tirosina hidroxilase (TH) e ao teste da apomorfina, respetivamente. Os resultados obtidos sugerem que o LPS e a histamina induzem ativação da resposta inflamatória periférica com consequente ativação da microglia, desencadeando memória imunitária inata. A longo prazo, verifica-se que estes estímulos inflamatórios protegem os neurónios dopaminérgicos num modelo da DP, bem como a recuperação motora, traduzindo-se num novo mecanismo de prevenção e melhoria celular e funcional da DP.

Palavras-chave

Doença de Parkinson; Histamina; Imunidade Inata; Inflamação; Lipopolissacarídeo; Microglia; Neuroproteção

Resumo Alargado

A Doença de Parkinson (DP) é a segunda doença neurodegenerativa mais prevalente na população a nível mundial, sendo caracterizada por uma perda progressiva dos neurónios dopaminérgicos na *substantia nigra* (SN) e pela degeneração das suas terminações nervosas no *striatum* (ST). Esta patologia está associada a mecanismos de neuroinflamação resultantes da ativação das células da microglia, que no seu estado ativo, libertam citocinas pro-inflamatórias (por exemplo: TNF-α, IL-1β, entre outras) e outros mediadores inflamatórios capazes de afetar a sobrevivência neuronal. As células da microglia são consideradas as células do sistema imunitário residentes no sistema nervoso central, encontrando-se particularmente em grande número na SN e, assim, podem aumentar a suscetibilidade dos neurónios dopaminérgicos a estímulos inflamatórios.

O lipopolissacarídeo (LPS) é uma molécula constituinte da parede de bactérias *Gram negativas* capaz de induzir respostas inflamatórias em modelos animais. Vários estudos referem que esta molécula tem a capacidade de induzir efeitos contraditórios na DP. A sua administração em regime crónico e a concentrações elevadas pode mimetizar esta patologia. Paradoxalmente, quando administrada a baixas concentrações, verifica-se que protege os neurónios dopaminérgicos da degeneração induzida por neurotoxinas. Estudos recentes mostram que a indução da neuroproteção depende da formação da memória imunitária inata. A memória imunitária inata é descrita como a capacidade de o organismo guardar informação de uma infeção prévia, através de células do sistema imunitário inato (células da microglia, macrófagos) para que quando ocorra uma reinfeção o organismo possa reagir de forma mais rápida e eficaz no combate à infeção. Recentemente, demonstrou-se que o LPS induz neuroproteção em modelos da Doença de Alzheimer e Acidente Vascular Cerebral através da ativação da memória inata em células da microglia ou monócitos/macrófagos.

A histamina, uma amina biogénica, está intimamente relacionada com os mecanismos de inflamação e encontra-se em elevadas concentrações no líquido cefalorraquidiano de paciente com DP. As terminações nervosas dos neurónios histaminérgicos também se encontram mais pronunciadas em modelos animais da DP. Desta forma, esta molécula poderá ser um mediador com uma função relevante nas respostas inflamatórias e na patologia da DP. Contudo, atualmente, desconhece-se o efeito da histamina na memória imunitária inata e o seu impacto em doenças neurodegenerativas.

O principal objetivo deste trabalho foi compreender se o LPS ou a histamina têm a capacidade de induzir memória imunitária inata e, consequentemente, neuroproteção nos neurónios dopaminérgicos num modelo animal da DP. Para avaliar a resposta inflamatória começou-se por quantificar a libertação de citocinas pró- e anti-inflamatórias no plasma sanguíneo de murganhos C57BL/6J, aos quais se administrou por via intraperitoneal (IP) uma ou quatro injeções consecutivas de LPS ou histamina. Verificou-se uma diminuição na concentração das citocinas pró-inflamatórias TNF-α e IL-1β no final das quatro injeções diárias, sugerindo o desenvolvimento de memória imunitária inata. De seguida, avaliou-se a ativação das células da microglia através da expressão do marcador Iba-1 por Western Blot. Verificou-se um aumento da expressão de Iba-1 em cérebro total após estimulação periférica com LPS ou histamina. De seguida, os animais foram sujeitos a injeções IP de LPS ou histamina durante quatro dias consecutivos e, após três semanas, administrou-se a neurotoxina 6-OHDA no ST. Este conjunto de experiências tinham como finalidade avaliar o efeito da memória inflamatória inata desencadeada pelo LPS ou histamina no comportamento motor e a sobrevivência dos neurónios dopaminérgicos. Verificou-se que os grupos pré-tratados com LPS ou com histamina apresentaram recuperação motora, assim como neuroproteção dos neurónios dopaminérgicos.

Em suma, os nossos resultados demonstram que a ativação periférica, com LPS ou histamina, induz resposta inflamatória periférica e ativação microglial, permitindo o desenvolvimento de memória inflamatória inata. A longo prazo, verifica-se que estes mesmos estímulos inflamatórios têm a capacidade de induzir neuroproteção dos neurónios dopaminérgicos expostos à neurotoxina 6-OHDA, bem como a recuperação motora. Com isto, podemos concluir que a estimulação periférica com os mediadores inflamatórios LPS ou histamina, induz mecanismos de neuroproteção dopaminérgica no modelo animal para a DP mimetizado com a administração de 6-OHDA.

Abstract

Lipopolysaccharide (LPS) is an inflammatory agent widely used to induce peripheral inflammatory responses in animal models. When administered at low concentrations and over a short time interval, it has the ability to induce neuroprotection in animal models of Alzheimer's Disease and stroke. This neuroprotective effect is due to the activation of the peripheral immune system and subsequently activation and induction of innate immune memory in microglial cells. Thus, the main objective of this work was to understand the effects of LPS and histamine, a biogenic amine also involved in peripheral inflammatory reactions, in the ability to induce innate immune memory and, consequently, to promote neuroprotection of dopaminergic neurons in an animal model of Parkinson's Disease (PD). For this, C57BL/6J mice were subjected to intraperitoneal administration with LPS or histamine and, after 3 weeks, were exposed to intrastriatal injury with 6-hydroxydopamine (6-OHDA) to mimetize PD. The inflammatory response was evaluated by the expression of proinflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10) in the blood, and by the expression of the ionised calcium binding adapter molecule 1 (Iba-1), a cell marker of microglia and macrophage cells, in the brain. To evaluate dopaminergic survival and motor behavior, immunohistochemistry for tyrosine hydroxylase (TH) and apomorphine test were use, respectively. The results suggest that LPS and histamine induce activation of the peripheral inflammatory response with consequent activation of microglia, triggering innate immune memory. In the long term, it is verified that these inflammatory stimuli protect dopaminergic neurons in a PD model, as well as motor recovery, resulting in a new mechanism of prevention and cellular and functional improvement of PD.

Keywords

Parkinson's Disease; Histamine; Inflammation; Innate Immunity; Lipopolysaccharide; Microglia; Neuroprotection

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List of Abbreviations

6-OHDA	6-Hydroxydopamine
AD	Alzheimer's Disease
AKT	Protein kinase B
BBB	Blood-Brain Barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DA	Dopaminergic
DAT	Dopamine membrane transporter
GDNF	Glial cell-derived neurotrophic factor
H_2O_2	Hydrogen peroxide
HDC	Histidine Decarboxylase
ICV	Intracerebroventricular
IP	Intraperitoneal
L-DOPA	3,4-dihydroxyphenylaline
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
MHC	Major histocompatibility complex
MPP+	1-methy-4-phenylpyridium
mRNAs	Messenger RNAs
MTPT	1-methy-4-phenyl-1,2,3,6-tetrahydropyridine
NAT	Noradrenaline membrane transporter
NGF	Nerve Growth Factor
NSCs	Neural Stem Cells
OB	Olfactory Bulb
PAMPs	Pathogen-associated molecular patterns
PD	Parkinson's Disease
PI3K	Phosphoinositide 3-kinase
PNS	Peripheral Nervous System
PQ	Paraquat
ROS	Reactive Oxygen Species
SGZ	Subgranular zone
SN	Substantia nigra
ST	Striatum
SVZ	Subventricular zone
TH	Tyrosine Hydroxylase
TLR-4	Toll-like 4 receptors
TLRs	Toll-like receptors
α-SYN	α-synuclein

Chapter 1

Introduction

1. Parkinson's Disease

1.1. Pathophysiology

Parkinson's Disease (PD) is the second most prevalent neurodegenerative disease affecting about 1 to 3% of the population over 65. It is estimated that by 2030 the number of individuals with this pathology will increase from 8.7 to 9.3 million. [1], [2]

PD is characterized by the loss of dopaminergic (DA) neurons present in the *substantia nigra* (SN) and degeneration of DA terminals in the *striatum* (ST). [3], [4] The formation of Lewy Bodies, due to the abnormal accumulation of α-synuclein (α-SYN) protein, is also considered a *hallmark*. [1]

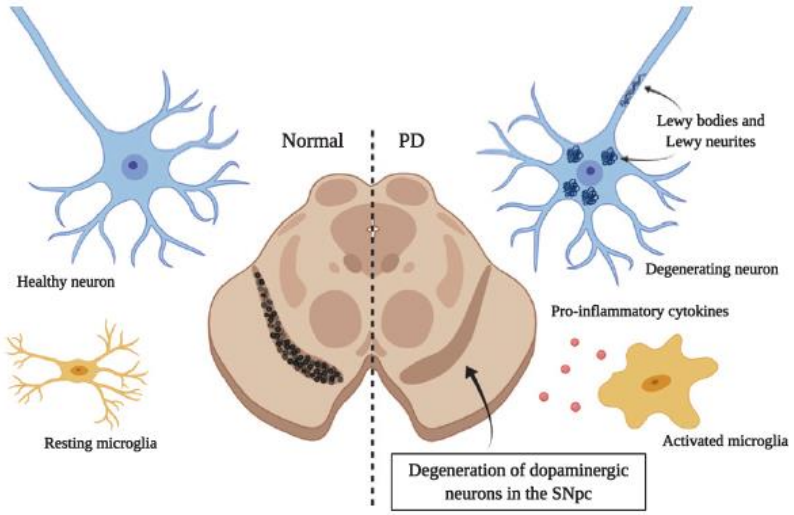

Figure 1 – Schematic representation of PD hallmarks. PD is characterized by the loss of DA neurons present in the *substantia nigra (*SN). The formation of Lewy Bodies, due the abnormal accumulation of α-synuclein protein, are present in affected neurons. Activated microglia are also present and release of pro-inflammatory cytokines promoting an inflammatory environment contributing to neuronal loss. Figure from [5].

