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Histamine modulates dopaminergic neuronal survival by boosting microglial activity

Tatiana Filipa Melo Saraiva

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Orientadora: Prof. Doutora Liliana Inácio Bernardino

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Resumo

As células microgliais são os principais intervenientes na resposta inflamatória inata no cérebro adulto. Num contexto de lesão cerebral, a resposta das células da microglia envolve mecanismos de fagocitose de neurónios mortos ou danificados, libertação de fatores tróficos e/ou inflamatórios, e a produção de espécies reativas de oxigénio (ROS).

A histamina é uma amina encontrada em grandes quantidades em mastócitos, neurónios histaminérgicos, e leucócitos. No Sistema Nervoso Central (SNC), a histamina também é libertada por células da microglia e exerce as suas funções através da ativação de quatro subtipos de recetores acoplados a proteínas G: H1, H2, H3 e H4. Previamente, mostramos que a histamina modula a motilidade microglial e a libertação de citocinas. Os principais objetivos deste trabalho foram: i) avaliar o papel da histamina na atividade fagocítica microglial e na produção de ROS; e ii) explorar as consequências da inflamação microglial induzida pela histamina na sobrevivência neuronal dopaminérgica.

Inicialmente, verificamos que a histamina, através da ativação do recetor H1R, induziu um aumento de fagocitose na linha celular N9 de microglia, quando comparada com a condição controlo. Este efeito foi acompanhado por um rearranjo do citoesqueleto microglial monitorizado através da imunomarcação para a faloidina e a tubulina acetilada. A histamina também induziu um aumento da produção de ROS através da ativação dos recetores H1R e do H4R. A apocinina, um inibidor do NADPH oxidase, foi capaz de inibir totalmente a fagocitose e a produção de ROS mediada pela histamina. A incubação com lipopolissacarídeo (LPS), utilizado como controlo positivo, também induziu um aumento significativo de fagocitose e produção de ROS, quando comparado com culturas controlo.

Por outro lado, a injeção estereotáxica de histamina ou LPS na substancia nigra de murganhos adultos da estirpe C57BL/6 durante 7 dias, induziu um aumento da reatividade glial e uma diminuição robusta na sobrevivência neuronal dopaminérgica. Tanto a apocinina como a anexina V (usada como inibidor de fagocitose induzida pela fosfatidilserina) inibiram completamente a toxicidade dos neurónios dopaminérgicos induzida pela histamina.

Surpreendentemente, valores semelhantes à condição controlo, nos parâmetros avaliados *invitro* (fagocitose e produção de ROS) e *in vivo* (sobrevivência neuronal dopaminérgica), foram encontrados quando se procedeu à co-administração de histamina e LPS.

Em geral, os nossos resultados sugerem que a histamina induz a reatividade da microglia e que este efeito pode modular a sobrevivência neuronal dopaminérgica. Histamina *per se* atua principalmente como um agente pro-inflamatório induzindo neurotoxicidade. Contudo, na presença de LPS, a histamina pode exercer atividade anti-inflamatória e neuroprotetora.

Palavras-chave

Microglia, Histamina, LPS, Fagocitose, Espécies Reativas de Oxigénio, Neurotoxicidade, Neurónios dopaminérgicos

Resumo Alargado

Em condições fisiológicas, as células microgliais apresentam uma estrutura ramificada caracterizada pela baixa expressão de moléculas imunológicas. Estas células gliais são os principais intervenientes na resposta inflamatória inata, participando na primeira linha de defesa em resposta a vários estímulos, tais como as infeções, trauma, doenças neurodegenerativas, entre outros. Num contexto de lesão cerebral, as células da microglia tornam-se reativas, libertando fatores tróficos e/ou inflamatórios, e produzindo espécies reativas de oxigénio (ROS). A sua morfologia também é alterada adquirindo um estado ameboide responsável por processos de migração em direção ao local de lesão e ativação de mecanismos de fagocitose de neurónios mortos ou danificados. A microglia expressa diferentes tipos de recetores na sua superfície que estão envolvidos, por exemplo, na eliminação de micróbios e de material apoptótico ou, na indução da fagocitose (processo que envolve o rearranjo do citoesqueleto).A ativação microglial em resposta a um estímulo neurotóxico está geralmente associada a um aumento da expressão de citocinas pro-inflamatórias capazes de provocar degeneração neuronal. Por outro lado, dependendo da natureza e da intensidade do estímulo, as células da microglia podem libertar citocinas anti-inflamatórias e factores neurotróficos envolvidos em mecanismos celulares de protecção e reparação neuronal.

A histamina é uma amina neurogénica detetada precocemente no cérebro em desenvolvimento. Esta molécula, para além de ser o maior mediador das reações de hipersensibilidade imediata é também um interveniente importante em respostas imunes celulares e humorais. No sistema periférico, a histamina é produzida principalmente por mastócitos e não é capaz de atravessar a barreira hematoencafálica. No cérebro humano, esta amina é sintetizada pelos neurónios histaminérgicos localizados especificamente no núcleo tuberomamilar. A histamina também é libertada por células da microglia e exerce as suas funções através da ativação de quatro subtipos de recetores acoplados a proteínas G: H1, H2, H3 e H4. Previamente, mostramos que a histamina modula a motilidade microglial e a libertação de citocinas.

Com este trabalho pretendemos determinar a papel da histamina na fagocitose microglial e na produção de ROS em linhas celulares de microglia. Pretendemos também avaliar o efeito da actividade microglial induzida por esta mina na sobrevivência neuronal dopaminérgica.

Inicialmente, verificamos que a histamina, através da ativação do recetor H1R, induz um aumento de fagocitose na linha celular N9 de microglia, quando comparada com a condição controlo. Este efeito foi acompanhado por um rearranjo do citoesqueleto microglial monitorizado através da imunomarcação para a faloidina e a tubulina acetilada. Em adição, também verificamos que a histamina induz um aumento da produção de ROS através da

ativação dos recetores H1R e do H4R. A pré-administraçãode de apocinina, um inibidor do NADPH oxidase, inibiu totalmente a fagocitose microglial e a produção de ROS mediada pela histamina. A incubação com lipopolissacarídeo (LPS), utilizado como controlo positivo, também induziu um aumento significativo de fagocitose e produção de ROS, quando comparado com culturas controlo.

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Abstract

Microglial cells are the main players involved in the innate inflammatory responses in the adult brain. The response of microglia to brain injury involves the phagocytosis of death or damaged neurons, release of trophic and/or inflammatory factors, and the production of reactive oxygen species (ROS). Histamine is an amine found in high amounts in mast cells, histaminergic neurons, and leukocytes. In the Central Nervous System (CNS), histamine is also released by microglial cells and exerts its functions through the activation of four subtypes of G-protein coupled receptors: H1, H2, H3 and H4. Previously, our group showed that histamine modulates microglial motility and cytokines release. The main aims of this work were: i) to evaluate the role of histamine in microglial phagocytic activity and ROS production and ii) to explore the consequences of histamine-induced microglia inflammation in dopaminergic neuronal survival.Initially, we showed that histamine induced an increase of phagocytosis via H1R activation in a N9 murine microglial cell line, as compared to control. This effect was accompanied by the rearrangement of microglial cytoskeleton monitored through phalloidin and acetylated tubulin immunostaining. Histamine also induced an increase of ROS production *via* H1R and H4R activation. Apocynin, a NADPH oxidase inhibitor, was able to fully inhibit phagocytosis and ROS production mediated by histamine. Incubation with lipopolysaccharide (LPS), used as a positive control, also increased phagocytosis and ROS production, as compared with control cultures.

On the other side, the stereotaxic injection of histamine or LPS in the *substantia nigra* of adult C57Bl6 mice for 7 days induced an increase of glial reactivity and a robust decrease in dopaminergic neuronal survival. Both apocynin and annexin V (used as inhibitor of phosphatidylserine-induced phagocytosis) fully abolished the histamine-induced neurotoxicity of dopaminergic neurons.

Surprisingly, values similar to controls were found in cells co-treated with histamine and LPS, both in *in vitro* (phagocytosis and ROS production) and *in vivo* (dopaminergic survival).

Overall, our results suggest that histamine induce microglial reactivity both and that this effect may modulate dopaminergic neuronal survival. Histamine *per se* may act as a pro-inflammatory stimulus leading to neurotoxicity, whereas, in the presence of LPS, it acts as an anti-inflammatory and neuroprotective agent.

Keywords

Microglia, Histamine, LPS, Phagocytosis, Reactive Oxygen Species, Neurotoxicity, Dopaminergic neurons

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List of Abbreviations

ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CCL	Chemokine (C-C motif)
CNS	Central nervous system
CRs	Complement Receptors
CXCL10	C-X-C motif chemokine 10
CCL21	Chemokine (C-C motif) ligand 21
CD11b	Alpha chain of αMβ2-integrin or cluster of differentiation molecule 11B
COX	Cyclo-Oxygenase
DA	Dopamine
FBS	Fetal Bovine Serum
FcR	Fc-Receptors
GDNF	Neurotrophic factor derived from a glial cell line
GFAP	Glial Fibrillary Acid Protein
HRs	Histamine receptors
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
i.v.	Intravenouse
LBs	Lewys Bodys
LPS	Lipopolysaccharide
NADPH (NOX)	Nicotinamida Adenine Dinucleotide Phosphate (NADPH Oxidase)
NGF	Nerve growth factor
NO	Nitric Oxide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
PBS	Phosphate Buffer Saline

PC	Phosphatidylcoline
PD	Parkinson's Disease
PFA	Paraformaldehyde
PKA	Protein kinase A
PLA2	Phospholipase A2
PLC	Phospholipse C
PRs	Purine receptors
PS	Phosphatidylserine
PSRs	Phosphatidylserine receptors
TGFβ	Transforming growth factor β
TH	Tyrosine Hydroxylase
TLR	Toll like receptors
TNF	Tumour necrosis factor
TREM	Triggering receptor expressed on myeloid cells
ROS	Reactive oxygen species
RT	Room temperature
SN	Substantia nigra
SNpc	Substantia nigra *pars compacta*
SRs	Scavenger receptors
VMAT	Vesicular Monoamine Transporter

CHAPTER I

Introduction

1. Microglial cells: the "housekeepers" of the brain

Microglial cells are originated from myeloid/mesenchymal progenitors that migrate from the yolk sac to the embryo and surround the neuroepithelium (Figure 1).

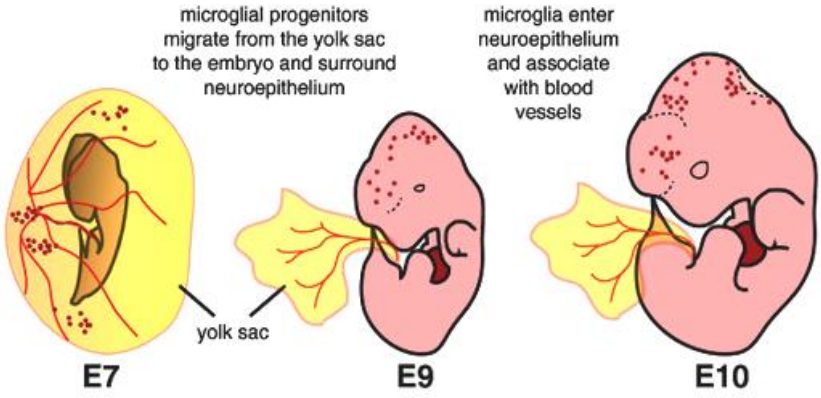


Figure 1 - Microglial cells origin. Microglial cells originate from myeloid precursors in the yolk sac, which migrate into the neuroepithelium by the embryonic day 10 (E10) (Adapted from Arnold and Betsholtz, 2013).

In the neuroepithelium, the microglial population rapidly expands and colonizes the brain from the dorsal to the ventral side (Figure 2). Over time, as early microglia move deeper into the developing parenchyma, they begin to differentiate, becoming more branched and expressing markers of mature microglia (*Pont-Lezica et al. 2011)*.

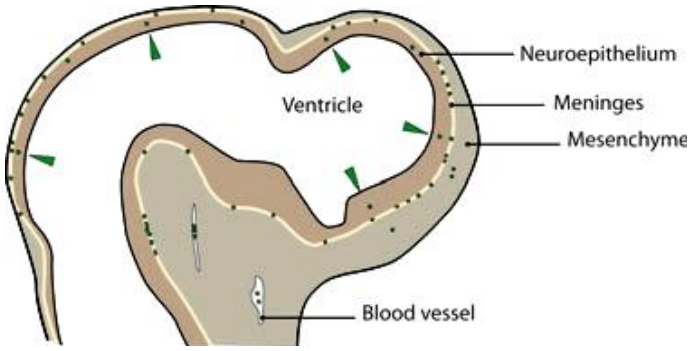


Figure 2 – Microglial colonization during the brain development. At E12, microglia can be detected in the brain mesenchyma, in the meninges and scattered in the neuroepithelium. (Adapted from *Pont-Lezica et al. 2011)*.

In the mature brain, approximately 12% of the total cells are microglial cells but they are not uniformly distributed *(Block et al., 2007; Walter and Neumann, 2009*). These cells exist in higher density in areas such as the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (SN) (*Block et al., 2007; Walter and Neumann, 2009*).

In physiologic conditions, these cells remain in a "resting" stage that is characterized by a ramified structure (Figure 3) and low expression of immunological molecules such major histocompatibility complex molecules (MHC), chemokine receptors, and several other markers (*Walter and Neumann, 2009; Zhang et al., 2010*). These receptors expressed constitutively at low levels are essential to the initiation and propagation of immune responses (Figure 4).

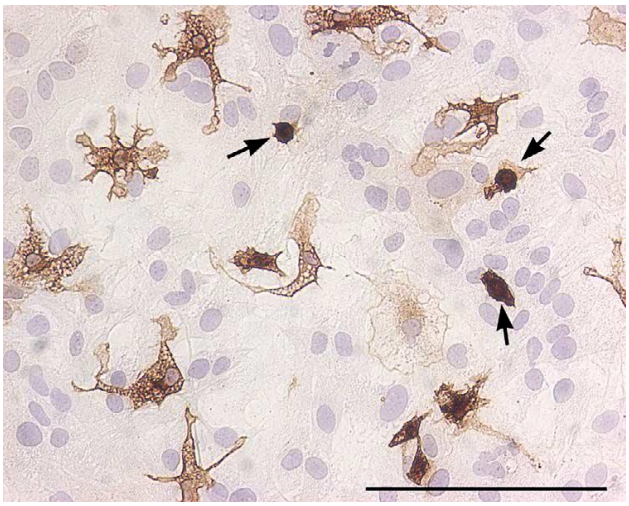


Figure 3 – Microglial morphology. Resting and ramified microglia in mixed glial cultures. Bright field image of a murine primary cortical mixed glial culture stained with the microglial marker Tomato lectin (brown) and counterstained with hematoxylin (blue). Three of them, identified with arrows, are round microglial cells with a strong lectin staining. In contrast, there are several microglial cells with ramified morphology and less intense lectin staining (*Adapted from Saura, 2007*).

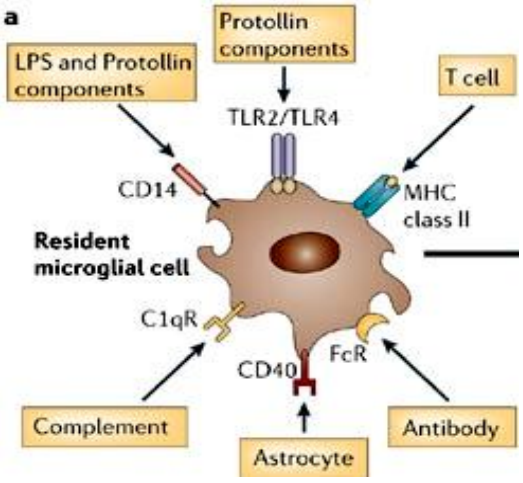


Figure 4 – Receptors on microglia cell surface responsible by the propagation of neuroimmune responses. Microglial cells can be activated by binding of various ligands to various cell-surface innate immune receptors: CD14 binds lipopolysaccharide (LPS) and components of Protollin; Toll-like receptor 2 (TLR2) and 4 (TLR4) bind Protollin components; MHC class II molecules interact with T-cell receptors; CD40 binds CD40 ligand expressed by T cells and astrocytes; complement receptors bind complement components such as C1q; and Fc receptors (FcRs) bind amyloid-ß-specific antibodies (*Adapted from Weiner et al., 2006)*

The ramified morphology is a cytoarchitectural reflection of their surveillance function in the healthy adult tissue. In fact, microglia cells are not passive agents. Instead, they are highly dynamic cells, always patrolling the brain parenchyma, extending and retracting their processes, searching for any neuronal lesion or infection (*Hanisch, 2013*). In addition, they contact with neighbouring cellular elements, including neurons and astrocytes, in order to maintaining the structural and functional integrity of the CNS (*Tremblay et al., 2011; Kettenmann et al., 2011*).

