UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Evaluation of dopaminergic degeneration influence on endothelial activity in experimental models of Parkinson's Disease

Patrícia Vanessa Almeida Lindeza

Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

Orientadora: Doutora Ana Clara Braz Cristóvão
Coorientadora: Profª. Doutora Liliana Bernardino
Coorientador: Prof. Doutor Yoon-Seong Kim

Covilhã, junho de 2015

"Para ser grande, sê inteiro; nada teu exagera ou exclui; sê todo em cada coisa; põe quanto és no mínimo que fazes; assim em cada lago, a lua toda brilha porque alta vive."

Fernando Pessoa

Acknowledgments

There are lots of people I would like to thank for a huge variety of reasons. This dissertation involved about 9 months, which seemed much more to me, given the way I was welcomed by all members of Brain Repair Group.

It is a pleasure to express my truthful gratitude to all who made this thesis possible with words of encouragement. It will be easy to name all the people that helped to get this done, but it will be tough to thank them enough. I will nonetheless try…

Firstly, I would like to thank my Supervisor, Doctor Ana Clara Cristóvão, for accepted me to integrate the best investigation group ever, for her friendship, guidance, help and support during the course of all this work. Her wise advices help me to grow as an investigator. I also acknowledge her for having believed in my capabilities all the time.

I would like to extend my gratitude to Doctor Liliana Bernardino, my Co-supervisor, for doing so much more than would be possible to do for me. Her friendship, prompt way of being and her work capacity are remarkable characteristics to pursue. Her optimism is an example of life. I would also like to thank to Doctor Yoon-Seong Kim for his help and support. I could not have imagined having a better Supervisor and Co-supervisors. It has been a privilege to be able to learn from their example.

I would like to thank my entire colleagues group for their support, knowledge sharing, force and courage in less good moments, but also for all the moments of fun and happiness that we had together, without a doubt, I have been blessed with a friendly and cheerful group.

I would like to special thank Márcia Fonseca, my snowflake, who as a very good friend, was always willing to help and give her best suggestions. It would have been a lonely lab without her. I thank you for have been present from the beginning and show me that even with a broken foot, we can achieve anything we want. Besides, that's why we're here!

To the exceptional Raquel Ferreira and the “impecável” Tiago Santos, a very special thank, they were part of this. It was because of their prompt availability and help that part of this project went forward. The lab was always more fun with you both! Is impossible to forget such good friends! I’ve learned so much! Thank you!

To Cláudia Saraiva and Catarina Rebelo, my favorite phD students, to all friendship, encouragement, advices for the future and help for the help in the laboratory work. For you, I wish all the best!

To my family, thanks for your endless love, support and encouragement. Thanks for never give up on believing me and for all your kindness even when you can't understand what I am doing so many hours in the lab. Mom, Dad, Sister and Samuel, I love you all.

To Luís Martins, my deep love, for all the care and support throughout my life. Thank you for giving me strength to reach for the stars and chase my dreams. You were part of this. I love you.

Resumo

A doença de Parkinson é uma doença neurodegenerativa cujas causas não se encontram totalmente compreendidas. Os modelos animais de Parkinson são ferramentas fundamentais em investigação permitindo o entendimento dos mecanismos envolvidos na patogénese da doença. O modelo animal ideal deve reproduzir muitas, se não todas, as características da doença de Parkinson. Os modelos atuais de Parkinson para além de não mimetizarem os modelos de exposição a toxinas que se pensa ocorrer nos humanos, não apresentam todas as características moleculares e bioquímicas fundamentais da doença de Parkinson, como por exemplo a acumulação de alfa-sinucleína, tornando-se assim restritivos no que se refere à sua aplicação. Deste modo, é necessário o permanente desenvolvimento de novos modelos que mimetizem a doença de Parkinson nos humanos de uma forma mais consistente. Neste projeto, desenvolvemos um novo modelo através da administração crónica de paraquato, via difusão lenta e prolongada de pequenas doses de paraquato assegurada por cápsulas de difusão osmótica. Este modelo desenvolvido por nós reproduz características importantes da doença humana, como é o caso da acumulação de alfa-sínucleina que não se observa nos anteriores modelos animais da doença de Parkinson induzidos por esta toxina. Em paralelo, estudos recentes têm-se focado no estudo da barreira hemato-encefálica que é uma estrutura pouco explorada nesta doença neurodegenerativa. Evidências clinicas demonstram que as disfunções nesta barreira estão associadas a um número elevado de doenças do sistema nervoso central. O HMGB1 tem sido demonstrado como um sinalizador de inflamação, sendo libertado por células necróticas durante estes processos, e tem como recetor, entre outros, o RAGE. Este recetor entre outras células está presente nas células endoteliais, que são o principal componente da barreira hemato-encefálica, e está envolvido em processos inflamatórios através da promoção da proliferação e migração celular. Apesar da importância deste recetor em doenças como AVC, tumores cerebrais e Alzheimer, não existe informação consistente acerca do seu envolvimento na doença de Parkinson. Em estudos anteriores, outro ligando do recetor RAGE (S100B) apresentou-se aumentando em doentes de Parkinson, e existem evidências de que a neuroinflamação observada em Parkinson compromete o funcionamento normal da barreira hemato-encefálica. Deste modo, existem boas indicações para analisar os níveis de expressão de HMGB1 e do recetor RAGE em modelos experimentais de Parkinson. Os resultados obtidos revelam um aumento da expressão de HMGB1 e RAGE com mais significado nos modelos *in vivo* de PQ e 6-OHDA. Os resultados obtidos sugerem que o papel do ligando HMGB1 tal como do seu recetor na doença de Parkinson, deve ser explorado como forma de perceber se serão marcadores consistentes da neuroinflamação observada nesta doença e se poderão constituir importantes alvos terapêuticos.

Palavras-Chave

Doença de Parkinson, Modelos animais, Paraquato, Neurónios dopaminérgicos, Barreira hemato-encefálica, RAGE, HMGB1

Resumo Alargado

No ano de 1817, James Parkinson descreveu pela primeira vez a doença de Parkinson. Esta patologia caracteriza-se por uma degeneração progressiva da via nigrostriatal dopaminérgica. Os mecanismos moleculares e celulares responsáveis pelo desenvolvimento da doença de Parkinson não se encontram totalmente compreendidos devido, em parte, ao facto de não existir um modelo que mimetize o que se passa nos humanos. Vários estudos apontam para que esta doença tenha uma causa multifatorial, incluindo disfunção mitocondrial, aumentos de stress oxidativo, neuroinflamação, disfunção do proteossoma e agregação de proteínas, como responsáveis pela degeneração dos neurónios dopaminérgicos na *substantia nigra pars compacta*. Esta doença foi a primeira doença neurológica a ter um modelo animal de estudo e a ser tratada através de terapia de substituição de neurotransmissores. Diversos estudos têm demonstrado uma relação entre a exposição a neurotoxinas ambientais e o desenvolvimento da doença de Parkinson. O Paraquato é um herbicida capaz de induzir toxicidade seletiva nos neurónios dopaminérgicos, levando à sua degeneração podendo assim ser utilizado para mimetizar o ambiente patológico observado na doença. Ainda outros modelos de Parkinson incluem o tratamento com 1-metil-4-fenil-1,2,3,6-tetraidropiridina (MPTP) e 6-hidroxidopamina. Contudo, até à data, não existe um modelo capaz de reproduzir completamente as características observadas na doença humana, que apresenta um caracter crónico. Nenhum dos modelos até hoje desenvolvidos apresenta consistentemente a característica fulcral da doença, a alfa-sínicleinopatia, limitando o estudo dos seus mecanismos patogénicos. O modelo ideal deve incluir, se não todas, as principais caraterísticas da patologia em estudo. Este é um desafio difícil, uma vez que surgem continuamente novos dados acerca da patologia. O desenvolvimento de um modelo que se aproximasse da doença crónica observada em humanos representaria um avanço para o estudo dos mecanismos envolvidos na doença de Parkinson. Neste projeto, foi desenvolvido um novo modelo animal da doença de Parkinson através da administração lenta e continua de paraquato ao longo de 4 semanas. Para este efeito recorreu-se a um método que por difusão osmótica garantiu a libertação gradual de pequenas doses do pesticida. Este novo modelo de Parkinson reflete caraterísticas fulcrais da patologia incluindo perda neuronal dopaminérgica e acumulação e agregação de alfa-sinucleína. O modelo animal desenvolvido representa uma ferramenta atrativa para estudar não só os diversos mecanismos envolvidos na doença, mas também, a aplicação de diversas terapias para a doença de Parkinson.

O ambiente inflamatório crónico observado em doentes de Parkinson leva à libertação de citocinas pro-inflamatórias que resultam em claros sinais de inflamação central e periférica do sistema nervoso. De acordo com a literatura, existem evidências de que a inflamação observada em Parkinson pode comprometer o normal funcionamento da barreira hemato-encefálica. Nos últimos anos, o envolvimento desta barreira tem sido estudado em diversas

patologias como o AVC, tumores cerebrais ou a doença de Alzheimer. Em ambientes de inflamação a libertação de citocinas tem-se revelado um forte alvo terapêutico. A molécula HMGB1 trata-se de um mediador de inflamação presente na maioria das células humanas e tem-se revelado fortemente envolvida em processos inflamatórios de diferentes naturezas. O seu recetor principal, RAGE, é um multi-ligando da superfamília das imunoglobulinas descrito pela primeira vez em 1992. Este é amplamente expresso em vários tipos de células, incluindo as células endoteliais conhecidas pelo seu papel fundamental na barreira hemato-encefálica. Os seus diversos ligandos são denominados de moléculas associadas ao dano e são libertadas por células necróticas. Após a sua ativação, esta molécula induz proliferação e migração celular. Estudos recentes têm desvendado o papel deste recetor em doenças como Alzheimer contudo, apesar da importância deste recetor em diversas doenças neurodegenerativas não existe nenhuma informação acerca do seu envolvimento na doença de Parkinson. A literatura indica ainda que as espécies reativas de oxigénio geradas na doença de Parkinson conduzem ao aumento de HMGB1, por outro lado, um estudo envolvendo outro ligando do recetor RAGE (S100B) revelou um aumento da sua expressão em doentes de Parkinson. Deste modo e de acordo com a literatura, existem fortes evidências que reforçam a necessidade de aprofundar a análise da expressão de HMGB1 e RAGE em modelos experimentais de Parkinson. Neste projeto avaliou-se a expressão de HMGB1 e RAGE em modelos *in vivo* e *in vitro* de Parkinson. Os níveis de expressão foram obtidos por Western Blot de tecidos das regiões *substantia nigra* e estriado, recolhidos dos cérebros de animais expostos a diferentes neurotoxinas (paraquato, MPTP e 6-hidroxidopamina), bem como de células neuronais dopaminérgicas (células da linha celular N27) expostas a paraquato, MPP^+ e 6-hidroxidopamina e células endoteliais (células endoteliais da veia umbilical humana) expostas a 6-hidroxidopamina. Os resultados obtidos, revelam um aumento mais expressivo de HMGB1 e RAGE nos modelos *in vivo* de paraquato e 6-hidroxidopamina.

Deste modo, os resultados obtidos indicam que um estudo mais detalhado do papel tanto do ligando HMGB1 como do seu recetor RAGE na doença de Parkinson, bem como dos seus mecanismos celulares e moleculares associados, seria uma mais-valia para melhor perceber se estes têm potencial para servirem como moléculas para monitorizar a inflamação neuronal, bem como para testar potenciais abordagens terapêuticas para o tratamento desta doença.

Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder with no known cure. The animal models, in particular the PD models, are important tools in experimental medical science to better understand the mechanisms involved in disease pathogenesis. The ideal animal model for PD should recapitulate most, if not all, features of the human disease. Actual PD animal models available do not show prominent alpha-synucleinopathy, an hallmark of the disease, leading to their restrictive application to undisclosed important molecular mechanisms responsible for PD development. In this way, the implementation of a new model that mimic PD in a more consistent way will be an important step to improve future investigations. In this work, we developed a novel paraquat (PQ)-based chronic PD model by using osmotic minipumps to assure the continuous administration of low doses of PQ for a longer period. Besides the fact that the exposure paradigm mimics in a closer way what happen in humans, this model also reproduces several key characteristics of the human PD, including the important alpha-synucleinopathy. Recent studies have implicated the Blood-Brain Barrier (BBB) as one of the underexplored brain structures in PD. Clinical evidences indicate that BBB dysfunctions are associated with a number of serious CNS diseases. HMGB1 has been shown to be a long-searched-for nuclear danger signal passively released by necrotic cells inducing inflammation. Its receptor, RAGE, is expressed in various cells, including endothelial cells that are largely present in BBB being involved in chronic inflammation and cell proliferation and migration. Despite the importance of this receptor in several diseases such as multiple sclerosis, stroke, brain tumors, AD and cancer, there is no consistent information about this receptor in Parkinson's disease neuroinflammation. In previous studies, another RAGE ligand (S100B) have been reported to be overexpressed in PD patients, despite there are evidences that neuroinflammation associated with PD can compromise the BBB. In this way, it will be of great importance to analyse the expression levels of HMGB1 and RAGE within PD experimental models. Our results revealed an increase of HMGB1 and RAGE expression levels with more significance *in vivo models,* specifically in PQ and 6-hydroxidopamine models. In this way, the obtained results indicates that a deeper study of the role of both HMGB1 and RAGE in PD will be of great interest to understand they role in PD neuroinflammation and to know if they targeting may serve as a neuroprotective approaches against the development and/or progression of PD.

Keywords

Parkinson's Disease, Animal models, Paraquat, Dopaminergic neurons, Blood-brain barrier, RAGE, HMBG1.

Index

Index of figures

Chapter I – Introduction

Chapter II- Materials and Methods

Chapter III- Results

List of acronyms

6-0HDA- 6-Hydroxidopamine

AD- Alzheimer's Disease

AGE- Advanced glycation end products

ANOVA- Analysis of variance

Aß- ß- Amyloid peptide

BBB- Blood-brain barrier

CNS- Central nervous system

DA- Dopamine

DAT - Dopamine active transporter

HMGB1 - High-mobility group protein 1

LRP-1- Lipoprotein receptor-related protein 1

MPP^+- 1-methyl-4-phenylpyridinium

MPTP- 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine

MTT- (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

NF-kB- Nuclear factor kappa B

PD- Parkinson's Disease

PQ -Paraquat

RAGE- Receptor for advanced glycation end products

ROS- Reactive oxigen species

SN- Substantia nigra

ST- Striatum

SNpc- Substantia nigra pars compacta

TH- Tyrosine hydroxylase

Chapter 1-Introduction

1.1. Parkinson´s Disease (PD)

1.1.1. Definition and Pathophysiology

Parkinson's disease (PD), described in 1817 by James Parkinson [1-3]. This neurodegenerative disease affect an important fraction of world population. It is estimated that 1-2% of the population over 55 years of age is affected by PD and its prevalence dramatically increases after this age, illustrating the effect of aging in this disease [4-6]. In the US it is previsted that in 2040, the population aged 65 years and older will be as high as 80 million [4, 7, 8]. There is no curative treatment [4, 9] and the current management is limited to supportive care and treatment that partially alleviates disease symptoms but does not slow the disease progression [10]. PD is the second most common neurodegenerative disease and is characterized by a severe loss (~50-70%) of dopaminergic neurons in the *substantia nigra* (SN) [1, 11] and of its fiber projections in *putamen* and *caudate nucleus* with the consequent loss of dopamine (DA) levels in the *striatum* (ST), resulting in motor control impairment [2] (Figure 1A and B). Another pathological hallmark of PD is the observation of intracytoplasmic inclusions called Lewy bodies, containing alpha-synuclein and ubiquitin in dopaminergic neuron, [1, 2, 4, 11] (Figure 1C).

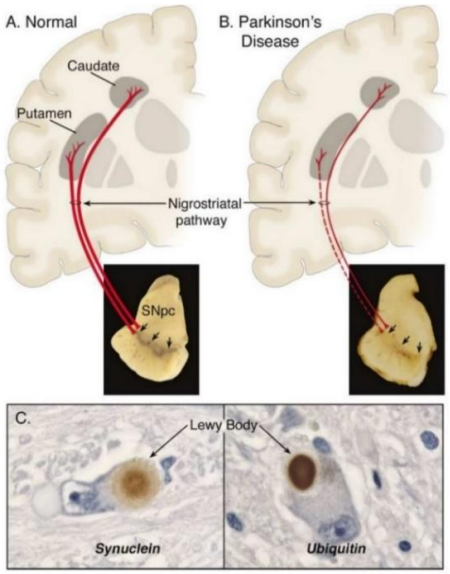

Figure 1- Schematic representation of PD hallmarks. (A) In a normal nigrostriatal pathway the pigmentation of the SNpc produced by neuromelanin within the DA neurons is projected to the ST. (B) In PD the degeneration of the nigrostriatal pathway result in loss of dark-brown pigment neuromelanin due to marked loss of DA neurons and decrease of fibers projecting to ST. (C) Intraneuronal inclusions (Lewy bodies) showing alpha-synuclein and ubiquitin. Figure from Dauer and Przedborski (2003) [12].

The PD pathogenesis is characterized by motor problems of patients and several non-motor features [3]. Impaired motor function is typically used to establish the clinical diagnosis of PD and result from nigral neuron degeneration and consequent decrease in dopaminergic striatal innervation, (Figure 1) [6, 11]. The main symptoms are bradykinesia, rigidity, falls, tremor, speech and swallowing difficulties and postural instability with an asymmetric onset spreading to become bilateral with time [3, 13]. The non-motor symptoms such as depression [14], anxiety, apathy [15, 16], psychosis [17], and sleep disturbance, have greater significance when assessed by quality-of-life measures or health economics [3, 13, 18].