There are two distinct forms of this pathology. Sporadic (or idiopathic) results from the interactions between genes and the environment, and genetically inherited through an autosomal dominant or recessive manner. [6] Epidemiologically, PD is related to some demographic factors, such as age and gender. Age is the primary risk factor for developing this disease, being the majority of cases found in individuals over 65 years of age, primarily in economically developed countries. [6], [7] Moreover, this disease predominantly affects the male gender, which indicates that estrogen may act as a neuroprotective agent. [8], [9]

James Parkinson, in 1917, described the motor symptoms of PD. Classic motor symptoms include bradykinesia, which corresponds to the slowness of voluntary movements; tremors when resting, characterized by an involuntary movement of a part of the body; rigidity, due to an increase in resistance in the execution of movements; and posture instability, which concerns balance oddities that compromise the ability to maintain the former. Typically, these symptoms are manifested unilaterally. [2], [10], [11] However, motor symptoms are not the only ones that manifest, and non-motor symptoms are equally important for diagnosis and are the first to manifest. Non-motor symptoms include, for example, depression, drowsiness, dementia, cognitive dysfunctions, sleep disorders, and constipation. [2], [11], [12]

Several experimental models mimic this pathology, such as pesticides, herbicides, and neurotoxins. The herbicide paraquat (PQ) is a toxin that can indue degeneration of dopaminergic neurons, due the production of superoxide radicals. [13] Rotenone, a member of a family of natural cytotoxic compounds extracted from tropical plants, is highly lipophilic and therefore has the ability to cross the Blood-Brain Barrier (BBB) without relying on transporters. Rotenone has the ability to inhibit mitochondrial membrane complex I, one of five enzymatic mitochondrial membrane complexes responsible for oxidative phosphorylation, promoting reactive oxygen species (ROS) formation. Administration of low doses of this compound intravenously induces the degeneration of DA neurons and the formation of Lewy bodies, due to the accumulation of the α-synuclein protein. [14] MPTP (1-methy—4-phenyl-1,2,3,6-tetrahydropyridine), is a toxin that can cross the BBB due to its lipophilic nature. After crossing the BBB, the toxin is metabolized into non-dopaminergic cells by the enzyme monoamine oxidase (MAO-B) in its active compound, MPP+ (1-methy-4-phenylpyridinium) which is subsequently released into the extracellular space. MPP+ has a high affinity for the dopamine transporter, managing to enter in DA neurons and damage them. [15]

6-OHDA is a neurotoxin incapable of crossing the BBB and the toxicity in the Central Nervous System (CNS) is achieved only when directly injected into the brain (SN, ST or medial forebrain bundle). The neurotoxic effects occur through a two-step mechanism involving accumulation of the toxin into catecholaminergic neurons, followed by alteration of cellular homeostasis and neuronal damage. Since this toxin is an analog of dopamine, due to its structural similarity with endogenous catecholamines, the intracellular storage is mediated by the dopamine or noradrenaline membrane transporters (DAT and NAT, respectively). When internalized in the

brain, 6-OHDA produces cytotoxic species, such as hydrogen peroxide (H_2O_2), ROS and catecholamine quinones, due the oxidation by monoamine oxidase (MAO-A). [16] Neuroinflammation and microglia activation were considered other effects associated with the toxic effect of 6-OHDA, contributing to the degeneration of DA neurons. [17]

In addition to the sporadic form, PD may appear due to genetic factors. Genetic models are used to understand the role of the different genes involved in the onset and progression of this pathology. Genetic changes may be due to dominant autosomal mutations such as mutations in the α-synuclein gene (the first gene identified associated with the familiar form of PD) [18] or mutations in the LRRK2 (more frequent mutations). [19] On the other hand, there are also mutations associated with the autosomal recessive form, such as the parkin gene (the first cause of recessive autosomal mutations) or the PINK1 gene. [20], [21]

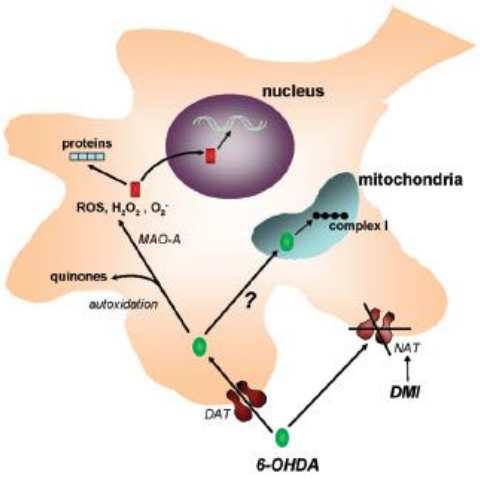

Figure 2 – Schematic representation of the mechanism of 6-OHDA – induced neurotoxicity. The neurotoxin is taken up from the extracellular space by DAT or NAT and is store in catecholaminergic neurons. Inside neurons, the toxin undergoes both enzymatic degradations, by MAO-A, and autoxidation, producing cytotoxic species which results in neuronal damage. Figure from [16].

1.2. Inflammatory Response Mediated by Microglia

The CNS is composed of two types of cells: neurons, whose function is to allow electrical transmission and processing of information, and glial cells, which are indispensable for the correct functioning of neurons. Although neurons are the most well-known out of pair, in turn, glial cells represent about 90% of brain cells. [22], [23]

The CNS contains three major glial cell types: astrocytes, oligodendrocytes, and microglia. In the Peripheral Nervous System (PNS), the predominant cells are Schwann cells. In the CNS, glial cell and neurons have their origin in neural precursor cells, namely the radial glial cell, located in the subventricular zone (SVZ). [23] In fact. CNS relies on a small number of neural stem cells (NSCs) to generate various cell types that proliferate and differentiate in neurons or glial cells during embryonic development. In the adult brain there are two niches containing neural stem cells: the SVZ, in the lateral ventricles, and the subgranular zone (SGZ), in the hippocampal dentate gyrus.

In the SVZ, there are three distinct types of cells: B, quiescent astroglial stem cells, that originate non-radial progenitor cells, type C cells, which subsequently originate neuroblasts (type A cells) that migrate through the olfactory bulb (OB). [24]

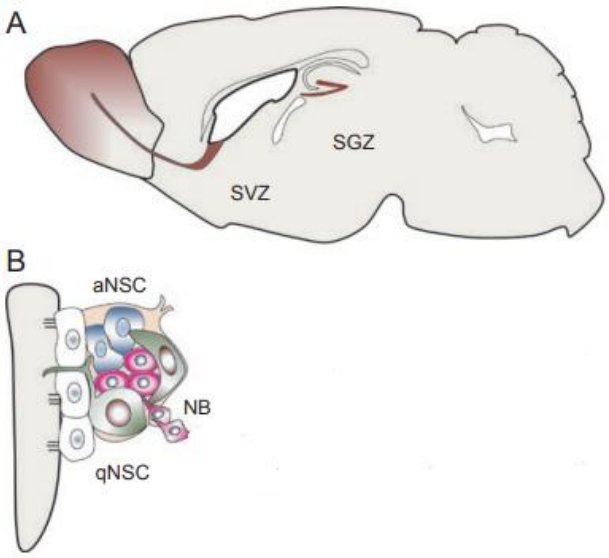

Figure 3 – Schematic representation of localization and composition of neural stem cells (NSCs) niches. (A) Sagittal rodent brain section. Adult NSCs are present in two neurogenic niches: the SVZ, in the lateral ventricles, and the subgranular zone (SGZ), in the hippocampal dentate gyrus. (B) quiescent astroglial stem cells (qNSC), originate adult progenitor cells, type C cells (aNSC), which subsequently originate neuroblasts (type A cells, or NB). Figure adapted from [25].

However, microglia originate from the hematopoietic system, migrating to the brain parenchyma during the post-natal period. [26] Microglia are considered the resident immune cells in the brain parenchyma, being able to respond to various stimuli. [27], [28]

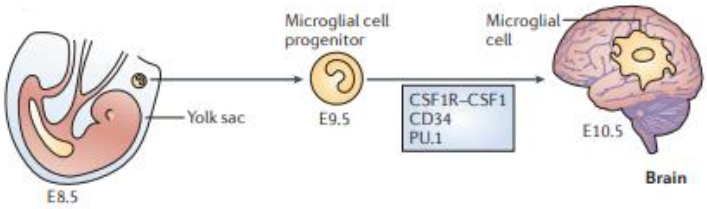

Figure 4 – Schematic representation of microglia formation and migration into the brain. Microglia are derived from the hematopoietic system, migrating to the brain parenchyma during the post-natal period. E8.5, embryonic day 8.5, E9.5, embryonic day 9.5. Figure adapted from [29]

In a healthy brain, microglia are in a resting state with a branched morphology. However, the resting state is extremely sensitive to any disturbance in the neuronal environment, such as infections or exposure to an inflammatory stimulus, leading to the active form. [28], [30] Microglia shares several functionalities with macrophages, including with regard to the production and release of proinflammatory cytokines, presentation of antigens, as well as is related to the phagocytosis process. Microglia contributes to the maintenance of CNS homeostasis

by providing the necessary trophic support through the secretion of neurotrophic molecules such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). [31] On the other hand, microglia are responsible for the innate immune response of the brain, called neuroinflammation. Neuroinflammation has, the main objective of containing the infection and eliminating pathogens, primarily playing a role in the resolution of these injuries and promoting the homeostasis of neuronal tissue. However, in neurological diseases, such as PD, neuroinflammation becomes persistent and harmful to neuronal cells. [32] When microglia are in a reactive state, cytotoxicity is mediated by releasing of cytokines that can potentiate the inflammatory response and thereby induce neurodegeneration. Therefore, there is an increase in the concentration of cytokines when microglia face an injury or infection in the brain parenchyma. [27]

One of the main inflammatory reactions associated with PD is the increased expression of the major histocompatibility complex (MHC). MHC plays an important role in the presentation of antigens, such as T cells, initiating the immune response. This molecule is divided into two classes, MHC class I that presents to CD8 cytotoxic T cells, and MHC class II that presents to CD4 T cells. Studies show that there is an increase in the number of HLA-DR (MHC-II) positive microglia in the SN and an increase in β2-microglobulin (MHC-I light chain) levels in the ST of PD. [33] In addition, in PD patients, there is an increase in the levels of pro-inflammatory cytokines, such as TNF-α, IL-1β and INF-γ, in the cerebrospinal fluid (CSF). In *post-mortem* studies of brains from patients an increase of these cytokines had also been found. Increased levels of these cytokines were also described in the CSF and in plasma of the 6-OHDA model. These cytokines promote the continuous activation of microglia, leading to a cycle of continuous activation and a chronic inflammatory response, which causes the progression of PD. [27], [33] These neuroinflammatory reactions ultimately induce the permeabilization of BBB and, therefore, the migration of peripheral inflammatory cells, which interact with microglia and trigger neurotoxic mechanisms. Once the density microglia is higher in the SN, this area becomes more susceptible to inflammation and, thus, to the progressive loss of DA neurons. [30], [34]

2. LPS

2.1. Role of LPS in Neuroinflammation

LPS is an endotoxin obtained from the outer membrane of gram-negative bacteria that can activate the immune system and is therefore used in experimental models to mimic bacterial infection and/or sepsis. LPS activates the Toll-like 4 receptors (TLR-4), which ultimately leads to the release of pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6. [35], [36]

Toll-like receptors (TLRs) are type I transmembrane proteins that are activated by a vast set of pathogen-associated molecular patterns (PAMPs), thus inducing an innate immune response. TLR-4 is a member of TLRs that recognizes and is activated by LPS. This receptor has a modular

structure composed, in the extracellular part, of a domain consisting of leucine-rich repeats (LRR), which are linked to the intracellular TIR domain, which allows the transmission of the signal. In fact, TLR-4 does not bind directly to the LPS and is it necessary the presence of an adaptor protein (MD-2), which binds directly to the lipophilic part of the LPS (called lipid A) and forms a complex thar is associated in a non-covalent way to the receptor and, later, the heterodimer formed is the one that initiates intracellular signaling. [37]

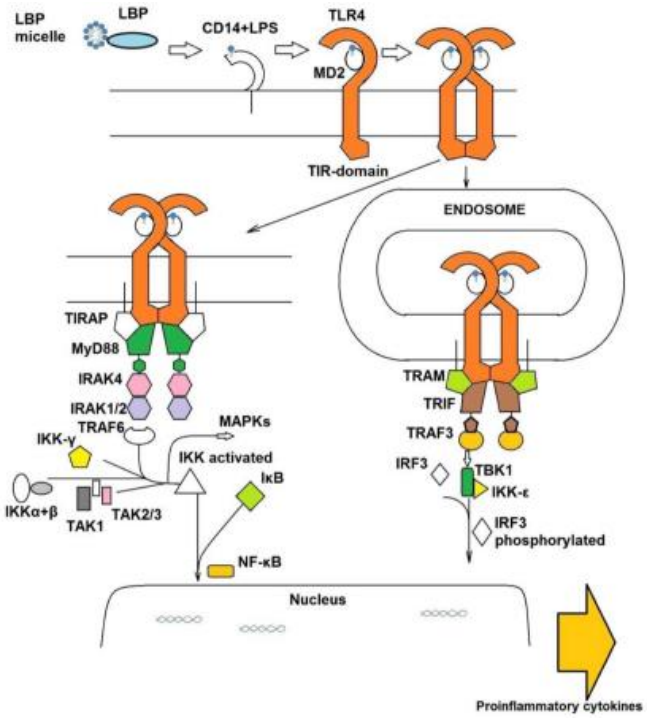

Figure 5 – Schematic representation of LPS/TLR-4 signaling pathway. The LPS-binding protein (LBP) binds a LPS monomer from LPS aggregates and transfers this molecule to cluster of differentiation 14 (CD14) protein, and then formation of the complex of LPS with MD-2/TLR4. After the formation of the heterodimer, the intracellular signal can follow two distinct directions: TLR4/MyD88/NF-kB or TLR4/TRIF/IRF3. MyD88 (myeloid differentiation primary response gene); TIRAP (TIR domain-containing adaptor protein); TRAM (TRIF-related adaptor molecule) and TRIF (TIR-domain-containing adapter-inducing interferon-β). Figure from [37].