Microglia are considered to be a first line of brain defence and respond quickly to diverse stimulus, such as infection, trauma, ischemia, neurodegenerative diseases, or altered neuronal activity which can cause changes in brain homeostasis (*Suzumura, 2013*). These changes that may be potentially dangerous to the CNS leads to "microglia activation", which is characterized by rapid change in the ramified structure to the amoeboid morphology, migration of these cells to the site of injury or invading pathogens where they proliferate to increase the number of fighter cells and phagocyte cell debris or invading agents (*Walter and Neumann, 2009; Kettenmann et al., 2011; Sierra et al., 2013)*

In a classic activation paradigm, the so-called M1 phenotype, microglia are activated by the detection of pathogen-associated molecular patterns (PAMP's) and pro-inflammatory cytokines resulting in an increased expression of Toll-like receptors (TLR), tumour necrosis factor α (TNFα), coregulatory molecules for antigen presentation and an increase of reactive species of oxygen (ROS) production (Figure 5). This phenotype leads mainly to a pro-inflammatory status. The administration of LPS, an endotoxin derived from Gram-negative bacteria, is the well-studied stimulus leading to a M1 microglia phenotype. LPS triggers microglial activation, release a variety of pro-inflammatory cytokines and chemokines (as IL-1ß, IL-1, IL-10), nitric oxide (NO), transforming growth factor ß (TGFß) and TNFα (*Kim et al. 2000; Kim and de Vellis 2005; Kettenmann et al., 2011*).

On the other hand, the alternative activation or M2 phenotype is induced by interleukin 4 (IL-4) or interleukin 13 (IL-13), resulting in an increased production of interleukin 10 (IL-10) and TGFß and, higher expression of scavenger receptors (*Sierra et al., 2013)* (Figure 5). It was proposed that this phenotype is associated with an anti-inflammatory and neuroprotective status.

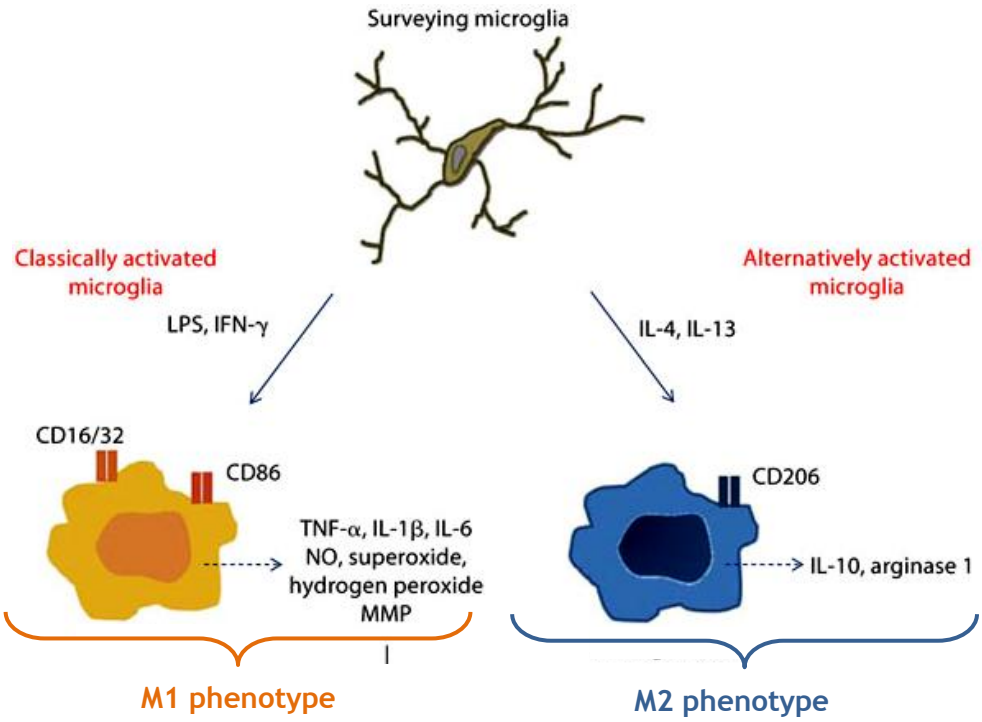


Figure 5 - Microglial phenotypes. Microglia can be classified in a simplified manner into two subsets of phenotypes and effector functions depending on the activation pathway (*Adapted from Czeh et al., 2011*)

1.1. Microglial migration/motility

The microglia migration plays a central role in many physiological and pathophysiological processes; with particular relevance on the clearance of microbes and other invading agents or neuronal debris (*Walter and Neumann, 2009; Kettenmann et al., 2011*).

The highly ramified microglial processes are remarkably motile, continuously and randomly undergoing cycles of filopodia-like protrusion formation, extension and withdrawal of bulbous tips (*Walter and Neumann, 2009; Kettenmann et al., 2011*). Due to this mobility, microglia are capable of monitoring the local microenvironment surroundings and possibly to endocytose small cellular debris or budded vesicular structures, including that from apoptotic cells (*Nimmerjahn et al., 2005; Kress et al., 2007; Neumann et al., 2009*).

During pathological processes, injured neurons release various signals responsible for the attraction of microglia to the sites of injury, such as a triphosphate (ATP), chemokines as C-X-C motif chemokine 10 (CXCL10) and C-C motif ligand 21 (CCL21), grown factors as nerve growth factor (NGF), ß-amyloid (Aß), cannabinoids, morphine, lysophosphatidic acid and bradykinin (*Neumann et al., 2009; Walter and Neumann, 2009; Kettenmann et al., 2011).*

Likewise, ion channels and transporters play an important role in controlling microglial cell migration, such as potassium (K^+) and chlorine (Cl^-) channels, sodium/hydrogen (Na^+/H^+) and chlorine/bicarbonate ($Cl^-/HCO3^-$) exchanger, and $Na^+/HCO3^-$ cotransporter, which all are linked to actin cytoskeleton dynamics (Figure 6) (*Kettenmann et al., 2011; Harry, 2013)*.

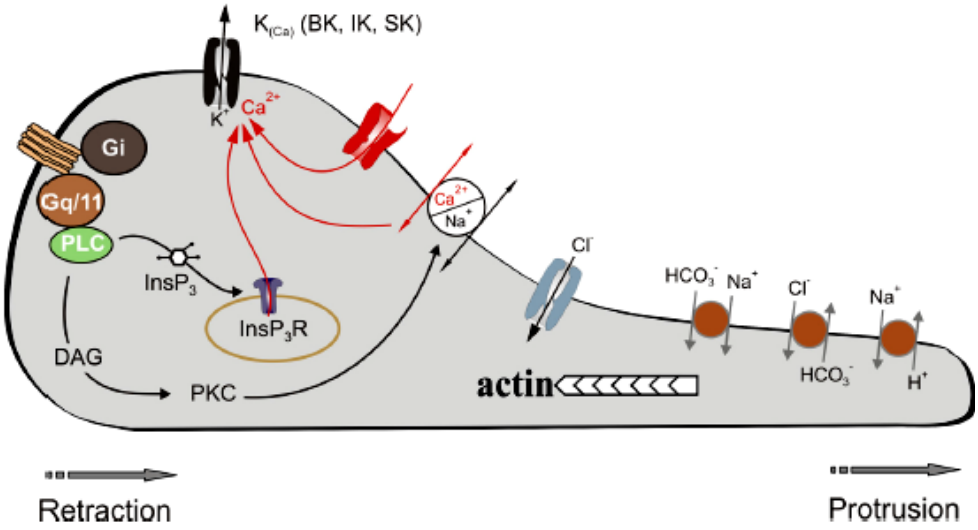


Figure 6 – Model summarizing the role of ion channels and transporters in controlling microglial migration. The cytosolic calcium (Ca^{2+}) signals induced by activation of metabotropic receptors and $InsP_3$ cascade and/or by Ca^{2+} entry through ionotropic receptors or reverse mode of Na^+/Ca^{2+} exchanger induces the retraction of the rear part of a migrating cell, which is paralleled by massive K+ efflux via Ca^{2+}-dependent K^+ channels and shrinkage of the cell at the rear (retraction site). Transporters such as Na^+/H^+ and Cl^-/HCO_3^- exchangers at the front of migrating cells (protrusion site) are reported to contribute to the extension of the actin projection (lamellipodium) by mediating salt and osmotically obliged water uptake (Adapted from *Kettenmann et al., 2011)*.

1.2. Release of soluble mediators

Another consequence of microglia activation is the release of inflammatory/neurotrophic factors which regulate the inflammatory response. The type of soluble factors released by microglial cells dependents on the initial stimulus that microglia cells receive.

Normally, the microglial activation in response to a strong neurotoxic stimulus results in the increase of the expression and release of pro-inflammatory cytokines, ROS and NO, that can cause further neuronal death (Figure 7; *Konsman et al. 2002; Walter and Neumann, 2009; Kettenmann et al., 2011; Fricker et al., 2012; Suzumura, 2013*).

However, microglial activation can also induce neuroprotective actions by the release of anti-inflammatory cytokines such as TGFß and IL-10, the release of neurotrophins such as NGF, brain-derived neurotrophic factor (BDNF) and neurotrophic factor derived from a glial cell line (GDNF) and/or inhibition of antigen presentation and release of pro-inflammatory

cytokines and reactive oxygen intermediates. The release of these trophic/anti-inflammatory factors contributes to the creation of an environment conducive for regeneration. These soluble factors can also attract phagocytic and repair-promoting effector and precursor cells, which are able to repair the damaged tissue (Figure 7; *Honda et al., 1999; Lai and Todd, 2008; Neumann et a., 2009; Garden and La Spada, 2012).*

A typical example of this duality of effects is the fact that the components of pathogens such as LPS are typically neurotoxic agents because it rapidly induce the production of interleukin-1 beta (IL-1ß) and TNFα by microglia; but when microglial cells are pretreated with IL-4, occurs a downregulation of TNFα and an upregulation of insulin-like growth factor-1 (IGF-1) gene transcripts, resulting in a neuroprotective effect (Figure 7; *Neumann et a., 2009*).

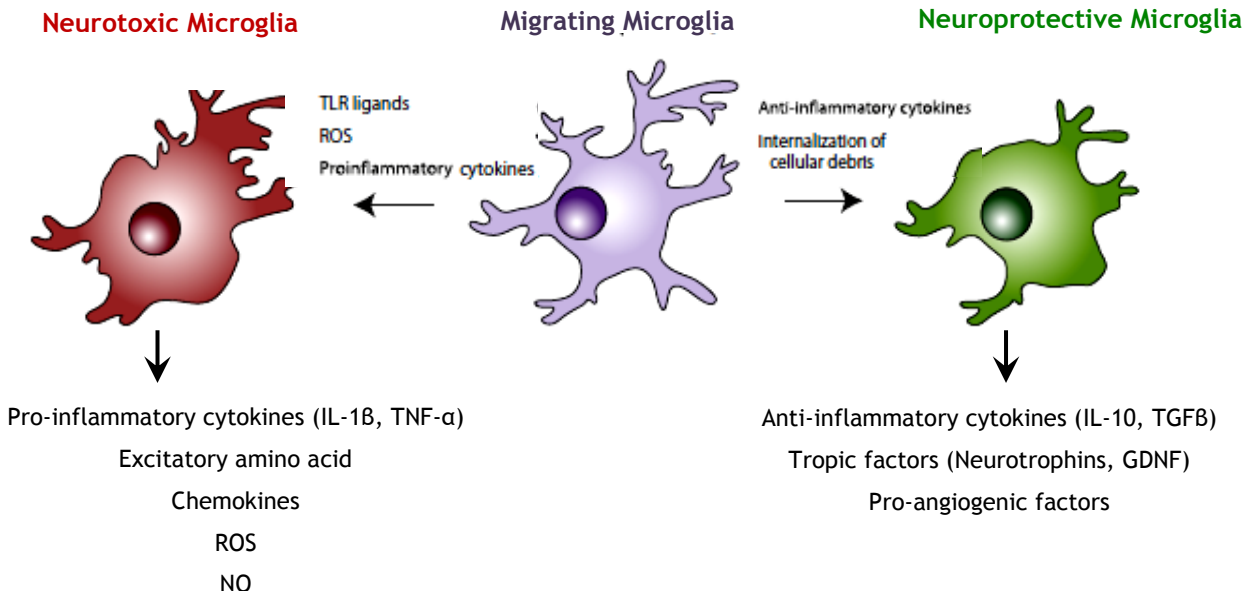


Figure 7 - Microglia play distinct roles depending on the stimulus. In the healthy CNS, microglia survey their microenvironment, and in this "resting state", do not express inflammatory mediators. However, after exposure to a number of chemical signals from damaged neurons, microglia respond rapidly and physically migrate to the site of injury. Responding microglia may then adopt a pattern of behavior similar to proinflammatory macrophages (left), as they release neurotoxic cytokines, chemokines, ROS, and NO. The release of cytokines and chemokines can lead to the recruitment of additional inflammatory cells from adjacent blood vessels, and may also engage astrocytes in the proinflammatory response. Alternatively, activated microglia may have neuroprotective behavior (right), secreting molecules that promote tissue repair, and internalizing cellular debris including aggregated, misfolded proteins such as β-amyloid, through phagocytosis. Whether two distinct populations of microglia exist that are committed to either of these response patterns, or all microglia can be induced to exhibit either response behavior when exposed to the correct combination of signals, remains to be determined (*Addapted from Lai and Todd, 2008; Neumann et al, 2009; Garden and La Spada, 2012)*

It is also known that microglia have an antimicrobial activity due to production and release of toxic oxygen-derived and nitrogen-derived products, which are generated in a process known

as the respiratory or oxidative burst. This production is due to situations of tissue damage or during defence against pathogens that have to be eliminated from brain (*Sun et al., 2008; Walter and Neumann, 2009; Hirsch and Hunot, 2009; Czeh et al., 2011; Fricker et al. 2012; Peterson and Flood, 2012).*

This oxidative process is regulated by several enzymatic systems, principally the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase/NOX) and inducible nitric oxide synthase (iNOS), and to a lesser extent by mitochondrial oxidases, cytochrome P450c, cyclooxygenases, myeloperoxidase (MPO) *(Qin et al., 2005; Barger et al., 2007; Drechsel and Patel, 2008; Hirsch and Hunot, 2009; Mead et al., 2012).*

After microglial activation, the four regulatory cytoplasmic subunits (p47 ^{phox}, p67 ^{phox}, p40 ^{phox} and Rac proteins) present in the NOX translocate to the plasma membrane linking to the other two subunits (p22 ^{phox} and gp91^{phox}/Nox2) present there, forming the functional enzyme that catalyses the reaction of NADPH and oxygen to form NAD^+, protons and O^{2-} (Figure 8; *Walter and Neumann, 2009; Sierra et al., 2013*). Due to acidic pH into phagosome, the O^{2-} is dismuted into hydrogen peroxide (H_2O_2) and, later, into hypochlorous ($HOCl^-$) that actively participate in the modulation of signalling pathways involving microglial phagocytosis, for example in the phagocytic neutralization of microorganisms and promotion of neuronal death in animal models of neurodegenerative diseases (Figure 8; *Block et al., 2007; Sun et al., 2008*; *Chéret et al., 2008; Walter and Neumann, 2009; Peterson and Flood, 2012; Sierra et al., 2013)*

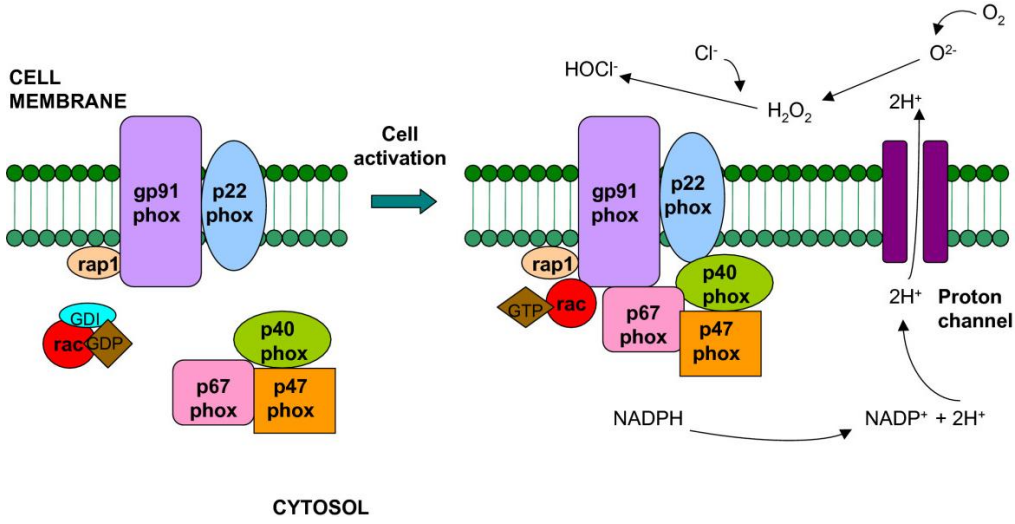


Figure 8 - NADPH oxidase enzyme. The integral membrane of the phagocyte consists of two subunits: p22phox and gp91phox which respectively produce the smaller and larger chain of the cytochrome-b558. Two cytosolic subunits: p67phox and p47phox; a p40phox accessory protein and a Rac-GTP binding protein then translocate to the cell membrane upon cell activation to form the NADPH oxidase complex which generates a respiratory burst. Superoxide can react to form hydrogen peroxide and hypochlorus acid, which together participate in bacterial killing *(Adapted from Assari T., 2006)*.

Several studies demonstrated that higher levels of inflammatory mediators due to activated microglial cells, particularly ROS and NO, are responsible for the loss of the majority of DA neurons in Parkinson's disease (PD) patients. This fact suggests that the oxidative stress response that comes from microglial activation may be an important component in the neurodegenerative diseases and in the maintenance of the chronic pro-inflammatory response in PD patients (Drechsel and Patel, 2008; Hirsch and Hunot, 2009; Peterson and Flood, 2012).

