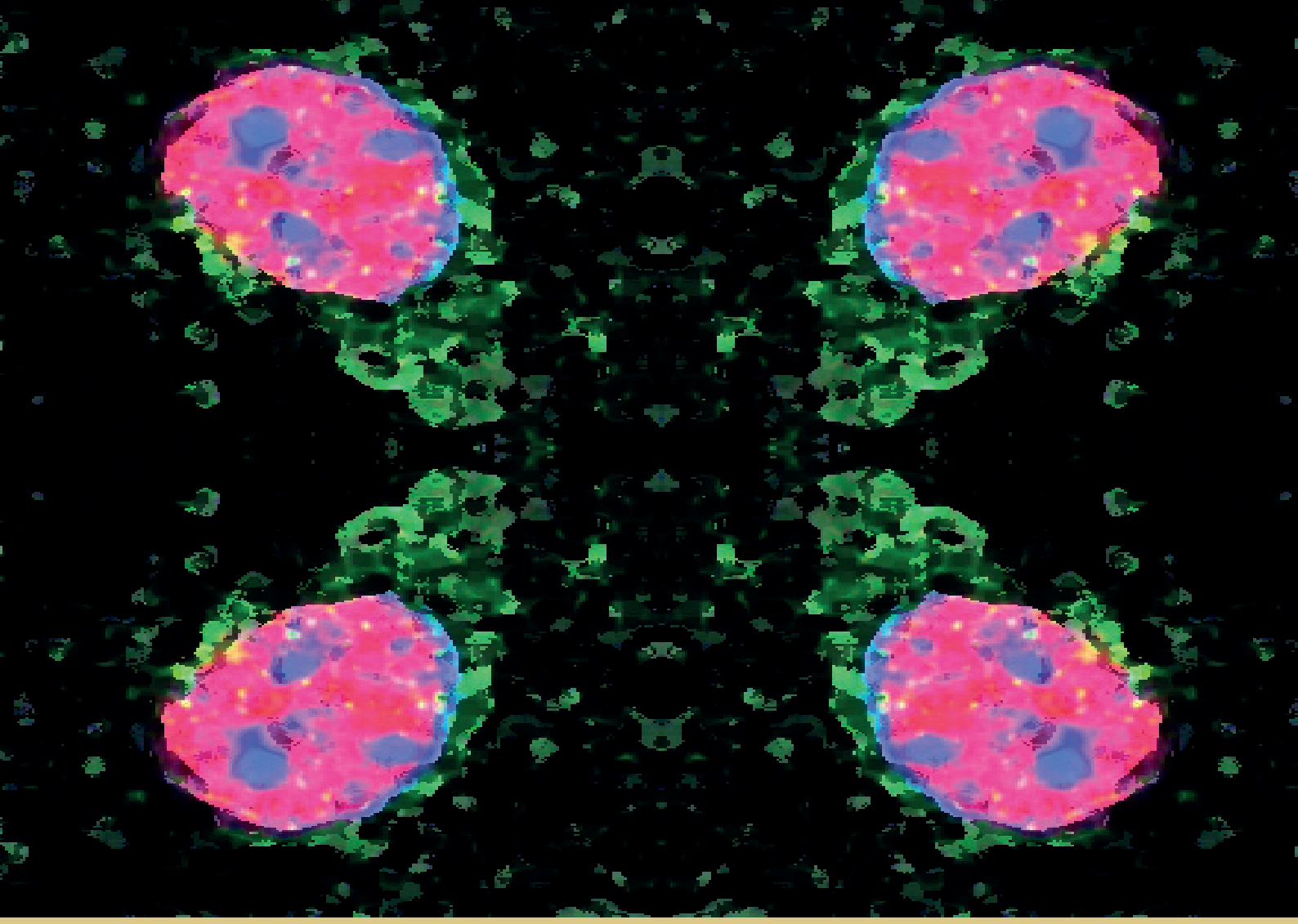

Sofia Andreia Domingues Viana

MODULATION OF RECEPTORS FOR ADVANCED GLYCATION ENDPRODUCTS NETWORK IN PARKINSON'S DISEASE

Doctoral Thesis in the Doctoral Program in Health Sciences branch of Biomedical Sciences, under supervision of Professor Frederico Guilherme Costa Pereira and Professor Carlos Alberto Fontes Ribeiro and presented to the Faculty of Medicine of the University of Coimbra.

July 2016

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Universidade de Coimbra

Front cover:
Image of striatal cells from C57BL/6 mice expressing receptor for advanced glycation endproducts (RAGE) N-terminus (green), RAGE C-terminus (red) and DAPI (blue)

Sofia Andreia Domingues Viana

Modulação da função dos receptores dos produtos de glicação avançada na Doença de Parkinson

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This work was conducted at the Laboratory of Pharmacology and Experimental Therapeutics, Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Portugal, under the supervision of Professor Frederico Guilherme Costa Pereira and Professor Carlos Alberto Fontes Ribeiro.

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"The important thing in science is not so much to obtain new facts
as to discover new ways of thinking about them"

(William Lawrence Bragg)

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Abbreviations

A

AGE's Advanced glycation endproducts

B

BBB Blood brain barrier

BDNF Brain derived neurotrophic factor

C

CML N^{ε}-(carboxymethyl)lysine

CN Caudate nucleus

CNS Central nervous system

COX-2 Cyclooxygenase-2

CSF Cerebrospinal fluid

ctRAGE C-terminal cytoplasmic tail

D

DAergic Dopaminergic

DAMP's Damage-associated molecular patterns

DAT Dopamine transporter

DOPAC 3,4-dihydroxyphenylacetic acid

F

flRAGE Full-length RAGE isoform, also denominated mRAGE

G

GABA γ-aminobutyric acid

GDNF Glial derived neurotrophic factor

GFAP Glial fibrillary acidic protein

Glu Glutamate

GP Globus pallidus

GS Glutamine synthetase

H

HNE 4-hydroxy-2-nonenal

HVA Homovanillic acid

I

i.n. Intranasal

i.p. Intraperitoneally

Iba-1 Ionized calcium-binding adapter molecule 1

IL Interleukine

iNOS Inducible nitric oxide synthase

L

LB Lewy bodies

L-DOPA Levodopa

LN Lewy neuritis

M

MAO-B Monoamine oxidase B

MHC Major histocompatibility complex

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

MSN Medium spiny neurons

N

NeuN Neuronal-specific nuclear protein

NF-κB Nuclear factor kappa B

NGF Nerve growth factor

NM Neuromelanin

NMS Non-motor symptoms

NO Nitric oxide

NSAID's Non-steroidal anti-inflammatory drugs

O

OCT's Organic cationic transporters

P

PAMP's Pathogen-associated molecular patterns

PD Parkinson's disease

PE Physical exercise

PET Positron Emission Tomography

PPARγ Peroxisome proliferator-activated receptor gamma

PRR's Pattern recognition receptors

R

RAGE Receptors for advanced glycation endproducts

ROS Reactive oxygen species

RPM Rotations per minute

RT-qPCR Real Time quantitative Polymerase chain reaction

S

SAID's Steroidal anti-inflammatory drugs

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SN*pc* Substantia nigra *pars compacta*

SN*pr* Substantia nigra *pars reticulata*

sRAGE Soluble RAGE isoforms

STN Subthalamic nucleus

α-syn α-synuclein

T

T2D Type II diabetes

TH Tyrosine hydroxylase

TLR's Toll-like receptors

TNF-α Tumor necrosis factor α

V

VMAT-2 Vesicular monoamine transporter

W

WB Western blot

Published work

The results presented in this Thesis have been published or submitted for publication in international peer-reviewed journals as follows:

Viana SD, Fernandes RC, Canas PM, Silva AM, Carvalho F, Ali SF, Fontes Ribeiro CA, Pereira FC (2016) Presymptomatic MPTP Mice Show Neurotrophic S100B/mRAGE Striatal Levels. CNS Neurosci Ther. 22(5):396-403 (doi: 10.1111/cns.12508) – **Chapter 2**

Viana SD, Valero J, Rodrigues-Santos P, Couceiro P, Silva AM, Carvalho F, Ali SF, Fontes Ribeiro CA, Pereira FC (2016) Regulation of striatal astrocytic RAGE variants in an early stage of experimental Parkinson's disease. J Neurochem [(doi:10.1111/jnc.13682 (in press)] – **Chapter 3**

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Resumo

A complexa relação entre quadros inflamatórios crónicos, provenientes de uma desregulação do sistema imune inato, e o processo neurodegenerativo associado à Doença de Parkinson (DP) é uma área emergente na comunidade científica. Através de um conjunto intricado de sensores imunes, onde os receptores dos produtos de glicação avançada (RAGE) aparentam desempenhar um papel preponderante, a imunidade inata regula a extensão e consequências da reacção inflamatória: é benéfica quando limitada no tempo mas podendo ser deletéria se perpetuada indefinidamente. Vários estudos *post-mortem*, clínicos e experimentais, evidenciam a acumulação de padrões moleculares associados a danos [Damage-associated molecular patterns – DAMP's (e.g. S100B, HMGB-1)], RAGE e co-receptores da imunidade inata em diferentes regiões cerebrais afectadas pelo processo neurodegenerativo. Estas evidências sugerem que uma cascata de eventos deletérios mediada por RAGE possa estar sobre-activada na DP. No entanto, este receptor apresenta simultaneamente uma importante função em processos homeostáticos e regenerativos pelo que se torna imperioso identificar estratégias que anulem os seus efeitos deletérios sem compromisso das suas funções benéficas. Este delicado balanço será porventura bem-sucedido após a clarificação dos parâmetros biológicos que ditam a resposta mediada por RAGE, nomeadamente em contextos experimentais associados à DP. Assim, o objectivo principal deste trabalho consistiu na caracterização biológica que determina o fenótipo de RAGE, recorrendo a distintos regimes de intoxicação da neurotoxina 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) em modelos animais, que representam paradigmas experimentais da DP.