Increased LPS levels induce systemic inflammation that activates immune system cells, including neutrophils, mast cells, monocytes, and macrophages. In the CNS, LPS also triggers microglia activation due to the activation of TLR-4. [38], [39] When microglial activation occurs, the release of pro-inflammatory cytokines is not a single consequence. In fact, the activation of TLR-4 by LPS induces the production of ROS and reactive nitrogen species. ROS production is the main responsible for the induction of phagocytosis. In addition, DA neurons are extremely sensitive to oxidative stress, since they have a lower concentration of glutathione compared to other cells, which implies that these mechanisms are associated with the loss of DA neurons. [40]

When administered in high doses, the inflammatory effect of LPS can lead to septic shock. Contrarily, when very low doses are administered, induces neuroprotection due to innate

immune memory. Although this immunological memory is not fully understood, it is assumed that it is due to the release of anti-inflammatory cytokines, especially IL-10. [41]

2.2. LPS: an experimental model of PD

LPS can be used as a model of PD as it mimics some of the pathologic and behavioral aspects of this disease. [42]

The *in vivo* models may be induced by the intracranial administration of the endotoxin in the ventricles (intracerebroventricular - ICV), striatum, pallium or peripherally by IP injection.

In 1998, Castaño and his colleagues first used stereotaxic surgery to administer LPS in the SN of rats. The results obtained showed that the LPS induced activation of microglia and, consequently, degeneration of dopaminergic neurons in SN and of their axonal projections into the ST. [43] Administration *via* ICV and intrapallidal also has the ability to induce microglial activation and degeneration of DA neurons. [44]

In 2007, Quin and his colleagues demonstrated for the first time that a single IP administration of LPS induces neuroinflammation and degeneration of DA neurons. [45] However, the presence and intensity of the disease depend on the dose administered and the number of injections. In male C57BL/6 mice, systemic administration of a single high-concentration dose (5 mg/Kg) induces a progressive degeneration of DA neurons about 7 months after treatment. Nevertheless, the same dose administered in female C57BL/6 mice did not induce dopaminergic neurodegeneration, probably due to the protective role of estrogen. [46] This process agrees with the epidemiology of this pathology, since it affects more men than women.

In systemic models of LPS administration, whether chronic or acute, the passage of LPS to the brain is limited by the BBB. In these conditions, LPS activates TLR-4 in peripheral macrophages, resulting in the release of pro-inflammatory cytokines and chemokines that are indispensable for inducing systemic inflammation and to induce neuroinflammation in the brain. [44], [45] Therefore, when LPS doses are low, BBB permeability does not change, and in this case, neuroinflammation appears indirectly. On the contrary, high doses of LPS increase the permeability of BBB and consequently allow the entry of the endotoxin into the brain. [38]

3. Histamine

3.1. Definition

Histamine is an endogenous biogenic amine being associated predominantly with peripheral allergies and inflammatory reactions. In the brain, histamine may act as neurotransmitter, neuromodulador and inflammatory agent. [47], [48] In a healthy brain, histamine is found at very low levels in the CSF and brain parenchyma. [49]

The histaminergic neuronal cell bodies are found in the tuberomammillary nucleus of the posterior hypothalamus, and the fibers are widely distributed throughout the brain, thus, being involved in various functions, such as the regulation of sleep/wakefulness, feeding and memory. Histamine does not cross the BBB; it is synthesized in the brain from the amino acid L-Histidine by the enzyme Histidine Decarboxylase (HDC). [50], [51] Histamine is metabolized very quicky and there are two enzymatic pathways that can lead to metabolism. By activating histamine *N-methyltransferase* – which is responsible for the most of the metabolization – and by diamine oxidase. [52] The production of this biogenic amine depends on the bioavailability of the precursor – histidine – which is not synthesized in the body and is obtained through the diet, having the ability to cross the BBB. [53]

In the CNS, histamine is produced in histaminergic neurons, mast cells, and microglia. In neurons, it is stored in synaptic vesicles that release histamine in the presence of an excitatory stimulus. In the PNS, this biogenic amine is produced and secreted by mast cells, basophils, neutrophils, platelets, monocytes, macrophages, and dendritic cells. [54]–[56]

Histamine performs its functions by activating four metabotropic receptors: two postsynaptic (H1R and H2R), one presynaptic (H3R), and H4R present mainly in the immune system. All receptors belong to the family of rhodopsin-like class A receptors coupled to G proteins. [57]

H1R is found in glial cells, blood vessels and sensory nerves, and its activation increase vascular permeability, and in the CNS induces an excitatory stimulation. This receptor is highly expressed in regions closely related to nutritional state, behavior, and neuroendocrine control, namely the hypothalamus, aminergic and cholinergic brainstem nuclei, thalamus, and cortex. [58] On the other hand, H2R it is located on the vascular bed, epithelium of the olfactory mucosa and submucosal glands. This receptor can also be found in several brain regions including the basal ganglia, amygdala, hippocampus, and cortex. The functions of this receptor are associated with cognitive performance, regulation of gastric acid, neuronal plasticity, and synaptic transmission. [58], [59]

H3R, located presynaptically in dendrites and axonal varicosities, has a fundamental role in modulating brain functions, mainly by inhibiting the formation of adenylyl cyclase and N- and P-type Ca^{2+} channels, just as inhibited the release of other neurotransmitters, like glutamate, acetylcholine and serotonin. Finally, H4R, is expressed mainly in immune cells (eosinophils, mast cells, basophils, neutrophils, and nerves in the nasal turbinate), therefore acting in peripheral inflammatory contexts. [59], [60]

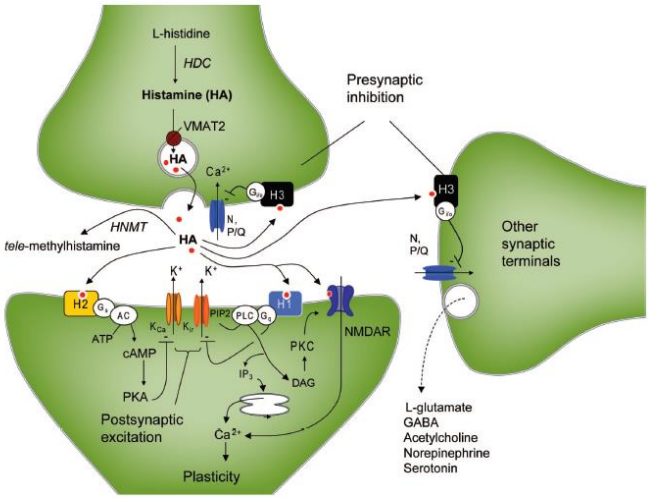

Figure 6 – Schematic representation of synthesis, metabolism and signaling pathways mediated by HRs. Histamine is synthesized from the amino acid L-Histidine by the enzyme Histidine Decarboxylase (HDC), stored in synaptic vesicles *via* the vesicular monoamine transporter-2 (VMAT-2), and release by excitatory stimuli, due the accumulation of Ca^{2+}. Histamine is release in the extracellular space and is metabolized by the histamine *N-methyltransferase* with production of tele-methlyhistamine. In the CNS, the effects of histamine are mediated by H1R, H2R (located both postsynaptically) and H3R (located presynaptically). Activation of H1R increases the intracellular Ca^{2+} and decreases K^+. H2R stimulates adenylyl cyclase (AC) and triggers cyclic adenosyl monophosphate (cAMP) – dependent activation of protein kinase A (PKA) which inhibits Ca^{2+} - activated K^+ channels (K_{Ca}). Finally, H3R inhibits AC and N- and P-type Ca^{2+} channels. Presynaptic H3 heteroreceptors inhibit the release of other neurotransmitters (glutamate, GABA, acetylcholine, norepinephrine, and serotonin). ATP – adenosine trisphosphate; DAG – diacylglycerol; IP3 – inositol triphosphate; PIP2 – phosphatidylinositol biphosphate; PKC – protein kinase C; PLC – phospholipase C. Figure from [56].

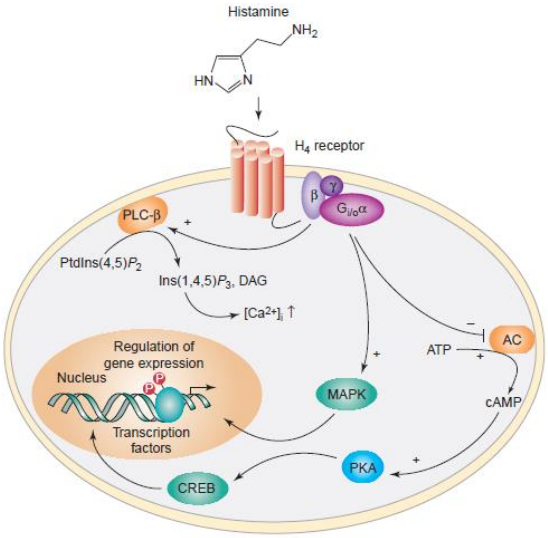

Figure 7 – Schematic representation of signaling pathways active by H4R. H4R interact with $G\alpha_{i/o}$, who have two subunits (α-subunit and βγ-subunit). The first, negatively regulates the activity of AC, which inhibits the formation of cAMP from ATP. The cAMP stimulates the activity of PKA, which leads to phosphorylation of cAMP responsive element-binding protein (CREB). The second subunit promotes the activation of phospholipase Cβ (PLC-β), which hydrolyzes the phospholipid phosphatidylinositol (4,5)-bisphosphate to the second messenger inositol (1,4,5)-triphosphate and DAG, which promotes the increase of intracellular Ca^{2+}. Finally, the activation of H4R can stimulate mitogen-activated protein kinase (MAPK) activity. Figure from [61].