1.3. Microglial phagocytosis

The phagocytosis comprises the first line of the innate immune defence against multicellular organisms and is mostly performed by specialized phagocytes, such as macrophages, dendritic cells, and neutrophils (*Sierra et al., 2013)*. In the CNS, the innate immune response is mediated by microglia (*Czeh et al., 2011; Sierra et al., 2013)*.

Microglia express different types of receptors on their surface that are involved in scavenging particles, debris, apoptotic material and microbes, or induction of phagocytic signaling, an active process involving rearrangement of the cytoskeleton (*Walter and Neumann, 2009*). More specifically, there are two functional types of phagocytic receptors, the receptors recognizing microbes such as to TLRs and Fc receptors (FcR's) which support removal of pathogens and simultaneously stimulates a pro-inflammatory response in the phagocytes, and, receptors recognizing apoptotic cellular material such as receptors that recognize phosphatidylserine (PS) and which are important for ingesting apoptotic cell and stimulate an anti-inflammatory response in phagocytes (Figure 9; *Ravichandran, 2003; Walter and Neumann, 2009)*. The phagocytosis of apoptotic debris is essential and beneficial for the CNS because it reduces the secretion of pro-inflammatory cytokines, chemoattraction and migration of T lymphocytes *(Tremblay et al., 2011*).

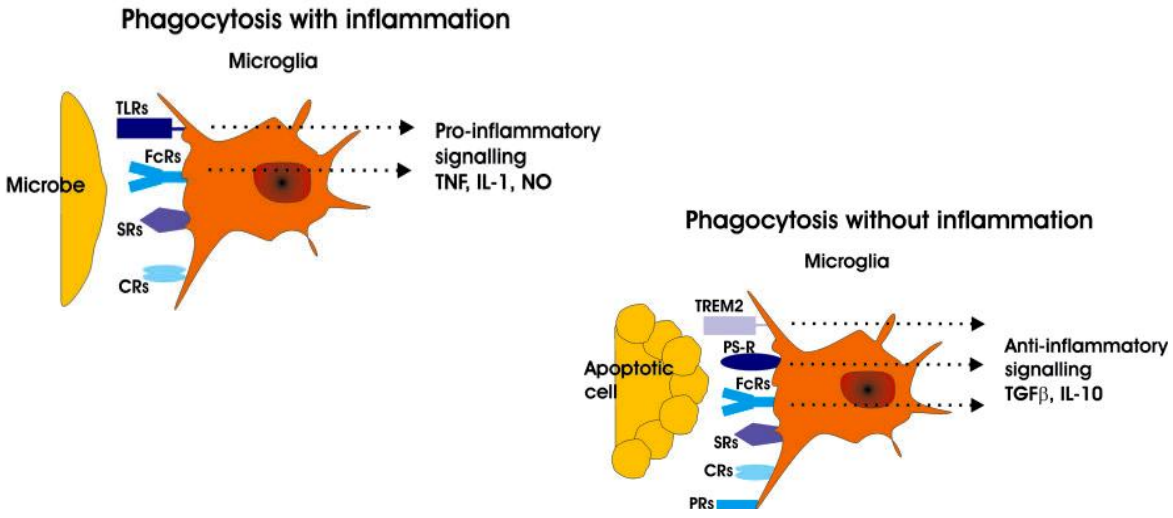


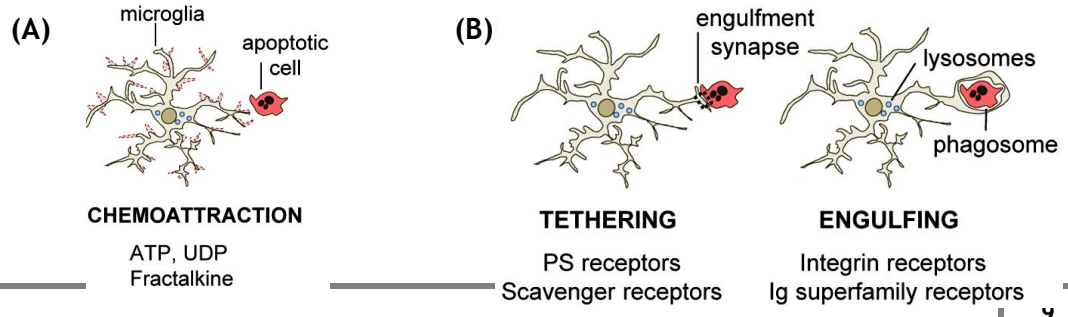
Figure 9 - Microglial phagocytic receptors. (Left) Phagocytosis is associated with inflammation during uptake of microbes, while phagocytosis of apoptotic cells is executed without inflammation (Right). Recognition and phagocytosis of apoptotic cells induces an anti-inflammatory cytokine profile in microglia (*Adapted from Neumann et al., 2009*)

The phagocytic process is mediated by a number of receptors. Actually, some studies that focus on the action of the FcR's that are responsible to generate signals that regulate phagocytosis of immunoglobulin G (IgG)-coated particles. This process occurs when the Fc regions of the IgG molecules, that are formed when a small particle (eg. beads) or erythrocyte is opsonized with IgG, bind to FcR in the macrophage plasma membrane and initiate a phagocytic response forming a cup-shaped folds of plasma membrane extend outward from the macrophage around the particle and constrict at its distal margin, closing in a few minutes into a plasma membrane-derived phagosome. During the next hour, interactions between the phagosome and other membranous organelles change its internal and surface chemistries in a maturation process that typically leads to degradation of the phagosome contents by acid hydrolases. Throughout this **event**, the reduced NADPH oxidase complex is activated to deliver ROS into the phagosome by producing O^{2-} from the oxidation of NADPH and reduction of molecular oxygen (Kerrigan and Brown, 2009; Jaumouillé and Grinstein, 2010)

1.3.1. The mechanisms of phagocytosis

Microglial phagocytosis is a highly efficient process that maintains brain homeostasis. Targets for phagocytosis include: apoptotic cells, synapses, degenerated neuronal debris, or proteins with very high turnover such as Aß protein.

Recent studies have demonstrated that damaged neurons are not merely passive targets but they regulate the microglia activity by releasing several signaling molecules. Specifically, degenerated neurons release nucleotides, cytokines and chemokines, to recruit microglia and enhance their activities. In literature, these molecules are described as “find-me”, “eat-me” and “digest-me” signals (Figure 10; *Tremblay et al, 2011; Suzumura, 2013; Sierra et al., 2013).*



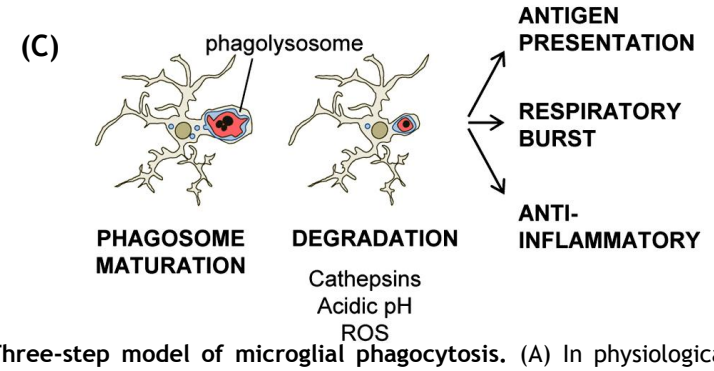


Figure 10 - Three-step model of microglial phagocytosis. (A) In physiological conditions, microglial processes are highly motile and respond to chemoattractant molecules released by damaged or apoptotic cells - "find-me" signals - such as fractalkine and extracellular nucleotides (ATP, UDP). (B) An engulfment synapse is formed between a series of microglial receptors and their ligands in the membrane of the apoptotic cell - "eat-me" signals, leading to the tethering and engulfing of the apoptotic cell in a phagosome. (C) The phagosome becomes mature by fusing with lysosomes and other organelles, and the apoptotic cell is fully degraded in the phagolysosome in less than 2h -"digest-me" signals (Adapted from Sierra et al., 2013).

- "Find-me" signals (Figure 10A)

It is known that the role played by phagocytic microglia occurs due to constant surveillance in the brain. So, phagocytosis is initiated when the phagocyte encounters a target cell by the presence of signals released by these cells. For instance, apoptotic cells release extracellular nucleotides (ATP and UTP) and other chemotactic signals fractalkine (CX3CL1) that are recognized by the receptors P2Y6 and CX3C chemokine receptor 1 (CX3CR1) respectively, on the surface of microglia, facilitating phagocytosis (*Sierra et al., 2013)*.

- "Eat-me" signals (Figure 10B)

Microglial cells have a series of receptors on their surface which are responsible for the different steps of phagocytosis. One group of receptors is responsible for recognition of target cells while another group is responsible for the internalization of these cells. These steps are the most important in the process of phagocytosis, leading to the formation of the phagocytic cup (*Fricker et al., 2012; Sierra et al., 2013)*.

There is a group of receptors which are called "pathogen-associated molecular patterns" (PAMPS) that are mediated through scavenger receptors in conjunction with TLRs such as the CD14/TLR4 complex, or receptors of the immunoglobulin superfamily (e.g., c-type lectins). On the other hand, there is another group of receptors called "apoptotic cells-associated cellular patterns" (ACAMPs) which detects PS residues on the surface of microglial cells; this process is regulated by receptors as brain-specific angiogenesis inhibitor 1 (BAI-1) and by

linking with molecules, as milk fat globule-epidermal growth factor (MFG-E8), soluble opsonins and peroxynitrite (*Armstrong and Ravichandran, 2011; Neher et al., 2011; Fricker et al., 2012; Sierra et al., 2013*).

These pathways lead to the remodeling of the microglial cytoskeleton through actin polymerization triggering the formation of pseudopodia that form a phagocytic cup engulfing the target *(Lee et al., 2007; Sierra et al., 2013)*

- "Digest-me" signals (Figure 10C)

The phagosome formation occurs after closing the phagocytic cup. The phagosome merges with the early and late endosome and lysosome forming the phagolysosome which contains hydrolases and proton pumps responsible for digestion of the target and acidification of the medium, respectively. The acidic pH (pH≤5) allows lysosomal degradation, besides being an optimum environment for hydrolases. In addition, the low pH deactivates the production of free radicals resulting from the oxidative burst *(Li et al, 2010; Sierra et al., 2013)*. This degradation process leads to subsequent antigen presentation, respiratory burst and release of anti-inflammatory factors.

The rapid elimination of apoptotic cells prevents them from becoming necrotic cells which can lead to loss of cell membrane permeability and spillover of intracellular contents. In fact, others have showed that the blockade of phagocytosis of microglia and polymorphonuclear neutrophils that infiltrate the brain parenchyma after focal ischemia, decreases neuronal viability in organotypic slices (*Neumann et al, 2008*).

Currently, the most recent method used to block microglial phagocytosis is the systemic administration of annexin V, which binds to the PS residues causing the accumulation of apoptotic debris *(Lu et al., 2011; Fricker et al., 2012; Sierra et al., 2013)*. Other known compounds able to inhibit microglia phagocytosis include vitronectin receptor blockers, such as mutant MFG-E8 and vitronectin antagonists *(Neher et al., 2011; Fricker et al., 2012)*

2. Histamine

Histamine is one of the first neuroactive molecules to be detected in the early development of the brain. This biogenic amine is the major mediator of immediate-type hypersensitivity reactions as well as a modulator of cellular and humoral immune responses occurring in the pherypheric vascular system, but it is not transported into the brain across the blood–brain barrier (BBB). Most of the histamine is stored in master cells but it is also present in basophils, gastric enterochromaffin-like cells, leukocytes, platelets and even tumor cells. In the brain, histamine is synthesized in histaminergic neurons distributed in a posterior basal hypothalamus region - the tuberomammillary nucleus- and their axonal ramifications covers all over the CNS (Figure 11; *M. L. Vizuete et al., 2000; R.E. Brown et al., 2001; N. Adachi, 2005; Molina-Hernández et al., 2012, 2013; Walker et al, 2013).*

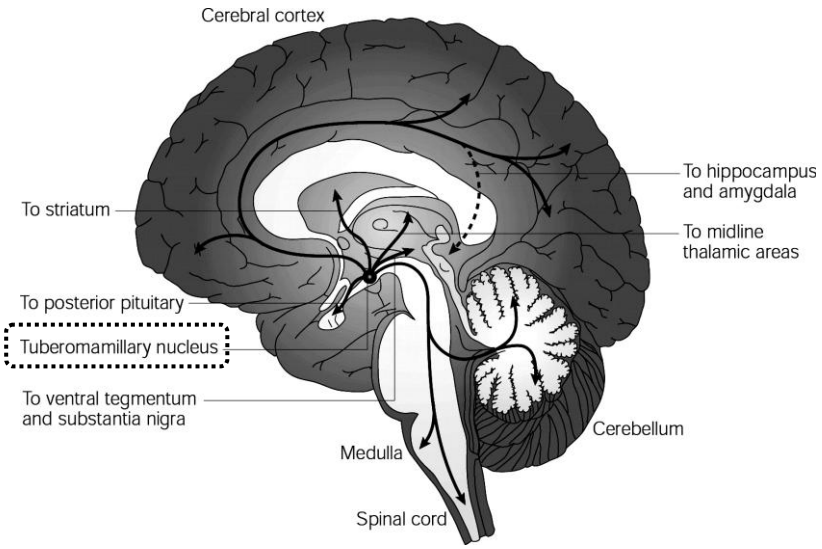


Figure 11 - The histaminergic system in the human brain. The histaminergic fibers emanating from the tuberomamillary nucleus project to and arborize in the whole central nervous system *(Adapted from Haas, Sergeeva, and Selbach, 2008).*

During neuronal differentiation in cerebral cortex, the fibers from the histaminergic neurons can be detected in the mesencephalon, passing through the ventral tegmental area and within the medial forebrain bundle and the optic tract, to reach the frontal and the parietal cortices, earlier than other monoaminergic systems *(Molina-Hernández et al., 2012).*

Histamine is synthesized from L-histidine by the enzyme L-histidine descarboxylase and converted into tele-methylhistamine by histamine-N-methyltransferase. By action of Monoamine oxidase B, tele-methylhistamine is converted into tele-methylimidazoleacetic acid (Figure 12; *Brown et al., 2001; Adachi, 2005).*

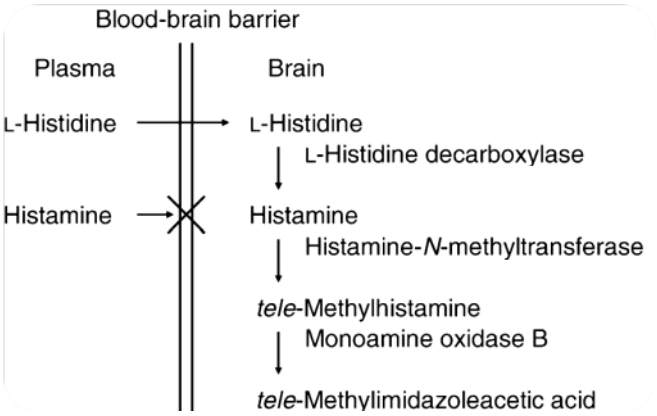


Figure 12- Biosynthesis and metabolism of brain histamine. In the brain, histamine is formed from l-histidine by a specific enzyme, l-histidine decarboxylase. There are two major pathways of histamine metabolism; ring methylation and oxidative deamination by diamine oxidase. In the brain, most of histamine is catalized by histamine- N-methyltransferase to form tele-methylhistamine, which is converted by monoamine oxidase B to tele-methylimidazoleacetic acid *(Adapted from Adachi, 2005)*.

Histamine exerts its functions through the activation of four subtypes of G-protein coupled receptors: H1R, H2R, H3R and H4R.

The H1Rs are expressed in regions related to behavioural, nutritional sate control and neuroendocrine but also plays an important role in inducing anaphylactic responses, such as bronchospasm, an increase in vascular permeability, and hypotensive shock. In contrast, the H2Rs mediates gastric acid production besides contributing to depress immunological processes by suppressing lymphocyte proliferation, cytokine production, and neutrophil accumulation. The H3Rs are heterogeneously distributed in brain and it is responsible to mediate feedback inhibition of the release and synthesis of histamine. Finally, the H4R is predominantly expressed in hematopoietic cells and is involved in or controlling the activities of eosinophils, master cells, monocytes, dendritic cells and T cells (Table 1) *(O'Reilly et al. 2002; Adachi, 2005; Dijkstra et al. 2008; Jadidi-Niaragh and Mirshafiey, 2010; Molina-Hernández et al., 2012)*.

Table 1 – Properties of histamine receptors *(Adapted from Jadidi-Niaragh and Mirshafiey, 2010)*

Characte -ristics	H1R	H2R	H3R	H4R
G-protein coupling	Gq/11	Gs	Gi/o	Gi/o
CNS expressio n	Thalamus, hippocampus, cortex, amygdala, basal forebrain	Basal ganglia, hippocampus, amygdala, pyramidal cells, raphe nuclei, SN	Nucleus, accumbens, striatum, basal ganglia, olfactory tubercles, SN, amygdala	Cerebellum, hippocampus
General function	Wakefulness, inflammatory responses, decreasing blood pressure	Regulation of gastric acid secretion, decreasing blood pressure, relaxation of airway and vascular smooth muscle, excitation, fluid balance, regulation of hormonal secretion	Regulation of production and release of histamine	Modulation of immune system
Signaling pathway	PLC	Activation of PKA	Inhibition of PKA, activation of PLA2, MAPK	Inhibition of PKA, activation of PLC, MAPK

In CNS, histamine can be also released by microglial cells (*Katoh et al., 2001*). Recently, our group showed a dual role for histamine in the regulation of microglia activity by modulating cell recruitment and the release of pro-inflammatory cytokines, such as IL-1β and TNF α *(Ferreira et al, 2012)*.