The precise ethiology of PD has been under investigation for almost two centuries [19]. Approximately 95% of PD cases are sporadic with no apparent genetic linkage to the pathology resulting in idiopathic PD. There are some risk factors for PD, such as, ageing or environmental exposure to toxins like the herbicides in example *(i.e)* paraquat (PQ) and the synthetic heroin analogue 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) resulting in acquired PD [1, 10, 19] . On the other hand, genetic risk factors include mutations in an ever increasing list of genes which affect either protein metabolism or mitochondrial function, such as Pink1 (PARK6) [20], Parkin (PARK 2) [21], DJ-1 (PARK7) [22] and alpha-synuclein (PARK 1) [23], thus highlighting that the dysfunction in either is sufficient to cause PD in 5% of the cases [12, 19, 24].

1.1.2. Mechanism of Neurodegeneration

Decades of investigation of toxin-based models and genetic models of PD, as well as of sporadic PD patients, have unveiled a number of potential molecular biomarkers of pathology that include protein aggregation [25], proteasomal stress, oxidative stress [26], mitochondrial dysfunction, lysosomal dysfunction and aberrant autophagy proving that PD is a multisystem disorder [6, 27].

Oxidative stress remains a keystone of the concepts underlying the loss of dopaminergic neurons in PD [6, 28, 29]. The mitochondria produces an important amount of reactive oxygen species (ROS). In addition, SN neurons are subjected to oxidative stress derived from their own endogenously occurring dopamine metabolism [30]. Also, it is known that Nox1-generated superoxide is implicated in the oxidative stress elicited by PQ in DA cells [29].

Oxidative stress may induce PD due to altered accumulation of iron in SNpc, changes in calcium channel activity, altered proteolysis (proteasomal and lysosomal), changes in alpha-synuclein aggregation, and the presence of mutant proteins (*i.e*. DJ-1) [6].

The effect of mitochondrial dysfunction has been discussed for over 3 decades and is considered an important contributor to the pathogenesis of PD [6]. The discovery that MPTP impair the mitochondrial electron transport chain by inhibiting complex I lead to subsequent studies that allowed the identification of abnormalities in the activity of this complex resulting in cellular oxidative stress [2].

There are several molecular mechanism contributing to the neurodegenerative process of dopaminergic neurons in the SN in PD. Some of these mechanism are represented in (Figure 2). It is important to consider not only the oxidative stress and mitochondrial dysfunction, but also the changes occurring in the inflammatory environment as important contributors to PD pathogenesis [30].

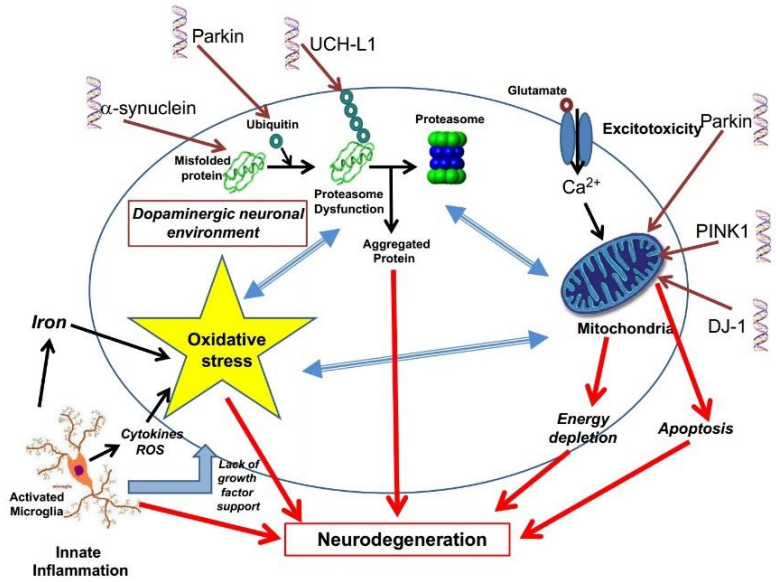

Figure 2 - Key molecular mechanisms that result in neurodegenerative processes in PD. The innate inflammation plays an important role trough activated microglia that in association with proteasome and mitochondrial disjunction lead to oxidative stress. In the cause of these dysfunctions are genetic mutations or neurotoxins exposure. Figure from D.T Dexter and P. Jenner (2013) [6].

1.1.3. **Inflammatory Response**

The term "inflammation" refer the complex biological response of the immune system to cell injury and tissue damage. Also, this response occurs after exposure to toxic proteins, infection, or abnormal molecular signals [31]. Chronic inflammation is an important feature of this neurodegenerative disease [32]. In PD patients, pro-inflammatory cytokines are increased in the brain and cerebrospinal fluid. Also in pre-clinical animal models of PD there are obvious signs of central and peripheral inflammation [33].

Microglia is thought to play a several role in the Central Nervous System (CNS) innate immune response [31]. They are the resident innate immune cells in the brain being only 5-15% of the

whole population of cells [32]. In healthy conditions, microglia generally exhibit a surveying phenotype and perform a scavenging role by removing debris and waste material from the parenchyma. These actions are enhanced following infection, tissue damage, accumulation of toxic protein, or other triggering signal [31, 34].

Activated microglia produce ROS during neuroinflammatory process through intracellular peroxidases, cell surface NADPH oxidase activity and oxidative processes in mitochondria [26, 34]. High levels ROS can damage or inactivate proteins leading to aberrant intracellular signalling, cellular degeneration and death [31]. Pro-inflammatory molecules released by activated microglia such as HMGB1 [35], interleukin-1 (IL)-1, tumour necrosis factor-alpha (TNF-α), and nitric oxide (NO) can be neurotoxic. For instance, the NO reacts with superoxide (O_2^-), produced by activated microglia, producing highly reactive peroxynitrite anions ($ONOO^-$) leading to DNA base modifications. These events lead to a disruption of enzymatic function by altered transcription due to DNA damage resulting in loss of structural protein integrity, which can generate cellular apoptosis or necrosis [26].

1.1.4. Animal models

Animal models of PD have been widely used to explore the pathogenesis and pathophysiology of this neurodegenerative disorder [7, 36]. Various pesticides, herbicides and drugs have been used in animals and *in vitro* models of PD [37]. Animal models are essential tools in experimental biomedical science to better understand pathogenesis of human diseases [28, 38], providing the opportunity to test different therapeutic approaches [28, 38]. The classification of these models depends on systemic or local (intracerebral) administration of neurotoxins that are capable to reproduce most of the pathological and phenotypic features of PD in mammals [36]. Actually, the major animal models used for PD are the ones induced by the toxins rotenone, PQ, MPTP and 6-Hydroxidopamine (6-OHDA), (Figure 3).

The ideal model should reproduce the clinical and pathological features of PD such as progressive loss of dopaminergic neurons and deposition of LB-like inclusions in brain, however no animal model reproduce all the features of the human disease [18]. Naturally, actual animal models of PD have their own specificities and limitations, which must be carefully taken into consideration when choosing the one to be used [12, 36].

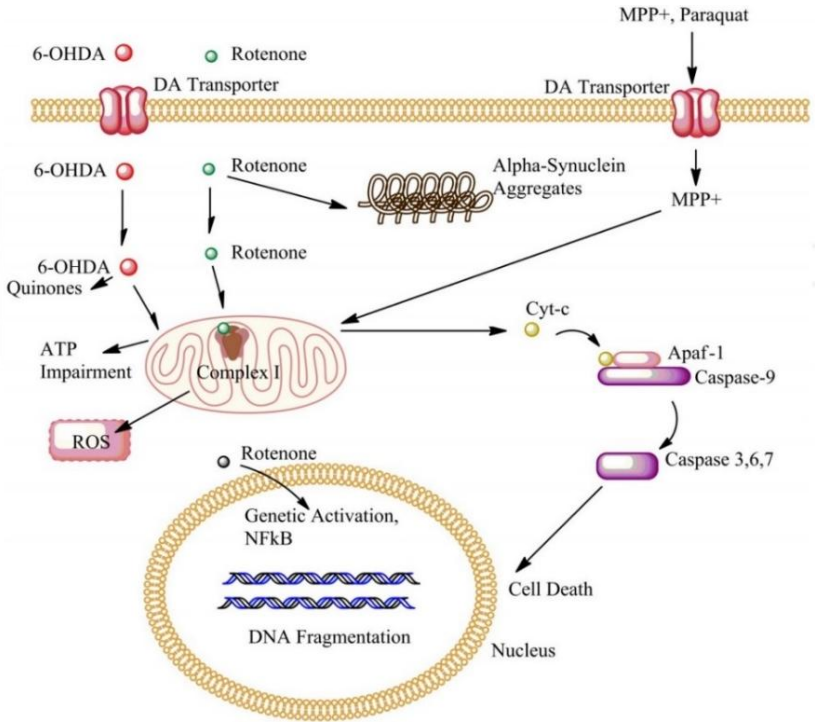

Figure 3- Molecules like PQ, rotenone, 6-OHDA and MPP^+ are currently used to produce experimental models of PD. These neurotoxins easily cross cell membrane through the dopamine transporter (DAT) thus inducing the formation of alpha-synuclein aggregates and mitochondrial impairment. These events result in ROS production and eventually in cell death. Figure adapted from Cabezas, R *et al*. (2013) [37].

PQ Model

Exposure to the herbicide 1,1'-dimethyl-4,4'-bipyridinium or PQ, used in agriculture, is considered a putative risk factor for PD [39]. The toxicity of PQ appears to be mediated by the formation of superoxide radicals [12, 40]. The effects of PQ through oxidative stress mediated by redox cycling, generates ROS. The superoxide radical, hydrogen peroxide, and hydroxyl radicals can lead to the damage of lipids, proteins, DNA and RNA [7, 41]. In this way, PQ can induce PD-like lesions in certain mouse strains and rats [24].

Recent studies have shown fundamental features of PD induced by PQ exposure, such as, the selective degeneration of dopaminergic neurons [42], dopamine depletion in the ST, alpha-synuclein up-regulation [43], as well as lipid peroxidation [44]. In result, the PQ-based model represent great importance to PD research due to its ability to induce increases in alpha-synuclein and Lewy body-like inclusions in dopaminergic neurons in the SN [12, 43].

PQ is structurally similar to 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP [7, 24, 28, 41] however their biochemical lesions are substantially different. The primary mechanism of MPP^+ toxicity is the impairment of mitochondria. This impairment result in excess radical formation and subsequent oxidative/nitrosative stress. The toxicity

induced by PQ is mainly by cellular redox cycling [24, 28, 41]. There are reports revealing that PQ also target complex I and III of mitochondria [24].

MPTP Model

The MPTP administration is one of the most common animal models used to study PD and has shown to produce permanent parkinsonism in humans, non-human primates and rodents, by exerting an effect primarily of mitochondrial complex I function [24, 28, 45]. The mechanism of MPTP toxicity has been extensively studied and characterized, [12, 46]. MPTP can rapidly cross the blood-brain barrier due to his lipophilic nature [12] being posteriorly metabolized in astrocytes by monoamine oxidase-B, and subsequently converted to the active toxic cation MPP^+ that is released from the nigral and striatal astrocytes through the organic cation transporter 3 into the extracellular space [12, 46]. MPP^+ is a polar molecule that is not able to enter dopaminergic cells freely, thus, its uptake depends on active plasma membrane carrier systems, being taken up by neighbouring dopaminergic neurons and terminals through the DAT [12, 46, 47]. Within the mitochondria, MPP^+ lead to production of ROS and decreases the synthesis of adenosine triphosphate by blocking the complex I that interrupt the transfer of electrons from complex I to ubiquinone [12].

The MPTP model of PD have some limitations, most of protocols of MPTP administration apply acute drug treatments and do not mimic the progressive nature of PD [28, 36]. The chronic MPTP model may overcome this limitation however long-term administration of MPTP in smaller doses may result in the recovery of motor behaviour deficits. In addition, the MPTP model does not directly mimic the systemic mitochondrial impairment found in PD [28, 36].

6-OHDA Model

The classic model based on local (*i.e.* intracerebral) injection of a neurotoxin is the 6-OHDA model, which was also the first PD animal model ever generated [36, 38]. This model was used to cause lesion of the nigrostriatal dopaminergic pathway in the rat, being used today for both *in vitro* and *in vivo* investigations [7, 48].

The neurotoxin, 6-OHDA, is structurally similar to dopamine and norepinephrine presenting high affinity for the plasma membrane transporters of these catecholamines [45]. 6-OHDA cannot cross the blood-brain barrier [12, 38], being most commonly injected unilaterally to the SN, medial forebrain bundle, or ST [46]. When delivered to the ST, 6-OHDA induces progressive and partial damage to the nigrostriatal pathway [46].

The 6-OHDA, once inside of neurons is readily oxidized and produces hydrogen peroxide and paraquinone, both of which are highly toxic and 24 hours after 6-OHDA injections

dopaminergic neurons start degenerating and die [12, 45]. The degree of loss is dependent on the injection location and dose of the toxin, as well as the survival time following the lesion. However, like many other PD models, this model lacks the progressive, age-dependent effects of PD and does not produce extra-nigral pathology or Lewy body-like inclusions [45, 46].

PD is a chronic disease developed gradually with the typical symptoms expressed over a long period of time [28]. Neurotoxins such as PQ, 6-OHDA and MPTP used to induce dopaminergic neurodegeneration, mainly by ROS generation, have received the most attention [12]. Few models so far reproduce the progression of extranigral and alpha-synuclein pathology pathology that characterizes PD [28, 45]. Eventually, the ideal model would exhibit all the clinical and pathologic features of PD, but this may be a difficult challenge.

1.2. HMBG1-RAGE

The HMGB1 previously known as amphoterin, is a DNA binding protein and an important mediator of inflammation via receptors of the innate immune system that are present in the cell nucleus of most mammalian cells [49-51].

There are a few number of identified receptors for HMGB1, among them the principals are the toll-like receptor (TLR) 4 and receptor for advanced glycation end products (RAGE) [52, 53]. These receptors are increased in neurons and glia cells resulting in acute and chronic CNS injuries [52, 54].

The functional role of HMBG1 depends upon its location. Inside the nucleus, HMGB1 acts as an architectural protein that binds DNA, where it exerts different roles and functions, once outside the cell it acts as a pro-inflammatory cytokine [55]. As an extracellular protein, HMGB1 exerts autocrine and paracrine effects. It is responsible for activation of nuclear factor kappa B (NF-κB), diffuse endothelial activation, systemic activation of inflammatory cells, stimulation of innate immune cell migration and activation [55]. To act as inflammatory mediator, HMGB1 must be released by active secretion from living inflammatory cells or from necrotic cells. HMGB1 is the only nuclear protein that have the capacity to induce cytokines and activate inflammatory cells when it is applied extracellularly [56]. After its release from stressed and necrotic cells, HMGB1 triggers inflammation, induces cell proliferation, migration and survival, mainly through interactions with RAGE [57].

RAGE was first described in 1992 [58] as a multi-ligand receptor of the immunoglobulin superfamily of cell surface molecules that can interact with several ligands named damage-

associated molecular pattern molecules, released by dying or necrotic cells during tissue damage [57, 59-62]. The ß-amyloid peptide (Aß), S100B, HMGB1 and advanced glycation end products (AGE) are examples of these molecules and are represented in (Figure 4) [59-61]. AGEs are a product of a series of reactions, the initial one being nonenzymatic glycoxidation that is followed by mechanisms involving ROS [61]. This receptor is expressed in cerebral endothelial cells, neurons, macrophages, monocytes and microglia [63-65]. In humans and mice, the gene encoding RAGE is located on chromosome 6 resulting in a protein with a molecular weight of about 55 kDa [66].

RAGE acts like a pattern recognition receptor being involved in inflammation resolution responsible for tissue repair or alternatively, through its perpetuation, may results in chronic inflammation [62]. Recently, a study developed by Li and colleagues [64], suggest that intracerebral Aß interaction with RAGE at the blood-brain barrier (BBB) up-regulates endothelial cognate ligand chemokine ligand 5 expression and causes circulating T cell infiltration in Alzheimer Disease (AD) brain [64]. The RAGE-ligand interaction lead to up-regulation of RAGE (Figure 4) via positive feedback loop. The RAGE activation increases pro-inflammatory cytokines secretion promoting inflammatory cell recruitment. RAGE also activates pathways responsible for acute and chronic inflammation [66] that have been associated with various diseases such as vascular disease, diabetes, cancer, and neurodegenerative disorders like AD [67, 68] suggesting that RAGE might be an effective target to treat many different diseases [62, 66].

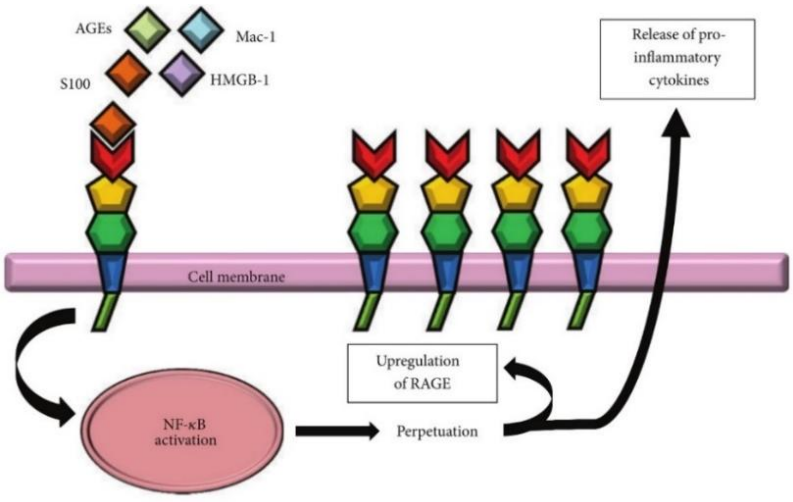

Figure 4- RAGE signalling resulting in sustained inflammation by activation of the transcription factor NF-kB. Figure from Chuah Y. *et. al.* (2013) [66].