A diversidade de isoformas RAGE é crucial para a regulação dos seus efeitos benéficos/deletérios. Efetivamente, a sinalização mediada por RAGE resulta da complexa combinação dos efeitos mediados por isoformas competentes no processo de sinalização (flRAGE) *versus* isoformas antagonistas [variantes truncadas (e.g. isoformas solúveis, sRAGE)]. Através da sua capacidade em sequestrar ligandos no espaço extracelular, as isoformas RAGE solúveis previnem a acumulação tóxica de ligandos, garantindo uma activação basal e trófica de flRAGE. No entanto, quadros caracterizados por stress oxidativo e inflamação crónica potenciam a acumulação de ligandos, perpetuando cascatas intracelulares de sinalização deletéria mediada por RAGE, com o aumento da sua expressão e subsequente sinalização

inflamatória. Assim, a primeira abordagem experimental deste trabalho consistiu na caracterização das isoformas RAGE e do seu ligando S100B no estriado de murganhos C57BL/6 submetidos a distintos protocolos de intoxicação sistémica (aguda e crónica) de MPTP. Esta estratégia permitiu-nos primeiramente observar que, num modelo de intoxicação crónico, níveis proteicos basais de S100B/flRAGE (considerados neurotróficos) e uma predominante expressão de flRAGE neuronal acompanham processos compensatórios (e.g. aumento do *turnover* de dopamina) recrutados como resposta à disrupção do sistema dopaminérgico estriatal em murganhos sem compromisso motor. Este fenótipo potencialmente protector motivou-nos a fazer uma caracterização completa das isoformas RAGE numa fase inicial de degenerescência dopaminérgica no estriado, induzida por um protocolo de intoxicação aguda de MPTP. Neste segundo paradigma experimental, observámos o aumento da expressão de isoformas inibitórias de RAGE. No entanto, encontrámos teores basais de isoformas sinalizadoras. Esta regulação potencialmente citoprotectora ocorreu em astrócitos pre-hipertróficos, exibindo sobre-expressão do ligando S100B. Estes resultados sugerem a activação de processos regulatórios conducentes a um perfil citoprotector de RAGE na lesão dopaminérgica estriatal, em fases pré-motoras do processo neurodegenerativo.

A complexidade da sinalização citoprotectora/citotóxica mediada por RAGE aumenta com o recrutamento de outros receptores de padrões moleculares (PRR's), nomeadamente os receptores da família Toll (TLR's). Tendo em conta que os DAMP's são reconhecidos por ambas as classes de receptores, a acumulação destes ligandos despoleta a activação de tríades funcionais formadas por DAMP's/RAGE/TLR's, exacerbando quadros inflamatórios com subsequente dano celular/tecidular. Neste contexto, a estratégia experimental seguinte consistiu num estudo abrangente de transcrição génica visando DAMP's, RAGE e TLR's, bem como efectores da imunidade inata. Foram incluídas nesta terceira abordagem experimental regiões cerebrais associadas a défices cognitivos e emocionais (e.g. hipocampo, córtex frontal), sintomatologia característica da fase prodrómica da DP. Numa fase pré-motora de um modelo de administração intranasal (i.n.) de MPTP a ratos Wistar, observámos uma supressão selectiva da transcrição de RAGE e outros elementos chave da imunidade inata (e.g. TLR5,7 e subunidade p22 do complexo NADPH oxidase) no córtex frontal de animais intoxicados por MPTP, e que apresentavam défices comportamentais do foro cognitivo e emocional. Este padrão coordenado de expressão génica sugere a activação precoce de mecanismos de retro-controlo como tentativa

de regular a resposta imunitária, prevenindo assim um excesso de reatividade imunitária e inflamação.

Por fim, recorremos a este mesmo paradigma experimental para avaliar a capacidade do exercício físico (EF) em modular centralmente o eixo RAGE e o fenótipo cognitivo e emocional. Neste contexto, observámos uma imunossupressão de RAGE, TLR's e efectores do sistema imune no córtex frontal de ratos sujeitos a um protocolo aeróbico de longa duração, em passadeira. Este perfil imunitário não se repercutiu em alterações de fenótipo não motor. Surpreendentemente, constatámos que ratos intoxicados com MPTP (i.n.) e submetidos ao mesmo protocolo de EF, recuperaram a expressão do eixo RAGE e aumentaram a expressão do gene neuroprotector DJ-1. Esta modulação imunitária acompanhou a recuperação dos défices cognitivos e emocionais observados nos ratos sedentários e intoxicados. Estas observações constituem um importante avanço dado que o conhecimento do impacto do EF no sistema neuroimune é manifestamente insuficiente num contexto fisiológico, e praticamente desconhecido na DP.

Em suma, as evidências observadas nos diferentes paradigmas experimentais implementados neste estudo convergem para um fenótipo citoprotector de RAGE em estadios inicias da DP. Abordagens complementares que identifiquem i) os mecanismos regulatórios subjacentes à repressão do fenótipo citotóxico de RAGE e ii) estratégias moduladoras das isoformas RAGE, priveligiando selectivamente as variantes citoprotectoras, são indispensáveis. Paralelamente, é desejável o aprofundamento da avaliação funcional da adaptação imunitária do eixo RAGE no córtex frontal promovida pelo EF, nomeadamente em estadios iniciais da DP. Este trabalho encoraja a existência de estudos complementares na esperança de se poder desenhar estratégias inovadoras na terapêutica da DP.

Palavras-chave: Doença de Parkinson; Exercício físico; Receptores dos produtos de glicação avançada; S100B; Receptores *Toll*

Abstract

A burgeoning research field in Parkinson's disease (PD) relies on the complex relationship between deregulated innate inflammation and neurodegenerative process. Through an elaborate network of immune sensors where receptor for advanced glycation endproducts (RAGE) seems to play a chief-regulatory role, innate immune system is strongly implicated in the fine regulation of the best balance between acute resolution of injury versus chronic amplification of noxious pathways. Strong evidence from humans and experimental PD settings points towards the accumulation of danger associated molecular patterns [DAMP's, (e.g. S100B, HMGB-1)], RAGE and immune co-receptors in distinct affected brain regions, strongly suggesting that an operative RAGE network participates in neurodegeneration. Yet, RAGE is simultaneously a central player in homeostasis maintenance and tissue regeneration upon short-limited injury. This "friend or foe" behavior suggests that strategies aiming to retain primal RAGE responses to stress, yet derailing deleterious RAGE outcomes would be of utmost importance in PD. Such fine tuning regulation can be achieved only with an in-depth understanding of RAGE biology in PD settings. Hence, the broad aim of this thesis is to provide a global characterization of key-aspects of RAGE biology using distinct neurotoxin-based 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) rodent models that experimentally replicate specific features of early PD.

The diversity of RAGE isoforms is pivotal in dictating RAGE protective or deleterious outcomes. In fact, when one speaks of RAGE signaling, one is referring to an outcome that results from the interplay between signaling-competent full length RAGE (flRAGE) and antagonistic RAGE isoforms [truncated RAGE variants (e.g. soluble RAGE, sRAGE)]. By acting as decoy receptors, sRAGE prevents RAGE ligands accumulation (e.g. S100B) thereby maintaining a low-grade of flRAGE trophic signaling. Conversely, chronic inflammatory/oxidant settings favor ligands accumulation with subsequent noxious flRAGE up-regulation. Hence, we initially addressed RAGE protein isoforms and its S100B ligand in long-lasting *versus* short-limited MPTP stressed striata (chronic and acute MPTP models, respectively). We newly provide experimental evidence showing neurotrophic S100B/flRAGE contents and major neuronal RAGE localization pairing compensatory changes (e.g. increased dopamine turnover) in chronic stressed striata from mice showing normal locomotor behavior.

This seemingly beneficial outcome prompted us to further dissect the overall balance of striatal RAGE variants shortly after acute MPTP. Noticeably, we found increased inhibitory RAGE variants combined with normal flRAGE contents. Regulation of RAGE isoforms towards a cytoprotective phenotype occurred in pre-hypertrophic astrocytes showing higher S100B density. These findings seem to converge towards RAGE trophic outcomes within MPTP-striatal dysfunction, an early event of dopaminergic neurodegenerative process that classically characterizes PD.