The more than two decades ago, Francis et al. demonstrated that, while, the receptors specific for the C3bi cleavage fragment of the third component of complem ent (CR3) promote adhesion, histamine and our receptors inhibited the ability of CR3 to cluster on plasma membranes of neutrophils adherent to C3-coated surfaces *(Francis et.el. 1991)*. Based on this fact, Azuma et al., demonstrated that histamine can inhibits phagocytosis through expression of complement receptor 3 in macrophages and it may affect the flow through the membrane and the expression of Fcγ receptors.

Other studies showed that histamine releasing peptide (HRP) promotes chemotaxis of leukocytes and enhances macrophage phagocytosis, and, in a presence of acute cutaneous inflammatory response promotes an increased of the level of HRP. These results suggested that HRP is a pro-inflammatory peptide that helps amplify and perpetuate the inflammatory response *(Jaumouillé and Grinstein, 2011)*.

3. Neuroinflammation in Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD) and it is the most common movement disorder that affects approximately 1% of the population at the age of 55/60 and increases in prevalence to 4/5% by the age of 80/85 *(Block et al., 2007; Hirsch and Hunot, 2009; Glass et al., 2010; Labandeira-Garcia et al., 2011)*.

PD is a proteinopathy, such as AD, characterised by the presence of intraneuronal proteinaceous cytoplasmic inclusions known as Lewy bodies (LBs) and, by progressive and selective degeneration of DA-containing neurons in the substantia nigra pars compacta (SNpc) (Figure 13). It is known that these effects result from multiple molecular and cellular alterations that might be induced by abnormal protein handling, mitochondrial dysfunction, excitotoxicity, apoptotic processes, oxidative stress, inflammation and impairment of the ubiquitin-proteosome system (*Hirsch and Hunot S., 2009; Glass et al., 2010; Neher et al, 2011; Labandeira-Garcia et al., 2011; Morroni et al, 2013)*.

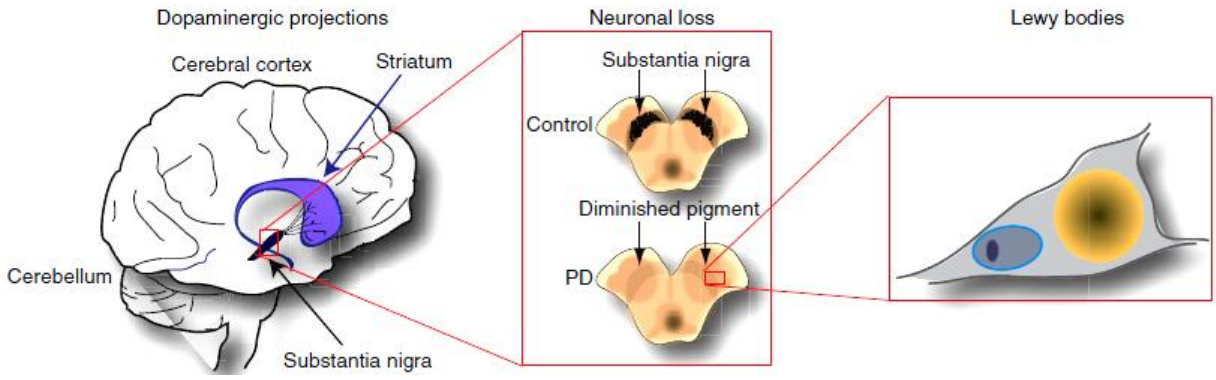


Figure 13 - The pathology of Parkinson's disease. The image represents the main neuropathological events in PD at three levels from left to right. At the level of the brain, a major pathway is degeneration of the dopaminergic projections from the SN (in black) to the striatum (in purple), both of which are in the midbrain underneath the cerebral cortex. At the level of SN, the neurons that form the presynaptic portion of this pathway are normally melanized and are easily identified by this pigment in control brains (upper panel). In contrast, the loss of neurons in this region is so substantial that the whole area becomes depigmented in PD cases (lower panel). Of the few remaining cells, many show pathological changes, including the accumulation of proteins and lipids in Lewy bodies (*Adapted from Cookson, 2012*).

The degeneration of the dopaminergic signalling present in the nigrostriatal pathway is responsible for the symptoms of motor dysfunction such as rigidity tremor, slowness of motion, difficulty to initiate movements and loss of balance. PD also presents non-motor-related symptoms as olfactory deficits, autonomic dysfunction, depression, cognitive deficits, and sleep disorders *(Pei at al., 2007; Block et al., 2007; Hirsch E.C. and Hunot S., 2009; Glass et al., 2010; Morroni et al, 2013).*

Several evidences support that neuroinflammation can be involved in the loss of DA neurons that occurs in PD (*Block et al., 2007; Brown and Neher, 2010; Glass et al., 2010; Neher et al, 2011; Labandeira-Garcia et al., 2011)*. Microglia activity can be detrimental to DA neurons by regulating the activity of several enzymatic systems, among which NOX, iNOS, and MPO, are responsible for the production of O^{2-}, NO free radicals, and $HOCl^{-}$. In PD, these compounds are increased in the SN (Figure 13; *Hirsch and Hunot, 2009; Glass et al., 2010; Brown and Neher, 2010; L'Episcopo et al. 2010; L'Episcopo et al., 2010)*. Moreover, the *SN* is highly enriched in microglial cells, making this brain region highly vulnerable to inflammatory reactions.

3.1. Animal models of Parkinson's disease

Over several decades, has been extremely important to use animal models of PD that allow the pathological study of the disease and the development of therapeutic strategies to treat motor symptoms or, even one day, prevent to some extent the development of this disease neurodegenerative (*J. Bové and C. Perier, 2012*).

All models of PD are formulated based on the loss of DA neurons in the SN, although many of them have similar characteristics to the disease itself, can't produce all the features presented in chronic neurodegenerative human PD. However, these animal models must possess special requirements such as having the ability to induce an injury replicable in the SN, the loss of DA neurons should be stable over time without the occurrence of spontaneous recovery, and must be able to "treated" based neuroprotective strategies (*Emborg, 2004)*.

The toxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) are two compounds commonly used and best characterized with respect to the development of PD in animals once they are responsible for loss of DA neurons. In recent decades have been discovered compounds able to produce similar effects as rotenone, paraquat, dieldrin and maneb *(Emborg, 2004; Bové et al, 2005; Dutta et al., 2008; Drechsel and Patel, 2008; Cristóvão et al., 2009; D.M. Crabtree, Zhang, 2012; Bové and Perier, 2012*).

In the same sense, the lipopolysaccharide (LPS) has been the most extensively used to determine whether direct activation of microglia promotes a progressive and selective degeneration of DA neurons in rodents.

LPS an endotoxin found in the outer membrane of gram-negative bacteria is known as a potent activator of the innate immune response. It is composed by the O-antigen with multiple repeating units of monosaccharides, a polysaccharide core with an unusual sugar (2-keto-3-deoxyoctonate), and lipid A consisting of a unique diglucosamine backbone to which six fatty acid chains are attached (Figure 14; *Qin et al, 2004; Dutta et al., 2008*).

The binding of LPS to the soluble LPS binding protein (LBP) and CD14, which is anchored to the outer leaflet of the plasma membrane, promotes signal transduction through the plasma membrane, making possible the interaction of the complex LPS-CD14 with the TLR4 and extracellular accessory protein MD2. This interaction leads to the activation of kinases of various intracellular signaling pathways and upregulation of gene transcription for a variety of proinflammatory factors and free radical-generating enzymes. Consequently, this endotoxin is a potent stimulator of the microglia that able to promote the release of various immunoregulatory and proinflammatory cytokines and free radicals (Figure 14; *Qin et al., 2004*; *Dutta et al., 2008*).

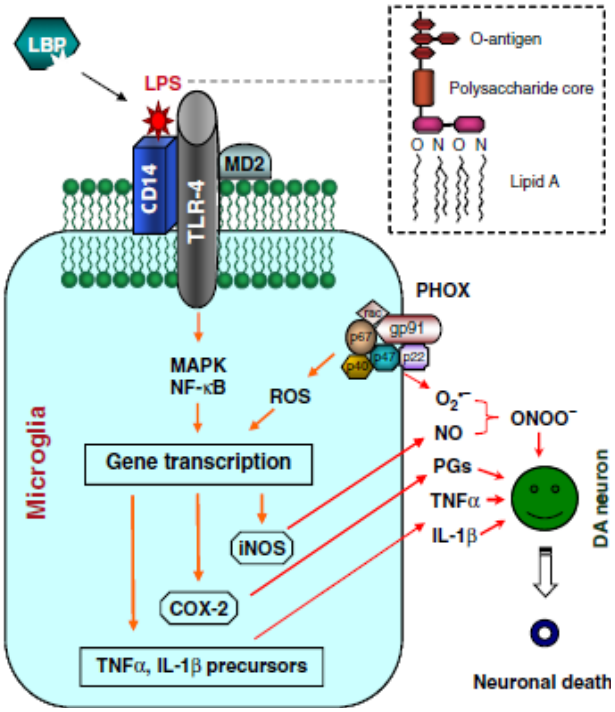


Figure 14 - Schematic representation of LPS-induced and glial activation-mediated DA neurodegeneration. LPS binding protein works as a chaperon that enhances the binding of LPS to its intermediate receptor CD14. The association of the LPS-CD14 complex with TLR4, together with the accessory adaptor protein MD2 initiates a plethora of downstream signalling events that involve mitogen-activated protein kinases (MAPK) and transcription factors such as nuclear factor-kappa B. Upregulation of gene transcription leads to the production and release of cytokines such as TNFα and IL1b. Induction of cyclo-oxygenase-2 and iNOS expression results in the biosynthesis and release of prostaglandins and NO. Activation of the multi-subunit phagocyte oxidase complex (PHOX), also called NADPH oxidase generates superoxide anion that combines with NO from iNOS to form the more

damaging peroxynitrite ($ONOO^-$) free radical. The collective insult of microglia-released cytokines, ROS and lipid metabolites eventually leads to the demise of the oxidative stress-vulnerable DA neurons *(Adapted from G. Dutta et al., 2008)*.

It is described that both the administration of LPS *in vitro* and *in vivo*, is responsible for the microglial cell activation, which the release of ROS promoting the selective and progressive degeneration of DA neurons. In the same way, some reports suggest that a brief episode (±2 weeks) of neuroinflammation that occurs early in life is capable of inducing significant glial activation accompanied by a delayed, progressive and preferential degeneration of SNpc DA neurons (*Pei et al., 2007; Neher et al., 2011; Sanchez-Guajardo V., 2013*).

Objectives

Microglial cells act as resident macrophages on the CNS. They are responsible for the constant monitoring of the brain microenvironment through elimination of toxic compounds and pathogenic substances. Several studies demonstrated that microglial activity can be related with the loss of DA neurons in the SN, a hallmark of PD.

In the brain, histamine is synthesized in histaminergic neurons present in the hypothalamus; however, it can be also released by microglial cells. Recently, our group showed a dual role for histamine in the regulation of microglia activity by modulating cell recruitment and the release of pro-inflammatory cytokines, such as IL-1ß and TNF-α (*Ferreira et al, 2012)*.

In general, this thesis aimed to determine the role of histamine in microglial phagocytosis and ROS production in a murine N9 microglia cell line. In the same way, we proposed to evaluate the effect of histamine-induced microglial activity on dopaminergic neuronal survival.

Specific aims included:

- The evaluation of the role of histamine and its receptors in microglia phagocytic activity and ROS production, with or without the presence of an inflammatory stimulus – LPS;
- The characterization of cytoskeleton alterations driven by histamine and /or LPS in microglial cells;
- To investigate the role histamine and/or LPS-induced microglia activation on DA neuronal survival.

Chapter III

Materials and Methods

In Vitro assays

3.1. Cell line cultures

A murine N9 microglia cell line (a kind gift from Prof. Claudia Verderio, CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy) was grown in modified RPMI medium during 24h to 37°C in a 95% atmospheric air and 5% CO_2 humidified atmosphere.

Cells were plated at a density of 2×10^4 cells per well in 24-well trays (phagocytic studies and immunocytochemistry), 5×10^5 cells per well in 6-well trays (protein extraction) or 5×10^4 cells per well in 96-well trays (ROS quantification).

Cell treatments included the following incubation setup: LPS (100 ng/ml, Sigma Aldrich), Histamine dihydrochloride (1–100 μM, Sigma), H1 receptor antagonist (mepyramine maleate, 1 μM), H4 receptor antagonist, (JNJ7777120, 5 μM), H1 receptor agonist, (2-Pyridylethylamine dihydrochloride, 100 μM) H4 receptor agonist, (4-methylhistamine dihydrochloride, 20 μM) (all from Tocris, Ballwin, MO, USA), apocynin (5 μM, Sigma). All histamine receptor antagonists/agonists and apocynin were added 30 min and 1h, respectively, prior to cell treatment and maintained during the course of experiments.

3.2. Phagocytosis assay

- ✓ **Beads**

The murine N9 microglia cell lines were plated in a MW24 with at the density of 2×10^4 cells per well containing sterile glass coverslips (10 mm). Cells were allowed to grew for 24h and then treated for further 6h with LPS (100 ng/mL) and/or histamine (100 μM). Latex beads (*Sigma Aldrich*) were opsonized with rabbit IgG (1 μg/ml, *Sigma Aldrich*) under constant agitation overnight at 4ºC. Then, the beads were ressuspended in modified RPMI medium without $NaHCO_3$ (Sigma Aldrich), and distributed at a density of 1×10^5 beads per well.
After 40 min of incubation, cells were washed with [1]PBS and fixed with 4% paraformaldehyde (PFA, *Sigma*) or methanol/acetone (1:1, *Fisher/Labsolve*) for 30 min at room temperature (RT) or at -20ºC, respectively.Extracellular and/or adherent beads were labeled with

[1] **PBS:** NaCl 140 mM, KCl 2.7mM, KH_2PO_4 1.5mM and Na_2HPO_4 8.1 mM; pH 7.4

secondary antibody Alexa Fluor 594 donkey anti-rabbit (1:500; *Molecular Probes, Oregon, USA*), in PBS, for 1h at RT. For nuclear labeling, cell preparations were stained with Hoechst 33342 (2 µg/ml) (Molecular Probes, Eugene, Oregon, USA) in PBS, for 5 min at RT. Coverslips were then mounted in Dako fluorescent medium (Dakocytomation Inc., California, USA). Fluorescent images were acquired using an AxioObserverLSM710 confocal microscope (Zeiss) under a 63×/1.40 oil objective. For each field, five photomicrographs were acquired in order to capture stained nuclei (in blue), extracellular and/or adherent beads (in red) and total number of beads (differential interference contrast image). The location of each bead was analyzed by comparing the three separate images simultaneously. Only beads without fluorescent labeling were considered as internalized particles.

✓ Phosphatidylserine/ Phosphatidylcholine containing liposomes

The murine N9 mcroglia cell lines were plated in a MW24 at the density of 2×10^4 cells per well containing sterile glass coverslips (10 mm). After 24h, cells were incubated for further 6h with RPMI medium fresh (control), LPS 100 ng/mL and/or histamine 100 µM. Then, fluorescent labelled PS or PC containing liposomes (5µL/well) were added, directly, for 2h. To block PS-induced phagocytosis, Annexin V (4µL/well) waadded 1h prior the incubation with the liposomes. At the end of liposomes incubations, cells were washed with RPMI medium and then fixed with PFA 4% (Figure 15).

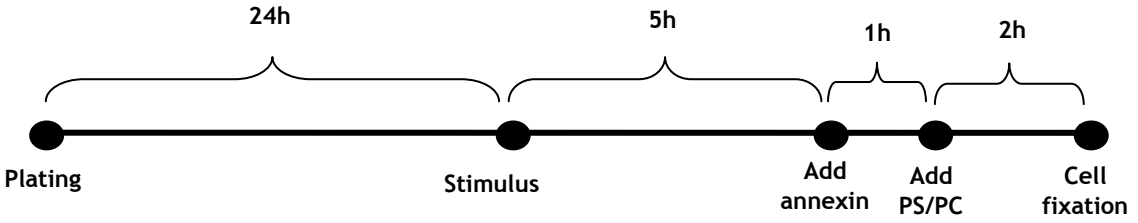


Figure 15 – Treatment of N9 microglia cell cultures for phagocytosis assays *in vitro*. The N9 microglia cell line was plated in glass coverslips placed on MW24 plates until they reach confluence (24h). Then, cells were treated with 100 ng/mL LPS and/or 100 µM histamine for 6h. Liposomes containing PS or PC (5 µL/well) were added to the cells for further 2h, followed by several washes with RPMI medium and fixation with PFA 4%. Annexin V (4 µL/well) was added prior the liposomes incubation to inhibit phagocytosis.