Antihistamines are antagonists of histamine receptors, i.e., they competitively inhibit histamine binding to their receptors. H1R antagonists are used to treat allergic symptoms, which can be divided into first-generation antihistamines, with lipophilic properties, and consequently, able to cross the BBB leading to drowsiness; and the second generation, which are selective for peripheral H1R and cannot cross the BBB. [62], [63] Other example of the use of antagonist of histamine receptors is the use of H2-receptor antagonists to treat gastric ulcers. [58]

Mepyramine, also known as pyrilamine, is an example of a first-generation H1R antagonist. [63]

3.2. Role of Histamine in the Innate Immune Response

In a healthy brain, histamine levels are very low in the CSF and brain parenchyma. [49] However, when brain lesions or inflammatory conditions occur, circulating histamine levels increase, both in the blood and in the CSF, leading to the permeability of BBB. With this, histamine can modulate the function of several immune cells, allowing them to cross the BBB and act on the cerebral parenchyma. [64]

In the CNS, histamine and its receptors can influence the activity of glial cell, specifically of astrocytes and microglia. In the astrocytes, histamine influence the ion homeostasis, energy metabolism and neurotransmitter clearance. [65] In vitro studies, with rat primary astrocytes, found that astrocytes express H1R, H2R and H3R but not H4R. Furthermore, the activation of these receptors suppressed the production of TNF-α and IL-1β and stimulated the synthesis of

glial cell-derived neurotrophic factor (GDNF). This mechanism my evoke the neuroprotective effect of astrocytes. [66]

On the other hand, N9 microglia cell line express low levels of H1R, H2R and H3R messenger RNAs (mRNAs), but high levels of H4R mRNA. [67] In a physiological context, histamine appears to have a pro-inflammatory phenotype, with the ability to modulate the brain inflammatory response since it induces the release of pro-inflammatory cytokines by activating H1R and H4R; promote microglial phagocytosis by activation of H1R; through the Nox1 signaling pathway it can produce ROS and increases the mobility of microglia by activating H4R. These inflammatory response may trigger neuronal degeneration. [47], [59]

Still, the role of histamine in the CNS is not fully understood, since it can assume a dual role depending on the receptor, the activation state of cells and the neuronal microenvironment. [47] Microglia expresses the four histamine receptors, and, with this, histamine can stimulate microglia activation. However, in the face of peripheral inflammation caused by LPS, histamine responds differently through a decrease in microglia migration and decreased production of pro-inflammatory cytokines, e.g., IL-1β and TNF-α. [68].

3.3. Role of Histamine in PD

Histamine has an impact on several physiological functions. Therefore, dysfunction in its system contributes to the onset and/or progression of neurodegenerative diseases, including PD. [59]

As mentioned earlier, PD is characterized by the progressive loss of dopaminergic neurons in the SN. This brain region is particularly vulnerable to neurotoxicity induced by microglia and histamine. In PD patients, histamine levels are extremely high in SN, putamen and globus pallidus. This increase was also observed in animal models using the neurotoxin 6-OHDA. This increase occurs in areas of the brain associated with motor behavior that present functional changes in PD. [69], [70]

Any change in the histamine system induce changes in dopamine concentration. One of the main roles of the histaminergic system is the control of sleep/wakefulness cycles. Since histamine levels are increased in this pathology it is plausible to hypothesize a relationship between abnormal histamine levels and the appearance of the first non-motor symptoms of this pathology, namely sleep disorders and insomnia. [69], [71]

One of the pharmacological treatments that has been tested for the treatment of PD is the use of histamine receptor antagonist molecules. Diphenylpyraline hydrochloride is a first-generation antihistamine, such as mepyramine, which is used in the treatment of PD. This H1R antagonist has the ability to bind to the dopamine transporter, allowing an increase in the concentration of this neurotransmitter in the *nucleus accumbens*. [72], [73] The use of famotidine (H2R antagonist) reduced bradyphrenia and improves the motor behavior of PD patients. On the other hand, the use of H3R antagonist improves motor coordination in neonatal rats treated with 6-

OHDA. The H3R agonist (imetit) attenuated 3,4-dihydroxyphenylalanine (L-DOPA)-induced increases in striatal extracellular dopamine and dyskinesia. [69], [70] With regard to the H4 receptor, in the animal model for rotenone-induced PD, an H4R antagonist injected directly into the ventricle resulted in the blockade of microglial activation with consequent decrease in the apomorphine-induced rotational behavior, which translates into the neuroprotection of DA neurons. [74]

4. Innate Immune Memory

4.1. Definition

The Immune System is composed of the innate immune system and the adaptative immune system. Innate immunity is induced by a wide variety of myeloid cells and is our body's first line of defense against foreign pathogens. It is a rapid mechanism (no longer than minutes) and is a non-specific defense and therefore is not always enough to fight invading microorganisms. [75] When the innate system is not sufficient to eliminate the pathogens occurs the activation of the adaptative immunity. The adaptative immunity is mediated essentially by B and T lymphocytes [76] which induce a specific recognition and elimination of the pathogen. There is a strong relationship between these two types of immunity, since the activation of the adaptative immunity depends on the antigen-presenting cells, from the innate immune system, such as dendritic cells. [77] In the CNS, microglia are the main innate immune cell population that has the ability to respond to peripheral inflammations. [78]

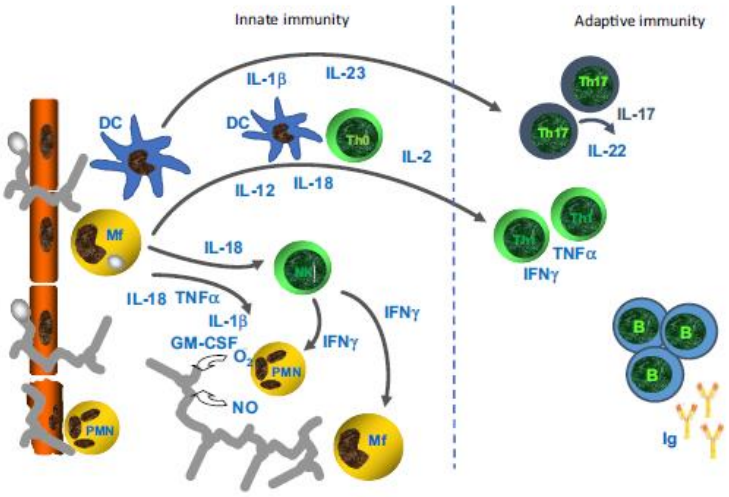

Figure 8 – Schematic representation of the Innate and Adaptative Immune responses. During the first hours of an infection or the invasion by pathogenic agents occurs the activation of the innate immune system which is mediated by macrophages, monocytes, and NK-cells. The infection agent is ingested and processed by antigen-presenting cells, who present the antigen and stimulate the T- and B-lymphocytes, leading to a clonal expansion and activation of effector mechanisms, thus involving the adaptative immune system. Figure from [77].

Immune memory is defined as the "ability of the immune system to store information of a primary infection and to recall the infection for an earlier, sometimes faster, and often more robust response against subsequent exposure to the same or an unrelated pathogen". [79] In the adaptative immune system, this immune memory is well known and presents itself as specific and durable protection against reinfection. Over the past few years, it has reported that innate immune cells, such as myeloid and microglia, also have the ability to generate immune memory. In fact, stimulus of a foreign agent induces an extensive reprogramming of these cells, leading them to present a faster and more intense response to reinfection and protect the tissue. [80]

There are two types of immune memory. Training, which improves the immune system's ability to respond to reinfection, associated with metabolic, epigenetic, and transcriptional reprogramming of these cells; and tolerance that is associated with suppression of immune responses in the face of reinfection. [78]

4.2. The Influence of Innate Immune Memory in Brain Diseases

Many neurological and neurodegenerative diseases are due, in part, to changes in innate immunity. However, the way these changes contribute to neurodegeneration is not fully understood. [78]

Wendel et al.,2018 [81] demonstrated that the application of peripheral inflammatory stimuli, such as LPS, can induce either acute immune training or immune tolerance, depending on the exposure paradigm. This research aimed to investigate whether immune training or tolerance of microglia could influence the progression of Alzheimer's Disease (AD) and Stroke. Using mouse models, they found that a single dose of LPS could induce microglia training, and the use of multiple LPS injections (in this case, four doses) induced a tolerant response. After six months, they collected the animal's brains and found that the immune training allowed the increase in the accumulation of β-amyloid (protein responsible for the appearance of AD). Contrarily, immune tolerance decreased the accumulation of this protein.

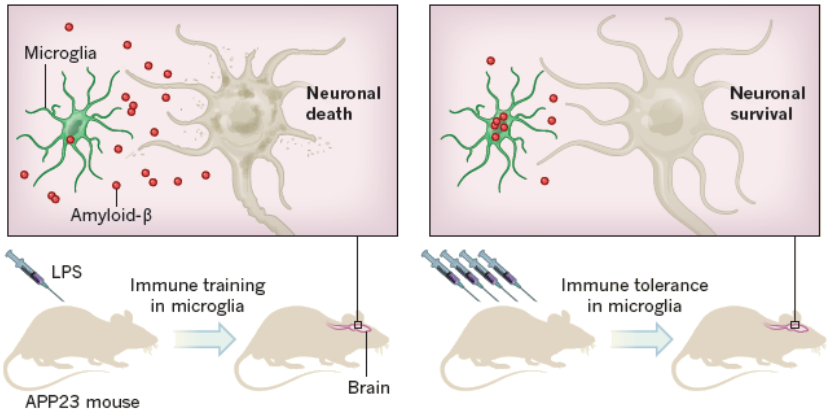

Figure 9 – Schematic representation of the innate immune memory in a model of AD. The innate immunity provides an immediate and nonspecific defense against invading pathogens. The main innate immune cells in the brain – microglia – provides innate immune memory. Wendeln et al., 2018, investigated innate immune memory in a mouse model of AD. The animals were injected with once or four times with LPS. A single dose of LPS induce microglia training, which exaggerate immune response, leading to neuronal death. Multiple LPS injections induce tolerance, which dampens the immune response, leading to an increased in microglia amyloid-β uptake and better neuronal survival. Figure from [78].

In the case of the stroke model, in Wendel et al.,2018 [81] mice were injected with 1xLPS or 4xLPS and focal brain ischemia was induced 1 month later. The results show that the animals subjected to 1xLPS present increased levels of IL-1β (pro-inflammatory cytokine), while the animals that were injected for four consecutive days (4xLPS) present a decrease in the concentration of this cytokine. Seven days after the insult, the neuronal damage and microglial activation was reduced in animals treated with 4xLPS. Nevertheless, the group 1xLPS was not affected. Thus, these results demonstrate that there is a long-term brain modulation of brain immune responses indicating that immune tolerance allow a reduction in neuronal death in this stroke model. [78] In other article, [82], in which the purpose of the study was to investigate the influence of innate immunity trained in stroke, they found that pre stimulation with LPS before the ischemic insult causes the appearance of immune memory.

In PD, a neurodegenerative disease associated with neuroinflammation mechanisms, the presence of innate immune memory, i.e., immune memory of microglia, is not described. However, since it is a disease with a strong inflammatory background, it is expected that immune memory may also play a role.

Chapter 2

Objectives

PD is a neurodegenerative disease associated with neuroinflammation processes. LPS is an endotoxin that has the ability to modulate the immune system, inducing inflammation and therefore can be used to mimic inflammation processes in experimental models, being widely used as a model of PD. On the contrary, in animal models of AD and stroke, the IP administration of LPS (low doses and short exposure paradigm) induces immunological memory and, consequently, neuroprotection.

The first objective of this project was to understand the role of the inflammatory response mediated by LPS in PD, since it can be used a model for this pathology or a trigger neuroprotection.

One the other hand, the levels of histamine are increased in PD patients. However, it appears to have a dual role in the CNS and may assume either a cytotoxic or anti-inflammatory role.

For this, the 6-OHDA model of PD was used with the objectives of:

- Evaluate the effect of peripheral stimulation with LPS or histamine on the peripheral release of cytokines (TNF-α, IL-1β and IL-10).
- Evaluate the impact that innate immune memory induced by peripheral LPS or histamine on dopaminergic survival and functional motor behavior.

Chapter 3

Materials and Methods

1. Animals

In this project, we used wild-type C57BL/6 male mice (three to five months-old). Mice were accommodated in the same room and similar cages under monitored conditions – 12h light/dark cycle at a temperature of 22ºC with free access to food and water.