1.3. Blood-Brain Barrier (BBB)

Recently, the role of the pathophysiological importance of the BBB in neurological disorders and the influence of its physiological changes in boosting the neurodegenerative process has been intensively investigated [69].

The BBB is localized at the interface between the blood and the cerebral tissue [70, 71], and it is formed by endothelial cells of cerebral blood vessels (Figure 5) which present intercellular tight junctions and the polarized expression of many transport systems. The transport systems at BBB are the carrier-mediated transport (glucose, amino acids, water-soluble vitamins), the active-efflux transport (low-molecular-mass metabolic products) and receptor-mediated transcriptosis (peptide-specific receptors) [70, 72-74]. The surface area of these microvessels is the largest interface for blood–brain exchange [72]. The BBB endothelial cells, together with pericytes, astrocytes and microglia, separates the components of the circulating blood from neurons and forms the functional neurovascular unit [75]. The crossing of components through the BBB in an uncontrolled way generates neurotoxic products compromising synaptic and neuronal dysfunction. This can happen due to an ischemic injury, intracerebral hemorrhage, neurodegenerative process, inflammation, or vascular disorder, this typically [75]. This barrier have several other functions in the brain. Some of that functions are the supply of the brain with essential nutrients and the efflux of waste products. Also, the BBB protects the brain from fluctuations in ionic composition that can occur after meals or exercise, which may disturb the synaptic and axonal signalling. Globally, the BBB plays an important role to maintain a tightly regulated microenvironment for reliable neuronal signalling [69, 72, 76].

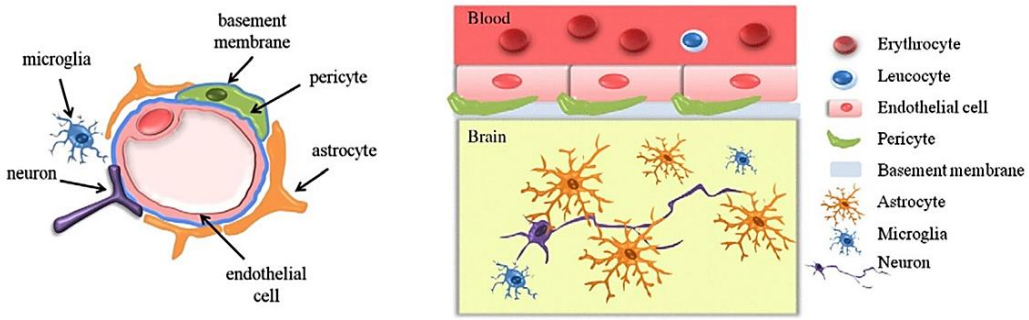

Figure 5- The BBB is composed by endothelial cells, basement membrane, astrocytes, microglia, neurons and pericytes. Brain microvascular endothelial cells acts as mediators between blood and brain interacting with the basement membrane and cells of the neurovascular unit such as neurons, astrocytes and microglia. Figure from Cardoso, F. *et al.* (2010) [73].

1.3.1. Blood-brain barrier in PD

BBB is one of the underexplored brain structures in ageing and PD and its dysfunction is associated to a number of CNS diseases such as multiple sclerosis, stroke, brain tumors, epilepsy or AD [70].

Recently, i*n vivo* studies have shown that BBB dysfunction is related to the course of PD [77]. A study developed by Gray, M. *et al*. [77], used histologic markers of serum protein, iron, and erythrocyte extravasation to demonstrate significant increased permeability of the BBB in the postcommissural putamen of PD patients. Another investigation in PD patients, reported an elevated uptake of the P-glycoprotein (Pgp) substrate [^{11}C] verapamil in the midbrain, which is consistent with disturbed Pgp function described by other authors such as Bartels, A. L., *et al*. [76, 78]. Altogether, these events could facilitate the accumulation of toxic compounds in the brain.

Taking as an example animal models of AD, Aß accumulation is first seen in the neighbourhood of blood vessels. The toxicity including the endothelium and astrocytes is observed before significant neuronal loss and disturbances of CNS homeostasis. These events happens as a result of barrier deficiencies contributing to exacerbate the later neuropathology [76]. Moreover, observations of post-mortem brain tissue from AD patients revealed a number of brain endothelium alterations, such as decreased number of mitochondria, increased number of pinocytosis vesicles, collagen accumulation in basal lamina and necrosis [70].

More recently, studies reached the identification and functional characterization of peptides and proteins transport through the BBB. The transport of Aß through cerebral endothelium is now well known, as shown in (Figure 6), Aß peptide influx into the brain is dependent on Aß chaperones and mediated by RAGE [79]. Due to a lower expression of lipoprotein receptor-related protein 1 (LRP-1) and increased expression of RAGE [79-81], the influx of Aß increases into the brain. Moreover, soluble forms of LRP-1 are detected in lower amounts conditioning the normal sequester of Aß peptide that increase in the brain [81]. In summary, these observations strongly suggest that AD progression may involve the age-dependent alteration of Aß transport across the BBB via RAGE and LRP-1 pathways [70].

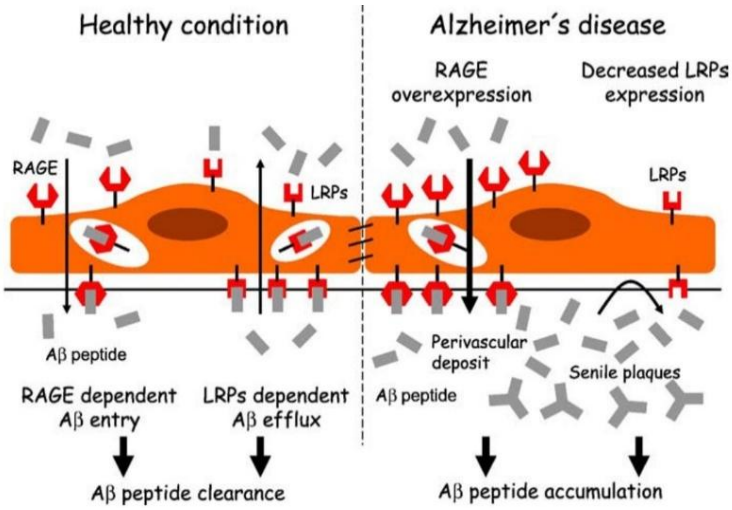

Figure 6- The BBB in AD. The Aß peptide is transported to brain by RAGE and cleared from the brain to the blood by LRPs, in healthy conditions (right). In AD (left), RAGE is overexpressed and the expression of LRPs is decreased, leading to the accumulation of Aß in the brain. Figure from Weiss, N. *et al*. (2009) [70].

In PD the involvement of ROS and inflammatory processes in neurodegeneration is evident [61]. Recent studies suggest that AGE-RAGE–induced cytosolic ROS production facilitates mitochondrial superoxide production. This fact show the evident role of the advanced glycation pathway in the development of disorders such as diabetic or nephropathy [82].

One of the principal factors linked to the induction of inflammation in PD pathogenesis is the NF-kB [83-85]. According to previous studies, RAGE ligation leads to a sustained activation of NF-kB pathway [86]. Due to an enhanced level of RAGE ligands in chronic disorders, this receptor is hypothesized to have a causative effect in a range of inflammatory diseases [57]. Moreover, ROS involved in PD pathogenesis such as increased levels of hydrogen peroxide, induces the secretion and release of HMGB1 by macrophages, monocytes [56, 87]. The use of antioxidants such as ethyl pyruvate [88] or green tea [89] have shown a protective effect in inflammatory response, by decreasing systemic HMGB1 accumulation. Although the interaction of HMGB1 with RAGE was shown to play a major role in oxidative stress-associated diseases its role in PD pathogenesis remains unclear [90].

1.4. Main Goals

There are several models for PD, however few *in vivo* models clearly exhibit the most important hallmarks of the disease which is alpha-synuclein pathology. With this in mind, we proposed to develop a new PQ-based rat PD model that closely recapitulates cardinal features of PD including dopaminergic neuronal loss and alpha-synuclein pathology markers providing an attractive tool to evaluate the pathologic mechanism as well as various therapeutics approaches for PD. In order to better understand PD pathogenesis, we also propose to evaluate the interplay between the BBB and the dopaminergic neurodegenerative process, induced by the activation of RAGE by its ligand HMGB1, which is so far underexplored in PD.

The main goals of this study were:

- Development a new animal model for PD by chronic administration of PQ using osmotic minipumps;

- Characterize the protein expression profile of HMGB1 and RAGE in experimental models of PD.

- Evaluation of the dopaminergic degeneration influence on endothelial activity, through HMGB1-RAGE pathway.

Chapter 2- Material and Methods

2.1. Animal Models and Treatment Paradigm

The experiments were carried out on rats and mice, in accordance with protocols approved by the national ethical requirements for animal research, and in accordance with the Directive 2010/63/EU of the European Parliament and the Council on the protection of animals used for scientific purposes. The experiments *in vivo* involved 8-10 weeks old male wistar rats for the PQ model and 8-12 weeks old C57BL6 mice for MPTP and 6-OHDA model. All animals were kept in appropriate cages, under temperature/humidity-controlled environment on a 12-hr light/dark cycle with free access to food and water. All efforts were made to reduce the number of animals to be used for the study and to minimize their suffering.

2.1.1. PD rat model induced by chronic exposure to low doses of PQ

The chronic administration of PQ was carried out using osmotic minipumps (Alzet Durect, Cupertino, CA) at a dose of 2.5 mg/kg/day with a fluid delivery rate of 0.25 μL/h for a period of four weeks (Alzet model 2004, large pumps). The pumps were implanted subcutaneously on the back, slightly posterior to the scapulae (shoulder blades). All the rats were weighed at day 1 and every other day for 5 weeks. One week after the end of infusion (5 weeks after implantation), all animals were anesthetized with 5 μl/g of ketamine and xylazine (900 and 500 μg in 4,9 ml 0,9% NaCL total volume, respectively) euthanized and the brains were then recovered (Figure 7). For Western blot analysis, eleven (5 saline and 6 treated) brains were collected and total protein lysates from SN and ST were prepared. For immunohistochemistry studies, animals were anesthetized, euthanized by transcardial perfusion with 0.9% NaCl followed by perfusion with 4% paraformaldehyde (PFA). Following perfusion with saline and 4% PFA, brains were removed, and immersion-fixed in 4% PFA overnight and cryoprotected in 30% sucrose. Serial coronal sections (40 μm) were cut on a cryostat, collected in cryopreservative solution, and stored at -20ºC until processed for immunohistostainings.

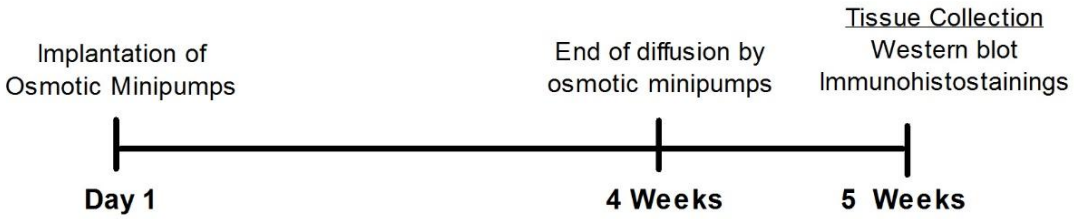

Figure 7- Timeline experiments for PQ chronic administration carried out *in vivo*.

2.1.2. MPTP Model

MPTP (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in sterile 0.9% NaCl and injected intraperitoneally (*i.p*) four times in the same day, each injection separated by 2 h intervals. The experiments were carried out on 8-12 weeks old male C57BL6 mice and the dose used was 15 mg/kg body weight, been the total dose after the 4 injections of 60 mg/kg [91]. Saline group (four mice) were exposed to the same procedure, receiving an equivalent volume of sterile 0.9% NaCl. Seven days after the MPTP exposure (Figure 8), all animals were anesthetized with 5 µl/g of ketamine and xylazine (900 and 500 µg in 4,9 ml 0,9% NaCL total volume, respectively) and euthanized by transcardial perfusion with 0.9% NaCl and the brains were then recovered. Afterward, for Western-blot analysis, brains were collected and total protein lysates from SN and ST were prepared.

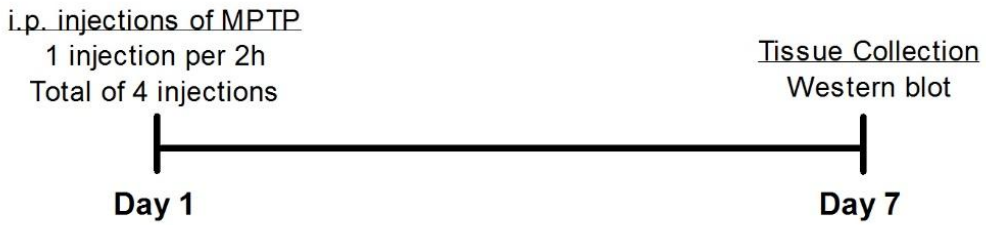

Figure 8- Timeline experiments for MPTP performed *in vivo*.

2.1.3. 6-Hydroxydopamine Model

6-OHDA (Sigma-Aldrich) was dissolved in sterile 0.02 % ascorbic acid. Mice were deeply anesthetized with 5 µl/g of ketamine and xylazine (900 and 500 µg in 4,9 ml 0,9% NaCL total volume, respectively) and placed in a mice stereotaxic apparatus, and a site in the right ST (coordinate: anteroposterior (AP), -0.6 mm; dorsolateral (DL), -2mm; dorsoventral (DV),-3 mm relative to *bregma*, in accordance to [92]), was selected to inject 10 µg of 6-OHDA at a rate was 0.2 µl/min [93]. Afterward, the syringe was kept in place for additional 5 min before

being slowly retracted. Mice were sacrificed after 3 and 7 days (3 and 2 mice respectively). All animals were anesthetized and euthanized by transcardial perfusion with 0.9% NaCl and the brains were then recovered (Figure 9). For Western blot analysis, brains were collected and total protein lysates from SN and ST Ipsi and Contralateral hemispheres were prepared. The ipsilateral side was considered the brain hemisphere injected with 6-OHDA and the contralateral side the non-injected one.

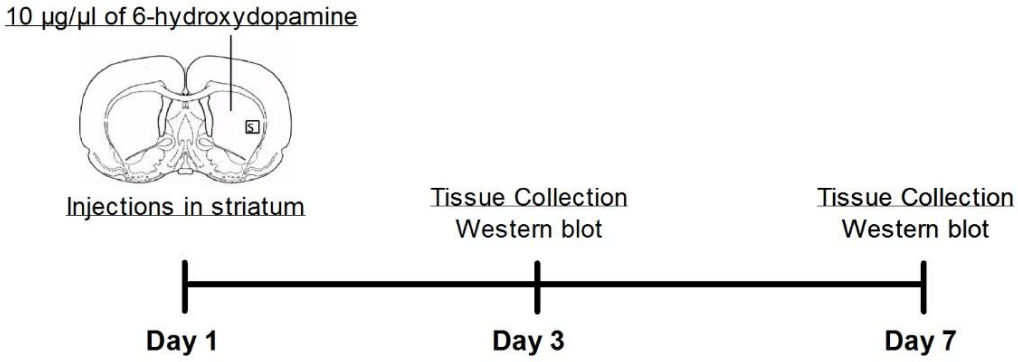

Figure 9- Timeline experiments for 6-hydroxydopamine administration carried out *in vivo*.

2.2. Immunohistochemistry

The TH immunohistostaining was carried out to determine the number of dopaminergic neurons in the SNpc of the PD rat model induced by PQ developed in the present project, by stereological count. We have also evaluated by immunohistochemistry the reactivity of p129 alpha-synuclein antibody in the SN of treated and untreated rats. In this way, sections were incubated at room temperature with blocking solution for 1 hour (5% FBS and 0.3 % Triton X-100 in PBS, pH 7.5). The endogenous peroxidase were inactivated by incubating the section in 3% H_2O_2 for 30 min and afterward with primary antibodies overnight. Finally, sections were incubated with secondary antibodies in blocking solution at room temperature for 1 hour. The primary antibodies used were mouse anti-TH (1:10,000) and rabbit anti-p129alpha-synuclein (1:250). The secondary antibodies used were, respectively, mouse and rabbit biotinylated secondary antibodies (1:200). The staining procedure was performed by the manufacturer's protocol (Vectastain ABC kit, Vectorlab, Burlingame, CA) and the reaction was visualized using 3,3'-diaminobenzidine (DAB) reagent in Tris buffer saline containing 0.02% H_2O_2. Unbiased counting of TH-positive dopaminergic neurons was performed in the SNpc using the optical fractionator method. For each rat brain, the TH+ neurons were count in the SNpc of eight coronal sections, serially selected with 200 µm apart representing the whole SNpc.