After several rinses with PBS, unspecific binding was prevented by incubating cells in a 3% BSA and 0.5% Triton X-100 solution (all from *Sigma Aldrich*) in PBS for 30min, at RT. Then, cells were incubated overnight at 4°C with the primary antibody: rat monoclonal anti-CD11b (1:600; *AbD Serotec, Oxford, UK*) diluted in PBS containing 0.3% BSA and 0.1% Triton X-100. After 3 washes with PBS (5min each), cells were incubated for 1h at RT with the corresponding secondary antibody: Alexa Fluor 488 goat anti-rat (1:200; *Molecular Probes)* diluted in PBS. For nuclear labeling, cell preparations were stained with Hoechst 33342 (10

μg/ml; *Molecular Probes, Eugene, Oregon, USA*) in PBS, for 5min at RT and mounted in Dako fluorescent medium (Dakocytomation Inc., California, USA).

Fluorescent images were acquired using an AxioObserverLSM710 confocal microscope (Zeiss) under a 40×/1.40 oil objective. For each coverslip, four photomicrographs were acquired in order to capture stained nuclei (in blue), PS/PC liposomes (in red) and microglial cells (in green). To quantify the fluorescence intensity of the liposomes in each condition (sixty-four cells per condition) we deducted the fluorescence intensity of background. The same confocal image acquisition settings were used in all experiments.

3.1. Determination of cellular ROS levels

ROS levels were measured using the probe dihydroethidium (DHE, *Molecular Probes*). In the DHE assay, blue fluorescent DHE is dehydrogenated by superoxide (O_{2^-}) to form a red fluorescent ethidium bromide. Cells exposed for 2h to the stimulus (histamine and/or LPS) were incubated for 4h with 100 μM DHE in culture medium at 37°C. The fluorescence emitted was read in a spectrofluorometer (SpetroMax GeminiEM; Molecular Devices) using Ex/Em 515/605 nm.

3.2. Immunocytochemistry

Cells were fixed with 4% PFA and unspecific binding was prevented by incubating cells in a 3% BSA and 0.5% Triton X-100 solution (all from *Sigma Aldrich*) for 30min, at RT. Then, cells were incubated overnight at 4°C with the primary antibodies: rat monoclonal anti-CD11b (1:600; *AbD Serotec, Oxford, UK*) and mouse monoclonal anti-acetylated α-tubulin (1:100; *Sigma Aldrich*), both diluted in PBS containing 0.3% BSA and 0.1% Triton X-100. After several washes in PBS (3x, 5min), cells were incubated for 1h at RT with the corresponding secondaryantibodies: Alexa Fluor 488 goat anti-rat (1:200; *Molecular Probes)* and Alexa Fluor 594 donkey anti-mouse *(*1:200*)* both diluted in PBS. Membrane ruffling was observed by using a marker for filamentous actin, phalloidin. Cells were incubated for 2h at RT with the phalloidin-Alexa Fluor 594 conjugate (1:100; *Molecular Probes*) in PBS, protected from light.

For nuclear labeling, cell preparations were stained with Hoechst 33342 (2 μg/ml) (Molecular Probes, Eugene, Oregon, USA) in PBS, for 5 min at RT and mounted in Dako fluorescent medium (Dakocytomation Inc., California, USA). Fluorescent images were acquired using an AxioObserverLSM710 confocal microscope (Zeiss) under a 63×/1.40 oil objective.

3.3. Western Blot

Cells were washed with ice-cold PBS and lysed on ice in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM sodium orthovanadate, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, containing 1% of a protease inhibitor mixture (AEBSF, pestatinA, E-64, bestatin, leupeptin and aprotinin) purchased from Sigma-Aldrich. After gentle homogenization, the total amount of protein was quantified using the Bradford method and bovine serum albumin as standard (Bio-Rad Protein Assay, Bio-Rad Laboratories).

Afterwards, samples were loaded onto 12% acrylamide/bisacrilamide gels (*BioRad, Hercules, CA, USA*). Proteins were separated by SDS-PAGE and then transferred to PVDF membranes *(Amersham Hybond*^{TM}-P, GE Healthcare). The membranes were then blocked with 5% non-fat milk (*Regilait, France*) in Tris buffer saline solution-Tween 20 ([2]TBS-T) for 1h at RT. Incubation with anti-acetylated α-tubulin (1:200) (Sigma) diluted in TBS-T was done overnight at 4ºC. After being rinsed three times with TBS-T, the membranes were incubated for 1h at RT with an anti-mouse antibody (1:10000) (GE Healthcare) diluted in TBS-T.

The membranes were then incubated with the ECF substrate (*ECF Western Blotting Reagent Packs, GE Healthcare*) for 5min. Protein bands were detected using the Molecular Imager FX system (Bio-Rad Laboratories) and quantified by densitometry analysis using the Quantity One software (Bio-Rad Laboratories).

[2] **TBS:** 20mM Tris and NaCl 137mM solution; pH 7.6

In Vivo assays

3.1. Animals

All experiments related to the use of experimental animal models were conducted in compliance with protocols approved by 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 (Directive number 2010-63-EU).

In this study were used 44 young adults (8-12 weeks-old) male C57BL6. All animals were kept in appropriate cages, under controlled conditions of temperature (20±2˚C) with a fixed 12h light/dark cycle (7:00 am/7:00 pm), with food and water freely available. All efforts were made to reduce the number of animals used and to minimize their suffering.

3.2. Stereotaxic injections

Young adult C57BL/6 mice (8-12 weeks-old) were injected intraperitoneally (i.p.) with 5µL/g of the mixed solution of Ketamine (90 mg/Kg, *Imalgene® 1000 Inyectable*) and Xilazine *(*10 mg/Kg, *Seton 2% injectable solution)*. In different set of animals, 4 mg/kg apocynin (i.p.) or annexin (10 µg per mice, intravenously) were administrated, one time, 1h before the stereotaxic injections begin and, other time in the next day. Mice were then placed on the stereotaxic frame (Stoelting 51600). Scalp was disinfected with Betadine and an incision was made along the midline with a scalpel. With the skull exposed, scales were taken after set zero at bregma (anterior-posterior (AP)+3.0 and mediolateral (ML)-1.4; corresponding to the right SN). The skull was drilled and the Hamilton syringe was lowered until the target Z, DV (-4.4). Injection of 2 µl of Histamine (100 µM in PBS) and/or 2µl of LPS (100 ng/mL in PBS) was performed at a speed of 0.2 µL/min during 10 minutes (Figure 16). After the needle was removed and the incision sutured, mice were kept warm during recovery (27ºC). Seven days after injections, mice were sacrificed and the brains were removed and frozen for further immunostainings.

3.8. Preparation of the brain tissue

Seven days upon the stereotaxic injections, the mice were transcardially perfused with NaCl 0.9% to clean systemic blood and, then were fixed with a 4% PFA solution. The brains were removed and postfixed in PFA 4% overnight at 4ºC. After this fixation protocol, brains were cryoprotected in 30% sucrose (in 0.1 M PBS) at 4ºC until sinking and were frozen in liquid

nitrogen (±20 sec) and maintained at -80˚C overnight. Before sectioning, the brains were embedded in optimal cutting temperature (O.C.T.) compound and were cut in 35 µm coronal sections using a cryostat-microtome (*Leica CM3050S, Leica Microsystems, Nussloch, Germany*) at -20ºC. The slices corresponding to the *SN* and striatum of each animal were collected sequentially in six weels of 24-well plates (*BD Biosciences, San Jose, California, USA),* free-floating in 0.1M PBS supplemented with 0.12 µM of sodium azide, at 4˚C until immunohistochemical processing.

3.9. Immunohistochemistry against glial markers

Microglia cells and astrocytes were revealed by CD11b (Alpha chain of αMß2-integrin or cluster of differentiation molecule 11B) and GFAP (Glial Fibrillary Acid Protein) markers, respectively.

SN slices were permeabilized with 1% Triton X-100 in 0.1 M PBS, for 45 min at RT. Then, non-specific binding sites were blocked with 10% FBS in PBS for 30 min at RT. Then, slices were incubated with the primary antibodies: rat monoclonal anti-CD11b (1:600; *AbD Serotec, Oxford, UK*) or Rabbit monoclonal anti-GFAP (1:500; *Chemicon, Temecula, USA*), diluted in 10% FBS in PBS, overnight at 4ºC. After several washes (3x, 15 min each) with 1% Triton in PBS, slices were incubated with the respective secondary antibodies Alexa Fluor 495 goat anti-rat or Alexa Fluor 488 anti-Rabbit (all 1:200; *Molecular Probes)* diluted in PBS, for 2h at RT. Sections were then mounted in glass slides with the Dako mounting medium (DAKO).

3.10. Free-Floating immunohistochemistry for Tyrosine Hydroxylase

Free-floating immunohistochemistry was initiated by incubating SN sections on a 10 mM citrate solution (pH 6.0) at 80ºC for antigen retrieval. After cooled to RT inside the solution, slices were placed in water for 5 min and then, were washed for 10 min in PBS-T. Once washed, the sections were blocked with 10% FBS in PBS containing 0.1% Triton X-100 (1h at RT) and then were washed twice for 10 min in PBS-T. For the inhibition of endogenous peroxidases activity brain sections were incubated for 10 min with 3% H_2O_2 in water (protecting slices from the light). This step was followed by two washes of 10 min with PBS-T.

Incubation with the primary antibody mouse monoclonal anti-TH antibody (1:1000; *Transduction Lab BD, San Jose, California, USA)* diluted in 5% FBS in PBS, was performed overnight at 4ºC. After being washed three times (10 minutes) with PBS-T, the slices were incubated for 1h at RT with a biotinylated goat anti-mouse antibody (1:200; *Vector Laboratories, Burlingame, CA*) diluted in 1% FBS in PBS.

The sections were washed three times (10min each) with PBS-T and were then incubated with the avidine–biotin peroxidase complex reagent (*ABC kit from Vector Laboratories, Burlingame, CA*) for 30min at RT. The sections were washed three times (10min) with PBS-T and developed using 3,3'-Diamine Benzidine (*DAB, Sigma-Aldrich, Saint Louis, MO, USA)* in TBS, with 12 μL of 30% H_2O_2. The reaction was stopped by removed of DAB and direct adding of PBS. Sections were then mounted onto Superfrost precleaned coated slides, dehydrated through a graded-ethanol series (70%, 80% 95% and 100%, 3min at each concentration) cleared using xylene and coversliped with a permanent mounting medium Entellan (*Merck, NJ, USA*).

3.10.1. Cell counting and quantitive analysis

The SN doesn't have weel-defined borders with adjacent brain structures so we defined the boundaries between *SN* and ventral tegmental area (VTA) for each brain slice. TH^+ cells were counted if they were stained perceptibly above the background level and only if they contained a nucleus surrounded by cytoplasm. The total number of TH^+ cells for each representative mesencephalic section (4 sections per animal) was calculated under the magnification of x10. All immunohistochemical analyses were performed in at least four animals per experimental group. The results are represented as the value of TH cells per section ± SEM.

3.10.1.1. 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 ± SEM of at least three independent experiments.

Densitometric quantification of immunoblots was obtained using Quantity One software. Statistical analysis was performed using Unpaired t Test or one-way ANOVA followed by Dunnet's Multiple Comparison 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).

Results

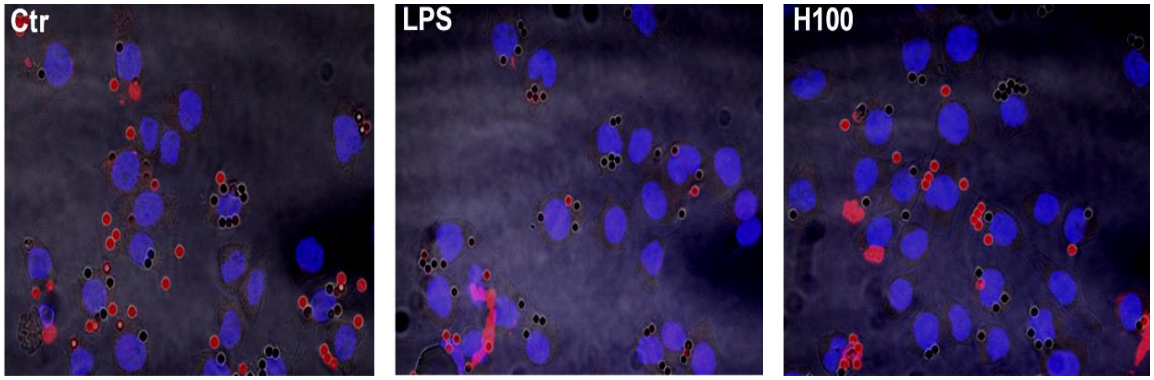
In Vitro assays

4.1. Histamine induced microglial phagocytosis of opsonized latex beads through H1 receptor activation

To evaluate the effect of histamine on microglial phagocytic activity, we quantified the number of phagocysed latex beads *per* cell in a murine N9 microglial cell line (Figure 16A). Microglial cells were treated for 6h with histamine (1, 10 and 100 µM) and/or LPS 100 ng/mL. Thereafter, IgG-opsonized latex beads were added to microglial cells at a density of 2×10^4 *per* well and allowed to be ingested for 40 min. After fixation, non-ingested beads were immunolabeled in order to distinguish extracellular and adherent particles from those internalized. Therefore, phagocysed beads were distinguished from non-phagocytosed beads by means of fluorescent labeling (none versus red, respectively) (Figure 16A). We observed that 100 µM histamine was the most effective concentration in significantly increasing phagocytosis when compared to control cultures ($mean_{Ctr}$=107.2 ± 4.6%; $mean_{H100}$=281.1 ± 28%). At this concentration, histamine did not interfere with microglial cell death or proliferation *(Ferreira et al., 2012)*. As expected, 100 ng/mL LPS also robustly triggered microglia phagocytosis ($mean_{LPS}$=291.3 ± 28.7%) (Figure16B). Histamine exerts its functions through the activation of four distinct receptors (H1R, H2R, H3R and H4R). In order to identify which histamine receptor is involved in phagocytosis, we then pre-treated microglial cells with antagonists for each receptor for 40 minutes before histamine (100 µM) treatment. Our results showed that only H_1R antagonist treatment (mepyramine maleate, 1 µM) significantly reduced histamine-induced phagocytosis when compared with histamine *per se* ($mean_{H100}$=281.2 ± 28.03%; $mean_{AntH1R+H100}$=112.8 ± 8.6%). Moreover, blockade of others receptors [H_2R antagonist, cimetidine (5 µM), H_3R antagonist, carcinine ditrifluoacetate (5 µM), H_4R antagonist, JNJ7777120 (5 µM)] did not abolished histamine-induced phagocytosis ($mean_{AntH2R+H100}$=237.1 ± 22.5%; $mean_{AntH3R+H100}$=236.2 ± 22.02%; $mean_{AntH4R+H100}$=259.9 ± 28.8%). Noteworthily, the treatment with an H_1R agonist (2-pyredylathylamine, 100 µM), mimicked the effect induced by histamine ($mean_{AgH1R}$=204.3 ± 19.3%) (Figure 16B). These data suggest that histamine induces microglial phagocytosis of opsonised beads via H_1R activation.

On the other hand, in the presence LPS-induced lesion, histamine can reduced the number of phagocytosed beads relatively to LPS and histamine *per se*; however, it presents a higher value in relation to the control ($mean_{H100+LPS}$=152,.3 ± 2,8%)

A



B

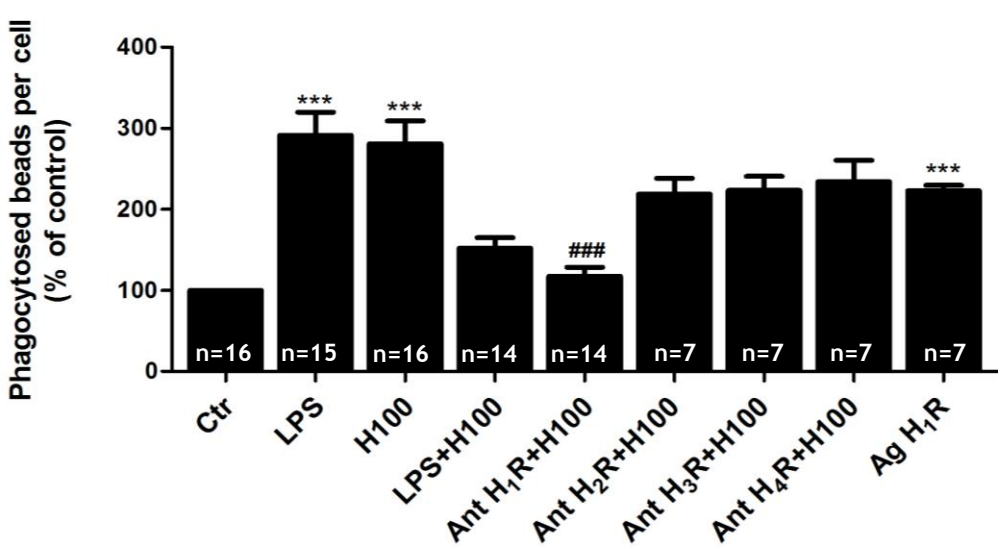


Figure 16 - Histamine induced bead phagocytosis by microglial cells. A) Representative photomicrographs illustrate phagocytosis of IgG-opsonized latex beads in the control, 100 μM histamine, and 100 ng/mL LPS conditions. Red fluorescence indicates non-ingested beads. Hoechst 33342 (blue) staining was performed to label cell nuclei. B) Quantification of fluorescent beads phagocytosed per microglial cell. The number of phagocytosed beads increased in presence of LPS (100 ng/mL) and histamine (100 μM). This effect is mimicked by H1R agonist and blocked by H1R antagonist, suggesting that microglial phagocytosis is mediated via H1R activation. The results are expressed as percentage of their controls (set to 100%). Data are shown as the mean ± SEM. Statistical analysis was performed by using one-way ANOVA followed by Dunnett´s Multiple Comparison Test (***$p<0.001$ compared with control; $^{###}p<0.001$ compared with Histamine 100 μM).