The fine tuning of repair/injury balance also involves RAGE cross-talk with other immune sensors, namely Toll-like receptors (TLR's). Since DAMP's are recognized by both class of receptors, accumulation of these ligands foster signaling pathways amplification through DAMP's/RAGE/TLR functional tripods engaged in exacerbated innate inflammation and injury. Hence, we underwent a comprehensive transcriptomic approach of ligands, RAGE, TLR's and intracellular effectors of immune response. We extended this study to frontal cortex and hippocampi regions regarding their role in cognitive and emotional deficits that typically characterizes PD prodromal phase. In a pre-motor phase of intranasal (i.n.) MPTP model, cognitive/emotionally impaired MPTP-animals showed a selective regional transcriptomic suppression of RAGE and core components of innate immunity (e.g. TLR5,7 NADPH oxidase p22-subunit) in frontal cortex. This coordinated expression pattern suggests counter-regulatory mechanisms aimed to restrain central innate immune response towards homeostasis.

Finally, we used i.n. MPTP model to experimentally address the aptitude of physical exercise (PE) to modulate central neuroimmune RAGE-network while correcting MPTP-elicited cognitive/emotional deficits. One of the most impressive findings of the present work is the selective and robust immunosuppression of RAGE, large majority of TLR's and immune effectors in frontal cortex of long-term treadmill-trained rats displaying normal behavioral phenotype. Noticeably, an almost recovery of neuroimmune RAGE-network and a significant up-regulation of neuroprotective PD-related gene DJ-1 paralleled normal cognitive and emotional/motivational phenotypes of trained-MPTP subjects. These findings reflect an important advancement in the research field as experimental data on the impact of PE on brain immune mediators is extremely scarce in physiological contexts, being almost ignored in PD pathology.

Overall, findings from all experimental paradigms are convergent and strongly suggest that RAGE has a protective rather tissue-destructive phenotype in early stages of PD neurodegeneration. Additional research is warranted to further dissect the regulatory mechanisms underpinning neuroimmune RAGE-network transcriptomic as well as strategies to specifically modulate RAGE variants towards cytoprotective isoforms in experimental PD. Besides, future studies should evaluate the functional consequences of RAGE/TLR's/immune effectors adaptation in frontal cortex associated with PE, particularly in early stages of PD. Hopefully, this will bring new innovative strategies for the disease.

Keyword: Parkinson's disease; Physical exercise; Receptors for advanced glycation endproducts; S100B; Toll-like receptors

Chapter 1

Introduction

General Introduction

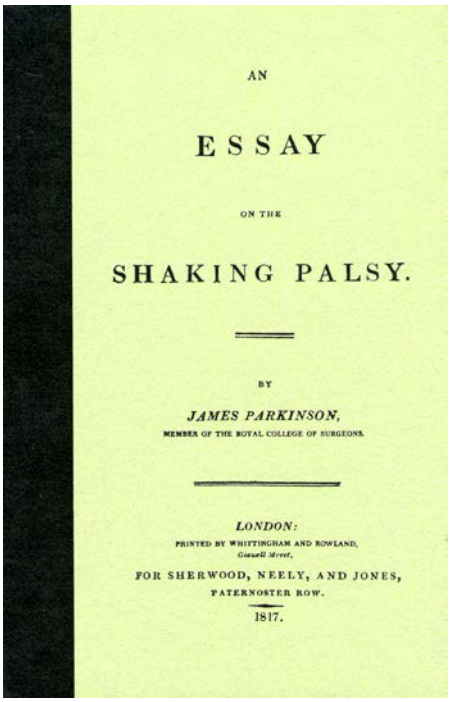
Aging is associated with an increased rate in neurologic diseases. Parkinson's disease (PD) is currently the second most common neurodegenerative disease, after Alzheimer's disease (Freeman & Vatz 2010). Most community based prevalence studies across Europe found crude PD prevalence rates between 100 and 200 per 100,000 inhabitants, a number predicted to double by 2030 particularly in developed countries where life expectancy is increasing (Dorsey *et al.* 2007, von Campenhausen *et al.* 2005). Although age is its strongest risk factor, sex also influences disease risk with PD incidence being 1.5 times higher in men than women (Tanner & Aston 2000, Taylor *et al.* 2007). The disease is found in all ethnic groups with no geographical differences in prevalence. Since it is mainly an illness of later life, the economic burden of PD will likely continue to grow in the future and will be a critical challenge for health, social and economic policies (Alves *et al.* 2008). There has been much progress over the last few decades in devising more accurate diagnostic criteria and in the development of treatments for PD. However, the etiology of disease is still poorly understood and PD remains incurable.

In the introduction of this Thesis, it will be covered a number of topics related to PD, including some historical aspects of this pathological condition, its clinical presentation and neuropathological features, available treatments and our current understanding of the etiology of this condition, with particular emphasis on immune system and the intricate involvement of Receptor for advanced glycation endproducts (RAGE) network. Finally, the main aims of the thesis, theoretical models used herein and research questions will be presented.

1.1 Historical aspects of Parkinson's disease

In 1817, James Parkinson, a medical doctor from London, delivered a clear description of the medical condition that would later be named after his name in his classical publication "An essay on the shaking palsy" (see Figure 1.1). Main clinical features of PD, or *paralysis agitans*, were reported as *"Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured."*

Interestingly, Parkinson also described non-motor symptoms that are now recognized as sleep behavior disorder *"…the sleep becomes much disturbed. The tremulous motion of the limbs occurs during sleep, and augment until they awaken the patient, and frequently with much agitation and alarm."* and hallucinations and cognitive impairments: "*…at the last, constant sleepiness, with slight delirium, and other marks of extreme exhaustion, announce the wished-for release" (*Parkinson 1817, reprinted in Parkinson 2002). More than 50 years later, Jean-Martin Charcot was more thorough in his description, having identifyied bradykinesia as a cardinal feature of the illness, and having coined the term "Parkinson's disease" (Goetz 2011).


AN

ESSAY

ON THE

SHAKING PALSY.

BY

JAMES PARKINSON,

MEMBER OF THE ROYAL COLLEGE OF SURGEONS.

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1817.

Figure 1.1 James Parkinson's original publication from 1817

1.2 Clinical presentation and staging of PD

Application of clinical diagnostic criteria algorithms has been shown to improve differential diagnosis of PD among a long list of different parkinsonism conditions such as multiple system atrophy, Lewy body dementia or drug-induced parkinsonism (Gelb *et al.* 1999, Hughes *et al.* 2002, Litvan *et al.* 2003). PD is, classically, diagnosed when the cardinal or so-called prototypical motor symptoms (bradykinesia, rigidity, resting tremor and postural instability) become explicit (Jankovic 2008). PD motor symptomatology is subtle at disease onset but tend to evolve from mild unilateral symptoms (stage I) through end-stage nonambulatory state (stage V; see Table 1.1).

Table 1.1 Modified Hoehn and Yahr staging of PD
Adapted from (Hoehn & Yahr 1967)

Stage	Criteria
0	No signs of disease
I	Unilateral disease
I.5	Unilateral disease plus axial involvement
II	Bilateral disease, without impairment of balance
II.5	Mild bilateral disease, with recovery on pull test
III	Mild to moderate bilateral disease; some postural instability; physically independent
IV	Severe disability; still able to walk or stand independently
V	Wheel chair bound or bedridden unless aided

The recognition of a myriad of non-motor symptoms (NMS) in PD patients represents an important development in the recent years (Gaig & Tolosa 2009). The NMS consist of neuropsychiatric symptoms (e.g. apathy, anxiety, depression, psychosis, cognitive impairment/dementia), autonomic disturbances (e.g. constipation, bladder and sexual disturbances), sensory symptoms [e.g. impaired olfaction (hyposmia), pain] and sleep disorders (i.e. rapid eye movement (REM)-sleep behavioral disorders, excessive daytime sleepiness)(Cummings & Masterman 1999, Lewis *et al.* 2003, Pfeiffer 2016, Stebbins *et al.* 1999, Tolosa *et al.* 2007). NMS are envisioned by PD patients at least as troublesome as motor features, and strongly impact daily patient's and carer's well-being. Moreover NMS treatment remains suboptimal (Chaudhuri & Schapira 2009, Chen *et al.* 2013, Deane *et al.* 2014). Importantly, some of these NMS (e.g. hyposmia, constipation, REM-sleep disorders, depression) predate the onset of classical motor features of PD. This phase has been defined as the "pre-

motor" or the "pre-clinical" phase/stage of PD, and is part of ongoing neurodegenerative disease processes (Winkler *et al.* 2011).