2.3. Protein Extraction and Western Blot Analysis

For the western blot protein analysis, the dissected SN and ST brain tissues (*in vivo* setting), and the N27 cells lines (*in vitro* setting), were lysed on ice in RIPA buffer (0.15 M NaCl, 0.05 M Tris-Base, 5mM E ethylene glycol tetraacetic acid, 1% Triton X-100 (Fisher Chemicals, Hampton, New Hampshire), 0.5% deoxycholic acid 0.1% SDS, 10mM dichlorodiphenyltrichloroethane containing a cocktail of proteinase inhibitors). The total protein concentration from lysates was determined using the Thermo Scientific Pierce BCA Protein Assay Kit (Massachusetts, USA) following the manufacturing instructions. To perform the western blot, samples (100 µg of protein of cell lysates or 40 µg of brain tissue lysates) were loaded to each lane of a 12% bis-acrylamide gel (Applichem, Darmstadt, Germany). The proteins were separated by a sodium dodecyl sulfate-polyacrylamigel gel. The running buffer used was Tris-glycine SDS: 25mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). After electrophoresis at 120 V proteins were transferred at constant 300 mA onto a polyvinylidene difluoride membrane (GE, Heathcare, Little Chanfont, UK), using transfer buffer (10 mM Tris-glycine and 20% methanol (Fisher Chemicals). Afterwards, the membranes were blocked for 1 hour or 15 min in 5% low fat milk or 0.1% gelatin (Fluka, St.Louis, Missouri, USA), respectively, in tris-buffer saline containing tween-20 (0.1%) (Fisher Scientific, Massachusetts, USA). The membranes were incubated overnight at 4ºC with the following primary antibodies: mouse anti-HMGB1 (1:500) from (HMGBiotech srl, Milano, Italy); goat anti-RAGE (1:300) and rabbit anti-p129alpha-synuclein (1:500) both from Santa Cruz Biotechnologies; mouse anti-β-actin (1:5000) and mouse anti-GAPDH (1:1000) from Millipore. Specific protein bands were detected using the appropriate secondary antibodies (goat anti-mouse (1:5000), donkey anti-rabbit (1:5000) or chicken anti-goat (1:5000) all from Santa Cruz Biotechnologies) conjugated to horseradish peroxidase and detected by Enhanced Chemiluminescence detection (Millipore). Densitometric analyses of the protein bands were performed using the ImageLab software (Bio-Rad, Hercules, CA, USA).

2.4. Cell Cultures and Treatments

2.4.1. Human Umbilical Vein Endothelial Cells (HUVECs)

The HUVECs were grown in EGM Plus Growth Medium (Lonza, USA) containing 2% FBS and BBE, 100 units penicillin, and 50 µg/ml streptomycin, growth factors, cytokines and supplements. The cells were maintained in a humidified atmosphere of 5 % CO_2 at 37°C. HUVEC cultures

were prepared for experiments by counting the number of viable cells by trypan blue-excluding cells and plating the cells on polystyrene tissue culture dishes at a density of $2x10^5$ cells/well in 6 well culture plates for 24 hours.

2.4.2. Immortalized rat mesencephalic dopaminergic cell culture (N27 Dopaminergic Cells)

The immortalized rat mesencephalic dopaminergic cell (N27 dopaminergic cells) were grown in RPMI 1640 Medium (Sigma-Aldrich) containing 10% foetal bovine serum, 100 units penicillin and 50 µg/ml streptomycin (Invitrogen, Barcelona, Spain), in a humidified atmosphere of 5 % CO_2 at 37°C. N27 cultures were prepared for experiments by counting the number of viable cells by trypan blue-excluding cells and plating the cells on polystyrene tissue culture dishes at a density of $0.5x10^4$ cells/well in 96 well culture plates and $3x10^5$ cells/well in 6 well culture plates for 24 hours.

2.4.3. Co-Culture

A co-culture can be defined as the growth of more than one distinct cell type in a combined culture. Such *in vitro* models can provide a more physiologically relevant way of demonstrating *in vivo*-like tissue morphology and function. Co-cultures can be employed to monitor intercellular communication between distinct brain cell types. In order to understand the intracellular communication between injured dopaminergic cells (N27 cells) and endothelial cells (HUVECs) both cells were growth in a co-culture system. The HUVECs and N27 cells were plated separately, both in 6 well plate, at $2x10^5$ per well. N27 cells were then growth in glass coverslip supported in solid paraffin droplets, and 24 hours after transferred to the 6 well plate containing HUVECs, resulting in co-culture. The co-cultures were then treated with 100 µM of 6-OHDA and kept for additional 24 hours (Figure 10). The cells were maintained in a humidified atmosphere of 5 % CO_2 at 37°C.

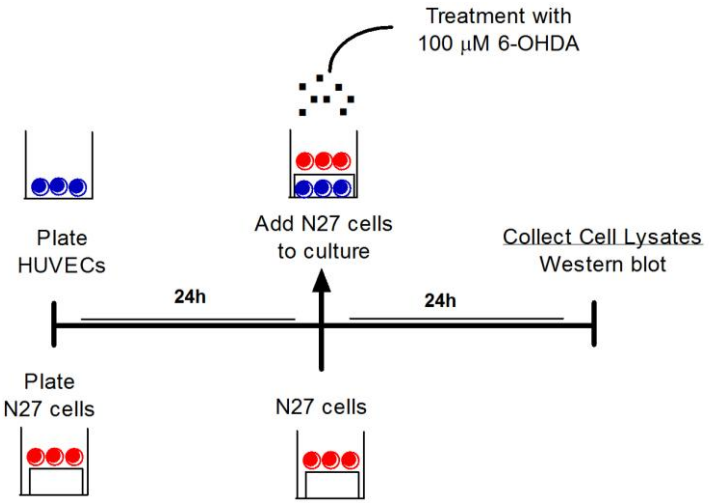

Figure 10 – Schematic representation of co-culture with N27 cells and HUVECs treatment.

2.4.4. N27 Cell Toxins Treatments

To estimate the response of the N27 cells to MPP^+, PQ and 6-OHDA (Sigma-Aldrich), these cells were grown at a density of $3x10^5$ cells/well in 6 well culture plates and then treated for 3 hours with 30 µM of MPP^+, 500 µM of PQ and 10, 25 and 50 µM of 6-OHDA. The treatments were prepared in RPMI 1640. Three hours after starting the treatment, all media containing toxins was removed, cells were quickly washed with sterile phosphate buffered saline (PBS) and fresh medium was added to the wells. Afterward, cells were incubated for further 21 hours (Figure 11). After this time, the cells were used to perform the cytotoxic studies using the MTT reduction assay or were collected to prepare cell lysates for western blot.

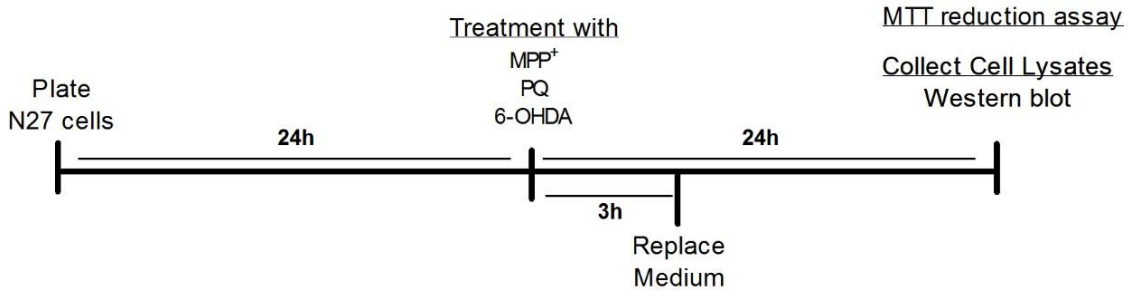

Figure 11- Toxin treatments applied to N27 Cells for a total of 2 days experiment

2.5. MTT Reduction Assay

To assess N27 cells viability after exposure to toxins, the levels of MTT reduction were measured. N27 cells were plated at a density of $0.5x10^4$ cells/well in 96 well culture plates. After exposure to MPP^+ 30 μM, PQ 500 μM or 6-OHDA 10, 25 and 50 μM for 3 hours the medium was replaced by new cell culture medium. The cells was incubated until preamble 24 hours. For the assay using MTT reduction cells were incubated with 0.5 mg/ml of MTT for 4 hours at 37ºC. MTT is converted by viable cells to a water-insoluble precipitate (formazan, presumably directly proportional to the number of viable cells) that is dissolved in 10% sodium dodecyl sulfate-hydrochloric acid (Across Organics) and colorimetrically quantified (O.D. 570-690 nm) using a microplate spectrophotometer (Xmark microplate spectrophotometer, Bio-rad) after overnight incubation at 37ºC with 5 % CO_2. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful marker for viable cells only.

2.6. Statistical Analysis

Statistical analysis was carried out with GraphPad Prism v.5 (GraphPad Software Inc., San Diego, CA). Data are expressed as mean ± standard error of mean (SEM) of at least three animals (*in vivo* studies) or at least three experiments in independent cell cultures. Statistical significance was determined by using one-way ANOVA or Student's *t*-test followed by Bonferroni's *post hoc* test for comparison with control. $P<0.05$ were considered to represent statistical significance.

Chapter 3- Results

In vivo assays

3.1. Chronic exposure to PQ induced the key features of PD in a novel animal model

Extensive efforts have been made to establish experimental animal models that recapitulate key pathologic hallmarks of PD, in order to obtain greater insight into the pathogenesis of disease as well as to test new therapeutic strategies. Here, we characterized important features of a novel animal model of PD. Specifically, we evaluated dopaminergic cell death by counting TH^+ neurons in SN and the distribution of these TH^+ neurons in sequential regions of SNpc.

The deposition of alpha-synuclein into Lewy bodies (LBs) is a major pathological feature of PD [94] and only a few rodent PD models recapitulate this pathological characteristic [43, 46, 95]. In order to evaluate if our new model developed LB-like alpha-synuclein aggregation, we also investigated the phosphorylated form of alpha-synuclein (pS129) expression and aggregation in the SN that was never achieved in any model before.

3.1.1. Chronic exposure to PQ by osmotic minipumps induced dopaminergic neurotoxicity

To investigate the extent of dopaminergic neuronal loss in our new PD rat model induced by slow infusion of a low dose of PQ, the number of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the SNpc were counted using unbiased stereological method.

As shown in (Figure 12 a and b), a significant decrease of 39 ±3.3% in dopaminergic neurons was observed in rats exposed to PQ, when compared with the ones exposed to vehicle. To evaluate if a specific region of the SNpc was preferentially affected by chronic exposure to PQ, we sequentially counted dopaminergic neurons in the representative sections of the entire SN from anterior to posterior (Figure 12 d). Even though significant a DA neuronal loss was observed over the entire SNpc, the most prominent loss (~2 fold reduction) was found in the middle sections of the SNpc (Figure 12 c) when compared with the same sections in vehicle animals.

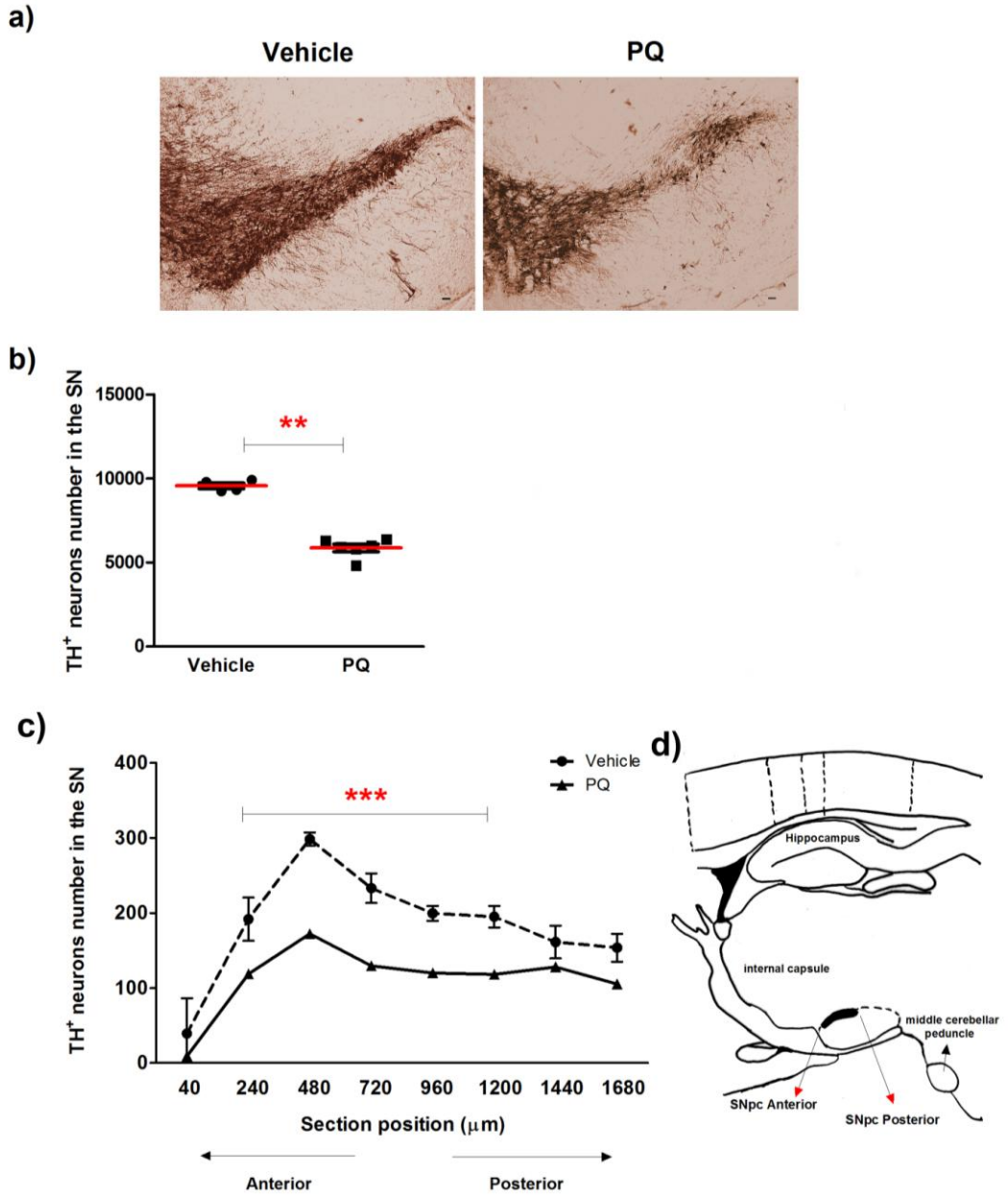

Figure 12- Dopaminergic neuronal degeneration induced by chronic exposure to PQ. (a) Representative photomicrographs of TH-immunostaining and quantitative analysis (b) of the number of TH-positive dopaminergic neurons in the SN of rats after 5 weeks exposure to PQ. TH-positive neurons were stereologically counted. (c) Quantitative analysis of TH-positive neurons, along the SN in section from anterior to posterior, collected with 240 μm apart. Scale bars = 500 μm. Data are shown as the mean ± SEM. (d) Sagittal representation of the rat brain depicting, among others, the SNpc. Adapted from [96]. Statistical analysis was performed using the Student *t* test. **P*<0.05; ***P*<0.01 and ****P*<0.001.

3.1.2. Chronic exposure to paraquat by osmotic minipumps induced alpha-synuclein phosphorylation

To investigate the expression of the phosphorylated form of alpha-synuclein in our new PD rat model, we then quantified the expression levels of pS129 alpha-synuclein by western blot. In parallel we obtained representative photomicrographs of pS129 alpha-synuclein immunoreactivity in the SN.

Western blot analysis of the SN tissues obtained by macroscopic dissection showed that PQ significantly increased the level of pS129 alpha-synuclein protein (296.7 ± 43.62%; n=4; *P*<0.01), when compared with vehicle (100.0 ± 18.49%) as shown in (Figure 13 a). The increase in pS129 alpha-synuclein protein was further confirmed in the SNpc region of the coronal tissue sections by immunohistochemistry (Figure 13 b). Taken together, these results show that a low-dose PQ induced dopaminergic neuronal loss in the SNpc and cytoplasmic inclusions of pS129 alpha-synuclein aggregates.

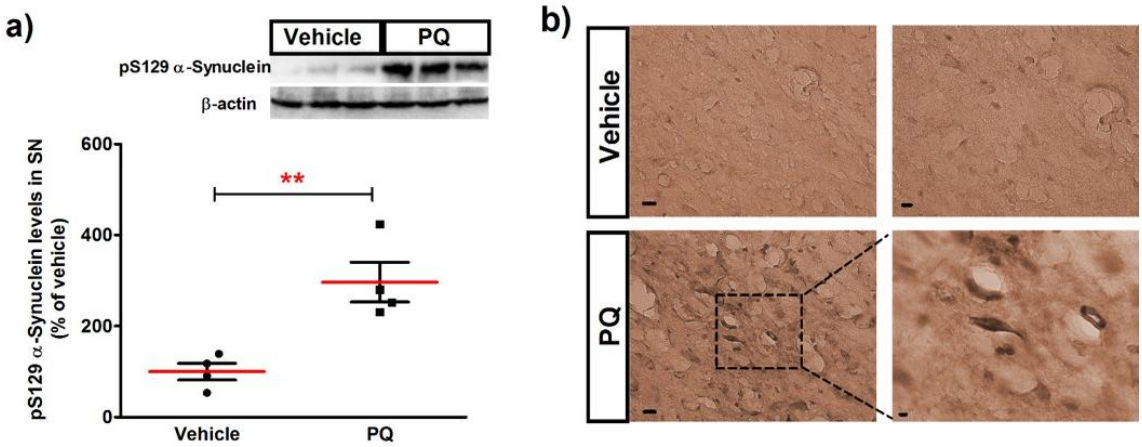

Figure 13- Levels of alpha-synuclein phosphorylated at serine 129 in the SN of rats exposed to chronic dose of PQ are increased. (a) Representative immunoblot and quantitative analysis of pS129 alpha-synuclein protein levels. pS129 alpha-synuclein protein was determined in total lysates of the rats SN tissues by immunoblot analysis. (b) Representative photomicrographs of pS129 alpha-synuclein immunoreactivity showing increased levels in the SN. Scale bars are: in the left column 20 μm, in the upper panel of the right column 10 mm and in the lower panel 5 μm. The results are expressed as percentage of vehicle (n=4). Data are shown as the mean ± SEM. Statistical analysis was performed using the Student t test. ***P*<0.01.