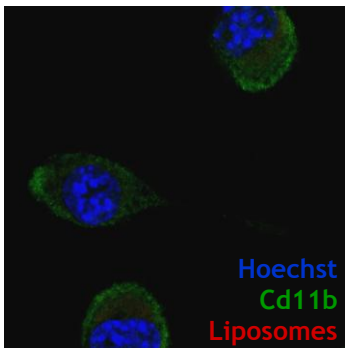
4.2. Histamine induced phagocytosis of PS-liposomes

Microglial cells have a series of phagocytic receptors on their surface which are responsible for recognition of target cells and their elimination. One type of these receptors is able to recognize residues of PS on the surface of apoptotic cells or cells that suffered stress (eg, stimulation with LPS).

In order to determine whether histamine also modulates microglial phagocytosis mediated by the recognition of PS residues, we then incubated cells with fluorescent PC or PS liposomes and quantified the intensity of fluorescence in each cell. For this propose, N9 microglial cells were exposed to LPS or/and histamine for 6 h, followed by an incubation with liposomes (PS or PC) for 2h. In a series of experiments, annexin V was added 1h before liposomes incubation in order to inhibit PS-induced phagocytosis. Finally, cells were fixed and proceed with immunocytochemistry against CD11b, in order to delimitate the microglial cell cytoplasm borders/limits. Four photos of each coverslip were acquired by confocal microscopy and the quantification of fluorescence intensity was performed by Image J (Figure 17).

We observed that both 100 μM histamine or LPS increased the intensity of PS-containing liposomes when compared to control cultures ($mean_{Ctr}$= 99.7 ± 4.7%; $mean_{H100}$= 172.9 ± 31.7%; $mean_{LPS}$= 197.6 ± 55.8%) (Figure 18). In the presence of annexin V (blocker of PS residues, inhibiting PS-induced phagocytosis) the histamine's effects is impaired ($mean_{H100+Ann}$= 89.2 ± 7.30%). Similar values as controls were also observed when a co-administration of LPS and histamine was performed ($mean_{H100+LPS}$= 118.1 ± 18.8%). Lower values of fluorescence intensity as compared with the control were observed in the control condition in which any liposomes were added to the cells (used as negative control) ($mean_{Ctr\ w/\ liposomes}$= 72.5 ± 4.7%). In all experiments, we always added experimental groups containing cells incubated with PC-liposomes (negative control for phagocytosis dependent on PS residues). No statistical differences were found in all experimental conditions as compared with the control. These results may suggest that histamine-induced phagocytosis depends on the presence of the PS presence on the surface of the liposomes (Figure 18).

Control without liposomes



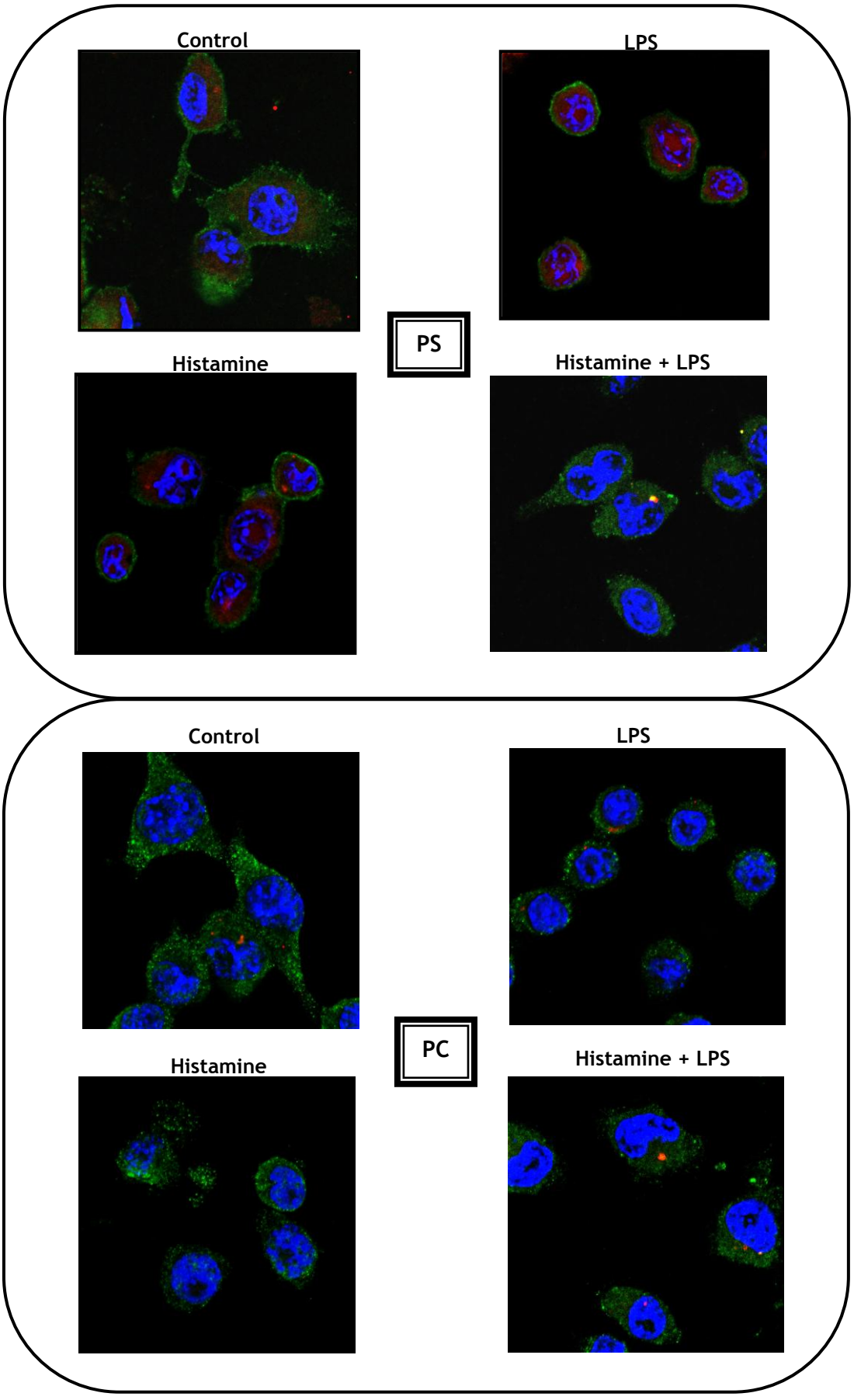

Control
LPS
PS
Histamine
Histamine + LPS
Control
LPS
PC
Histamine
Histamine + LPS

Figure 17 – Fluorescent immunostainning to reveal phagocytosed PS or PC liposomes (in red) in microglial cells. Representative confocal photomicrographs of microglial cells treated with LPS (100ng/mL) or/and 100 µM histamine and, histamine plus annexin V. The immunohystochemistry was performed against CD11b (green) and nuclei staining with Hoechst 33342 (blue). Snaps were taken with the same confocal exposure parameters.

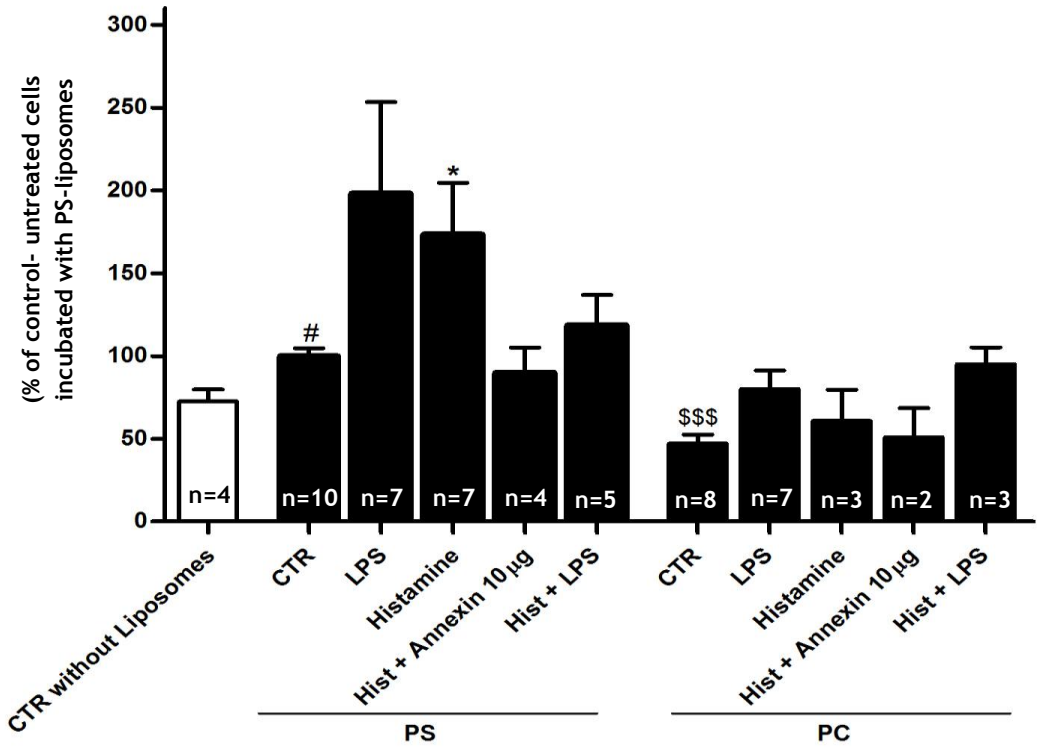


Figure 18 – Quantification of the fluorescence intensity of the liposomes staining (red) per cell. The percentage of intensity of lipossomes phagocytosed increased in presence of LPS (100 ng/mL) or histamine (100 µM). The histamine-induced phagocytosis is blocked by the presence of annexin V (blocker of PS residues, inhibiting PS-induced phagocytosis). In the presence of PC residues (negative control for phagocytosis) the intensity of liposomes is similar in all the conditions. The results are expressed as percentage of their controls (non-treated cells exposed to PS-liposomes; set to 100%). Data are shown as the mean ± SEM. Statistical analysis was performed by Student's t-test (*$p<0.05$ compared with control.PS; $^{\#}p<0.05$ compared with control without liposomes; $^{\$\$\$}p<0.05$ compared with control.PS).

4.3. Histamine induced ROS production via H1R/H4R activation

It was reported that the release of ROS and RNS is a direct consequence of microglial phagocytosis and it is able to cause neuronal death (*Drechsel and Patel, 2008; Hirsch and Hunot, 2009; Peterson and Flood, 2012)*. To evaluate the effect of histamine on ROS release, we stimulated microglial cells with LPS (100 ng/mL) or histamine (1, 10 and 100 μM) for 6h (Figure 18). ROS levels production by microglial cells were measured by the DHE assay. In this assay, blue fluorescent DHE can be dehydrogenated by superoxide (O^{2-}) to form a red fluorescent ethidium bromide. The emitted fluorescence was read in a microplate spectrophotometer plate reader at Emission/Excitation 515/605. As shown in Figure 19, 100 μM histamine significantly increased ROS levels when compared to control ($mean_{H100}$=132.7 ± 3.4%). As expected, LPS at 100 ng/mL also increased significantly ROS levels ($mean_{LPS}$=136.9 ± 2.98%). In order to identify which histamine receptor is involved in ROS release, we then pre-treated microglial cells for 40 minutes with respective agonists or antagonists for each receptor before adding histamine (100 μM) for further 6h. Our results showed that H_1R and H_4R antagonists (mepyramine maleate (1 μM) and JNJ7777120 (5 μM), respectively) significantly reduced histamine-induced ROS release when compared with histamine *per se* ($mean_{H100}$=132.7 ± 3.4%; $mean_{AntH1R+H100}$=111.5 ± 2.6%; $mean_{AntH4R+H100}$=114.9 ± 3.6%). Blockade of H_2 and H_3 receptors did not abolish histamine-induced ROS levels ($mean_{AntH2R+H100}$=125 ± 4.4%; $mean_{AntH3R+H100}$=126 ± 4.3%). It should be noticed that the treatment with H1R or H4R agonists [2-pyredylathylamine (100 μM) and 4-methylhistamine dihydrochloride (20 μM), respectively], mimicked the effect induced by histamine ($mean_{AgH1R+H100}$=122.2 ± 3%; $mean_{AgH4R+H100}$=123.4 ± 1.9%).These data suggest that histamine induced ROS release via H1R and H4R activation.

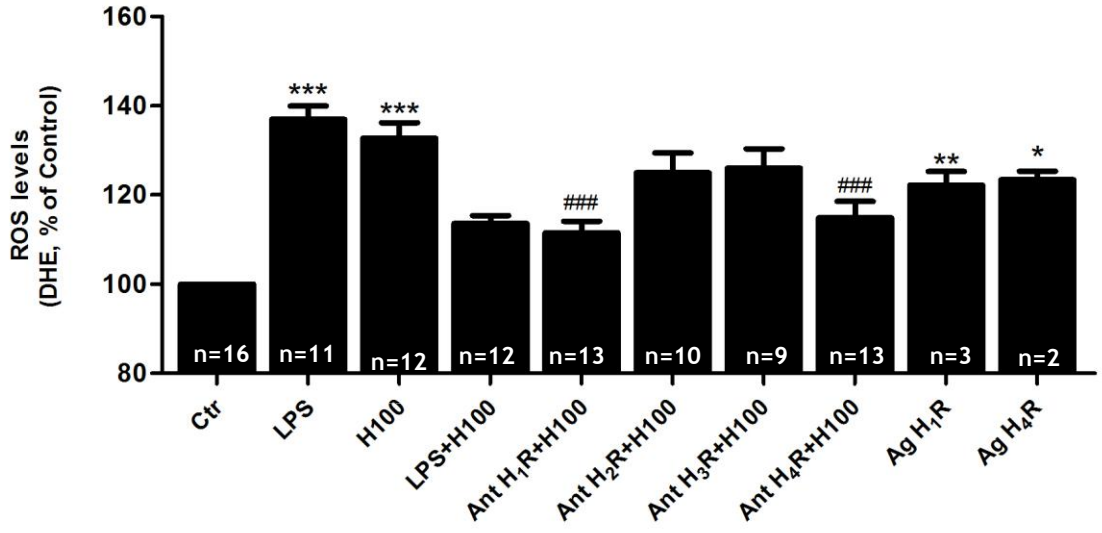
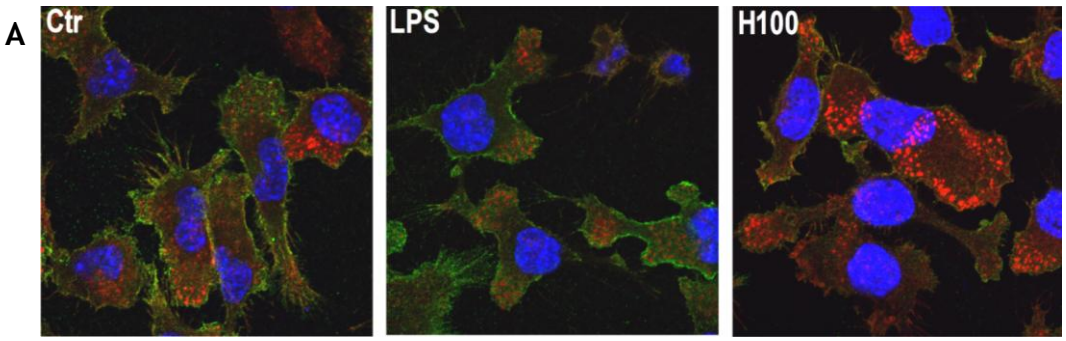


Figure 19 - Histamine increased ROS production via H_1R and H4R activation. ROS levels release by microglial cells were measured by DHE assay. The levels of ROS increased in presence of LPS (100 ng/mL) and histamine (100 μM). This effect is mimicked by H1R/H4R agonist and blocked by H1R/H4R

antagonist, suggesting that ROS production is mediated via H1R and H4R activation. The results are expressed as percentage of their controls. Data are shown as the mean ± SEM. Statistical analysis was performed by using one-way ANOVA followed by Benferroni´s Multiple Comparison Test (*p<0.05; **p<0.01 and ***p<0.001 compared with control; $^{###}$p<0.001 compared with Histamine 100 μM).

4.4. Histamine-induced phagocytosis requires cytoskeleton alterations

Phagocytosis is a process that causes remodeling of the microglial cytoskeleton through actin polymerization. Therefore, we hypothesized that the effect of phagocytosis induced by histamine was accompanied for cytoskeleton alterations. To test this hypothesis, microglial cells were stimulated with LPS (100 ng/mL) or histamine (100 μM) for 1 h for actin evaluation and, 12 and 24 hours for acetylated tubulin evaluation. Microglia cell morphology was assessed using staining against the surface marker CD11b, which is expressed by microglial cells. After fixation, cells were labelled with phalloidin to visualize the alterations in the actin cytoskeleton (Figure 20A, red). In fact, cells treated with LPS or histamine showed a more intense punctuate staining on the phagocytic cups, structures involved in the initiation of phagocytosis. Labeling with acetylated α-tubulin to visualize the alterations in the reorganization of the tubulin cytoskeleton (Figure 20B, red) showed that there was an increase of acetylation of α-tubulin in microtubules in cells treated with LPS or histamine. To confirm these results, we then quantified the expression of acetylated α-tubulin in microglial cells treated with LPS or histamine for 12 and 24 hours. Histamine induced an increase of α-tubulin acetylation levels, showing the highest effect at the 24h incubation timepoint ($mean_{H100\ 3h}$=115.2 ± 7.6%; $mean_{H100\ 6h}$=126.5 ± 15.5%; $mean_{H100\ 12h}$=145.874 ± 2.8%; $mean_{H100\ 24h}$=183.921 ± 36.2%) (Figure 21). LPS showed the higher effect on acetylated α-tubulin levels after 12h treatment. We used GAPDH as housekeeping gene because, it was found that this gene maintains its expression constant with the different stimuli used in these experiments.