All animal experiments were conducted in accordance with protocols approved by the Direção-Geral de Alimentação e Veterinária (DGAV) and Órgão de Bem-Estar e Ética Animal (ORBEA) from the Health Sciences Research Centre (CICS-UBI). All efforts were made to minimize the number of animals used in this study and potential suffering, according to the 3R's principles.

2. Intraperitoneal and Stereotaxic Injections

2.1. Intraperitoneal Injections

Mice were initially injected intraperitoneally for four consecutive days, with: LPS (from *Escherichia coli* L2880, Sigma-Aldrich), at 500 μg/Kg and 300 μg/Kg; Histamine (H7250, Sigma-Aldrich), at 10 μg/Kg and 50 μg/Kg; Histidine (H8125-0025, Sigma-Aldrich), at 25 mg/Kg and 50 mg/Kg; Mepyramine (T0-0660, Tocris Biotechnebican) at 10 mg/Kg. These compounds were diluted in 0.1 M of sterile phosphate buffered-saline (PBS; NaCl 140mM, KCl 2.7mM, KH_2PO_4 1.5mM and Na_2HPO_4 8.1 mM, pH=7.4). Mice injected intraperitoneally with 0.1 M of sterile PBS were considered the control group. (Figure 11)

2.2. Stereotaxic Injections

Three weeks after the IP injections, the mice were anesthetized with an IP injection of a mixture of ketamine and xylazine (90 mg/Kg and 10 mg/Kg of mouse weight, respectively), before proceeding to stereotaxic injection of 6-OHDA (Sigma-Aldrich, dissolved in sterile 0.02% ascorbic acid) (Figure 11). Then, mice were positioned in the digital stereotaxic frame (51900 Stoelting, Dublin, Ireland), and their scalp disinfected with Betadine® and a local anesthetic Lidocaine. An incision was made with a scalpel along the midline to expose the skull and define the coordinates after setting the zero at bregma point. A stereotaxic injection, using a Hamilton syringe, of 7 μg of 6-OHDA at a rate 0.2 μL/min, was performed in the right ST (coordinates: anteroposterior (AP), -0.6 mm; dorsolateral (DL), -2.0 mm; dorsoventral (DV), -3.0 mm relative

to bregma). The syringe was kept in place for additional 5 minutes before slowly retracting. The incision was sutured, and mice were kept warm (37ºC) until they recovered.

3. Apomorphine – Induced Rotation Test

Two weeks after stereotaxic injections, the animals were subjected to the Apomorphine – Induced Rotation Test (Figure 11). This test consists of administering a subcutaneous injection of apomorphine (0.5 mg/Kg). Apomorphine is a dopamine-like compound that is administered to animals with unilateral injury, having the ability to induce contralateral rotation, i.e., in the opposite direction to that of the lesion, due to the sub under activation of dopamine receptors.

Immediately after the injection, the animals were put into cylindric individual cages. The number of rotations in the direction opposite to the lesion (contralateral rotations) were measured for 45 minutes.

4. Brain Slices Preparation

After the Apomorphine – Induced Rotation Test, the mice were anesthetized with an IP injection of a mixture of ketamine and xylazine (90 mg/Kg and 10 mg/Kg of mouse weight, respectively). An incision through the thoracic midline was made, and the heart was exposed. A needle was introduced in the left ventricle and the right aorta was cut. The animals were rapidly transcardially perfused using sterile PBS, followed by perfusion with 4% Paraformaldehyde (PFA). Then, the brains were removed and fixed in 4% PFA overnight at 4ºC, followed by immersion in a 30% sucrose solution at 4ºC. Then, the brains were frozen and stored at -80ºC until used.

For slide preparation, the previously mentioned brains were embedded in optimal cutting temperature gel (Bio-Optica) and cut into coronal sections with a thickness of 40 µm on a freezing cryostat-microtome (Leica CM 3050S, Leica Microsystems). The sections corresponding to the ST and SN of each animal were collected sequentially in six wells of a 24-well plate and were kept in an anti-freeze solution (30% of ethylene glycol, 30% glycerol, 30% water and 10% phosphate buffer solution) until they were used for immunohistochemistry.

5. Immunohistochemistry for Tyrosine Hydroxylase (TH)

Brain slides of ST and SN sections were rinsed several times with PBS and once, for 10 minutes, with PBS – Tween 20 0.1% (PBS-T). Next, the brain slides were incubated with permeabilization/blocking solutions (10% FBS serum and 0.1% Triton X-100 in PBS) to prevent unspecific bindings at RT for 1 hour. Then, the slides were rinsed two times, for 10 minutes, with PBS-T. To inhibit endogenous peroxidase activity, the brain sections were incubated with 3% hydrogen peroxide (H_2O_2) in water for 10 minutes at RT. After washing with PBS-T, brain slides were further incubated in the primary antibody – Mouse anti-TH (1:2500) – diluted in 5% FBS serum in PBS, overnight at 4ºC.

After primary antibody incubation, the slides were rinsed three times, for 10 minutes, with PBS-T. Next, they were incubated whit biotinylated secondary antibody – anti-mouse (1:200) – diluted in 1% FBS serum in PBS for 1 hour at RT. After washing with PBS-T, brain slides were incubated with Avidin/Biotin (AB) solutions (1:500 in PBS) for 30 minutes at RT. This step was followed by three washes with PBS-T and one with PBS. Then, the sections were incubated with DAB solution until color developed (approximately 10 minutes). After the DAB reaction was complete, the sections were washed again with PBS-T. Finally, the sections were mounted on microscope slides, dehydrated through a graded ethanol series, cleared using xylene, and coverslipped with Entellan Mounting Medium.

6. Enzyme – Linked Immunosorbent Assay (ELISA)

To evaluate the effects of LPS and histamine in the release of pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10), mice received one or four consecutive IP injections of LPS (300 μg/Kg or 2 mg/Kg) or histamine (10 μg/Kg), and 3 hours after the last injection, mice were anesthetized. An incision through the thoracic midline was made, and the heart was exposed. A needle was introduced in the left ventricle, and then the blood was collected with EDTA Blood Collection Tubes (S-Monovette ® - Sarstedt). The samples were centrifuged for 20 minutes at 2988 rpm at RT, and the plasma was stored at -20ºC. The levels of cytokines were evaluated with ELISA Kits according to the manufacturer's instructions and the quantification were read at 450 nm. (Figure 10)

In the Mouse TNF ELISA Kit (BD OptEIA™), a monoclonal antibody specific for mouse TNF-α coated on a 96-well plate was used. Standards and samples were added to the wells. After washing, the biotinylated polyclonal anti-mouse TNF-α antibody is added, producing an antibody-antigen-antibody sandwich. This step was followed by a wash step and streptavidin-horseradish peroxidase conjugate was added. The TMB substrate solution was added which produce a blue color in direct proportion to the amount of TNF-α present in the initial sample. Finally, the stop solution is added, which change the color from blue to yellow. The experimental procedure for Mouse IL-10 ELISA Kit (Sigma-Aldrich Millipore®) was identical.

In the Mouse IL-1β uncoated ELISA (ThermoFisher Scientific), the experimental procedure is similar to the above mentioned. However, before starting the procedure the 96-well plate was first coated with the capture antibody, overnight at 4ºC, and blocked with the ELISA Diluent reagent before adding the samples and the standard.

7. Western Blot

7.1. Preparation of brain tissue extracts

To evaluate the effects of LPS and histamine in neuroinflammation and microglia activation, mice was challenged with one or four consecutive IP injections of LPS (300 μg/Kg or 2mg/Kg) or

histamine (10 μg/Kg) and euthanized 3 hours or 24 hours after the last injection. Brains were removed, frozen in liquid nitrogen, and stored at -80ºC. For Western Blotting, one of the hemispheres was mechanically dissociated, lysed on ice in RIPA Buffer (50 mM Tris-Base, pH=8.0; 150 mM NaCl; 1% Triton X-100; 0.5% Sodium Deoxycholate; 0.1% SDS), containing a cocktail of protease inhibitors (Sigma-Aldrich P8340) and sonicated for 30 seconds. Next, a centrifugation was made at 14000 rpm, for 20 minutes, at 4ºC, to obtain the protein´s soluble fraction, the supernatant. The total protein concentration was determined using the Pierce Bicinchoninic Acid Protein Assay Kit (BCA; Thermo Scientific).

7.2. Immunoblot Assay

A total of 40 μg of protein was loaded into the 12.5% SDS polyacrylamide gels and were separated using a running buffer solution (25 mM Tris, 190 mM glycine, 0.1% SDS; pH=8.3) by SDS-PAGE electrophoresis at 120V, 90 minutes, at RT. After the electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes through a semidry transfer in the following conditions: 100 V, 1 hour, on ice using transfer buffer (25 mM Tris, 190 mM glycine, 20% Methanol; pH=8.3). Then, membranes were blocked in Tris Buffer Saline (TBS) containing 0.1% Tween 20 and 0.05% gelatin for 1 hour at RT. Membranes were incubated with primary antibody – Mouse anti-IBA 1 (1:200) – overnight at 4ºC. After washing three times with distilled water, they were further incubated with their respective secondary antibody – Goat anti-Mouse (1:5000) – for 90 minutes at RT. To normalize the expression of the target protein, they were incubated with housekeeping antibody – Mouse anti-Actin (1:5000) – for 90 minutes and the corresponding secondary antibody – Goat anti-Mouse (1:5000) – for another 90 minutes, both at RT. Finally, the membranes were incubated, for 5 minutes, with Pierce™ ECL Western Blotting Substrate and protein levels were detected by ChemiDocTM MP and quantified using the software *ImageLab* (Bio-Rad Laboratories).

8. Data Analysis

All experimental conditions were performed at least with three different animals. Statistical analysis was made using the GraphPad Prism 8.0 Software, using one-way ANOVA followed by the Dunnett's multiple comparisons test. Values of $P<0.05$ were considered significant and data are shown as the mean ± standard error of the mean (SEM).

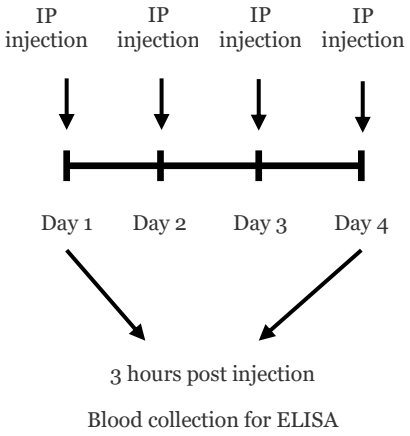

Figure 10 – Timeline for ELISA experiments. The mice received one or four consecutive IP injections and 3 hours after the last injection, the blood was collected to perform the ELISA.

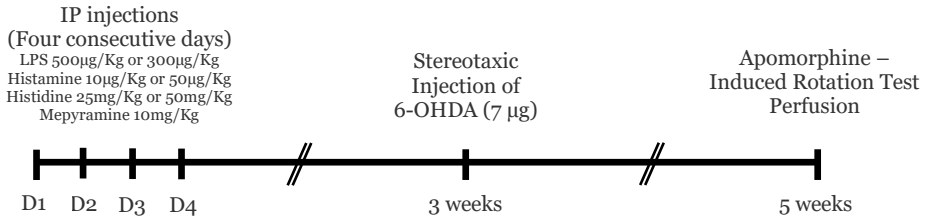

Figure 11 – Experimental paradigm to analyze the impact of innate immune memory induced by LPS or histamine in a 6-OHDA animal model of PD.