3.2. Expression of HMGB1 and RAGE in PD Animal Models

The dopaminergic cell death observed in PD lead the activation and development of neuroinflammatory reactions. In the inflammatory environment, there is an increased release of pro-inflammatory cytokines that activate immune cell proliferation and migration to the brain. This is a cycling mechanism that easily culminates in the activation of more inflammatory cascades increasing the susceptibility of neuronal cells to further degeneration. In spite of clear evidences regarding the importance of HMGB1 expression in inflammatory processes and the role of RAGE in neurodegenerative diseases, there is no consistent information concerning the expression of this receptor and its ligands in PD. Here we present the results of the protein expression levels of HMGB1 and RAGE in three different PD models: the novel model of PQ develop in this project, the classic MPTP model and the 6-OHDA model. To our knowledge, this is the first time that animal models of PD are evaluated to the expression of this ligand and receptor. This characterization will be helpful to understand the role of HMGB1 and RAGE in the neuroinflammation observed in PD.

3.2.1. *In vivo* chronic exposure to PQ induced significant changes in the expression levels of HMGB1 in ST and RAGE in both SN and ST

In order to characterize the behaviour of HMGB1 and RAGE protein expression levels in our new PD rat model induced by PQ, we tested the effect of a low dose of PQ infused at concentration of 2.5 mg/kg/day during 4 weeks, on the expression of these molecules in the SN and ST.

In the presence of PQ, the expression of HMBG1 does not show differences statistical significant, being slightly decreased (88.94 ± 10.02%; n=5) when compared to vehicle (100.0 ± 16.69%; n=4) in SN. In the ST, the expression of this ligand is significantly reduced (68.68 ± 6.732%; n=5; $P<0.01$) comparative to vehicle (100.0 ± 2.577%; n=4) as presented in (Figure 14 a). However, a significant increase of the expression of RAGE in SN was observed (142.0 ± 14.88%; n=5; $P<0.05$) relatively to the vehicle (100% ± 12.05%; n=4). The expression levels of RAGE in ST (179.0 ± 20.00%; n=5; $P<0.01$) was also significantly increased when compared to vehicle (100.0 ± 9.836%; n=4) as showed in (Figure 14 b).

These results suggest that in our new PD rat model the expression of HMGB1 decreases in both in SN and ST. The same does not happened with RAGE expression that may be influenced by the dopaminergic degeneration induced by chronic exposure to PQ.

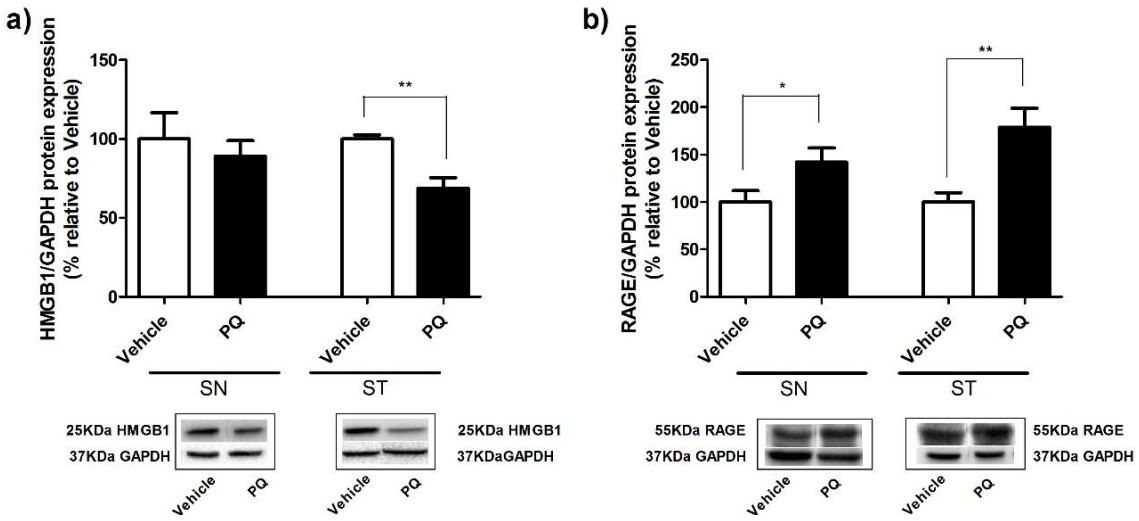

Figure 14- Expression of HMGB1 (a) and RAGE (b) in brain tissues from SN and ST of rat treated with a chronic administration of PQ. Graphs depict the percentages relative to control of 25 kDa HMGB1 and 55 kDa RAGE protein expression normalized to total 37 kDa GAPDH (n=5). Protein expression from vehicle treated animals was set to 100%. Data are shown as the mean ± SEM of five rats. Statistical analysis was performed using unpaired Student *t*-test. **P*<0.05 and ***P*<0.01.

3.2.2. The *in vivo* acute MPTP exposure does not induces changes in the HMGB1 and RAGE protein expression profile in both SN and ST

Similarly, to understand the effect of MPTP on HMGB1 and RAGE expression levels, we evaluated by western blot the protein expression profile of these molecules in the SN and ST of mice seven days after being exposed to a total of 60 mg/Kg of MPTP.

In MPTP treated animals, the expression of HMBG1 does not show statistical significance neither in SN (116.0 ± 8.076%; n=4) or ST (100.0 ± 2.513%; n=4), as compared with vehicle animals (100.0 ± 12.51%; n=4 and 82.55 ± 11.78%; n=4), respectively (Figure 15 a).

Likewise, RAGE expression levels were no statistically significant increase in the SN (112.0 ± 11.14%; n=4) when compared to vehicle (100.0 ± 6.495%; n=4). In the ST, although the expression levels of RAGE show a tendency to increase (133.5 ± 14.60%; n=4) when compared to vehicle (100.0 ± 21.73%; n=4), these levels did not reach statistical significance (Figure 15 b). These data shown that at the used end point for the exposure to MPTP (seven days after MPTP injections), this toxin seems to have no effect on the expression levels of HMGB1 and RAGE in either SN or ST.

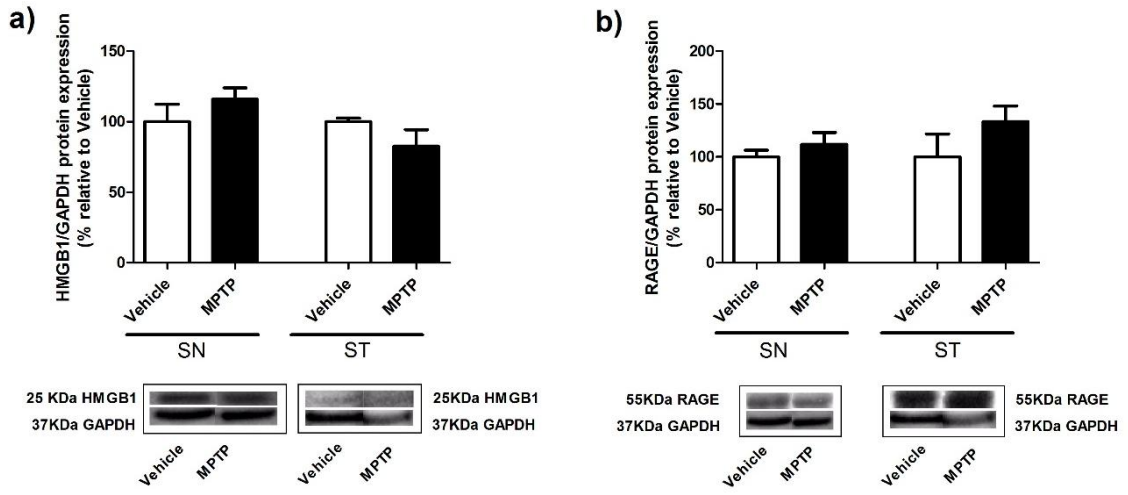

Figure 15- Expression of HMGB1 (a) and RAGE (b) in brain tissues from SN and ST of mice treated with acute administration of MPTP. Graphs depict the percentages relative to control of 25 kDa HMGB1 and 55 kDa RAGE protein expression normalized to total 37 kDa GAPDH (n=4). Protein expression from vehicle treated animals was set to 100%. Data are shown as the mean ± SEM of four mice. Statistical analysis was performed using unpaired Student *t*-test.

3.2.3. Dopaminergic damage induced by the intrastriatal injection of 6-OHDA increased both HMGB1 and RAGE expression in ST

The protein expression profile of HMGB1 and RAGE was also characterized in the 6-OHDA model. In this model, the toxin was directly injected in mice ST to overcome with the fact that 6-OHDA does not cross the BBB. The expression levels of these proteins were evaluated 3 and 7 days after 6-OHDA injections.

In animals exposed to 6-OHDA (10 µg) for 3 days there was a slightly non statistical decrease in HMBG1 protein levels expression in the SN (98.05 ± 9.937%; n=3) and statistically significant increase (129.4 ± 16.68%; n=3) in ST, when compared to untreated (Contralateral), data shown in (Figure 16 a).

Concomitantly, the protein expression levels of RAGE in the SN did not significantly decreased (86.26 ± 29.37%; n=3) when compared to the untreated contralateral SN (Contralateral considered 100%). In ST, the expression of RAGE significantly increased (267.1 ± 31.11%; n=3; $P<0.01$) relatively to untreated (Figure 16 b).

Altogether these data reveals that striatal exposure to 6-OHDA influences the protein profile of both HMGB1 and RAGE only in ST and not in SN.

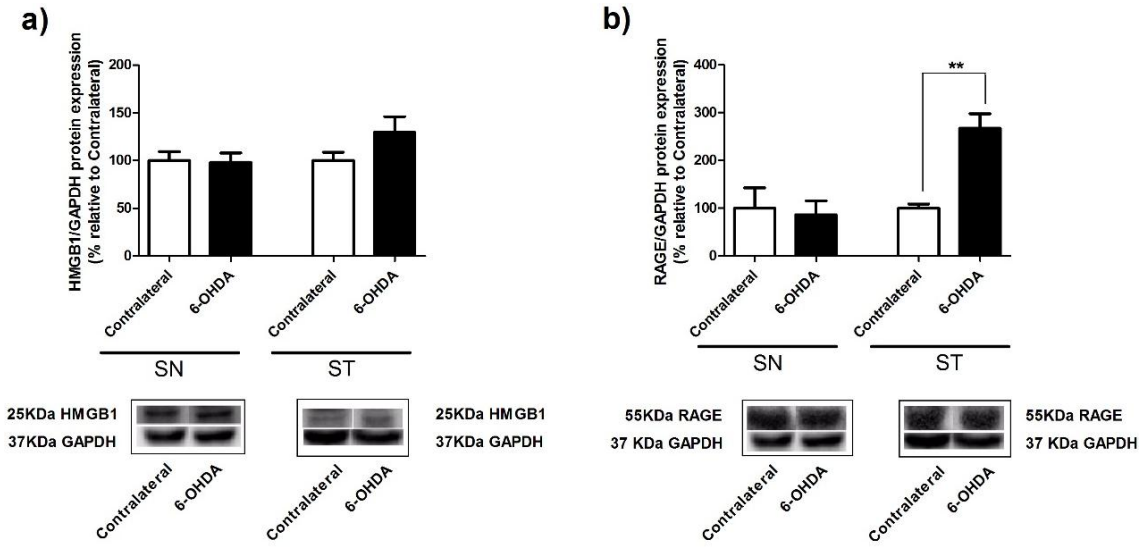

Figure 16 - Expression of HMGB1 (a) and RAGE (b) in tissues from SN and ST of mice brain treated with acute administration of 6-OHDA. The expression levels were measured after 3 days of exposure. Graphs depict the percentages relative to control of 25 kDa HMGB1 and 55 kDa RAGE protein expression normalized to total 37 kDa GAPDH (n=3). Protein expression from vehicle treated animals was set to 100%. Data are shown as the mean ± SEM of tree mice; $^{**}P<0.01$. Statistical analysis was performed using unpaired *t*-test.

Seven days after 6-OHDA striatal injection, the expression of both HMGB1 and RAGE shown to be restored to basal levels. There is a slight decrease in HMBG1 protein levels in the SN (92.59 ± 8.929%; n=2) as well as in the ST (76.34 ± 11.23%; n=2) relative to respectively contralateral hemisphere (100.0 ± 10.04%) and (100.0 ± 17.93%), shown in (Figure 17 a).

Concomitantly, the protein expression levels of RAGE in the SN were very similar of contralateral (103.7 ± 2.724%, n=2 and 100.0 ± 1.007%). In the ST, RAGE protein levels decreased (79.75 ± 6.42%) relatively to untreated (100.0% ± 18.86%), as shown in (Figure 17 b).

Altogether these data shown that after 7 days of 6-OHDA exposure, this neurotoxin seems to have no effect on the expression levels of HMGB1 and RAGE in either SN or ST.

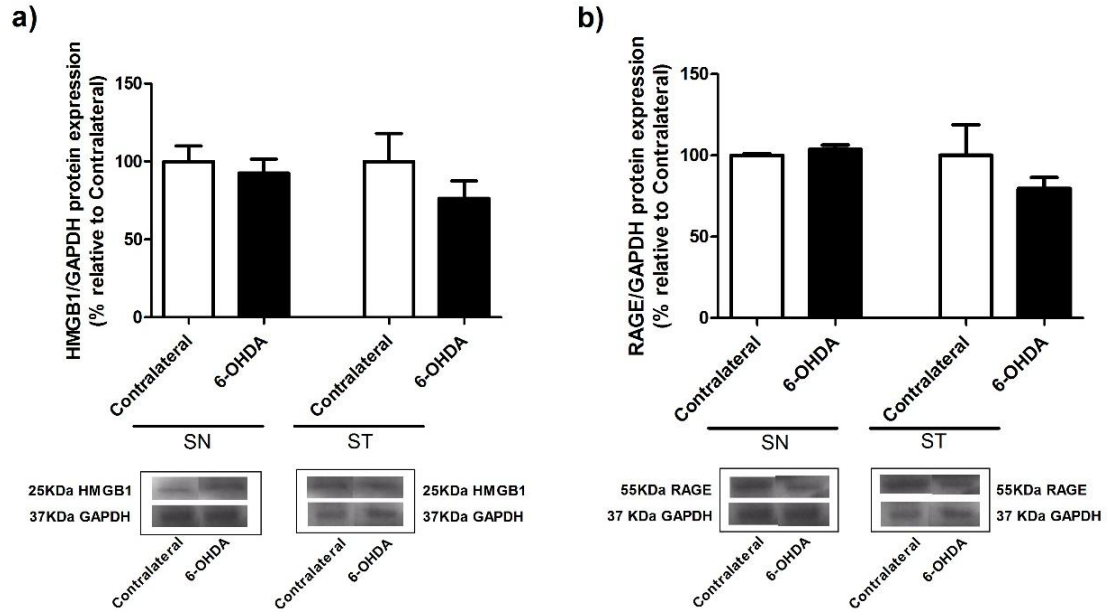

Figure 17- Expression of HMGB1 (a) and RAGE (b) in tissues from SN and ST of mice after intrastriatal injection of 6-OHDA. The expression levels were measured after 7 days of exposure Graphs depict the percentages relative to control of 25 kDa HMGB1 and 55 kDa RAGE protein expression normalized to total 37 kDa GAPDH (n=2). Protein expression from vehicle treated animals was set to 100%. Data are shown as the mean ± SEM of two mice.

In vitro assays

3.3. Dopaminergic damage induced by 6-OHDA *in vitro* show a tendency to increase the expression levels of RAGE in Co-Culture

To determine the best condition for our experiments using the co-culture model with HUVECs and N27 cells, we then perform a preliminary assay using a high concentration of the neurotoxin 6-OHDA (100 μM) and evaluated the effect of this dose on the protein expression levels of RAGE on both cell types. The exigent cell requirements only allow us to detect RAGE expression levels, since a very low amount of total protein was recovered from the two type of cell lysates. The expression levels of RAGE were measured in both HUVEC and N27 cell lysates treated for 24h hours with the neurotoxin. The main goal of this experiment was to understand the optimizations needed for co-culture procedures and treatments, so the sample size of this analysis was only n=1. The results shown an increased tendency in the protein expression levels of RAGE in the presence of the neurotoxin (Figure 18). To notice, the RAGE expression within HUVECs in non-treated samples was not detected, which made us speculate that in endothelial cells 6-OHDA may induce the increase of RAGE expression. In

contrast, neuronal cells (N27) seem to express the receptor even in basal conditions. After 6-OHDA insult RAGE expression levels in N27 cells seem to increase. Of note these results need to be further investigated, by increasing the number of independent experiments.

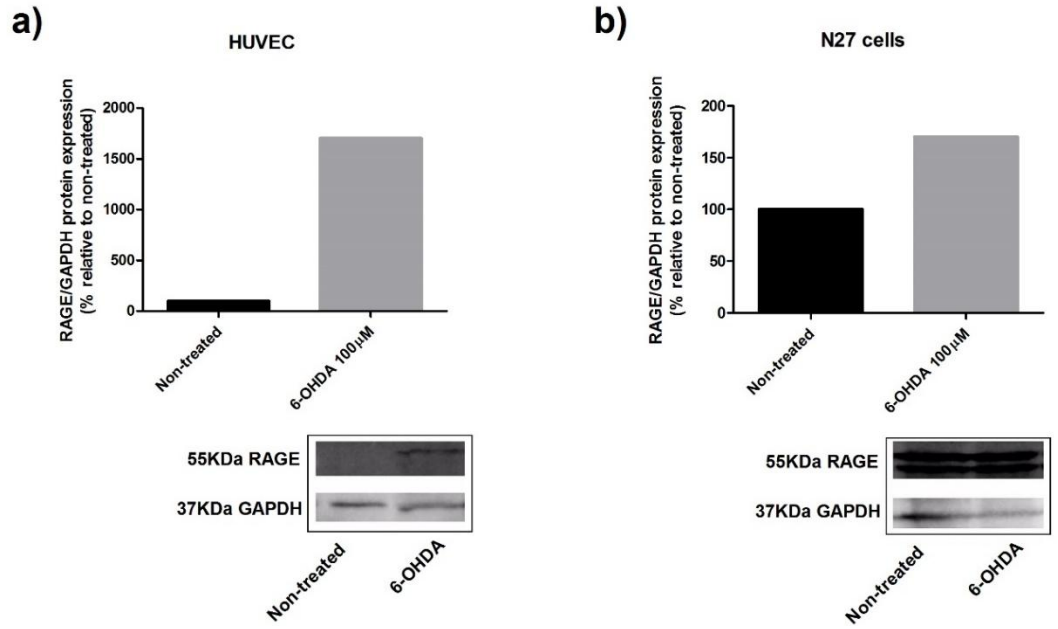

Figure 18- Expression of RAGE on HUVEC (a) and N27 cells (b) treated with 100 µM of 6-OHDA for 24 hours. The two co-cultured cell lines were then separated and protein was extracted from the recovered cells. Protein expression is normalized to GAPDH levels and is represented as a percentage of non-treated cells (n = 1).