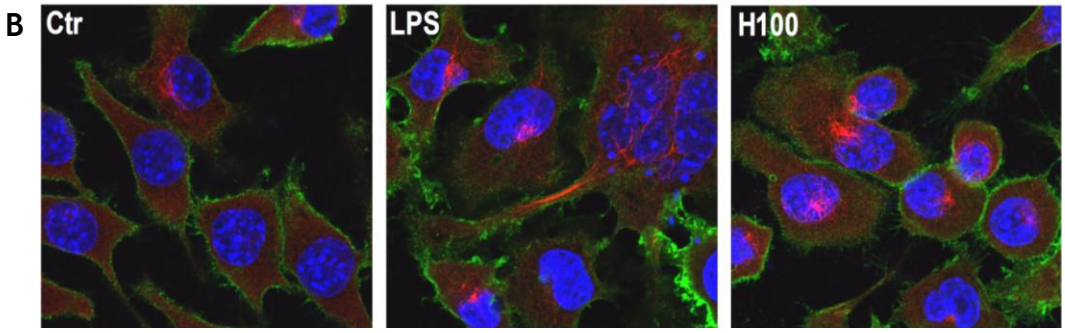


Figure 20 - Immunostaining against cytoskeleton proteins (phalloidin and α-acetylated tubulin) in microglial cells. Representative confocal photomicrographs of microglial cells treated with LPS (100ng/mL) and 100 μM histamine and stained for F-actin filaments (A, red) or acetylated α-tubulin (B, red), CD11b (green) and Hoechst (nuclei in blue). Immunocytochemistries were performed in three independent cell preparations.

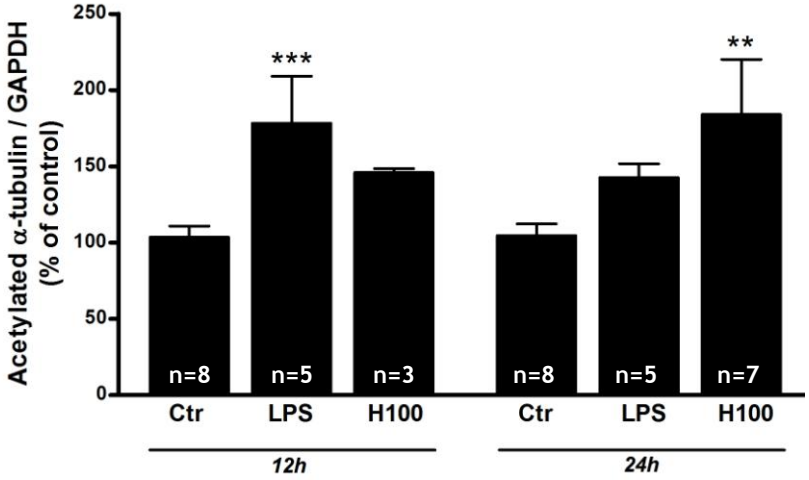


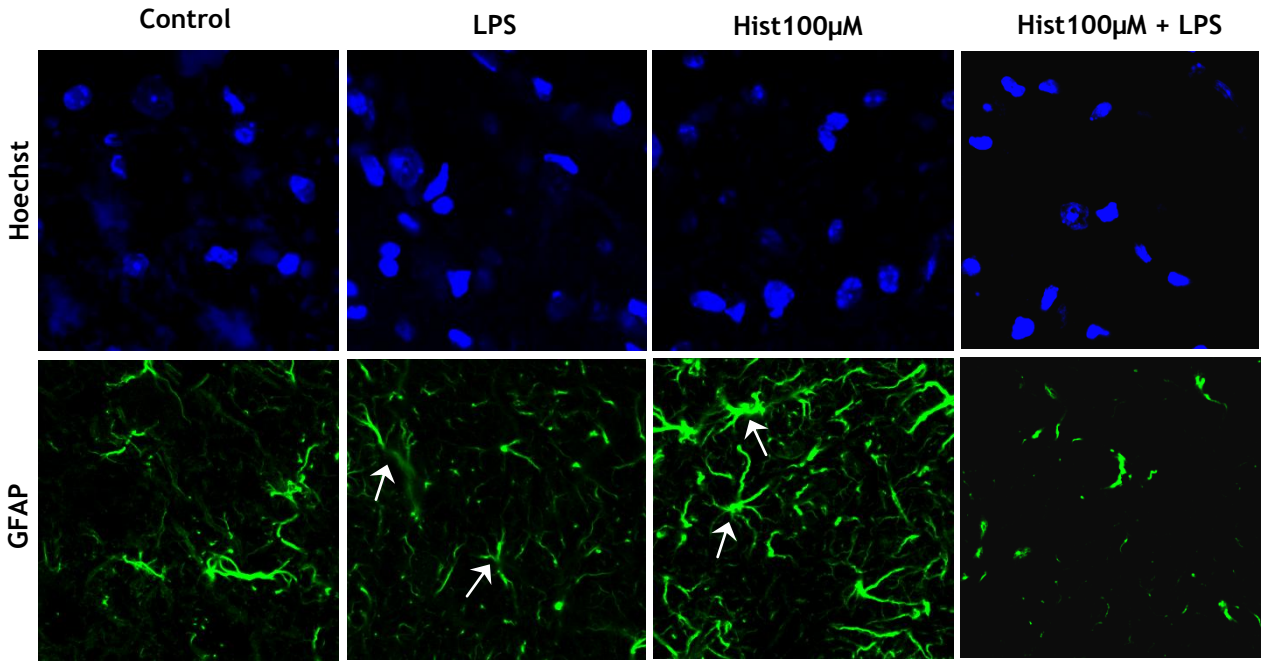
Figure 21 – Quantification of the acetylated α-tubulin protein levels in microglia cells exposed with LPS or histamine. Densitometric quantification of acetylated α-tubulin protein levels obtained in N9 microglial cells treated with LPS (100 ng/mL) and histamine (100 μM) for 12 and 24 hours. The results are expressed as percentage of their controls (set to 100%). Data are shown as the mean ± SEM. Statistical analysis was performed by using two-way ANOVA followed by Dunnett´s Multiple Comparison Test (**p<0.01 and ***p<0.001 relative to control).

In Vivo assays

4.1. Histamine increased glial reactivity *in vivo*

When exposed to brain injury or pathogen invasion, microglial cells proliferate, transform into reactive microglia (amoeboid structure), migrate to the lesion area and recognize and eliminate damaged neurons, apoptotic or stressed cells by phagocytosis (*Kreutzberg 1996; Stence et al., 2001; Dihne et al., 2001; Eugenin et al., 2001*).

Immunostaining against microglial and astrocytes markers is often used to determine glial cell reactivity. First, we analysed whether histamine and/or LPS injected in the SN of adult mice could induce glial reactivity by performing immunostainings against Cd11b (microglia) or GFAP (astrocytes). Representative confocal images of each staining were taken in comparable SN areas of each mouse. Control images concerns the respective uninjected brain hemisphere (contralateral) of the same animals. In presence of histamine or LPS, we observed an increase of intensity of microglial cells and astrocytes staining as compared with control. Moreover, in the presence of each stimulus *per se*, astrocytes showed a very ramified morphology whereas the majority of microglial cells adopted the ameboid shape. In contrast, reduced levels of CD11b and GFAP reactivity were found in the SN of mice that were co-injected with both LPS and histamine, similar to the levels found in the respective control hemisphere (Figure 22). These data suggest that LPS and histamine *per se* induce a dramatic increase in glial reactivity as compared with the control hemispheres; whereas the co-administration showed a glial cellular fenotype similar to the control.



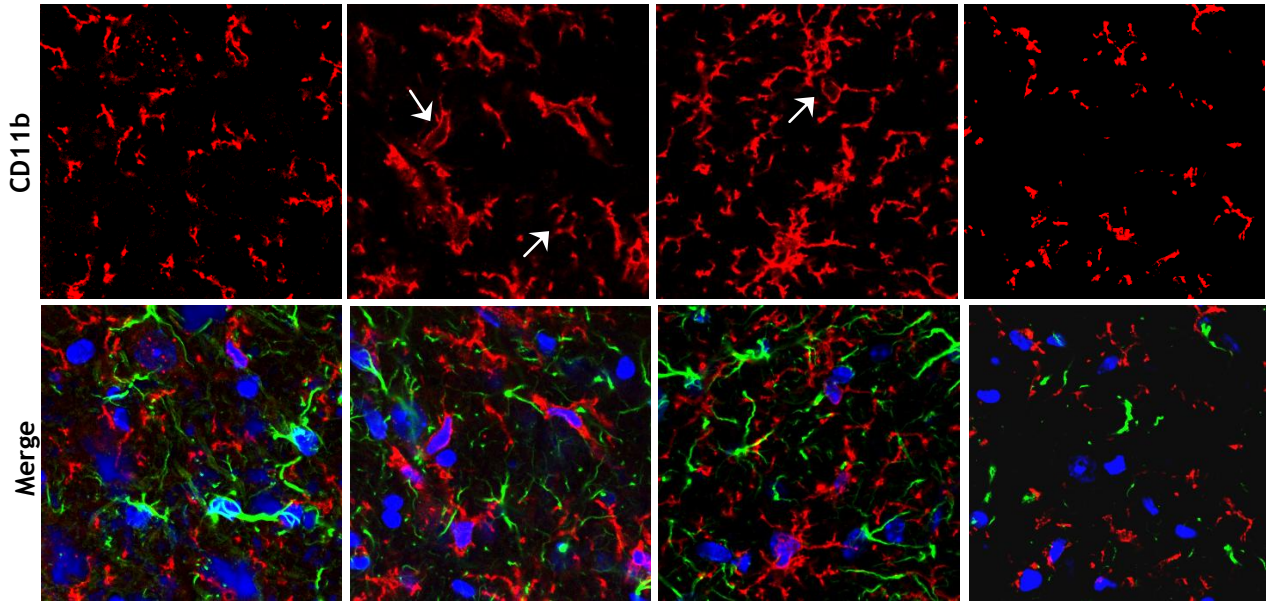


Figure 22 - Immunostainings to reveal astrocytes and microglia in the SN of mice injected with histamine and/or LPS. Representative images of immunocytochemical analysis of brain slices of adult mice stimulated with LPS (100 ng/µL) and/or Histamine (100 µM) for 7 days. The immunohystochemistry was performed against GFAP protein (green) and CD11b (red) and was followed by nuclei staining with Hoechst 33342 (blue). In white arrows have reactive astrocytes in green panel and amoeboid microglia in red panel. Stainings were performed in three independent cell preparations.

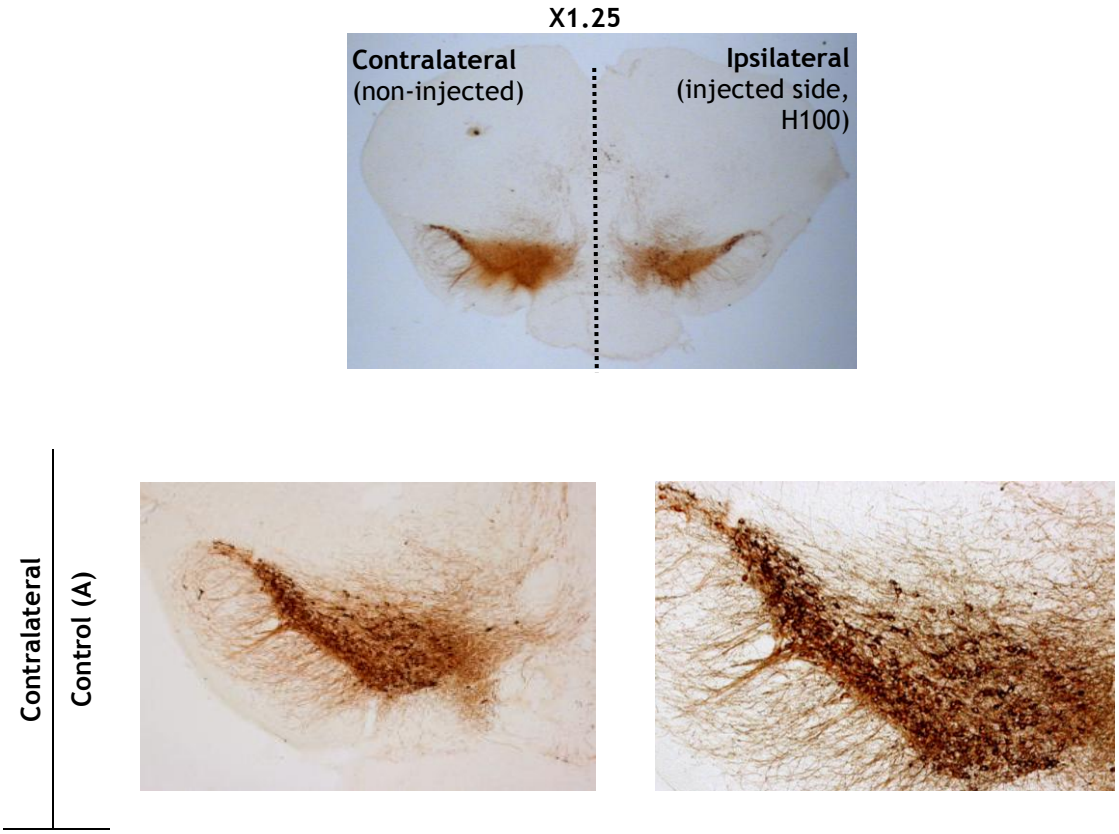
4.2. Histamine modulates dopaminergic neuronal survival

Several studies have been shown that SN is a brain region highly vulnerable to the neurotoxic actions of microglia, mainly due to the release of ROS/RNS and pro-inflammatory cytokines *(Brown and Neher, 2010; Glass et al., 2010; Neher et al, 2011; Labandeira-Garcia et al., 2011)*. Previous studies demonstrated that histamine injected in the SN of adult mice induced neuroinflammation and DA neuronal toxicity. Also, it can cause behaviour alterations characteristic of PD, suggesting that the changes that occur in the production of histamine can be related to the course of PD (*Rinne et al., 2002; Adachi, 2005; García-Martín et al, 2008; Shan et al., 2012; Molina-Hernández et al., 2012; Walker et al, 2013)*.

To evaluate whether histamine-induced inflammation modulates DA neuronal survival *in vivo*, we proceeded to sterotaxic injections with 2 µL of histamine (100 µM) in the SN of mice. LPS (2µL of LPS 1mg/mL) was used as a positive control. After collecting brains we performed immunohistochemistries against TH, a marker of DA neurons. TH^+ cells were quantified on both sides of the brain (Contralateral – Control; Ipsilateral - injection site) using the Image J software (Figure 24). As shown in the Figures 24 and 25, both histamine or LPS *per se* were responsible for the lost of DA neurons in the SN as compared with control ($mean_{Ctr}$=100 ± 3%; $mean_{LPS}$=74.9 ± 6.87%; $mean_{H100}$= 60,6 ± 8%).

Phagocytosis is normally secondary to the target cell dying by other means such as apoptosis (*Savill et al., 2002; Ravichandran, 2003*); however, cell death can be caused by phagocytosis of viable PS-exposed target cells *(Fricker et al. 2012)*. To verify if ROS production and phagocytosis can be involved in the histamine-induced DA toxicity, we then injected apocynin i.p. (ROS production inhibitor) or annexin V i.v. (blocker of PS residues, inhibiting PS-induced phagocytosis) 1h prior histamine injection in the SN. Both pre-treatments fully abolished the histamine-induced DA neurotoxicity (Figure 24 and 25; $mean_{Apocynin+H100}$= 94,701 ± 12,11%; $mean_{Annexin+H100}$= 108,599 ± 4,22%). The injection *per se* of both inhibitors did not change the numbers of TH+ as compared with the controls ($mean_{Apocynin}$= 100.7 ± 12.1%; $mean_{Annexin}$= 120.3± 3,3%). These findings suggest that histamine induces DA toxicity by increasing ROS levels as well as by inducing phagocytosis of vulnerable DA neurons in the SN.

Then, in a set of animals, we co-administrated histamine together with LPS in the SN and counted the surviving TH^+ neurons. Interestingly, the number of TH^+ cells found these mice were similar to the controls (Figures 24 and 25; $mean_{Histamine+LPS}$= 93 ± 6.5%). These data suggests that histamine *per se* acts mainly as a pro-inflammatory agent, inducing the loss of DA neurons in the SN; whereas, in the presence of a strong inflammatory stumulus, such as LPS, histamine induces a neuroprotective effect, reducing neuroinflammation and protecting DA neurons.