Research on pre-motor symptoms of PD may offer an excellent opportunity to better understand PD etiology (Chen *et al.* 2013, Noyce *et al.* 2016). Nevertheless, a definitive diagnosis of the pre-motor stage of PD is still far from being well established. Indeed, understanding of pre-motor symptoms of PD is in its infancy and faces many obstacles. These symptoms are often not specific to PD and have low positive predictive value for early PD diagnosis (Chen *et al.* 2013). The Movement Disorder Society modified the unified Parkinson's disease rating scale (UPDRS, the gold standard of motor measurement in PD) to include validated screening questions for non-motor symptoms (e.g. anxiety, depressed mood, psychosis) - Movement Disorder Society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS) (Chaudhuri *et al.* 2006). Notably, this scale is currently recommended as a "clinician-friendly" screening instrument in the new European guidelines on PD diagnosis, developed in the frame of a Task Force convened by the European Federation of Neurological Societies and Movement Disorder Society-European Section Scientist Panel on PD: EFNS/MDS-ES. This valuable and large-scale work was fostered by recent efforts of the PD research community focusing on the development of screening tools capable of identifying individuals *at risk* for PD. Groups of experts were asked to provide evidence-based recommendation level for nine sections addressing key aspects of the diagnostic work-up of patients presenting with parkinsonism. Interestingly, some selected investigations (e.g. genetic, olfactory and neuroimaging studies) were proposed to have an ancillary role in confirming the diagnosis and/or effectively used in the near future in the attempt to identify people in a preclinical phase of the disease (Berardelli *et al.* 2013).

1.3 Neuropathological features

PD is nowadays defined as a clinical condition of progressive parkinsonism of undetermined course without features suggestive of an alternative diagnosis, responding to dopaminergic (DAergic) treatment, and associated with depletion of approximately 30-50% of brain stem neuromelanin containing-dopaminergic neurons and with Lewy body (LB) inclusions in some of the remaining cells (see Figure 1.2) (Tolosa *et al.* 2006). Neuromelanin (NM) is a granular, dark brown pigment produced in some but not all of the dopaminergic neurons of the human SN, being a byproduct of catecholamine catabolism. The function of NM within the pigmented neurons is unknown but other melanins are believed to play a protective role via attenuation of free radical damage (Gerlach *et al.* 2003). This definition combines clinical features, response to anti-parkinsonian medication and ultimately pathological confirmation (Tolosa *et al.* 2006).

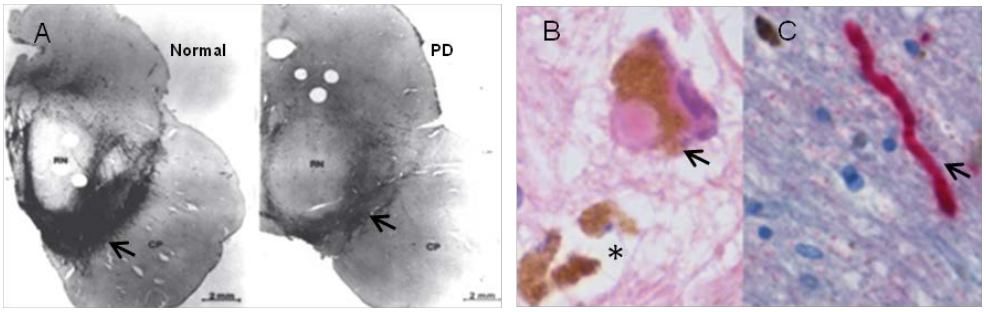

Figure 1.2 A. Axial cut of human mesencephalon. The substantia nigra *pars compacta* (SNpc) identified by tyrosine hydroxylase immunostaining (arrow) reflecting the presence of DAergic neurons. Cell loss in the parkinsonian SNpc can be appreciated macroscopically by decrease of staining density compared to the control. RN, red nucleus; CP, cerebral peduncle **B. SNpc neuron with a Lewy body** (arrow) and pigment-laden macrophages (asterisk, 500x haematoxylin/eosin stain). **C. Alpha-synuclein positive Lewy neurite** (arrow, 400x haematoxylin/eosin stain) (A. Taken from Hartmann 2004; B,C. from Werner *et al.* 2008)

1.3.1. PD neuron loss

1.3.1.1 Basal ganglia physiology – brief review

PD is known primarily as a movement disorder originating in the basal ganglia, which comprises the following structures: *striatum* (caudate nucleus and putamen), *globus pallidus* (internal and external segments - GPi and GPe, respectively), *nuclei of dienchepalon* (thalamus and subthalamic nucleus) and the *mesencephalic structures* including substantia nigra and pedunculopontine nucleus (see Figure 1.3).

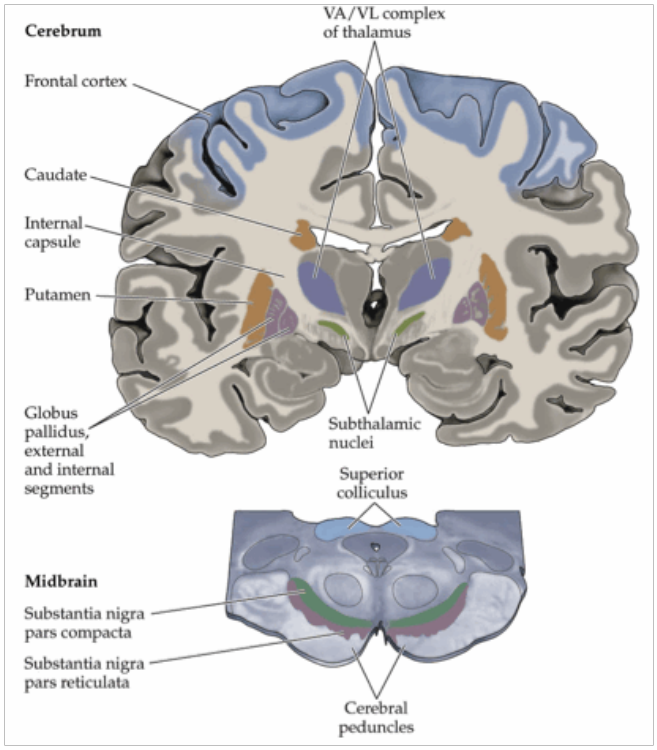

Figure 1.3 Idealized coronal section through the brain showing anatomical locations of structures involved in the basal ganglia pathway. Most of these structures are in the telencephalon, although the substantia nigra is in the midbrain and the thalamic and subthalamic nuclei are in the diencephalon. The ventral anterior and ventral lateral thalamic nuclei (VA/VL complex) are the targets of the basal ganglia, relaying the modulatory effects of the basal ganglia to upper motor neurons in the cortex (Taken from Purves 2008)

Normal function of basal ganglia requires fine tuning of neuronal excitability within each nucleus to determine the exact degree of movement facilitation or inhibition at any given moment (Obeso *et al.* 2008). This is mediated by the complex organization of striatum, the major input nucleus of basal ganglia. Striatal neuronal inputs come mainly from the cerebral cortex, thalamus and substantia nigra pars compacta (SNpc), the latter being topographically organized as the nigrostriatal pathway. The striatum contains mainly inhibitory γ-aminobutyric acid (GABA) neurons [over 90% of the cells are medium spiny neurons (MSN)] which project to the basal ganglia output nuclei [composed by GP and *substantia nigra pars reticulata* (SNr)] via direct and indirect pathways (Haber 2016). The direct pathway projects to the GPi and SNr that inhibit thalamus. The indirect pathway projects to the GPe that controls GPi/SNr either directly or via the subthalamic nucleus (STN), who projects excitatory (glutamatergic) fibers to the pallidum. Thus, activation of the direct or indirect pathway have antagonistic effects on basal ganglia output: inhibition versus activation of the output neurons, respectively (see Figure 1.4).

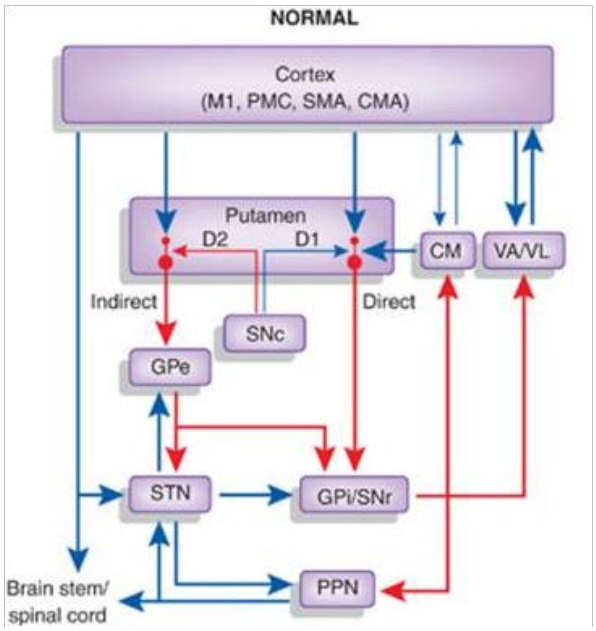

Figure 1.4 Basal ganglia circuitry in physiological state. Schematic diagram of the direct and indirect pathways of the basal ganglia motor circuits. Red arrows indicate inhibitory projections, and blue arrows indicate excitatory projections. Note that many connections have been purposefully omitted from this diagram. CM, centromedian nucleus; CMA, cingulate motor area; GPe, globus pallidus, external segment; GPi, globus pallidus, internal segment; M1, primary motor cortex; PMC, pre-motor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral nucleus (Adapted from Smith *et al.* 2012)

1.3.1.1.1 Dopamine life cycle and neuronal modulatory actions

SNpc efferents strongly regulate cortico-basal-ganglia loop by releasing the neuromodulator dopamine (DA). The biosynthesis of DA takes place in the cytosol of dopaminergic neurons and includes tyrosine hydroxylase (TH) which is the rate-limiting step in DA synthesis (Meiser *et al.* 2013) (see Figure 1.5). The cytoplasmic newly synthesized DA is sequestered into vesicles by the vesicular monoamine transporter (VMAT-2) or undergoes through metabolic degradation. For extensive review of DA homeostasis and intra and extra-synaptic routes, please see Chapter 1 and 2 from Pereira and colleagues work (Pereira *et al.* 2012).