3.4. Toxins-mediated N27 cell death

These previous assay involving co-culture were helpful to understand that we have a valid model to study the communication between endothelial and neuronal cells. Moreover, these experiment lead us to reformulate some of the aspects involving the co-culture. First we will optimize the treatments concentrations in order to understand the cytotoxicity induced in N27 cells by each individual toxin. Second, the future treatments applied would not be performed once the cells are in co-culture, but only in N27 cells. In this way, to define the cytotoxicity of the treatments applied to N27 cells, the levels of MTT reduction were measured in N27 cell culture treated for 3h hours with 30 µM MPP^+, 500 µM PQ and 10, 25 and 50 µM 6-OHDA, to access cell viability. As seen in (Figure 19), we can observe that N27 cells response differently to the different toxins added to the medium, which globally lead to ~40-50% cell death.

The treatment with 30 µM MPP^+ induced significantly lower levels of MTT reduction (52.28 ± 11.08%; n=3; $P<0.05$) as compared with their respective control cultures (untreated,

considered 100%, Figure 19 a), indicating that cells which were treated with this toxin were vulnerable to death. The treatment with 500 μM PQ presented similar results with significantly lower levels of MTT reduction (50.57 ± 9.347%; n=3; $P<0.05$), comparatively to the respective control cultures (untreated, considered 100%, Figure 19 b). The treatment with 6-0HDA was performed for three different concentrations, 10, 25 and 50 μM, in order to determinate the most adequate concentration to apply in the subsequent experiments. The cytotoxic effect of the three concentration of 6-OHDA was very similar. We observed that MTT reduction by N27 cell exposed to 10, 25 and 50 μM of 6-OHDA was respectively 44.44 ± 10.34%; n=3; $P<0.01$; 45.90 ± 11.10%; n=3; $P<0.01$ and 39.86 ± 11.08%; n=3; $P<0.01$, and significantly lower than the control cultures that receive no treatment (considered 100%) as shown in (Figure 19 c).

These results indicate that the concentrations of MPP^+, PQ and 6-OHDA selected to perform the N27 cells treatments can induce dopaminergic lesion and apoptosis and thus lead to a reduction in the number viable cells in culture, nevertheless lower than approximately 40%, allowing to perform the following experiments in co-culture with HUVECs.

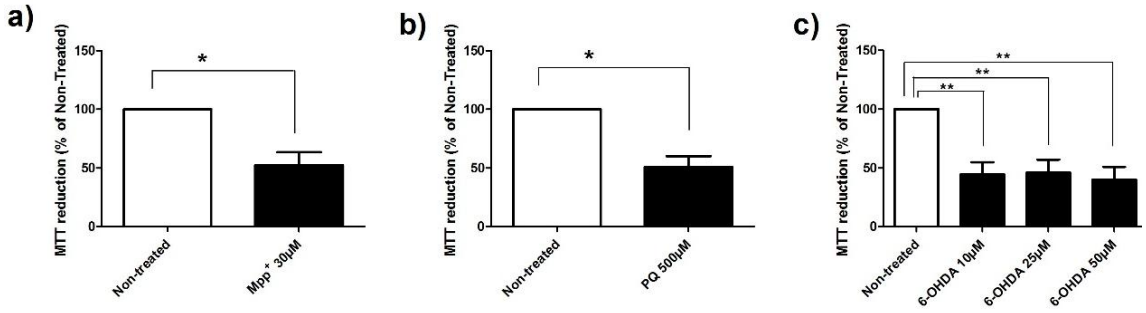

Figure 19- Toxins-mediated N27 cells death. Cell death was accessed by measuring the levels of MTT reduction in N27 cells culture with 24 hours *in vitro*, incubated with MPP^+ 30 μM (a), PQ 500 μM (b) and 6-OHDA 10, 25 and 50 μM (c) for 3 hours. The results are expressed as percentage of control (n=5). Data are shown as the mean ± SEM of three independent experiments. Statistical analysis was performed using Student *t*-test for a) and b) and one-way ANOVA followed by Bonferroni's Multiple Comparison test for c). * $P<0.05$; ** $P<0.01$ vs. control cultures.

3.5. Dopaminergic degeneration induced by toxins increased the expression of HMGB1-RAGE in N27 cells

In order to explore if the dopaminergic lesion applied to N27 cells by the toxins used before, (see figure 19), affect the protein expression levels of RAGE and HMGB1 we then evaluated the levels of these proteins in treated N27 cells by Western Blot.

In general, the increases observed for HMGB1 protein levels induced by the treatments are higher than the ones observed for RAGE expression (Figure 20 a, b). In the presence of MPP^+ 30 µM, the HMGB1 protein levels increases was 136.4 ± 12.80%; n=5, relatively to non-treated (100%). When compared with controls, the presence of 500 µM PQ and 10 µM 6-OHDA induced a significant increase of HMGB1 protein expression levels (138.7 ± 19.89%; n=5; $P<0.05$) and (154.0 ± 11.59%; n=5; $P<0.05$), respectively.

The effect of the toxins in the expression of RAGE are shown in (Figure 20 b). MPP^+ 30 µM treatment induced a slight increase of RAGE expression, with no statistical significance, (108.7 ± 7.171%; n=5) as well as in PQ 500 µM treatment (111.3 ± 7.924%; n=5). As for 6-OHDA exposure of N27 cells to 10 µM of the toxin, induced a significant increase in RAGE protein expression levels (131.3 ± 15.96%; n=5; $P<0.05$) when compared to control (Non-treated, considered 100%). Overall, toxins-mediated dopaminergic degeneration induced an increase of both HMGB1 and RAGE protein expression levels in N27 cells.

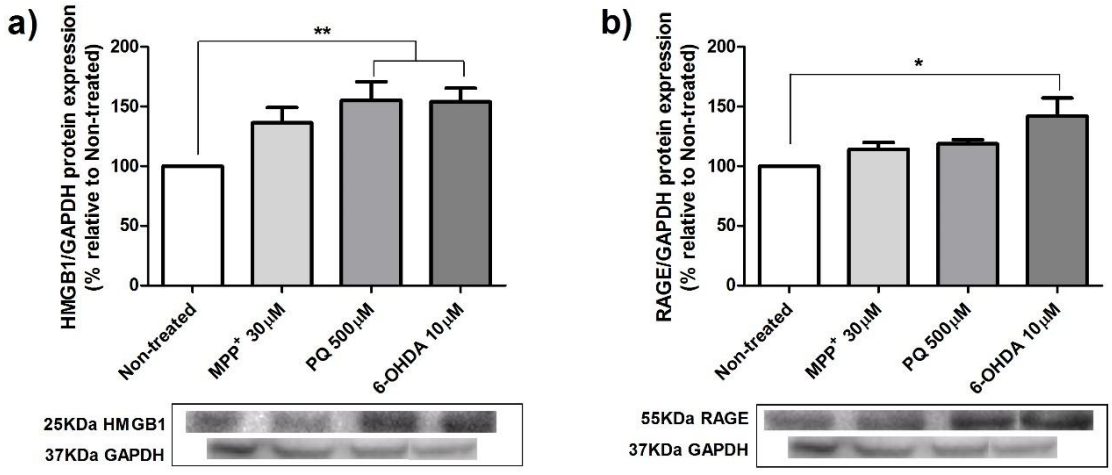

Figure 20- - Expression of HMGB1 (a) and RAGE (b) in N27 cells culture treated with 30 µM MPP+, 500 µM PQ, 10 µM 6-OHDA during three hours and maintained until 24 hours in medium without toxins. Graphs depicts the percentages relative to control of 25 kDa HMGB1 (a) and 55 kDa RAGE (b) expression normalization to total 37 kDa GAPDH (n=5). Proteins expression controls was set to 100%. Data are shown as the mean ± SEM of five independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. *$P<0.05$, **$P<0.01$ vs. non-treated.

Chapter 4- Discussion

The present work is the first report of a rat PD model induced by the chronic exposure to a low dose of PQ using osmotic minipumps that exhibits strong α-synucleinopathy, an important hallmark of PD. There are several PD models described in the literature, however none of the models fully recapitulate all the key clinical and neuropathologic features of PD. This animal model, depict a clear nigrostriatal dopaminergic neurodegeneration and strong α-synucleinopathy, as shown by decreased number of TH^+ cells in the SNpc and the increased levels of phosphorylated alpha-synuclein form (pS129), respectively.

The effect of PQ in dopaminergic neurons was shown in several studies, including in previous works from our group [7, 25, 29]. In this model, the chronic low dose of PQ induced a significant 39% of dopaminergic cell loss in the SN. This is a great improvement when compared with other PQ-based PD models in which nigrostriatal degeneration is usually minimal in spite of high doses of PQ [43, 97, 98]. Differential vulnerability of nigrostriatal dopaminergic neurons to PQ has been reported [99, 100]. Indeed, the loss of dopaminergic neurons is more severe in the intermediates and posterior sections of the SNpc. Our topographical distribution of the PQ-induced neurodegeneration within the SN demonstrated that depletion of dopaminergic neurons was higher in the intermediate and caudal part of the SNpc which is in agreement with what was reported in *postmortem* human PD brains [99, 100]. The distinct expression patterns of genes implicated in PD pathogenesis could be related to differential vulnerability of dopaminergic neurons [101]. Moreover, one of the most important neuropathological features of PD is the accumulation of alpha-synuclein that may induce several cellular dysfunctions leading to dopaminergic neurodegeneration [43, 98]. However, most animal models of PD have failed to reproduce alpha-synuclein pathology [7, 48]. Herein, we found signs of increased pS129 alpha-synuclein levels and aggregation in individual dopaminergic neurons in the SN. These results raise the possibility that our model with strong pS129 alpha-synuclein pathology may lead to progressive degeneration of the nigrostriatal pathway through aggregation. Thus, it will be of interest to perform long-term studies to investigate whether this model recapitulates chronic and progressive features of human PD. In other models, PQ-induced systemic toxicity was reported to have major toxic effects on lungs, followed by loss of body weight [102]. We have previously reported [103] that animals exposed to PQ showed alterations motor dysfuntion as well as depression and anxiety-like behaviour meaning that the low and continuous exposure to PQ is important to mimic the motor and emotional behaviour presented in human PD. The previously reported results were the first ones describing the possibility to perform behavioural tests in a PD rat model induced by PQ. Those results, together with the strong α-synucleinopathy found, leads us to believe that we are one step closer to achieve the ideal model to further investigate the

pathogenesis of this disease. Eventually, the ideal model would exhibit all the clinical and pathologic features of PD, but this may be a difficult challenge. Nevertheless, the use of our new animal model of PD will clearly lead to a better understanding of the mechanistic relationships between PD and associated alpha-synuclein abnormalities.

In this work, besides the development of an animal model of PD we have also proposed to characterize the protein expression profile of HMGB1 and RAGE in different *in vivo* and *in vitro* models of PD. To the best of our knowledge, we were the first to investigated the protein expression levels of these two proteins in the PQ and 6-OHDA experimental models of PD, although they behaviour in other pathologies were already reported.

In this way, we first evaluated HMGB1 and RAGE protein expression profile in three animal models of PD: the PQ model recently developed, the MPTP model and the 6-OHDA model. These models are different from each other. The PQ and MPTP models are characterized by the systemic application of the neurotoxin, by minipumps or intraperitoneal injections, respectively. In turn, in 6-OHDA model the neurotoxin was directly injected in ST, due to the incapability of this toxin to cross the BBB. Besides that, there are differences according to the time that protein levels expression of HMGB1 and RAGE were measured in each model. The PQ being a chronic model the levels were measured 5 weeks after continuous exposure to the neurotoxin and the MPTP 7 days after the *i.p.* injection of the toxin. The exposure to 6-OHDA was for 3 days, representing an intermediate end-point of the model were most of the neuroinflammation features are expected to be present and 7 days after were neuroinflammation markers should be less present. The HMGB1 and RAGE expression levels were measured both in SN and ST of the different models.

Overall, we can settle that HMGB1 is expressed in non-treated animals, but is only increased in the SN in MPTP model and in the ST in 6-OHDA model. RAGE is expressed in non-treated rodents as well, and increased in all PD models, mainly in the ST, with statistical significance in PQ and 6-OHDA model.

The paradigm of PQ exposure to induce a PD model is in accordance with a chronic exposure to this herbicide. In this way, the results obtained for HMGB1 and RAGE shown that the receptor expression is elevated after 5 weeks of exposure to PQ and for the ligand the observed decrease could be justified by an immediate mechanism of action for this type of cytokine. A previous study reported the active release of HMGB1 by macrophages and monocytes between the 3 and 24 hours after cell injury induced by hydrogen peroxide treatment. This report indicate that the release of HMGB1 from cells is a time- and dose-dependent process [104]. The cell damage through continuous exposure to PQ lead to increases on RAGE expression in both SN and ST, which may indicate that a previous release of RAGE ligand occurred. Changes in RAGE expression are observed in different disease states. Such changes in RAGE expression elicit concurrent increases in the expression of RAGE ligands, which themselves are also disease-associated [53]. In this case, HMGB1 is decreased

in the brain suggesting that at this time point the changes in the protein expression levels of this pro-inflammatory cytokine were back to basal levels. We believe that at earlier time-point, such as within the first week after minipump implantation it is possible that the expression levels of HMGB1 would be increased.

In the MPTP induced model of PD, no statistical differences were found in the levels of HMGB1 and RAGE in both SN and ST. Nevertheless we could see a tendency in increasing levels of these protein in the SN and ST, respectively. Different studies have reported that nitric oxide is an important mediator of MPTP toxicity in dopaminergic neurons [12, 84] with a preferential loss of neurons in the SNpc [12]. A recent study from our group using this model, have shown a significant decreased (approximately 50%) of TH protein levels in the SN [91]. The increased tendency observed on HMGB1 and RAGE expression profile in the SN and ST of the MPTP induced model of PD, may be related to the accentuated cell damaged and inflammation in the SN that typically occurs with this toxin. The RAGE expression on ST also shown a tendency to increase, although no statistical significance were obtained for this increase. The tendency discussed above may allow us to speculate that in the MPTP mouse model of PD the inflammation process may happen firstly in ST where the increase of the receptor was more accentuated and gradually expand to the SN. The fact that we were not able to detect increased levels of HMGB1 in ST may also be a consequence of the loss of dopaminergic fibers terminal in the ST as we have shown in a previous study [91] that could compromise the release of HMGB1 in the striatal region.

In the 6-OHDA model of PD, 3 days after neurotoxin exposure, HMGB1 and RAGE expression levels increased significantly in ST. These results emerge as a possible effect of the deposition of the neurotoxin in this particular location in the brain leading to inflammation in this area and are in accordance with others [102, 105] which showed apoptotic dopaminergic cells in the SN after intrastriatal injection of 6-OHDA in this location. Also, 6-OHDA accumulates in the ST after stereological injection and as a potent oxidant 6-OHDA can lead to local ROS that are known to induce cell damage and apoptosis [12, 102] and in increasing the expression levels of HMGB1 [56, 87]. Our results suggest that 6-OHDA injected in ST, seems to boost the increase expression of HMGB1 and RAGE protein levels only locally. The HMGB1 release by cells seems to be fast, in response to cell damage and inflammation. In a recent study [106] a significant increase of HMGB1 levels were observed between 6 to 36 hours in the supernatant of HUVECs cultures exposed to low discontinuous stress. In the same model, animals sacrificed 7 days after the neurotoxin exposure, shown no effect on the expression levels of HMGB1 and RAGE in either SN or ST revealing that an earlier time-point such as 3 days is better to target these molecules.

Taken all together, these results show that HMGB1 is increased in the 6-OHDA model (3 days after exposure) of PD and RAGE expression is increased in all *in* vivo PD animal models with the exception of 6-OHDA (7 days after exposure). In this way and exploring our hypothesis,

the neuronal damaged induced by the neurotoxins can lead to the release of HMGB1. The increase of extracellular HMGB1 may then induces the expression of RAGE mainly by endothelial cells. Nevertheless this needs further investigation.