Chapter 4

Results

1. Effects of LPS and histamine in the release of peripheral pro- and anti-inflammatory cytokines

Innate immune memory is described to mediate a decrease in the concentration of pro-inflammatory cytokines, like TNF-α and IL-1β, and an increase in anti-inflammatory cytokines, namely IL-10 when innate immune memory cells are challenged with a second stimulus. To evaluate the effects of LPS and histamine on the release of pro- and anti-inflammatory cytokines we used the ELISA technique. The animals received one or four consecutive IP injections of LPS 300 μg/Kg or histidine 10 μg/Kg, and blood was collected three hours after the last injection. The control group received IP injections of PBS and, to verify the functionality of the kits, a positive control that received an injection of 2 mg/Kg of LPS was also included. [47]

First, we found that the release of the cytokines TNF-α, IL-1β and IL-10 is residual in the control groups treated 1x or 4x with PBS. The concentration of TNF-α increased significantly after an injection with LPS 2 mg/Kg and LPS 300 μg/Kg. After the fourth IP injection the concentration decreases dramatically. (Figure 12A, $mean_{CTR\ 1x}$= 100.2 ± 12.7, n=3; $mean_{CTR\ 4x}$= 48.7 ± 7.5, n=3; $mean_{LPS\ 2mg/kg\ 1x}$= 1954.0 ± 110.5, n=4; $mean_{LPS\ 300\ 1x}$= 1353.0 ± 439.0, n=3; $mean_{LPS\ 300\ 4x}$= 116.1 ± 20.2 n=3). IL-1β, is a pro-inflammatory cytokine showed a significant increase in the group treated with LPS 2 mg/Kg 1x compared with the control. Although not significant, there is an increase in IL-1β concentration in the LPS 300 μg/Kg 1x treated group. In contrast to TNF-α, the IL-1β concentration did not decreased at the end of the four with LPS 300 μg/Kg (4x). (Figure 12C, $mean_{CTR\ 1x}$= 12.3 ± 1.2, n=2; $mean_{CTR\ 4x}$= 14.5 ± 3.4, n=3; $mean_{LPS\ 2mg/kg\ 1x}$= 102.7 ± 10.6, n=4; $mean_{LPS\ 300\ 1x}$= 72.8 ± 0.7, n=2; $mean_{LPS\ 300\ 4x}$= 72.4 ± 17.0, n=3). Finally, regarding IL-10 cytokine, an anti-inflammatory cytokine associated with the immune memory process, we found that animals treated with LPS 300 μg/Kg 1x or LPS 300 μg/Kg 4x showed a significant increase in IL-10 concentration compared with the respective controls. However, there is no significant difference in the IL-10 levels between the LPS 300 μg/Kg 1x and LPS 300 μg/Kg 4x treated animals. (Figure 12E, $mean_{CTR\ 1x}$= 91.1 ± 9.9, n=3; $mean_{CTR\ 4x}$= 70.6 ± 4.0, n=3; $mean_{LPS\ 300\ 1x}$= 162.2 ± 5.5, n=3; $mean_{LPS\ 300\ 4x}$= 105.0 ± 21.1, n=3).

The levels of the same cytokines were also evaluated in animals treated with histamine (10 μg/Kg and 50 μg/Kg) 1x and 4x. No significant differences were found in the levels of TNF-α when animals were challenged for 1x or 4x with histamine as compared with the respective control. (Figure 12B, $mean_{CTR\ 1x}$= 100.2 ± 12.7, n=3; $mean_{CTR\ 4x}$= 48.7 ± 7.5, n=3; $mean_{Hista\ 1x}$= 138.9 ± 62.5,

n=3; $mean_{Hista\ 4x}$= 44.3 ± 8.8, n=3). In contrast, animals exposed with 1x and 4x with histamine showed a significant increase in IL-1β levels as compared to their respective controls. Still, at the end of the fourth injection there is a small reduction in IL-1β concentration as compared with the single exposure. (Figure 12D, $mean_{CTR\ 1x}$= 12.3 ± 1.2, n=2; $mean_{CTR\ 4x}$= 14.5 ± 3.4, n=3; $mean_{Hista\ 1x}$= 42.9 ± 5.1, n=3; $mean_{Hista\ 4x}$= 36.9 ± 2.0, n=3; $p<0.05$). No differences were found in the levels of IL-10 between all experimental groups. (Figure 12F, $mean_{CTR\ 1x}$= 91.1 ± 9.9, n=3; $mean_{CTR\ 4x}$= 70.6 ± 4.0, n=3; $mean_{Hista\ 1x}$= 79.4 ± 7.0, n=3; $mean_{Hista\ 4x}$= 70.6 ± 1.1, n=3).

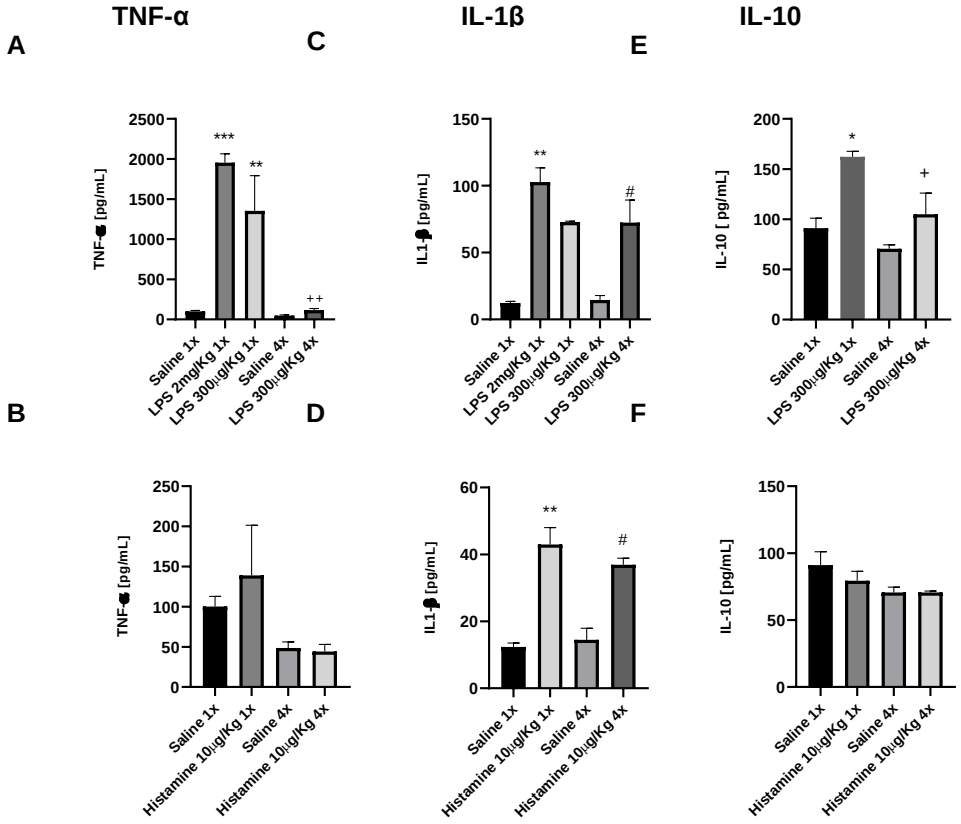

Figure 12 – The effects of LPS and histamine in the release of peripheral pro-inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10). Mice were injected IP with LPS or histamine one (1x) or four (4x) consecutive days. Three hours after the last injection the blood was collected. The control group received PBS. ELISA was used to analyze the levels of the levels of cytokines in the blood serum. Bar graph depicts the concentration [pg/mL] of TNF-α (A and B), IL-1β (C and D), IL-10 (E and F). Data are shown as the mean ± SEM. Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. *p<0.05, **<p0.05 and ***p<0.05 when compared with control 1x, ++p<0.05 when compared with LPS 300μg/Kg 1x and #p<0.05 when compared with control 4x.

2. Effects of LPS and histamine in microglia activation

Microglia is a pivotal trigger inflammatory responses in the CNS and, in the face of an insult, undergoes several epigenic modifications, become reactive and release pro-inflammatory cytokines, as well as produce ROS.

The second task of this study was to evaluate the effects of LPS and histamine on microglial activation by analyzing the expression of Iba-1 by Western Blot. The animals received an IP injection of LPS 300 µg/Kg or histamine 10 µg/Kg, and the brains were collected 3 or 24 hours after the last injection. The control group received IP injection of PBS.

First, we found that LPS at both concentrations (2 mg/Kg and 300 µg/Kg) did not change Iba-1 expression levels at 3 hours post injection as compared with the control. On the contrary, histamine induced an increase in the relative levels of this protein, although not reaching statistical significance. (Figure 13A, $mean_{CTR}$= 100.0 ± 12.0, n=2; $mean_{LPS\ 2mg/kg}$= 69.1 ± 24.8, n=2; $mean_{LPS300}$= 79.8 ± 12.0, n=2; $mean_{Hista\ 10}$= 183.5 ± 63.1, n=2). At 24 hours after IP injections, both LPS and histamine induced an increase of Iba-1 expression. (Figure 13B, $mean_{CTR}$= 100.0 ± 1.6, n=2; $mean_{LPS\ 300}$= 200.7 ± 18.8, n=2; $mean_{Hista\ 10}$= 257.4 ± 70.0, n=2).

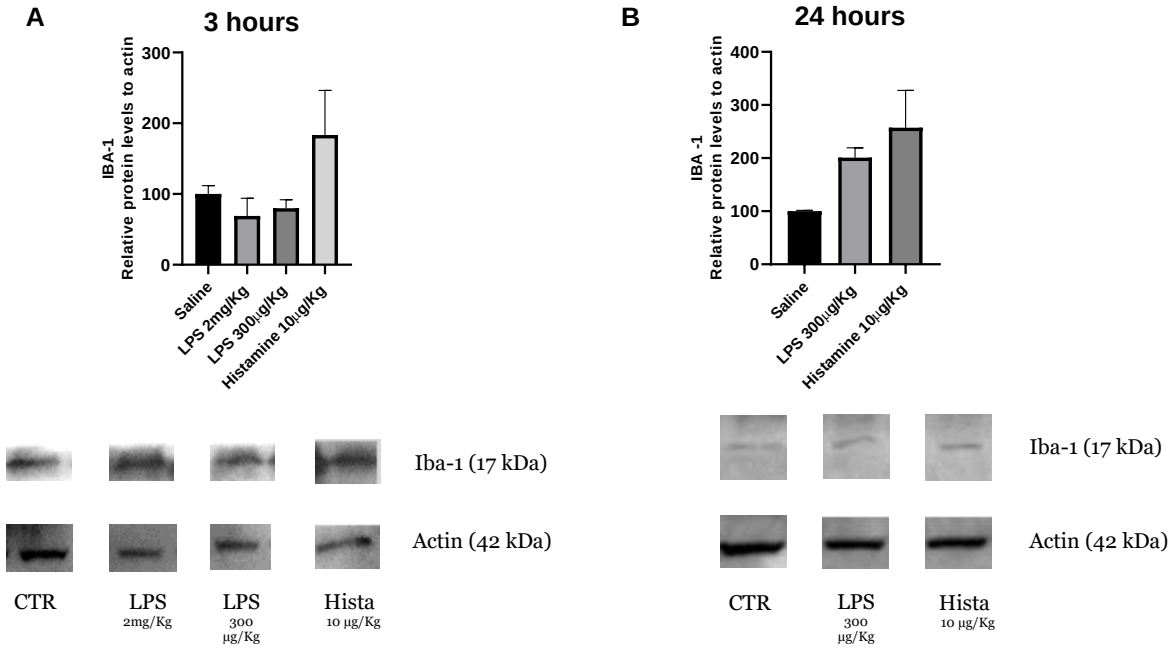

Figure 13 – The effects of LPS and histamine on the expression of the microglial marker Iba-1 in the whole brain. Mice were injected IP with LPS or histamine once. Three and twenty-four hours after the IP the brains were removed. The control group received PBS. To analyze microglia activation, we performed a Western Blot (WB) for Iba-1. Bar graphs depicts the relative protein levels of Iba-1 3 hours (A) or 24 hours (B) after the injections. Bellow the graphs, representative western blots for 17 kDa Iba-1 and 42 kDa actin are shown. Data are shown as the mean ± SEM. Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. No significant difference was obtained.