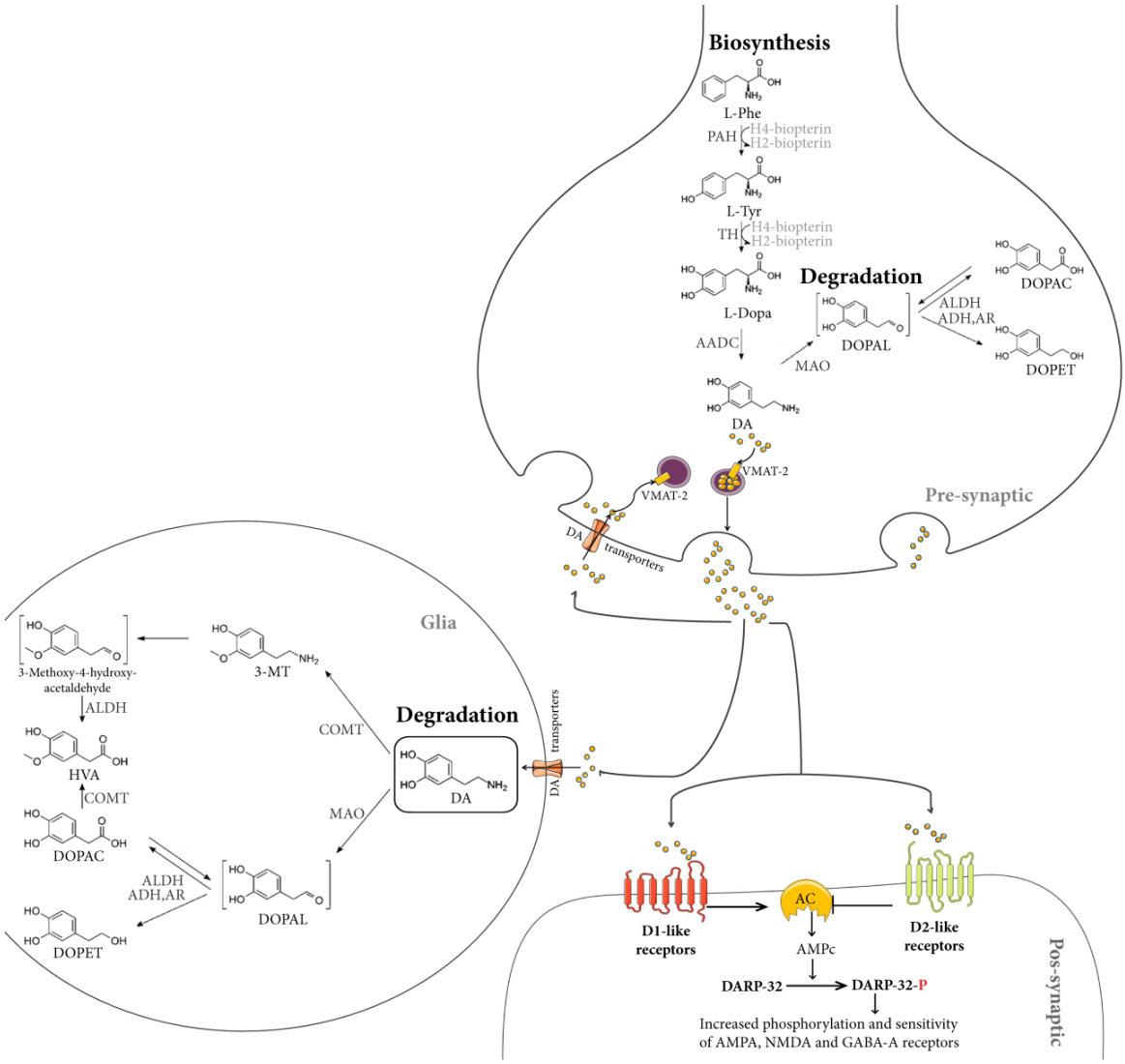

Figure 1.5. Dopamine life cycle and neuronal modulatory actions. The major pathway for dopamine (DA) biosynthesis starts at L-phenylalanine (L-Phe), which can be hydroxylated by phenylalanine hydroxylase (PAH). L-tyrosine (L-Tyr) is hydroxylated into L-dihyrophenylalanine (L-DOPA) that bears the cathechol moiety, by BH4-dependent tyrosine hydroxylase (TH). Decarboxylation of DOPA by aromatic L-amino acid decarboxylase (AADC) leads finally to DA. Newly synthesized DA is mainly sequestered into vesicles by vesicular monoamine transporter (VMAT-2). However, a small amount of DA remains in the cytosolic compartment where it undergoes metabolic degradation. Released DA can activate both D1-like or D2-like receptors with important downstream consequences in basal ganglia function. DA clearance from extracellular space is promoted by DA re-uptake transporters [e.g. DA transporter (DAT, high affinity); Organic cationic transporter-3 (OCT-3, low affinity) and plasma membrane monoamine transporters (PMAT, low affinity)] being DAT the major route for the rapid recycling back into DAergic terminals (Cui *et al.* 2009, Naganuma *et al.* 2014, Riddle *et al.* 2005). Enzymes involved in DA degradation are monoamine oxidase (MAO), alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), aldose reductase (AR) and catechol-O methyl transferase (COMT) which lead to 3,4-dihydroxyphenylacetaldehyde (DOPAL), 3,4-dihydroxyphenylethanol (DOPET), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), 3-methoxytyramine (3-MT) and 3-methoxy-4-hydroxyacetaldehyde by-products, being DOPAC and HVA the main DA metabolites. Since there is no COMT activity in DAergic nigro-striatal neurons, HVA is mainly synthesized in glial cells (AC, adenylate cyclase, AMPc, Cyclic adenosine monophosphate)

Human SNpc contains approximately between 3 to $6x10^5$ dopaminergic neurons which mainly project to the striatum (German & Manaye 1993). Although dopaminergic boutons represent only 10% of all striatal synapses, they form postsynaptic axospinous and axodendritic synapses with MSN's, and presynaptic axoaxonic synapses with the terminal boutons of corticostriatal fibers, which synapse mainly on the spines of MNS's (Kreitzer 2009, Smith & Kieval 2000, Yung & Bolam 2000). Unlike glutamate (Glu) and GABA, DA does not cause itself depolarization or hyperpolarization of the post-synaptic membrane (Greengard 2001). DA, being a neuromodulator, rather exerts a powerful influence over the efficacy of converging synaptic actions of the fast-acting neurotransmitters Glu and GABA through D1 class (D1,D5) or D2 class (D2,D3,D4) seven-transmembrane dopamine receptors. These two classes are based on their coupling to G-proteins that either increase (D1-like) or decrease (D2-like) cytoplasmic cyclic adenosine monophosphate (cAMP), which in turn regulate the phosphorylation of DARP-32 that controls the sensitivity of glutamate and GABA receptors (Alexander 2004). Importantly, direct and indirect striatal pathways are known to be under differential dopaminergic control from nigrostriatal pathway: while neurons of the direct pathway express excitatory-D1 dopamine receptors, MSN's of indirect pathway express inhibitory-D2 dopamine receptors (Haber 2016). Additionally, DA regulates corticostriatal synaptic plasticity which is essential in the regulation of voluntary movement, in addition to behavioural control, cognitive function and reward mechanisms (Calabresi *et al.* 2007).

1.3.1.2 Basal ganglia neurodegeneration

One major neuropathological signature of PD is striatal dopamine depletion arising from dopaminergic neuronal dysfunction/loss, which leads to an hypo-activity of the direct pathway and an over-activity of the indirect pathway. This functional imbalance disinhibits STN and over-activates GPi/SNr. In this PD state, the thalamo–cortical, premotor and prefrontal areas and brainstem are over-inhibited, resulting in PD symptomatology (Obeso *et al.* 2008, Smith *et al.* 2012) (see Figure 1.6). Yet, motor deficits are not a "sudden" event, but rather a mid-term stage of a continuous pathophysiological process (Winkler *et al.* 2011). In humans, dopaminergic neuronal loss has to exceed 30% and striatal dopamine contents to drop by 80% before PD motor symptoms are observed. These observations suggest the existence of

compensatory and adaptive processes during the pre-motor period (Agid 1991, Burke & O'Malley 2013, Hornykiewicz 1998, Tabbal *et al.* 2012, Vezoli *et al.* 2014).