To try to clarify the interplay between dopaminergic neuronal damage and endothelial cells and they role in the protein expression profile of HMGB1 and RAGE in PD, we also studied the expression variations of these molecules *in vitro*. First we developed a co-culture model to mimic the BBB environment, including neuronal cells (N27 cells) and endothelial cells (HUVECs). Although the results reflect only the analysis of n=1 we can see an increased tendency in the protein expression levels of RAGE induced by 6-OHDA. To notice, the RAGE expression within HUVECs in non-treated samples was not detected, revealing that the increase is due to the treatment applied. In contrast, untreated neuronal cells (N27) express the receptor which levels increases in the presence of 6-OHDA. These assay was helpful to understand that we have a great model to study the communication between endothelial and neuronal cells but there are some adjustments that are required. The future treatments applied would not be performed with the cells already in co-culture, but affecting only neuronal cells, to induced dopaminergic damage. In this way, to define the cytotoxicity of the treatments applied to N27 cells, the levels of MTT reduction were measured in N27 cell culture treated for 3 hours with 30 µM MPP^+, 500 µM PQ and 10, 25 and 50 µM of 6-OHDA to assess cell viability. The MTT results indicate that all toxins used (30 µM MPP^+, 500 µM PQ and 10, 25, 50 µM 6-OHDA), induced statistical increase of approximately 40-50% N27 cell death. In previous works from our research group, the same effect of 500 µM PQ was described with (62 ± 1.4%) of cell viability in N27 cells treated for 24 hours [29]. The MPP^+ treatment at concentration of 30 µM lead to ~48% of cell death. As for 6-OHDA, we tested three concentrations (10, 25 and 50 µM) and the results obtained shown a decrease of cell viability with increased concentration of the toxin. These data are in accordance with other report [107] where 6-OHDA exhibited significant cell damage at 50 µM when compared to 10 µM (that already had induced cell damage of about 56%). In this way, 10 µM was chosen for future experiments, since there is no benefit of having more than 50% of cell death because we need a healthy population to evaluate the evaluate the HMGB1 and RAGE protein expression levels.

The evaluation of the cytotoxic effect of the selected toxin also allowed us to determine the best condition to the future study on the effect of dopaminergic damage in endothelial cells using co-culture. After injury, these neuronal cells can release pro-inflammatory cytokines like HMGB1 [55] that we hypothesized to have an effect on HUVECs through RAGE [57, 61] (experiments in progress). The main goal of the co-culture with HUVECs, is to understand the effect of dopaminergic degeneration on endothelial activity, and the role played by HMGB1-RAGE signalling pathway in this effect.

Using the defined neurotoxins concentrations we further evaluated in N27 cells the expression levels of HMGB1 and RAGE by western blot. The interaction between HMGB1 and its receptor

may lead to multiple consequences to the immune system and neurons [59]. Notably, N27 cells treated with 30 μM MPP^+, 500 μM PQ and 10 μM 6-OHDA showed significant increases in both HMBG1 and RAGE expression levels revealing their functions as neuroinflammatory signals. These results are in accordance with other studies that also reveal an increase of these molecules in acute and chronic CNS diseases [52, 54]. As an example, in AD the RAGE-Aß interactions induce neuronal toxicity that is mediated by oxidative stress and NF-κB activation, via RAGE activation [67]. Also, the treatment of PC12 cell line with Aß induces neuronal toxicity leading to RAGE overexpression as well [108]. In our results, the protein levels expression of HMGB1 were more accentuated comparatively to RAGE. This may indicate that dopaminergic neurons are mainly responsible for the expression of HMGB1 which then will have an impact in HUVEC cells through RAGE signalling pathway, as shown previously [109]. RAGE is constitutively expressed at high levels in the lung and epithelial cells and in low expression in monocytes/macrophages and neurons [110]. In this way RAGE mediates interactions of its ligands with endothelium and other cells types [109]. Damaged dopaminergic cells undergoing necrosis release HMGB1 that is tightly bound to chromatin [110]. After its release, HMGB1 have the capacity to induce cytokines release, activate inflammatory cells and bind to or associate with other molecules such as DNA and RNA (signalling through RAGE) [56, 89, 110]. The RAGE binds to several classes of molecules and is linked to a variety of signal transduction pathways that include the activation of NF-kB, mitogen-activated protein kinases, PI3K/Akt, Rho GTPases, Jak/STAT [110], specially involved in inflammation [111]. The completed model, including damaged dopaminergic cells and HUVECs is important to evaluate the interaction involving neurons and endothelial cells during inflammatory processes and is in already in progress at the lab.

As the HMGB1-RAGE pathway has been reported to result in the activation of various inflammatory mechanisms, the same seems to be occurring in the PD experimental models used in this work. According to the literature, cytokines like HMGB1 are examples of a diverse set of endogenous molecules with known danger-signalling capacities that can induce more cytokines and activate inflammatory cells when is released from damaged cells [56, 110]. A study [112], has previously shown that a variety of nuclear molecules are released from cell during apoptotic death. These nuclear molecules may vary with cell type, inducing stimulus as well as a stage in the death process showing the difficulty to target the HMGB1 release. The role of HMGB1 in PD is not well studied, however some studies reported that ROS involved in PD pathogenesis such as hydrogen peroxide may induce HMGB1 expression and release in macrophages and monocytes [56, 87].

There are compelling data supporting that RAGE plays a major role in many inflammatory disorders. Several studies described that RAGE is involved in inflammation resolution leading to tissue repair or alternatively to its perpetuation leading to chronic inflammation by cell proliferation and migration [62]. It is well-known that RAGE mediates HMGB1-induced neuroinflammation and necrosis [113]. Moreover, in a study were RAGE was blocked there was

a protective effect on nigral dopaminergic neurons in the MPTP mouse model of PD [114]. The involvement of RAGE has been reported in several diseases over the last years. For example, it has been described that in a diabetic microenvironment, RAGE has a central role in sensory neuronal dysfunction by the activation of NF-κB [86]. As demonstrated on literature, one of the principal factors linked to the induction of inflammation in PD pathogenesis is the activation of this nuclear factor [83-85]. Also in diabetes, the AGE-RAGE–induced cytosolic ROS production lead to mitochondrial superoxide production showing the important role of this receptor in the development and progression of diabetic nephropathy [82]. There is a study that reported elevated RAGE levels in the ischemic hemisphere in stroke patients, suggesting a role for RAGE in this pathology as well [59]. The RAGE involvement in AD is well described so far. The Aß peptide influx into the brain is mediated by RAGE receptor. The expression of LRP-1 that efflux Aß of the brain is decreased in AD and at the same time RAGE expression is increased [79-81], resulting in accumulation of the Aß peptide in to the brain. Other report showed evidences that Aß peptide interaction with RAGE at the BBB up-regulates endothelial cognate ligand chemokine ligand 5 expression and causes circulating T cell infiltration in the brain. This study shows a new insight into the understanding of inflammation in the progress of AD by revealing the important role of RAGE in this disease [64].

Our present findings suggest that neuroinflammation observed in PD could be related to the increase of RAGE similarly to what occurs in other neurodegenerative diseases. Increases in HMGB1 expression levels could be related to PD as well, but it seems to be more evident in early stages of inflammation. In the assays performed in this work, there were slight increases of HMGB1 and RAGE expression with more statistical significance in *in vivo* models, specifically in the PQ and 6-OHDA model. This was the first time that HMGB1 and RAGE protein expression levels were evaluated in PD models. Given these results, our *in vivo* and *in vitro* findings indicate that HMGB1 and RAGE may have a crucial role in PD neuroinflammation and may represent a suitable targets for CNS disorders where neuroinflammation occurs as well as for neuroprotective treatment in PD by using a pharmacological approach.

Chapter 5- Conclusions

This work presents a novel PQ-based chronic PD model that reproduces several key characteristics of human PD. Most importantly, this model shows prominent alpha-synuclein pathology (including pS129 alpha-synuclein) that is not consistently observed in other toxin-based rodent PD models. This new rat model of PD may serve as an attractive novel tool for unveiling the molecular mechanism of alpha-synucleinopathy as well as for evaluation of new pharmacologic strategies for PD.

The precise ethiology of PD has been under investigation for more than two centuries and despite of the important role of RAGE in neurodegenerative disorders like AD, there is no consistent information of his involvement in neuroinflammation observed in PD. The same occurs to the RAGE ligand HMGB1, were the mediated pathological mechanisms have remained largely elusive. Recently, a great research effort has been devoted to understand the pathophysiological influence of the BBB in neurological disorders, including its influence in the initiation of neurodegenerative process due to changes in its physiologic condition. In PD there are evidences that neuroinflammation can compromise the BBB structure and function. Knowledge of these mechanisms is likely to lead to therapeutic targets for neurological diseases.

In this work we evaluated for the first time the protein expression levels of HMGB1 and RAGE *in vivo* and *in vitro* PD models. Our results shown that HMGB1 and RAGE expression levels are increased in PD models. These results suggest that both HMGB1 and RAGE may become suitable targets to reduce neuroinflammation and develop neuroprotective treatments.

The present work have open a new window on the possible influence of HMGB1 and RAGE signalling in PD. This has also raised several questions that needs to be further investigated in order to understand the details of this mechanism. Moreover, the co-culture assays will be fundamental to understand the dopaminergic damage influence on endothelial activity and to clarify the BBB dysfunction occurring in PD.

Chapter 6- References

[1] A. J. Lees, J. Hardy, and T. Revesz, "Parkinson's disease," *Lancet,* vol. 373, p.2055-66, Jun 13, 2009.

[2] S. Przedborski, "Parkinson´s Disease: Mechanisms and Models," *Neuron,* vol. 39, p889-909, 2003.

[3] K. R. Chaudhuri, D. G. Healy, and A. H. V. Schapira, "Non-motor symptoms of Parkinson's disease: diagnosis and management," *The Lancet Neurology,* vol. 5, p. 235-245, 2006.

[4] S. Montes, S. Rivera-Mancia, A. Diaz-Ruiz, L. Tristan-Lopez, and C. Rios, "Copper and copper proteins in Parkinson's disease," *Oxid Med Cell Longev,* vol. 2014, p.147251, 2014.

[5] A. McNeill, R. M. Wu, K. Y. Tzen, P. C. Aguiar, J. M. Arbelo, P. Barone*, et al.*, "Dopaminergic neuronal imaging in genetic Parkinson's disease: insights into pathogenesis," *PLoS One,* vol. 8, p.69190, 2013.

[6] D. T. Dexter and P. Jenner, "Parkinson disease: from pathology to molecular disease mechanisms," *Free Radic Biol Med,* vol. 62, p. 132-44, 2013.

[7] J. Blesa, S. Phani, V. Jackson-Lewis, and S. Przedborski, "Classic and new animal models of Parkinson's disease," *J Biomed Biotechnol,* vol. 2012, p. 845618, 2012.

[8] D. F. Boland and M. Stacy, "The economic and quality of life burden associated with Parkinson's disease: a focus on symptoms," *Am J Manag Care,* vol. 18, p. 168-75, 2012.

[9] J. Kulisevsky, "Advanced Parkinson's disease: Clinical characteristics and treatment (part 1)," *Neurologia,* 2013.

[10] G. Leyva-Gomez, H. Cortes, J. J. Magana, N. Leyva-Garcia, D. Quintanar-Guerrero, and B. Floran, "Nanoparticle technology for treatment of Parkinson's disease: the role of surface phenomena in reaching the brain," *Drug Discov Today,* vol.0, 2015.

[11] N. E. Mencacci, "Parkinson's disease in GTP cyclohydrolase 1 mutation carriers," *Brain: AJ ournal of Neurology,* vol. 136, p.2480-2492, 2014.

[12] W. Dauer and S. Przedborski, "Parkinson's disease: mechanisms and models," *Neuron,* vol. 39, p. 889-909, 2003.

[13] L. V. Kalia and A. E. Lang, "Parkinson's disease," *Lancet,* Seminar, 2015.

[14] D. Burn, "Beyond the iron mask: towards better recognition and treatment of depression associated with Parkinson's disease.," *Mov Disord,* vol. 17, 2002.

[15] M. Weisskopf, H. Chen, M. Schwarzschild, I. Kawachi, and A. Ascherio, "Prospective study of phobic anxiety and risk of Parkinson's disease.," vol. 18: 646–51, 2003.

[16] M. Shiba, J. Bower, and D. Maraganore, "Anxiety disorders and depressive disorders preceding Parkinson's disease: a case-control study.," *Mov Disord,* vol. 15: 669–77, 2000.

[17] N. Diederich, C. Goetz, and G. Stebbins, "Repeated visual hallucinations in Parkisnon's disease as disturbed external/internal perceptions: focused review and a new integrative model.," *Mov Disord,* vol. 20, p.130–40, 2005.

[18] V. N. Uversky, "Neurotoxicant-induced animal models of Parkinson's disease: understanding the role of rotenone, maneb and paraquat in neurodegeneration," *Cell Tissue Res,* vol. 318, p. 225-41, 2004.

[19] S. J. Chinta, C. A. Lieu, M. Demaria, R. M. Laberge, J. Campisi, and J. K. Andersen, "Environmental stress, ageing and glial cell senescence: a novel mechanistic link to Parkinson's disease?," *J Intern Med,* vol. 273, p. 429-36, 2013.

[20] E. Valente, P. Abou-Sleiman, V. Caputo, M. Muqit, K. Harvey, S. Gispert*, et al.*, "Hereditary early-onset Parkinson's disease caused by mutations in PINK1," *Science,* vol. 304, p.1158–1160, 2004.

[21] M. C. Zanellati, V. Monti, C. Barzaghi, C. Reale, N. Nardocci, A. Albanese*, et al.*, "Mitochondrial dysfunction in Parkinson disease: evidence in mutant PARK2 fibroblasts," *Front Genet,* vol. 6, p. 78, 2015.

[22] V. Bonifati, P. Rizzu, M. van Baren, O. Schaap, G. Breedveld, E. Krieger, *et al.*, "Mutations in the DJ-1 gene associated with autosomal recessive early-onset Parkinsonism.," *Science,* vol. 299, p.256–259, 2003.

[23] M. Polymeropoulos, J. Higgins, L. Golbe, W. Johnson, S. Ide, G. Di Iorio, *et al.*, "Mapping of a gene for Parkinson's disease to chromosome 4q21–q23.," *Science,* vol. 274, p. 1197–9, 1996.

[24] C. Berry, C. La Vecchia, and P. Nicotera, "Paraquat and Parkinson's disease," *Cell Death Differ,* vol. 17, p. 1115-25, 2010.

[25] A. C. Cristovao, S. Guhathakurta, E. Bok, G. Je, S. D. Yoo, D. H. Choi, *et al.*, "NADPH oxidase 1 mediates alpha-synucleinopathy in Parkinson's disease," *J Neurosci,* vol. 32, p. 14465-77, 2012.

[26] A. Hald and J. Lotharius, "Oxidative stress and inflammation in Parkinson's disease: is there a causal link?," *Exp Neurol,* vol. 193, p. 279-90, 2005.

[27] I. Celardo, L. M. Martins, and S. Gandhi, "Unravelling mitochondrial pathways to Parkinson's disease," *Br J Pharmacol,* vol. 171, p. 1943-57, 2014.

[28] R. Betarbet, T. B. Sherer, and J. T. Greenamyre, "Animal models of Parkinson's disease," *Bioessays,* vol. 24, p. 308-18, 2002.

[29] A. Cristovão, D.-H. Choi, G. Baltazar, F. Beal, and Y. Kim, "The Role of NADPH Oxidase 1–Derived Reactive Oxygen species in Paraquat-Mediated dopaminergic cell death," *Antioxidants & Redox Signaling,* vol. 11, p. 2105-18, 2009.

[30] A. Reeve, E. Simcox, and D. Turnbull, "Ageing and Parkinson's disease: why is advancing age the biggest risk factor?," *Ageing Res Rev,* vol. 14, p. 19-30, 2014.

[31] I. Stojkovska, B. M. Wagner, and B. E. Morrison, "Parkinson's disease and enhanced inflammatory response," *Exp Biol Med (Maywood),* vol. 0, p.1-9, 2015.

[32] M. T. Herrero, C. Estrada, L. Maatouk, and S. Vyas, "Inflammation in Parkinson's disease: role of glucocorticoids," *Front Neuroanat,* vol. 9, p. 32, 2015.

[33] N. Dzamko, C. L. Geczy, and G. M. Halliday, "Inflammation is genetically implicated in Parkinson's disease," *Neuroscience* ,2014.

[34] M. Block and J. Hong, "Chronic microglial activation and progressive dopaminergic neurotoxicity.," *Biochem Soc Trans,* vol. 35, p. 1127-32, 2007.

[35] H. M. Gao, H. Zhou, F. Zhang, B. C. Wilson, W. Kam, and J. S. Hong, "HMGB1 acts on microglia Mac1 to mediate chronic neuroinflammation that drives progressive neurodegeneration," *J Neurosci,* vol. 31, p. 1081-92, 2011.

[36] F. Blandini and M. T. Armentero, "Animal models of Parkinson's disease," *FEBS J,* vol. 279, p. 1156-66, 2012.

[37] R. Cabezas, M. Fidel, D. Torrente, R. Santos El-Bach, L. Morales, J. Gonzalez, *et al.*, "Astrocytes Role in Parkinson: A Double-Edged Sword," *Neurodegenerative Diseases,* chapter 20, 2013.

[38] M. Gerlach and P. Riederer, "Animal models of Parkinson's disease: an empirical comparison with the phenomenology of the disease in man," *J Neural Transm,* vol. 103, p. 987-1041, 1996.

[39] T. M. Dawson and A. S. Mandir, "Animal Models of Parkinson's Disease: Pieces of the same puzzle?," *Neuron,* vol. 35, 2002.

[40] K. Shimizu, K. Ohtaki, K. Matsubara, K. Aoyama, T. Uezono, O. Saito, *et al.*, "Carrier-mediated processes in blood-brain barrier penetration and neural uptake of paraquat," *Brain Res,* vol. 906, p. 135-42, 2001.

[41] R. J. Dinis-Oliveira, F. Remiao, H. Carmo, J. A. Duarte, A. S. Navarro, M. L. Bastos, *et al.*, "Paraquat exposure as an etiological factor of Parkinson's disease," *Neurotoxicology,* vol. 27, p. 1110-22, 2006.

[42] A. L. McCormack, M. Thiruchelvam, A. B. Manning-Bog, C. Thiffault, J. W. Langston, D. A. Cory-Slechta, *et al.*, "Environmental Risk Factors and Parkinson's Disease: Selective Degeneration of Nigral Dopaminergic Neurons Caused by the Herbicide Paraquat," *Neurobiology of Disease,* vol. 10, p. 119-127, 2002.