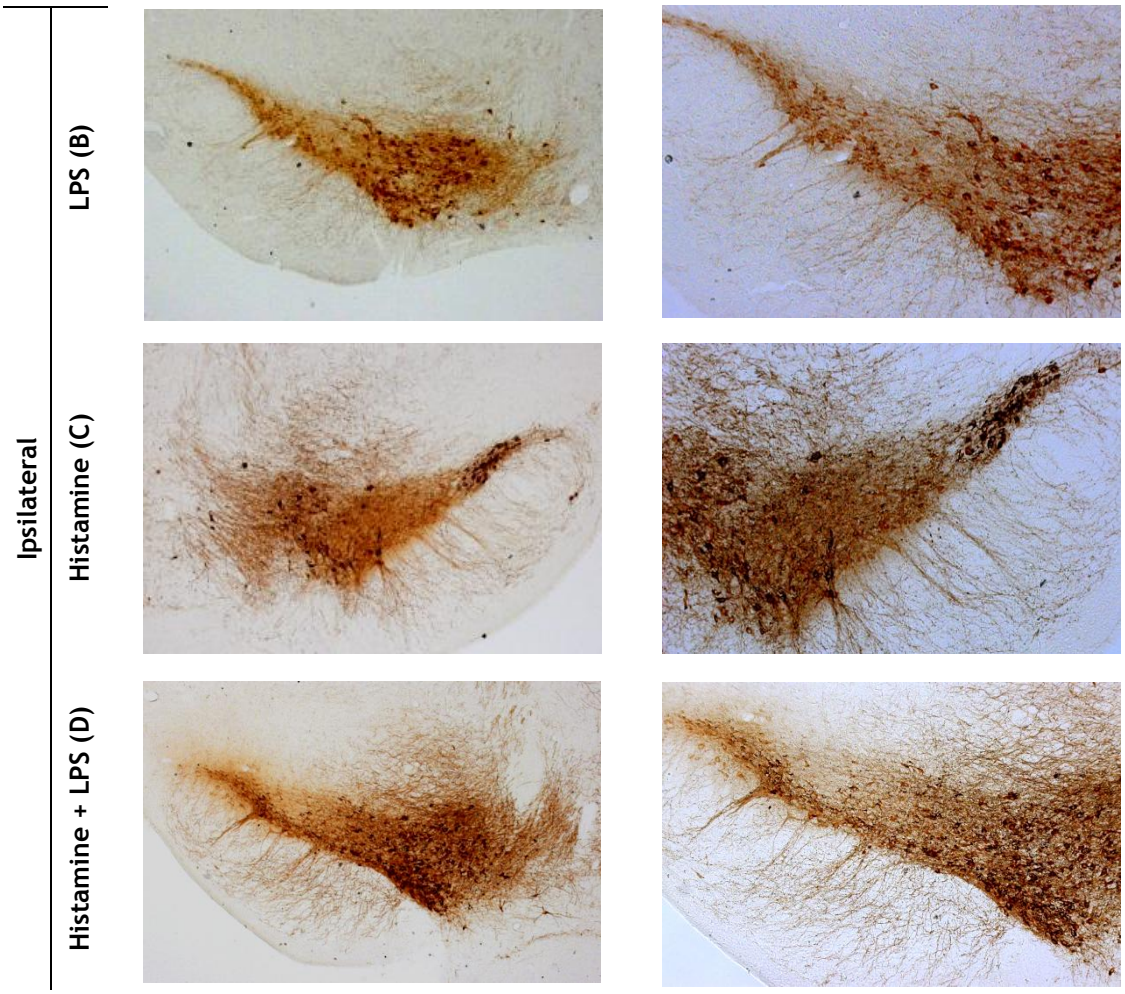


Figure 23 - Representative immunostainings for TH in the SN of adult mice. (A) High numbers of TH^+ cells were found in the controls- contralateral sections (non-injected) of the SN. A notable decreased in the number of TH^+ cells could be observed in mice injected with LPS (B) or Histamine (C) when compared with the control. Similar levels of TH+ cells were found in mice co-exposed to both LPS and histamine (D) as compared with the controls. The TH^+ neurons were counted in four sections of the SN per mouse. The ventral tegmental area – VTA was not included in the quantifications.

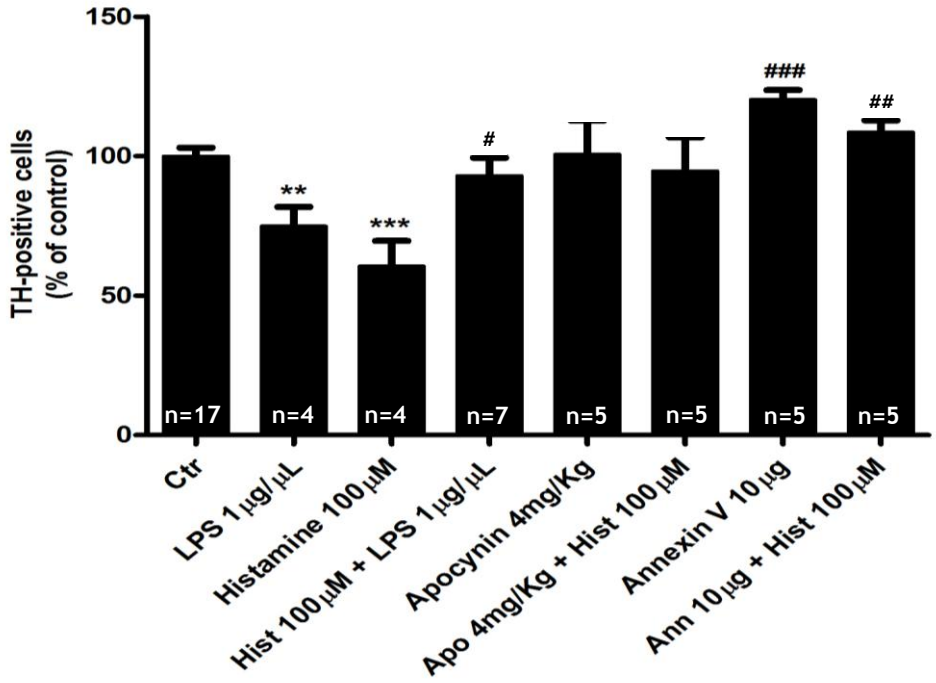


Figure 24 – Quantification of the percentage of TH$^+$ cells in the SN of mice. A significant reduction in the number of TH$^+$ neurons in the SN was found in histamine (100 μM) or LPS treated mice as compared with control. The toxic effect induced by histamine involves the production of ROS and PS-induced phagocytosis, since both apocynin ("Apo", ROS production inhibitor) and annexin V ("Ann", phagocytosis blocker) could blocker the DA toxicity. Surprisingly, the co-administration of histamine plus LPS reverted the loss of DA neurons to levels similar to controls. The results are expressed as percentage of their controls (set to 100%). Data are shown as the mean ± SEM. Statistical analysis was performed by Student's t-test (**p<0,001 and ***p<0,0001 relative to control; $^{\#\#}$p<0,001 and $^{\#\#\#}$p<0,0001 relative to Histamine 100μM).

Chapter V

Discussion

Microglia are generally the first cells of the innate immune system to detect the presence of invading pathogens or injury (*Azuma et al., 2001)*. In these conditions, microglia became amoeboid, migrate to sites of injury, release cytokines, acquire a phagocytic phenotype and generate bactericidal ROS *(Azuma et al., 2001; Konsman et al. 2002; Walter and Neumann, 2009; Kettenmann et al., 2011; Fricker et al., 2012; Suzumura, 2013)*. Microglia activity can be modulated by several soluble and membranar mediators present in the healthy or injured milieu. Recently, our group showed that histamine can modulate microglia migration and cytokines release *(Ferreira et al., 2012)*. In fact, histamine is released by microglial cells. Furthermore, was reported that histaminergic activity was increased in presence of LPS and IL-1ß in microglia primary cultures (*Katoh et al., 2001*). Based on these arguments, the main aims of this study were to investigate the role of histamine and its receptors in microglia phagocytosis and ROS release, and to disclose the consequence of histamine-induced microglia activity on the survival of dopaminergic neurons.

To the best of our knowledge, we were the first showing that histamine significantly increases microglial phagocytosis via the activation of the H1 receptor. This is in accordance with other reports showing that histamine can also induce phagocytosis in other immune cells, such as macrophages. Sternberg and colleagues showed that histamine potentiated interferon gamma IFNγ induced phagocytosis in murine bone marrow macrophages (Sternberg et al., 1987). In addition, the histamine releasing peptide (HRP), known for its ability to stimulate histamine release from isolated mast cells, could also increased macrophage phagocytosis (Cochrane et al., 2003). In contrast, some other reports argue that histamine do not have a role or inhibit macrophage phagocytosis, mainly by the activation of H2R (Radermecker et al., 1989; Azuma et al., 2001). These contradictory reports may be due to the different type of cells used, range of histamine concentrations tested, and different experimental protocols.

Microglial cells express different types of receptors on their cell surfaces that are involved in phagocytic signaling (*Walter and Neumann, 2009*). One of this type of phagocytic receptors (eg, FcRs) that are responsible to generate signals that regulate phagocytosis of immunoglobulin G (IgG)-coated that are formed when the Fc regions of the IgG molecules, that are formed when a small particles (eg. beads) are opsonized with IgG, bind to FcR in the macrophage plasma membrane and initiate a phagocytic response forming a cup-shaped folds around the particle, closing in a few minutes into a phagosome. Then, the interactions between the phagosome and other membranous organelles change its internal and surface chemistries in a maturation process that typically leads to degradation of the phagosome

contents by acid hydrolases. During this process, the NADPH oxidase complex is activated promoting the release of ROS into the phagosome that, consequently, leads to pro-inflammatory reaction *(Kerrigan and Brown, 2009; Jaumouillé and Grinstein, 2010)*.
In this sense, we used oponized latex beads in order to be able to determine this type of recognition and phagocytosis exercised by microglia.

On the other hand, microglial cells have other group of surface's receptors that recognize PS residues present at the surface of some cells that, for example, were subject to certain stressing agent (eg, LPS). These targets will be recognized by microglia leading to their respective elimination (*Ravichandran, 2003; Walter and Neumann, 2009; Armstrong and Ravichandran, 2011; Fricker et al., 2012; Sierra et al., 2013*). The phagocytosis of apoptotic debris is essential and beneficial for the CNS because it reduces the secretion of pro-inflammatory cytokines, chemoattraction and migration of T lymphocytes *(Tremblay et al., 2011*). In our case, we used histamine as stressor agent in microglial cells and we added fluorescent PS-containing liposomes (describe by *Lu et al., 2012* as important residues able to promote phagocytosis). We found that histamine could potentiate the phagocytosis of PS and not PC-containing liposomes. Annexin V was able to inhibit this histamine-induced phagocytosis, demonstrating that this process depends specifically on the exposition of PS residues by liposomes.

It was reported that activated microglia promoted the release of the phagocytic adaptors proteins (eg, MFG-E8) and ROS, responsible by the reversible exposition of the PS residues on surface's neurons that function as "eat-me" signals that can be recognized by microglial cells as targets to be eliminated (*Brown and Neher, 2012; Fricker et al., 2012)*. In general, microglial phagocytosis is beneficial because it removes pathogens or potentially pro-inflammatory debris and apoptotic cells; however, it appears that inflammatory activation of microglia impairs their ability to discriminate between apoptotic and viable neurons for phagocytosis. It is known that inflammation-activated microglia is accompanied by neuronal death and actually, this process is known by phagoptosis or "primary phagocytosis". This cellular lost is very important to remove excess or defective cells, and protects against pathogens and cancer; however it can contribute to the formation of a certain type of diseases *(Neher et al., 2012; Brown and Neher, 2012; Fricker et al., 2012)*.

As previously mentioned, the presence of specific types of surface receptors in microglia is important for the constant immune surveillance of the CNS. The signalling pathways induced by these receptors may lead to the remodeling of the microglial cytoskeleton, through actin polymerization, triggering the formation of pseudopodia that form a phagocytic cup engulfing the target (*Lee et al., 2007; Walter and Neumann, 2009*; *Sierra et al., 2013)*. In fact, we observed that histamine induced a robust actin-staining in the phagocytic cups and an increased acetylation of α-tubulin in microtubules, leading us to believe that histamine-

induced phagocytosis promotes reorganization of microglial cytoskeleton. This data are in accordance with a previous report showing that LPS promote microglial phagocytosis by modulating actin cytoskeleton remodelation and the formation of phagocytic cups *(Ferreira et al., 2011)*.

After exposure to certain chemical signals released from damaged neurons, microglia migrate to the site of injury and becomes activated, adopting proinflammatory behaviour by releasing neurotoxic cytokines, chemokines, an increase of ROS production. The release of cytokines and chemokines can lead to the recruitment of additional inflammatory cells from adjacent blood vessels, and may also engage astrocytes in the proinflammatory response (*Kim et al. 2000; Kim and de Vellis 2005; Kettenmann et al., 2011*). Additionally, activated microglial cells release high levels of ROS which are generated during an oxidative burst that is regulated, principally, by the enzyme NADPH oxidase *(Qin et al., 2005; Barger et al., 2007; Drechsel and Patel, 2008; Hirsch and Hunot, 2009; Mead et al., 2012)*. In the presence of oxygen, this enzyme form NAD^+, protons and O^{2-} *(Walter and Neumann, 2009; Sierra et al., 2013);* and, due to acidic pH into phagosome, the O^{2-} is dismuted into H_2O_2 and, later, into $HOCl^-$ that actively participate in the modulation of signalling pathways involving in microglial phagocytosis *(Block et al., 2007; Sun et al., 2008; Chéret et al., 2008; Walter and Neumann, 2009; Peterson and Flood, 2012; Sierra et al., 2013)*. In our study, we observed that histamine significantly increased ROS levels and this increase occurs via H1R and H4R activation. So far, this is the first report showing that histamine can modulate ROS production by microglial cells.

Reactive microglia contain numerous lysosomes and phagosomes that helps the elimination of damaged neurons, apoptotic or stressed cells. During this process, microglia migrates and accumulates at the site of injury *(Dihne et al., 2001; Eugenin et al., 2001)* where they play a neuroprotective role phagocytosing damaged cells and debris. However, the overactivation of these reactive cells may be associated with neuroinflammation and subsequent brain injury exacerbation. Several studies suggest an involvement of neuroinflammation in the pathological process progression of several neurodegenerative disorders; including in PD (*Hirsch et al., 2012)*. For instance, a single intranigral injection of LPS has been used widely as a model of PD by overactivating microglia and selectively reducing the numbers of DA neurons in the ventral midbrain *(Neher et al., 2011; Sanchez-Guajardo et al, 2013)*. Furthermore, previous results showed that histamine caused death of DA neurons in the SN, suggesting that the changes that occur in the production of histamine are related to the course of PD (*García-Martín at al, 2008; Shan et al., 2012; Molina-Hernández et al., 2012; Walker et al, 2013*). Moreover, during the development of PD, the accumulation of the LB's and LN's occurs mainly in the TMNS, the brain region that produces histamine (*Shan et al., 2012*). In our work, we evaluate whether histamine could induce glial reactivity in the SN of adult mice and how this reaction could modulate dopaminergic neuronal survival/death. We

found that histamine induced the loss of DA neurons and this effect was blocked by pre-administration of annexin V (blocker of PS residues, inhibiting PS-induced phagocytosis) and apocynin (NADPH oxidase inhibitor). This result suggests that histamine was able to promote the reversible exposition of PS residues on DA neurons's surface becoming targets for phagocytosis and killed. Thus, histamine-induced dopaminergic toxicity depends of the activation of the NADPH oxidase complex and the presence of PS residues on target cell's surface.

Previously results obtained by our group suggested that histamine has a dual role for the regulation of microglia activity. Histamine *per se* induced microglia activation, whereas, in the presence of a robust pro-inflammatory stimulus, mimicked by LPS, histamine had an inhibitory action in microglia migration and in the release of IL-1β *(Ferreira et al., 2012)*. Surprisingly, we found that in the presence of LPS, histamine can prevent microglial phagocytosis and ROS production *in vitro* and the number of DA neurons to levels similar to the contralateral hemisphere (non-injected). These data suggests that histamine is able to become an anti-inflammatory agent in the course of neurodegenerative diseases that are accompanied by an inflammatory milieu. We may hypothesize that histamine may inhibit the activity of the NADPH oxidase preventing the ROS release and phagocytosis into by microglial cell and, consequently, revert the loss of DA neurons in the SN. On the other hand, histamine can block, in a certain way, the "eat-me" signs that target cells expose at their surfaces when they are subject a stress stimulus, such as LPS.

Chapter VI

Conclusions

Our results showed that histamine *per se* is a pro-inflammatory agent since it promotes microglial phagocytosis and ROS production (*in vitro),* resulting in a reduction of dopaminergic neurons in the SN of adult mice.

On the other hand, in the presence of LPS, histamine becomes an anti-inflammatory agent since it inhibits microglial phagocytosis, ROS production and DA neuronal cell death induced by LPS.

Altogether, these data give us new perspectives for the future therapeutic use of histamine and/or histamine receptors agonists to treat inflammatory-associated brain diseases, such as PD.

Future Perspectives

- To determine if histamine can also induces microglial phagocytosis *in vivo*. For that, we will inject liposomes (PS/PC) in SN of adult mice and we will evaluate if microglial cells (stained with CD11b) ingested specific types of liposomes.
- To disclose the signalling pathways induced by histamine in the presence or absence of LPS. Knockout mice for NOX could be used to prove that this complex is vital to histamine-induced effects.
- To assess whether protection observed in the presence of histamine and LPS only occurs with this type of LPS-induced injury or, if also occurs with other types of proinflammatory stimulus (eg., zymosan).

Chapter VII

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