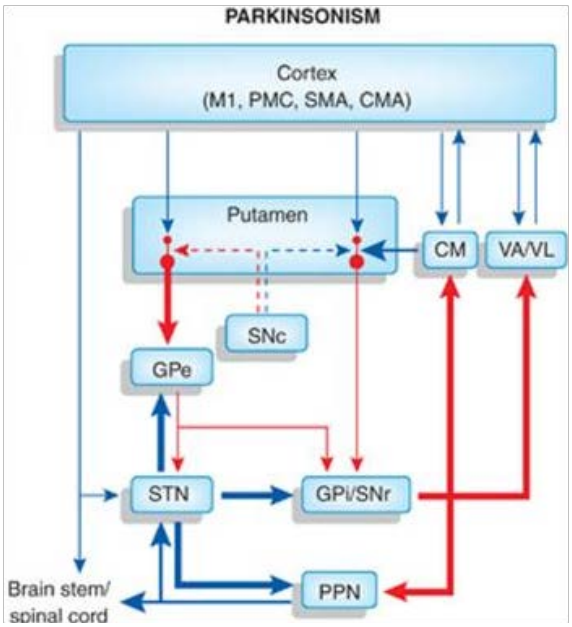

Figure 1.6 Basal ganglia circuitry in PD. Schematic diagram of the direct and indirect pathways of the basal ganglia motor circuits. Red arrows indicate inhibitory projections, and blue arrows indicate excitatory projections. The changes in the thickness of the arrows in the parkinsonian state indicate the proposed increase (larger arrow) or decrease (thinner arrow) in firing-rate activity of specific connections. The dashed arrows used to label the dopaminergic projection from the SNc to the putamen in parkinsonism indicate partial lesion of that system in this condition. Note that many connections have been purposefully omitted from this diagram. CM, centromedian nucleus; CMA, cingulate motor area; GPe, globus pallidus, external segment; GPi, globus pallidus, internal segment; M1, primary motor cortex; PMC, pre-motor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral nucleus (Adapted from Smith *et al.* 2012)

Although not fully understood, evidence from experimental studies suggest that functional compensation in the partially denervated striatum can be achieved by several, not necessarily mutually exclusive, mechanisms involving: i) dopamine homeostatic compensatory mechanisms (e.g. upregulation of DA turnover, MSN's dendritic remodeling); ii) non-dopamine mediated compensatory mechanisms in basal ganglia output structures (e.g. increased electrophysiological activity of STN and GPi) as well as iii) non-basal ganglia compensatory mechanisms (e.g. motor and non-motor cortical structures) (Bezard *et al.* 2003, Pifl & Hornykiewicz 2006, Rajput *et al.* 2004, Vezoli *et al.* 2014). In fact, the convergence of multiple cortical metabolic defects with specific transcriptomic changes observed in prefrontal cortex of experimental and human PD strongly suggest the involvement of frontal cortical regions in early compensatory processes within PD pathology (Ferrer 2009, Storvik *et al.* 2010). These mechanisms could also play a role in prodromal PD manisfestations (Storvik *et al.* 2010).

Nevertheless, changes in striatal dopamine metabolism within PD pre-motor period are still the most extensively studied. I*n vivo* Positron Emission Tomographic (PET) imaging incorporating different radioligands [e.g. 11C(±)dihydrotetrabenazine, [11C]d-threo-methylphenidate and 6-[18F]fluoro-L-DOPA] has been a valuable tool to study the functional changes in DA metabolism (e.g. synthesis, storage and DA turnover) in the presynaptic dopaminergic nerve terminals in human PD. Consistent with observations of PD animal models, Lee and co-workers found that loss of DA neurons is functionally compensated by increased DA synthesis, release and presynaptic DA reuptake downregulation in the remaining DAergic nerve terminals (Lee *et al.* 2000). Dopamine synthesis in the surviving presynaptic terminals through upregulation of the biosynthetic machinery and DAT downregulation - the principal mechanism of termination of dopamine action at the synapse - are likely to represent adaptative and restorative attempts to maintain near-optimal dopaminergic transmission in the face of progressive neuronal loss. Noteworthy, longitudinal multi-tracer PET imaging designed to assess presynaptic dopaminergic integrity as PD evolves showed failure of such putaminal dopaminergic compensatory responses in more advanced disease stages. This suggests that breakdown of restorative mechanisms may contribute to the onset of motor symptoms (Nandhagopal *et al.* 2011).

1.3.2 α-Syn inclusions

Intracytoplasmatic and intraneuritic eosinophilic inclusions including Lewy bodies (LB) and Lewy neuritis (LN) are typical hallmarks in the PD brain. These structures are composed of insoluble filamentous protein aggregates, mainly containing ubiquitinated α-synuclein (α-syn), in association with other proteins (phosphorylated *tau*, neurofilaments and other cytoskeletal proteins) (Bellucci *et al.* 2016, Vernier *et al.* 2004). Whilst LB are sensitive markers for PD, they are not disease-specific and can be found in a number of other conditions commonly referred to as α-synucleinopathies [e.g. Lewy body dementia (LBD), Parkinson's disease with dementia (PDD), multiple system atrophy (MSA)] (Spillantini & Goedert 2000). Although native unfolded α-syn is present in erythrocytes and platelets (Barbour *et al.* 2008, El-Agnaf *et al.* 2003), it is predominantly neuronal where it redistributes into the axonal and presynaptic compartments. Its main neuronal function is likely to promote a soluble regulating *N*-

ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex which is instrumental in supporting synaptic vesicle recycling and neurotransmitter release (Anichtchik *et al.* 2013, Benskey *et al.* 2016).

The continuous formation of α-syn inclusion bodies observed in PD occurs in susceptible neuronal types (e.g. projection cells with a disproportionately long, thin, and weakly myelinated or nonmyelinated axon) within certain areas of the CNS (Del Tredici & Braak 2016). Yet, Lewy pathology can also be found outside the brain including spinal cord and peripheral nervous system: vagus nerve, sympathetic ganglia, cardiac plexus, enteric nervous system, salivary glands, adrenal medulla, cutaneous nerves, and sciatic nerve (Beach *et al.* 2010, Iwanaga *et al.* 1999, Kalia & Lang 2015). Braak and collaborators suggested that Lewy pathology may follow a six stages-stereotyped sequence, starting in peripheral nervous system and progressively affecting the central nervous system in a caudal-to-rostral direction within the brain, rather than evolving simultaneously at all nigral and extra-nigral sites (Braak & Del Tredici 2009, Del Tredici & Braak 2016). Despite the obvious inherent complexities, the neuropathology appears the same in each of the regions affected, suggesting that PD is a multiorgan disease process with a common underlying pathogenic process, and not merely a CNS disorder (Alexander 2004, Beach *et al.* 2010). This rational has fed the debate on cell-to-cell transmission of misfolded and slightly aggregated α-syn seeds that can spread trans-synaptically along multisynaptic pathways. Some authors even hypothesized the spread of α-syn aggregates from one neuron to another, in a prion-like manner, contribute to PD progression (Goedert *et al.* 2014, Jucker & Walker 2013, Lamberts *et al.* 2015, Reichmann 2011). In further support of this thesis is the observation that fetal mesencephalic intrastriatal transplants to treat PD have been shown to develop Lewy body pathology, possibly by cell-to-cell transmission (Dickson 2012).

Although the Braak staging scheme is attractive, it should be remembered that it is not based on distribution of neuronal loss, but on distribution of abnormal α-syn deposits. How it relates to progression of neuronal loss has not been rigorously studied so far (Dickson 2012).

1.3.3 Retrograde degeneration: new vistas on old debate

Braak introduced the following question: where does PD begins in brain at the *regional level?* There is a second challenge under debate: where does PD begin at the *cellular level*? Although dopaminergic neuronal axons and cell bodies both degenerate in the course of PD, a "*chicken or the egg*" causality dilemma emerges: is it a dysfunctional cell soma that results in anterograde degeneration of the axons or is dysfunctional pre-synaptic and/or axonal compartments that retrogradly culminate in soma degeneration?

Even though PD clinical manifestations are related to striatal DAergic denervation, evidence of noticeable neuropathological changes in neurons or neuropil of striatum were far less obvious than those indisputably observed in nigral neuropathological examination (Forno & Norville 1979, Irizarry *et al.* 1998). Yet, a dying-back process of nigral DAergic neurons in PD initial phases was suggested years ago by Professor Oleh Hornykiewicz, a reputed neuroscientist that discoverd DA deficits in PD patients brain and initiated treatment with levodopa (Hornykiewicz 1998, Lees *et al.* 2015). Hornykiewicz correlated the assymetric loss of 80% of putamen DA content *versus* 30% of nigral neurons in the onset of PD motor symptoms with a similar mismatch observed in the experimental 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin-treated primates (Hornykiewicz 1998). This observed discrepancy in MPTP primates was explained by this neurotoxin having triggered a neurotoxic condition that primarily targeted striatal DA terminals. This mechanism was further transposed to PD itself. Although this assumption does not necessarily prove a retrograde mechanism occurring in PD, it was further supported by electron microscopy analysis of PD caudate biopsy specimens: dystrophic changes in distal ends of axons including presynaptic terminals from multiple CN afferent systems undergoing dying-back degeneration and in dendrites were shown. Distrophic neuritis were composed of dense bodies and *degenerated mitochondria* as well as *disorganized intermediate filaments and microtubules*, suggesting impaired local cellular respiration and faulty microtubule-based axonal transport. Such axonal changes in PD basal ganglia were accompanied by astrocytic gliosis and a focal increase of macrophages (Lach *et al.* 1992). A subsequent body of work employing new antibodies and staining protocols extended Lach and co-workers findings by showing that dystrophic neuritis and astrocytes of basal ganglia did also accumulate α-syn in PD, beyond nigral neuronal soma (Cheng *et al.* 2010, Duda *et al.* 2002, Shoji *et al.* 2000, Tagliaferro & Burke 2016).