3. The effects of LPS and histamine peripheral preconditioning in the motor recovery of 6-OHDA lesioned animals

Next, we assessed the impact of peripheral stimulation with LPS or histamine in a mouse model of PD, the 6-OHDA lesioned animals. For that, mice were submitted to IP injections for four

consecutive days of the various compounds, detailed in the Figure 11 (Materials and Methods). Subsequently, the animals underwent stereotaxic surgery for the injection of the 6-OHDA in the *striatum*. The control group received an injection of 0.02% ascorbic acid. Two weeks after the surgeries, the animals were submitted to the Apomorphine – Induced Rotation Test. In this test, the number of contralateral rotations, that is, the number of rotations for the uninjured side, was evaluated for 45 minutes, and the larger the injury, the greater the number of rotations.

As expected, animals injured with 6-OHDA showed a significant increase in the number of contralateral rotations compared with the saline animals. (Figure 14A, $mean_{CTR}$= 0.7 ± 1.3, n=6; $mean_{6\text{-}OHDA}$= 107.7 ± 23.6, n=6; $p<0.05$). LPS, at both concentrations, significantly reduced the number of contralateral rotations as compared with the 6-OHDA group, suggesting a neuroprotective effect on dopaminergic neurons. (Figure 14A, $mean_{LPS\ 500}$= 30.8 ± 19.2, n=6 and $mean_{LPS\ 300}$= 10.3 ± 6.2, n=3; $p<0.05$).

Animals treated with histamine at both concentrations (10 μg/Kg and 50 μg/Kg) showed a significant reduction in the number of contralateral rotations as compared with the 6-OHDA group, suggesting a neuroprotective mechanism in dopaminergic neurons. In contrast, peripheral stimulation with histidine, a histamine precursor able to cross the BBB, induced a significant increase in the number of contralateral rotations (in both concentrations, 25 mg/Kg and 50 mg/Kg) compared to the saline group. The highest number of contralateral rotations was found in animals challenged with mepyramine, an antihistamine that inhibits the H1 receptor of histamine. (Figure 14B, $mean_{Hista\ 50}$= 22.2 ± 11.9, n=5; $mean_{Hista\ 10}$= 15.5 ± 5.7, n=6; $mean_{Histidine\ 50}$= 130.2 ± 18.7, n=5; $mean_{Histidine\ 25}$= 59.5 ± 24.3, n=4; $mean_{Mepyramide}$= 205.4 ± 43.6, n=7; $p<0.05$).

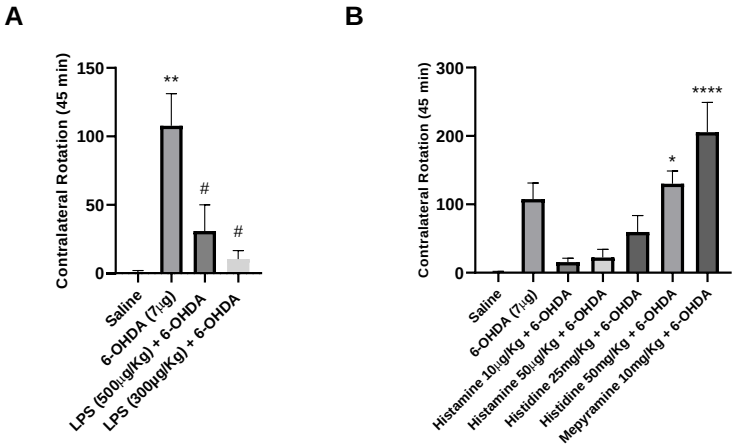

Figure 14 – The effects of LPS and histamine modulators preconditioning in the motor behavior of 6-OHDA lesioned animals. Mice were initially injected IP with LPS, histamine, histidine, mepyramine for four consecutive days. Three weeks after received a stereotaxic injection of 6-OHDA. Two weeks after recovery the animals were subjected to the Apomorphine – Induced Rotation Test. The control group received PBS and 0.02% Ascorbic Acid. (A) and (B) Bar graph depicts the number of contralateral rotations for 45 minutes, for LPS and histamine modulators, respectively. Data are shown as the mean ± SEM. Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. *p<0.05, **p<0.05 and ***p<0.05 when compared with control condition and #p<0.05 when compared with 6-OHDA group.

4. LPS precondition counteract dopaminergic degeneration induced by 6-OHDA

We then evaluated dopaminergic survival using the immunohistochemistry for TH, which stains dopaminergic neurons in the SN and its axonal fibers in the ST.

As expected, 6-OHDA induced a decrease in the number, area, and TH fibers of dopaminergic neurons in the SN and ST, respectively. (Figure 15B, $mean_{6\text{-}OHDA}$= 49.8 ± 5.3, n=3; Figure 15C, $mean_{6\text{-}OHDA}$= 73.9 ± 0.8, n=3; Figure 15D, $mean_{6\text{-}OHDA}$= 69.5 ± 1.0, n=3).

LPS, at both concentrations, increased the staining and area occupied by TH fibers in the ST and the number of TH neurons in the SN as compared with 6-OHDA lesioned animals. (Figure 15B, $mean_{LPS500}$= 118.5 ± 16.6, n=5; $mean_{LPS300}$= 74.2 ± 17.1, n=3; Figure 15C, $mean_{LPS500}$= 89.6 ± 4.2, n=5; $mean_{LPS300}$= 88.1 ± 2.5, n=3; Figure 15D, $mean_{LPS500}$= 92.4 ± 11.4, n=3; $mean_{LPS300}$= 80.6 ± 4.6, n=3).

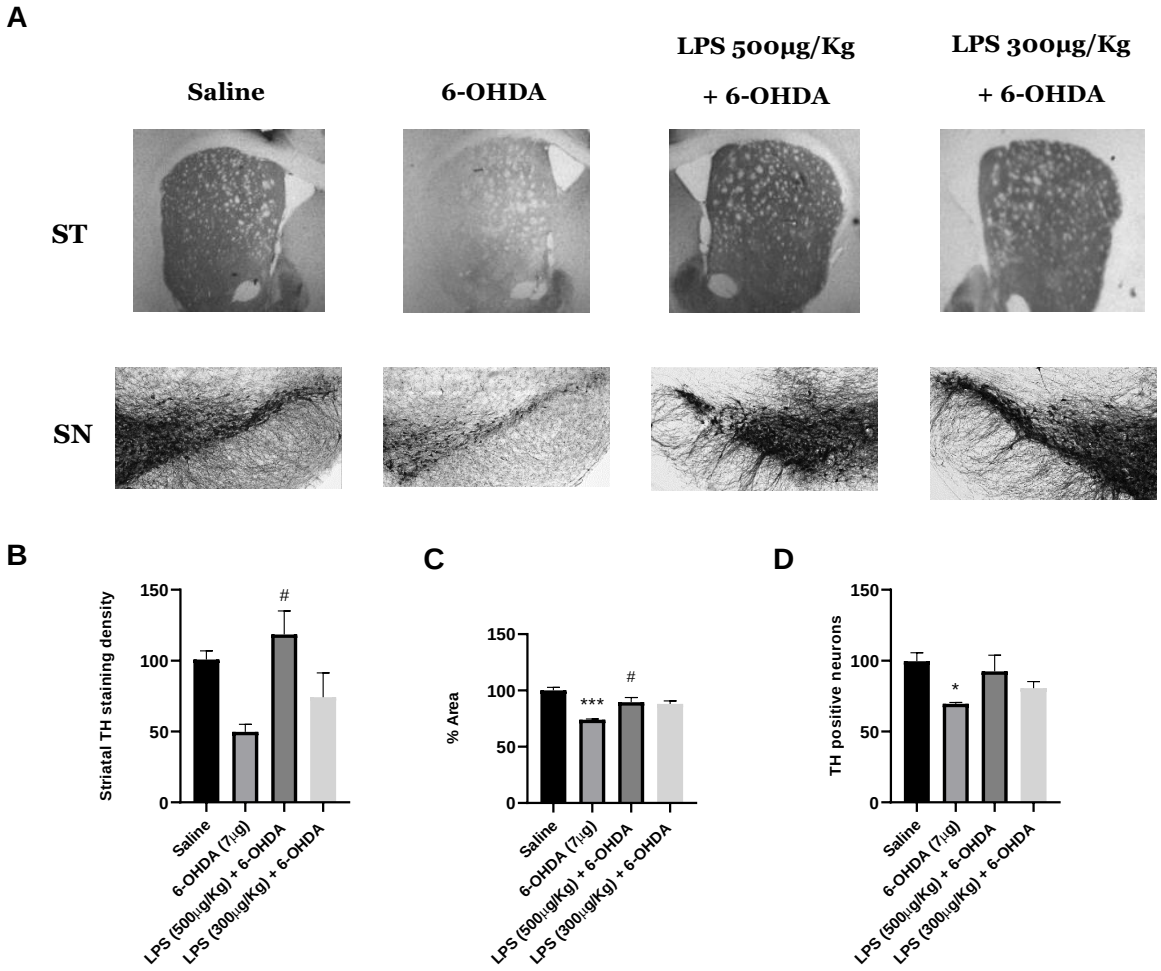

Figure 15 – LPS precondition counteract dopaminergic degeneration induced by 6-OHDA. Immunohistochemistry for TH in the ST and SN, two weeks after the stereotaxic injection of 6-OHDA. (A) Representative photomicrographs of SN and striatal sections immunostained for TH. Bar graphs depict TH staining density (B) and % area (C) of TH fibers in the *striatum*. (D) Bar graph depicts the number of TH positive neurons in the SN. The control group was set to 100%. Data are shown as the mean ± SEM. Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. *p<0.05, ***p<0.05 when compared with control condition and #p<0.05 when compared with 6-OHDA group.

5. Histamine precondition counteract dopaminergic degeneration induced by 6-OHDA

The last task was to understand whether histamine, histidine, and mepyramine peripheral priming can influence dopaminergic survival. The analysis was performed in the same way as described in the previous section.

In accordance with the motor recovery findings, we found that histamine, at both concentrations (10 µg/Kg and 50 µg/Kg) increased the intensity TH fibers in the ST. A less robust effect was found regarding the area occupied by TH fibers in the ST and the number of TH neurons in the SN, particularly when using the lowest dose of histamine. (Figure 16B, $mean_{Hista50}$= 63.8 ± 20.0, n=4; $mean_{Hista10}$= 92.7 ± 22.3, n=6; Figure 16C, $mean_{Hista50}$= 60.5 ± 18.2, n=4; $mean_{Hista10}$= 84.8 ± 7.1, n=6; Figure 16D, $mean_{Hista50}$= 76.4 ± 24.3, n=3; $mean_{Hista10}$= 76.3 ± 11.8, n=5).

Overall, animals treated with histidine (both concentrations) and mepyramine showed a decrease in the number, area, and TH fibers of dopaminergic neurons in SN and ST, respectively. (Figure 16B, $mean_{Histidine50}$= 31.0 ± 6.6, n=4; $mean_{Histidine25}$= 30.9 ± 1.7, n=3; $mean_{Mepyramide10}$= 49.5 ± 8.4, n=4; Figure 16C, $mean_{Histidine50}$= 60.2 ± 4.3, n=4; $mean_{Histidine25}$= 57.0 ± 0.4, n=3; $mean_{Mepyramide10}$= 68.6 ± 8.2, n=4; Figure 16D, $mean_{Histidine50}$= 51.6 ± 4.8, n=3; $mean_{Histidine25}$= 55.3 ± 7.5, n=3; $mean_{Mepyramide10}$= 54.9 ± 6.8, n=3). This data are in accordance with the motor behavioral analysis.