[43] A. B. Manning-Bog, A. L. McCormack, J. Li, V. N. Uversky, A. L. Fink, and D. A. Di Monte, "The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein," *J Biol Chem,* vol. 277, p. 1641-4, 2002.

[44] A. L. McCormack, J. G. Atienza, L. C. Johnston, J. K. Andersen, S. Vu, and D. A. Di Monte, "Role of oxidative stress in paraquat-induced dopaminergic cell degeneration," *J Neurochem,* vol. 93, p. 1030-7, 2005.

[45] G. E. Meredith, P. K. Sonsalla, and M. F. Chesselet, "Animal models of Parkinson's disease progression," *Acta Neuropathol,* vol. 115, p. 385-98, 2008.
[46] K. Tieu, "A guide to neurotoxic animal models of Parkinson's disease," *Cold Spring Harb Perspect Med,* vol. 1, p. 009316, 2011.
[47] A. Schober, "Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP," *Cell Tissue Res,* vol. 318, p. 215-24, 2004.
[48] D. Blum, S. Torch, N. Lambeng, M. Nissou, A. L. Benabid, R. Sadoul, *et al.*, "Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease," *Prog Neurobiol,* vol. 65, p. 135-72, 2001.
[49] N. M. Agalave and C. I. Svensson, "Extracellular High-Mobility Group Box 1 Protein (HMGB1) as a Mediator of Persistent Pain," *Mol Med,* vol. 20, p. 569-78, 2015.
[50] P. Asavarut, H. Zhao, J. Gu, and D. Ma, "The role of HMGB1 in inflammation-mediated organ injury," *Acta Anaesthesiol Taiwan,* vol. 51, p. 28-33, 2013.
[51] K. B. Batkulwar, S. B. Bansode, G. V. Patil, R. K. Godbole, R. S. Kazi, S. Chinnathambi, *et al.*, "Investigation of phosphoproteome in RAGE signaling," *Proteomics,* vol. 15, p. 245-59, 2015.
[52] V. Iori, M. Maroso, M. Rizzi, A. M. Iyer, R. Vertemara, M. Carli, *et al.*, "Receptor for Advanced Glycation Endproducts is upregulated in temporal lobe epilepsy and contributes to experimental seizures," *Neurobiol Dis,* vol. 58, p. 102-14, 2013.
[53] G. Fritz, "RAGE: a single receptor fits multiple ligands," *Trends Biochem Sci,* vol. 36, p. 625-32, 2011.
[54] Y. M. Allette, M. R. Due, S. M. Wilson, P. Feldman, M. S. Ripsch, R. Khanna, *et al.*, "Identification of a functional interaction of HMGB1 with Receptor for Advanced Glycation End-products in a model of neuropathic pain," *Brain Behav Immun,* vol. 42, p. 169-77, 2014.
[55] V. Chirico, A. Lacquaniti, V. Salpietro, C. Munafo, M. P. Calabro, M. Buemi, *et al.*, "High-mobility group box 1 (HMGB1) in childhood: from bench to bedside," *Eur J Pediatr,* vol. 173, p. 1123-36, 2014.
[56] H. Erlandsson Harris and U. Andersson, "Mini-review: The nuclear protein HMGB1 as a proinflammatory mediator," *Eur J Immunol,* vol. 34, p. 1503-12, 2004.
[57] K. Dzaman, M. Zagor, M. Molinska-Glura, and A. Krzeski, "High motility group box 1 (HMGB1) protein and its receptor for advanced glycation end products (RAGE) expression in chronic rhinosinusitis without nasal polyps," *Folia Histochem Cytobiol,* vol. 53(1), p. 70-8, 2015.
[58] E. Leclerc, G. Fritz, S. W. Vetter, and C. W. Heizmann, "Binding of S100 proteins to RAGE: an update," *Biochim Biophys Acta,* vol. 1793, p. 993-1007, 2009.
[59] D. X. Zhai, Q. F. Kong, W. S. Xu, S. S. Bai, H. S. Peng, K. Zhao, *et al.*, "RAGE expression is up-regulated in human cerebral ischemia and pMCAO rats," *Neurosci Lett,* vol. 445, p. 117-21, 2008.
[60] J. Xie, J. D. Mendez, V. Mendez-Valenzuela, and M. M. Aguilar-Hernandez, "Cellular signalling of the receptor for advanced glycation end products (RAGE)," *Cell Signal,* vol. 25, p. 2185-97, 2013.
[61] P. Teismann, K. Sathe, A. Bierhaus, L. Leng, H. L. Martin, R. Bucala, *et al.*, "Receptor for advanced glycation endproducts (RAGE) deficiency protects against MPTP toxicity," *Neurobiol Aging,* vol. 33, p. 2478-90, 2012.
[62] L. Sessa, E. Gatti, F. Zeni, A. Antonelli, A. Catuci, M. Koch, *et al.*, "The Receptor for Advanced Glycation End-Products (RAGE) Is Only Present in Mammals, and Belongs to a Family of Cell Adhesion Molecules (CAMs)," *PLoS One,* vol. 9, 2014.
[63] J. Song, W. T. Lee, K. A. Park, and J. E. Lee, "Receptor for advanced glycation end products (RAGE) and its ligands: focus on spinal cord injury," *Int J Mol Sci,* vol. 15, p. 13172-91, 2014.
[64] M. Li, D. S. Shang, W. D. Zhao, L. Tian, B. Li, W. G. Fang, *et al.*, "Amyloid beta interaction with receptor for advanced glycation end products up-regulates brain endothelial CCR5 expression and promotes T cells crossing the blood-brain barrier," *J Immunol,* vol. 182, p. 5778-88, 2009.
[65] R. Bianchi, E. Kastrisianaki, I. Giambanco, and R. Donato, "S100B protein stimulates microglia migration via RAGE-dependent up-regulation of chemokine expression and release," *J Biol Chem,* vol. 286, p. 7214-26, 2011.

[66] Y. K. Chuah, R. Basir, H. Talib, T. H. Tie, and N. Nordin, "Receptor for advanced glycation end products and its involvement in inflammatory diseases," *Int J Inflam,* vol. 2013, p. 403460, 2013.

[67] S. H. Han, Y. H. Kim, and I. Mook-Jung, "RAGE: the beneficial and deleterious effects by diverse mechanisms of actions," *Mol Cells,* vol. 31, p. 91-7, 2011.

[68] B. R. Choi, W. H. Cho, J. Kim, H. J. Lee, C. Chung, W. K. Jeon, *et al.*, "Increased expression of the receptor for advanced glycation end products in neurons and astrocytes in a triple transgenic mouse model of Alzheimer's disease," *Exp Mol Med,* vol. 46, p. 75, 2014.

[69] W. Zheng, M. Aschner, and J.-F. Ghersi-Egea, "Brain barrier systems: a new frontier in metal neurotoxicological research," *Toxicology and Applied Pharmacology,* vol. 192, p. 1-11, 2003.

[70] N. Weiss, F. Miller, S. Cazaubon, and P. O. Couraud, "The blood-brain barrier in brain homeostasis and neurological diseases," *Biochim Biophys Acta,* vol. 1788, p. 842-57, 2009.

[71] P. T. Ronaldson and T. P. Davis, "Targeting transporters: Promoting blood-brain barrier repair in response to oxidative stress injury," *Brain Res,* vol. S0006-8993(15), p. 00200-0, 2015.

[72] N. J. Abbott, A. A. Patabendige, D. E. Dolman, S. R. Yusof, and D. J. Begley, "Structure and function of the blood-brain barrier," *Neurobiol Dis,* vol. 37, p. 13-25, 2010.

[73] F. L. Cardoso, D. Brites, and M. A. Brito, "Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches," *Brain Res Rev,* vol. 64, p. 328-63, 2010.

[74] W. M. Pardridge, "Drug and gene targeting to the brain with molecular Trojan horses," *Nat Rev Drug Discov,* vol. 1, p. 131-9, 2002.

[75] B. V. Zlokovic, "The blood-brain barrier in health and chronic neurodegenerative disorders," *Neuron,* vol. 57, p. 178-201, 2008.

[76] N. J. Abbott, L. Ronnback, and E. Hansson, "Astrocyte-endothelial interactions at the blood-brain barrier," *Nat Rev Neurosci,* vol. 7, p. 41-53, 2006.

[77] M. T. Gray and J. M. Woulfe, "Striatal blood-brain barrier permeability in Parkinson's disease," *J Cereb Blood Flow Metab,* vol. 35, p. 747-50, 2015.

[78] A. L. Bartels, A. T. Willemsen, R. Kortekaas, B. M. de Jong, R. de Vries, O. de Klerk, *et al.*, "Decreased blood-brain barrier P-glycoprotein function in the progression of Parkinson's disease, PSP and MSA," *J Neural Transm,* vol. 115, p. 1001-9, 2008.

[79] S. R. Deane, R. K. Du Yan, B. Submamaryan, S. LaRue, E. Jovanovic, D. Hogg, *et al.*, "RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain," *Nat. Med.,* vol. 9, 2003.

[80] B.V. Zlokovic, C.L. Martel, E. Matsubara, J.G. McComb, G. Zheng, R.T. McCluskey, *et al.*, "Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid beta at the blood-brain and blood-cerebrospinal fluid barriers" *Proc. Natl. Acad. Sci.U.S.A.,* vol. 93, 1996.

[81] A. Sagare, R. Deane, R. D. Bell, B. Johnson, K. Hamm, R. Pendu, *et al.*, "Clearance of amyloid-beta by circulating lipoprotein receptors," *Nat Med,* vol. 13, p. 1029-31, 2007.

[82] M. T. Coughlan, D. R. Thorburn, S. A. Penfold, A. Laskowski, B. E. Harcourt, K. C. Sourris, *et al.*, "RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes," *J Am Soc Nephrol,* vol. 20, p. 742-52, 2009.

[83] A. Ghosh, A. Roy, X. Liu, J. H. Kordower, E. J. Mufson, D. M. Hartley, *et al.*, "Selective inhibition of NF-kappaB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease," *Proc Natl Acad Sci U.S.A,* vol. 104, p. 18754-9, 2007.

[84] T. Dehmer, M. T. Heneka, M. Sastre, J. Dichgans, and J. B. Schulz, "Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with IκBα induction and block of NFκB and iNOS activation," *Journal of Neurochemistry,* vol. 88, p. 494-501, 2003.

[85] L. Perrone, O. Sbai, P. P. Nawroth, and A. Bierhaus, "The Complexity of Sporadic Alzheimer's Disease Pathogenesis: The Role of RAGE as Therapeutic Target to Promote

Neuroprotection by Inhibiting Neurovascular Dysfunction," *Int J Alzheimers Dis,* vol. 2012, p. 734956, 2012.

[86] A. Bierhaus, K.-M. Haslbeck, P. M. Humpert, B. Liliensiek, T. Dehmer, M. Morcos, *et al.*, "Loss of pain perception in diabetes is dependent on a receptor of the immunoglobulin superfamily," *Journal of Clinical Investigation,* vol. 114, p. 1741-1751, 2004.

[87] Tang D, Shi Y, Kang R, Li T, Xiao W, Wang H, *et al.*, "Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1. ," *J Leukoc Biol,* vol. 81, 2007.

[88] L. Ulloa, M. Ochani, H. Yang, M. Tanovic, D. Halperin, R. Yang, *et al.*, "Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation," *Proc Natl Acad Sci U S A,* vol. 99, p. 12351-6, 2002.

[89] W. Li, M. Ashok, J. Li, H. Yang, A. E. Sama, and H. Wang, "A major ingredient of green tea rescues mice from lethal sepsis partly by inhibiting HMGB1," *PLoS One,* vol. 2, p. e1153, 2007.

[90] D. Tang, R. Kang, K. M. Livesey, H. J. Zeh, 3rd, and M. T. Lotze, "High mobility group box 1 (HMGB1) activates an autophagic response to oxidative stress," *Antioxid Redox Signal,* vol. 15, p. 2185-95, 2011.

[91] M. Esteves, A. C. Cristovao, T. Saraiva, S. M. Rocha, G. Baltazar, L. Ferreira, *et al.*, "Retinoic acid-loaded polymeric nanoparticles induce neuroprotection in a mouse model for Parkinson's disease," *Front Aging Neurosci,* vol. 7, p. 20, 2015.

[92] Paxinos G and F. KBJ, "The Mouse Brain in Stereotaxic Coordinates, 2nd edn," *San Diego: Academic Press.,* 2001.

[93] A. Virgone-Carlotta, J. Uhlrich, M. N. Akram, D. Ressnikoff, F. Chretien, C. Domenget, *et al.*, "Mapping and kinetics of microglia/neuron cell-to-cell contacts in the 6-OHDA murine model of Parkinson's disease," *Glia,* vol. 61, p. 1645-58, 2013.

[94] M. R. Cookson, "Alpha-Synuclein and neuronal cell death," *Mol Neurodegener,* vol. 4, p. 9, 2009.

[95] R. Betarbet, T. B. Sherer, G. MacKenzie, M. Garcia-Osuna, A. V. Panov, and J. T. Greenamyre, "Chronic systemic pesticide exposure reproduces features of Parkinson's disease," *Nat Neurosci,* vol. 3, p. 1301-6, 2000.

[96] G. Paxinos and C. Watson, "The Rat Brain in Stereotaxic Coordinates, 4th Edn.," *San Diego, Academic Press.,* 1998.

[97] A. L. McCormack, M. Thiruchelvam, A. B. Manning-Bog, C. Thiffault, J. W. Langston, D. A. Cory-Slechta, *et al.*, "Environmental risk factors and Parkinson's disease: selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat," *Neurobiol Dis,* vol. 10, p. 119-27, 2002.

[98] M. G. Purisai, A. L. McCormack, S. Cumine, J. Li, M. Z. Isla, and D. A. Di Monte, "Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration," *Neurobiology of Disease,* vol. 25, p. 392-400, 2007.

[99] P. Damier, Hirsch EC, Agid Y, and G. AM., "The substantia nigra of the human brain. I. Nigrosomes and the nigral matrix, a compartmental organization based on calbindin D(28K) immunohistochemistry.," *Brain,* vol. 122, p. 1421-36, 1999.

[100] Damier P, Hirsch EC, Agid Y, and G. AM., "The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. ," *Brain,* vol. 122, p. 1437-48, 1999.

[101] A. Hartmann, "Postmortem studies in Parkinson's disease," *Dialogues Clin Neurosci,* vol. 6, p. 281-93, 2004.

[102] Y. He, T. Lee, and S. K. Leong, "6-Hydroxidopamine induced apoptosis of dopaminergic cells in the rat substantia nigra," *Brain Research,* vol. 858, 2000.

[103] F. L. Campos, M. M. Carvalho, A. C. Cristovao, G. Je, G. Baltazar, A. J. Salgado, *et al.*, "Rodent models of Parkinson's disease: beyond the motor symptomatology," *Front Behav Neurosci,* vol. 7, p. 175, 2013.

[104] D. Tang, Y. Shi, R. Kang, T. Li, W. Xiao, H. Wang, *et al.*, "Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1," *J Leukoc Biol,* vol. 81, p. 741-7, 2007.

[105] M.J. Marti, C.J. James, T.F. Oo, W.J. Kelly, and R. E. Burke, "Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of the substantia nigra," *J. Neurosci.,* vol. 17, p. 2030–2039, 1997.

[106] W. D. Qin, S. H. Mi, C. Li, G. X. Wang, J. N. Zhang, H. Wang, *et al.*, "Low shear stress induced HMGB1 translocation and release via PECAM-1/PARP-1 pathway to induce inflammation response," *PLoS One,* vol. 10, p. e0120586, 2015.

[107] A. Asaithambi, M. Ay, H. Jin, A. Gosh, V. Anantharam, A. Kanthasamy, *et al.*, "Protein kinase D1 (PKD1) phosphorylation promotes dopaminergic neuronal survival during 6-OHDA-induced oxidative stress," *PLoS One,* vol. 9, p. e96947, 2014.

[108] I. G. Onyango, J. B. Tuttle, and J. P. Bennett, Jr., "Altered intracellular signaling and reduced viability of Alzheimer's disease neuronal cybrids is reproduced by beta-amyloid peptide acting through receptor for advanced glycation end products (RAGE)." *Mol. Cell. Neurosci. ,* vol. 29, p. 333-343, 2005.

[109] O. Hori, J. Brett, T. Slattery, R. Cao, J. Zhang, J. X. Chen, *et al.*, "The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of RAGE and amphoterin in the developing nervous system," *J. Biol. Chem.,* vol. 270, p. 25752–25761, 1995.

[110] G. P. Sims, D. C. Rowe, S. T. Rietdijk, R. Herbst, and A. J. Coyle, "HMGB1 and RAGE in inflammation and cancer," *Annu Rev Immunol,* vol. 28, p. 367-88, 2010.

[111] E. S. Calay and G. S. Hotamisligil, "Turning off the inflammatory, but not the metabolic, flames," *Nat Med,* vol. 19, p. 265-7, 2013.

[112] C. W. Bell, W. Jiang, C. F. Reich, 3rd, and D. S. Pisetsky, "The extracellular release of HMGB1 during apoptotic cell death," *Am J Physiol Cell Physiol,* vol. 291, p. 1318-25, 2006.

[113] Fang P, Schachner M, and S. YQ., "HMGB1 in development and diseases of the central nervous system.," *Mol Neurobiol.,* vol. 45, p. 499-506, 2012.

[114] Gao J, Teng J, Liu H, Han X, Chen B, and X. A., "Association of RAGE gene polymorphisms with sporadic Parkinson's disease in Chinese Han population," vol. 24, p. 559:158-62, 2014.