A wealth of literature using innovative techniques is consistent with aforementioned studies and argue against the previous notion of "no definite ultrastructural abnormalities" within PD striatal pathology (Forno & Norville 1979, Irizarry *et al.* 1998). For example, semiquantitative studies demonstrated that MSN's dendritic spine loss is as an early pathological feature of experimental PD (McNeill *et al.* 1988, Smith & Villalba 2008, Villalba & Smith 2011). Also, reduced cell count and striatal atrophy were more recently confirmed with magnetic resonance imaging by Pitcher and colleagues, who showed that caudate and putamen undergo volume loss even early in PD course (Bugiani *et al.* 1980, Pitcher *et al.* 2012). In further support of a retrograde cellular sequence of events, recent animal models (eg. viral vector transduction of A53T α-syn gene into rat SN dopaminergic neurons) suggested to more efficiently recreate some of the cardinal morphological features of the disease display striatal axonopathy early within nigrostriatal degeneration (Chung *et al.* 2009, Tagliaferro & Burke 2016). Finally, it was recently shown that the magnitude of the nigral neuromelanin signal loss was smaller than the decrease in striatal DAT signal of neuromelanin in PD patients, by *in vivo* imaging (Hansen *et al.* 2016). These authors also made the case for dying back pathology in striatum in early stages of PD degeneration.

Although nigrostriatal pathway have been most extensively studied in PD, this pathology is a multi-organ disease affecting also non-dopaminergic and non-motor systems. For example, peripheric autonomic neurons were also explored to address the timing of events of α-syn pathology in PD at the cellular level (Orimo *et al.* 2008). Based on patterns of α-syn pathology and TH immunostaining, authors concluded that α-syn accumulation begins in distal axons of cardiac sympathetic neurons and proceeds retrogradly to neuronal somata in sympathetic ganglia. The revision presented herein highlights a robust wealth of evidence showing synaptic collapse and axonal damage as an early and predominant feature of PD, likely to be a retrograde form of degeneration. This seemingly strenghtens the Hornykiewicz' proposal of a PD dying-back process. Noteworthy, the molecular mechanisms of axon degeneration are separate and distinct from the canonical pathways of programmed cell death associated with soma destruction (Cheng *et al.* 2010, Tagliaferro & Burke 2016). Based on these observations, a more in-depth understanding of events that are culprits of axonal dysfunction/degeneration of remaining nerve cells may become the research spotlight in PD. This will putatively provide new therapeutic targets (Schulz-Schaeffer 2015).

1.3.4 Gliosis

1.3.4.1 Glia in healthy CNS – an overview

Under non-diseased conditions, glia are highly active and play critical roles in CNS homeostasis. For example, microglia perform essential roles in synapse development and turnover, neurogenesis, phagocytosis, antigen presentation and regulated cytokine expression to maintain homeostasis (Catalin *et al.* 2013, Kettenmann *et al.* 2011, Michell-Robinson *et al.* 2015, Valero *et al.* 2016). Their small somata display long and ramified processes that are continuously moving, surveying the microenvironment for foreign antigens (Catalin *et al.* 2013). Astrocytes, by the other hand, display important functions in ion homeostasis (e.g. potassium, calcium), antioxidant action (e.g. producing high levels of glutathione and its precursors, detoxifying enzymes), trophic effects [e.g. synthesis of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF)], metabolism and gliotransmission (e.g. lactate and glutamate synthesis and release), neurogenesis, regulated cytokine expression and modulation of blood brain barrier (BBB) permeability, to name a few (Ben Haim *et al.* 2015, Cabezas *et al.* 2014, Iglesias *et al.* 2016, L'Episcopo *et al.* 2014).

Any disturbance or loss of brain homeostasis indicating real or potential danger to the CNS (e.g. infection, trauma, ischemia, neurodegenerative diseases) can evoke rapid and profound changes in glial cell shape, gene expression and functional behavior, a process generally described as glial activation, reactive gliosis or, simply, gliosis (Burda & Sofroniew 2014, Kettenmann *et al.* 2011). It is important to emphasize that there is no single program of "reactive gliosis" that is triggered in an *all-or-none* fashion and similarly in all situations (Burda & Sofroniew 2014). For instance, microglial cells, the primary immune effectors of the nervous system, start to change their morphology by extending their processes towards the target, followed by cell bodies migration. Microglia can unfold their phagocytic activity acquiring an amoeboid appearance and increase their rate of proliferation to provide more cells for the defense against insult and to organize for the protection and restoration of tissue homeostasis (Catalin *et al.* 2013, Kettenmann *et al.* 2011, Michell-Robinson *et al.* 2015). One should stress that there is growing interest about diverse microglia morphology and density across brain regions (Hart *et al.* 2012). This adds further complexity to glia biology.

Astrocytes are also key-players in the coordinated multicellular response to CNS insults. Changes in astrocytic gene expression, functions and appearance that are associated with astrogliosis occur along a seamless graded continuum of intensity which has been categorized by Sofroniew (2015) as mild to moderate astrogliosis, severe diffuse astrogliosis and proliferation. Briefly: i) mild to moderate astrogliosis is characterized by variable degrees of hypertrophy of cell body and stem processes, increased expression of the astrocyte intermediate filament protein (GFAP, somewhat proportional to the degree of reactivity) without substantive loss of individual astrocyte domains and without astrocyte proliferation ii) Severe diffuse reactive astrogliosis consists in cellular hypertrophy and GFAP up-regulation, dispersed astrocyte proliferation, and some loss of individual astrocyte domains with overlapping of neighboring astrocyte processes and iii) Compact astroglial scars derive almost entirely from newly proliferated astrocytes with elongated shapes, whose cell processes overlap and intertwine extensively to form compact borders that surround and demarcate areas of tissue damage, necrosis, and inflammation after trauma, stroke, infection, autoimmune-triggered inflammatory infiltration, or neurodegenerative disease. The increased magnitude in astrogliosis is inversely correlated with the potential for resolution and return to normal structure (Sofroniew 2015). However, an uniform measure including upregulation of GFAP expression does not simply translate into an uniform concept of astrocyte reactivity. For example, certain inflammatory signals can substantively alter reactive astrocyte transcriptome profiles without inducing further changes in GFAP. This is in line with the growing interest in the potential for heterogeneity among astrocytes across different CNS regions (Sofroniew 2015).

Glial morphological changes are engaged in gene expression remodeling (up or down) towards the rearrangement of surface molecules for cell to cell and cell to matrix interactions, modulation of intracellular enzymes as well as release of multiple factors and compounds with inflammatory activities (pro- and/or anti-) and immunoregulatory effects (Burda & Sofroniew 2014, Kettenmann *et al.* 2011, Sofroniew 2015). Hence, even though brain parenchyma has been long considered to be an immune-privileged site mainly because of the presence of the BBB, it is now well established that it is fully capable of mounting an inflammatory response (Tansey *et al.* 2007). Reactive gliosis, the cellular manifestation of neuroinflammation, can therefore be beneficial or detrimental, depending on the nature, intensity and duration of the insult (Iglesias *et al.* 2016, Niranjan 2014).

1.3.4.2 Glia reactivity in PD

Neuropathological evidences linking microgliosis to PD have been extensively outlined by Whitton (2007). Briefly, upregulation of major histocompatibility complex (MHC) class II (a readout of activated microglia) was described in SN and striatum of *post-mortem* PD patients long years ago (McGeer *et al.* 1988, Mogi *et al.* 1995, Whitton 2007). Subsequent studies aimed to address microglia functionality further described the presence of pro-inflammatory cytokines (e.g. TNF-α, IL-6) and glia molecular effectors (NF-κB, iNOS and COX-2) in those brain regions (Hunot *et al.* 1997, Knott *et al.* 2000, Nagatsu *et al.* 2000). This pro-inflammatory profile was also observed in cerebrospinal fluid (CSF) and serum of PD patients (Brodacki *et al.* 2008, Koziorowski *et al.* 2012, Mogi *et al.* 1994, Nagatsu *et al.* 2000). Nevertheless, how inflammation timely-follow the neurodegenerative process was a missing question. To address this issue, Sawada and colleagues (2006) analyzed the pattern of activated microglia and inflammatory profile in *post-mortem* samples in distinct stages of PD progression: they found activated microglia mainly associated with TH-positive neurites in the putamen at the early stages of disease and with damaged TH-positive neurons in SN at more advanced stages of degeneration (Sawada *et al.* 2006). This observation gives additional credence to Hornykiewicz' postulate of dopaminergic retrograde degeneration (see 1.3.3).