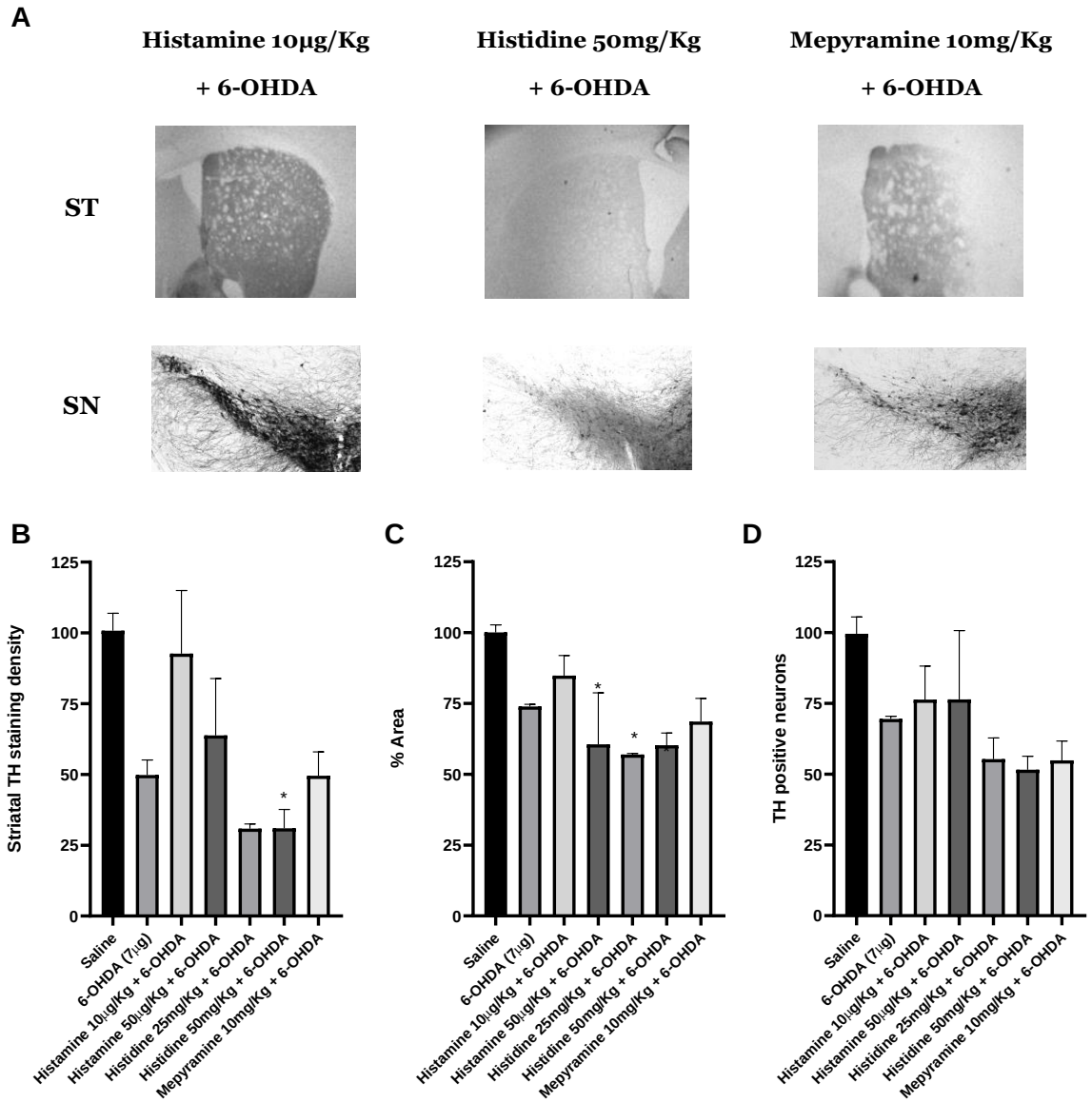

Figure 16 – Histamine precondition counteracts dopaminergic degeneration induced by 6-OHDA. Immunohistochemistry for TH in the ST and SN, two weeks after the stereotaxic injection of 6-OHDA. (A) Representative photomicrographs of SN and striatal sections immunostained for TH. Bar graphs depict TH staining density and % area in the *striatum* (B and C). (D) Bar graph depicts the TH positive neurons in the SN. The control group was set to 100%. Data are shown as the mean ± SEM. Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. *p<0.05 when compared with control condition and #p<0.05 when compared with 6-OHDA group.

Chapter 5

Discussion

Peripheral inflammatory stimuli such as LPS and histamine can induce neuroinflammation and, depending on the doses administered and the number of injections, can be considered as neurotoxic or neuroprotective stimuli. In fact, high doses of LPS and histamine induce neuronal death. [45] However, when administered consecutively at a lower dose, it induces neuronal recovery. [44] The objective of this study was to evaluate the protective priming of both these molecules on a subsequent injury induced by 6-OHDA.

Previous studies have shown that the peripheral administration with LPS at a low concentration for four consecutive days induced innate immune memory. This effect was due to the decreased concentration of pro-inflammatory cytokines (TNF-α and IL-1β) and an increase in IL-10 (an anti-inflammatory cytokine) concentration found in the serum. Moreover, LPS-induced innate immune memory protected against neurodegeneration in AD and stroke models. [81] These results demonstrate that the gradual use of low concentrations of LPS leads to beneficial effects, since it promotes neuronal protection mechanisms in several pathologies. However, there are no evidences of the protective effect mediated by innate immune memory in PD. Based on previous studies, we used LPS to induce innate immunity, Two doses were selected, 500 μg/Kg, reported previously by others, and a lower dose (300 μg/Kg) [81]. In parallel, we also used the same paradigm to test the effect of histamine, a well-known inflammatory mediator. In a healthy brain the amount of endogenous histamine circulating in the CSF and parenchyma is relatively low. Upon injury the amount of this biogenic amine increases, leading to an increase in blood and CSF concentration, allowing an increase in the permeability of BBB and, consequently, the entire inflammation process begins. In the case of PD, histamine levels are extremely high in SN, a brain area that is extremely vulnerable to neurotoxicity and where there is a higher density of microglia. [69], [70] Due to the predominance of microglia in SN, it is believed that the activation of microglia is related to the pathogenicity of PD, by releasing inflammatory mediators, namely histamine, that have the ability to activate microglial motility due to activation of H4 receptor and the activation of the signaling pathway associated with α5β1 integrin, p38 MAPK and Akt. [68] Histamine plays a dual role in the inflammatory response driven by macrophages, since the activation of H1 receptor boosts the release of IL-6 by these cells, while the activation of H2R inhibited chemotaxis, phagocytosis, and the production of TNF-α. [83], [84] In addition to activating these cells of innate immune system, it also has the ability to act on adaptative immune system cells, particularly in T cells and its activation/inhibition depends on the receptor subtype involved and on the disease and context. [85]. Therefore, there are evidence that histamine may be a promising target to induced innate immune memory as LPS and, thereby, develop a

neuroprotective mechanism. Finally, two other compounds were used in this study. Histidine, an essential amino acid precursor of histamine present. [86] Moreover, the involvement of H1 receptor was evaluated by using the receptor antagonist, mepyramine, which has the ability to inhibit the binding of endogenous histamine to H1R and, consequently, prevents its activation. [87]

To understand if this innate immune memory occurs in PD, we first evaluated the levels of cytokines in the blood plasma of mice submitted to 1x or 4x injections of LPS or histamine. Our results show that there is a decrease in the production of pro-inflammatory cytokines at the end of the four injections, and this decrease is more evident in TNF-α. In relation to IL-10, at the end of the four injections, there is no increase in concentration, but rather a non-significant decrease from the 1st to the 4th injection in the different stimuli. The administration of these inflammatory agents causes the activation of the peripheral inflammatory system through the activation of the cells responsible for the immune response as is the case of monocytes and macrophages. After the activation of these cells occur the release of inflammatory mediators, such as the release of cytokines that has the ability to cross the BBB and subsequently induce microglial activation. [38], [39] We also observed an increase of Iba-1 expression upon LPS/histamine challenge, indicating that there is an activation of the microglia after peripheral priming with LPS or histamine.

After evaluating the effects of peripheral stimulation with LPS or histamine on peripheral innate memory at the short term, we then evaluated the impact of innate memory on dopaminergic survival and motor behavior in the 6-OHDA animal model for PD. Three weeks after stereotaxic administration of 6-OHDA, the animals were submitted to the Apomorphine – Induced rotation test, that evaluates the number of rotations to the uninjured side (contralateral). Thus, this test allows evaluating the extent of the lesion, since the greater the lesion in the animal, the greater the number of rotations given to the contralateral side. Our results show that in the control group there is no tendency of rotation to either side, contrary to what is found in the 6-OHDA group, where there is a clear trend for contralateral rotation, thus indicating that this group presents a high dopaminergic lesion. In the groups treated with the inflammatory stimuli, LPS or histamine, the tendency of rotation to the uninjured side does not occur so often, so it is suggesting that, even if there is some neuronal lesion, this is reduced as compared with the 6-OHDA lesion mice. On the other hand, the groups treated with histidine 50 mg/Kg (precursor of histamine) and mepyramine (antagonist for the H1 receptor) have an evident lesion. After the behavior test, we evaluated dopaminergic survival in SN and ST using the immunohistochemistry for TH, a marker of dopaminergic neurons. In the 6-OHDA group there was a reduction in TH density in the ST by about 50% and a decrease in the number of neurons in the SN by 40%, compared with the control. These results are in accordance with the behavior test performed and indicate that the 6-OHDA model correctly mimic PD, like in previous papers from our group. [4] In the animals pre-treated with LPS, there is no lesion in SN or ST, since the values are like the control group, indicating that innate immunity induced by LPS may have a protective mechanism in the survival of dopaminergic neurons. A higher variability was found in the experimental groups where histidine

was used, which depends on its concentration and in the number of animals tested. It would be necessary to do more tests for a better conclusion. Finally, in the groups treated with the histidine and with mepyramine, the results are in agreement with the behavior test, since they present a clear lesion in ST and SN.

Considering the possible potential of these inflammatory stimuli in the neuroprotection-found in PD models, it is necessary to investigate several questions that remained unanswered and, with this, draw more precise conclusions. Having said that, we consider that it is essential to understand how the innate immune response is performed in the short term, which induces innate immune memory, as well as to evaluate the activation of the different receptors of histamine in several immune cells, as well as specific signaling pathways. In addition, is it also known that astrocytes are also closely related to the neuroprotection effect on PD and, therefore, it is essential to understand the role of these stimuli in these glial cells. [66] Finally, microglial activation was only evaluated after one injection, however, it is essential to evaluate whether this activation occurs at the end of the four injections.

Chapter 6

Conclusions

Our results suggest that the peripheral administration of inflammatory stimuli (LPS and histamine) induce an innate immune memory due to decreased production of pro-inflammatory cytokines. Although the stimulation is peripheral, it was found that induces microglial activation in the whole brain.

This peripheral activation and the consequent activation of microglia, may protect dopaminergic neurons against the insult caused by the neurotoxin 6-OHDA, as shown by the motor behavior and dopaminergic survival analysis in SN and ST.

However, is it important to note that the number of animals per group are very low and further studies are needed to corroborate these conclusions.

Chapter 7

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