Remarkably, activated microglia was not confined to nigro-striatal region but was also observed in other brain regions including cortex, hippocampus and olfactory bulb of PD patients (Doorn *et al.* 2014a, Sawada *et al.* 2006). In line with Sawada's work, anatomically widespread microglial activation (e.g. brainstem, basal ganglia and frontal cortical areas) was found in early and late stages of PD patients using *in vivo* [^{11}C](R)-PK11195 PET (a radiotracer of peripheral benzodiazepine binding sites (PBBS) expressed by activated microglia) (Gerhard *et al.* 2006). Even though this *in vivo* PET analysis warrants further validation, these findings strengthen the theory of PD being a "multisystem" disorder (Alexander 2004, Beach *et al.* 2010). It is important to emphasize that widespread microglia activation was independent of disease severity or duration (rated both clinically and with [^{18}F]-DOPA PET). A similar lack of correlation was found in MPTP-exposed humans and primates who showed a long-lasting pattern of active microglia, present up to 16 years after exposure (Langston *et al.* 1999, McGeer *et al.* 2003). Taken together, these studies suggest that reactive microglia is very likely to represent a marker of ongoing disease activity.

In terms of astrocytes, their role in PD is mainly studied in the context of MPTP intoxication and is far less understood in PD human pathology. Yet, PD *post-mortem* analyses showed some cases of astrocytic glial scars in the SN and also increased GFAP-immunoreactive astrocytes in caudate and frontal cortex (see Figure 1.7) (Forno *et al.* 1992, Lach *et al.* 1992, Mythri *et al.* 2011).

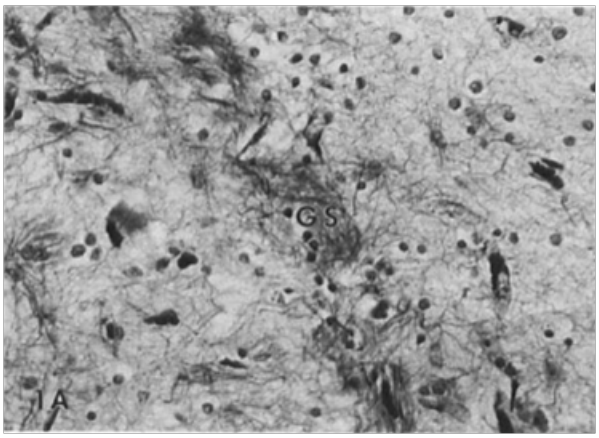

Figure 1.7 Glial scarring in ventral lateral cell group of SNpc in PD. The glial scar (GS) consists of glial fiber bundles coursing from top to bottom of photograph. Holzer stain. Magnification, x470 (Taken from Forno *et al.* 1992)

In SNpc, brightly GFAP-immunoreactive stellate astrocytes were observed in the immediate vicinity surrounding degenerating nigral neurons (Forno *et al.* 1992, Knott *et al.* 1999). Many of these showed moderate nestin immunoreactivity, a marker of reactive astrogliosis which in rodents are normally expressed in immature astrocytes and re-expressed following lesion, perhaps responding to signals released from neurons (Knott *et al.* 1999). Interestingly, a rearrangement of neuronal-glial contacts was noted in PD degenerated nigra: whilst astrocytic processes extended over cell bodies of nigral healthy neurons forming a dense astrocytic carpet, they were withdrawn from fragmented somata particularly when associated with small clusters of amoeboid microglia in PD. Authors argued that an inability to maintain a neuron might be associated with withdrawal of astrocyte processes, with further microglial proliferation/activation and subsequent neuronal destruction and phagocytosis. Moreover, the density of glutathione peroxidase positive-astrocytes (the major protective enzyme against peroxide toxicity) increased in relation to the severity of nigral neuronal injury, likely to be an attempt to protect the surviving dopaminergic neurons against an excess of toxic species. In fact, this buffer function is consistent with the decreased contents of glutathione (the substrate of glutathione peroxidase) found on homogenates of parkinsonian substantia nigra (Perry *et al.*

1982). Such putative protective phenotype was also proposed by Forno and colleagues (1992), who found neuromelanin-positive astrocytes in striatum. Since striatal neurons are not catecholaminergic and do not contain neuromelanin, authors speculated that in human PD, astrocytes may be able to sequester harmful byproducts of DA metabolism released by injured DAergic terminals, thereby protecting them from further damage. Actually, a protective role for astrocytes in DA metabolism has been reported thereafter (for further reading, please see 2.3 of Pereira *et al.* 2012). Conversely, the presence of α-syn positive astrocytes in human pathological samples was correlated with nigral neuronal loss (Wakabayashi *et al.* 2000). Moreover, high levels of astrocytic S100B protein, an important mediator of astroglial inflammatory responses, were found in SN of *post-mortem* samples and in CSF of a large cohort of PD patients within stage 2 of Hoehn and Yahr scale (Sathe *et al.* 2012). These emergent evidences support a role for astrocytic neuroinflammatory responses within PD neurodegeneration. It is known that the dual role of astrocytes in mediating protective (e.g. release of trophic factors, antioxidant glutathione) or degenerative functions (e.g. release of intracellular calcium bursts, inflammatory cytokines) on surrounding neural and non-neural cells largely depends on the microenvironment that astrocytes and neurons commonly share (Rappold & Tieu 2010). Thus, multifaceted astrocytic reactivity may be initially beneficial although later corrupted by disease-specific brain regions alterations (Ben Haim *et al.* 2015).

Finally, it is important to highlight the importance of astrocytes in BBB function considering clinical evidence for increased BBB permeability during PD development (Kortekaas *et al.* 2005, Pisani *et al.* 2012). While BBB tightness is largely regulated by soluble BBB-promoting factors (e.g. Sonic hedgehog - SHh) secreted by astrocytic end-feets, reactive astrocytes can also produce BBB-disrupting factors with subsequent decreased expression or altered localization of tight-junction proteins at endothelial domains which have the capacity to promote barrier function (Alvarez *et al.* 2013, Gimsa *et al.* 2013). Altered permeability can also allow greater influx of peripheral immune cells propagating inflammation and inducing neuronal injury (if not already present) (Kannarkat *et al.* 2013). Thus, increased BBB permeability could also be a catalytic factor in the development of PD. A better understanding of the astrocytic functions in PD BBB dysfunction may have important prospective consequences (Cabezas *et al.* 2014).

1.4 Etiology

Despite an intense research over the last few decades, the etiology of PD remains poorly understood. Approximately 5-10% of PD patients have monogenic forms of the disease, exhibiting a classical Mendelian type of inheritance. However, the majority PD cases are sporadic, probably caused by an interplay of genetic and environmental risk factors (Kalinderi *et al.* 2016, Spataro *et al.* 2015). In the following sub-sections (1.4.1 and 1.4.2), it will be summarized the evidence supporting both the genetic and environmental hypothesis underlying PD degeneration.

1.4.1 Genetic hypothesis

The last decade has seen a revival in identifying causes of familial PD mainly through genetic linkage analysis and subsequent positional cloning. Point mutations and duplication or triplication of α-syn gene were the first to be identified and defined PD as a synucleinopathy (Polymeropoulos *et al.* 1997, Spillantini *et al.* 1997). Other autosomal dominant [e.g. ubiquitin-C-terminal hydrolase-L1 (UCH-L1); leucine-rich repeat kinase (LRRK2)] and recessive [Parkin, PINK1, DJ-1] point mutations were subsequently identified and are presented in Table 1.2. In the current PD genetics nomenclature, 18 specific chromosomal regions are termed PARK, and 18 PD-related genetic loci (PARK1-18) were identified in chronological order (Klein & Westenberger 2012).

Monogenetic forms of PD are rare and account for about 30% of familial and 3-5% of sporadic cases. Thus, it is evident that PD is a genetically heterogeneous disease with several susceptibility genes and numerous risk loci associated with disease onset (Kumar *et al.* 2011). In this regard, unbiased genome-wide studies (GWAS) have shed new light into the identification of risk loci associated with PD. An elegant review from Hernandez and colleagues (2016) summarizes the most recent findings from genome-wide association in assessing PD risk. In brief, two collaborative studies examining Caucasian and Asian subjects were the first to reveal genome-wide significant risk alleles (e.g. SNCA, LRRK2, PARK16) and risk loci [e.g. histocompatibility complex class II, DR beta 5 (HLA-DRB5) and cyclin G associated kinase (GAK)] for PD (Hamza *et al.* 2010, Hernandez *et al.* 2016, Pankratz *et al.* 2009, Satake *et al.* 2009, Simon-Sanchez *et al.* 2009). A large-scale meta-analysis of GWAS data was taken by a

great number of PD consortiums to provide risk loci identification (Nalls *et al.* 2014). Notably, this study identified loci which appear to contain more than one risk allele. For instance, each loci containing SNCA, HLA-DRB5, GAK and synaptogamin 11 (SYT11), includes two identified common risk alleles that act independently of one another. This notion of multiple types of risk alleles at the same locus is consistent with the *pleomorphic risk locus hypothesis* and reinforces the notion that mutated genes can be involved in the pathogenesis of typical, apparently sporadic disease, through yet undisclosed overlapping mechanisms (Hernandez *et al.* 2016, Singleton & Hardy 2011).

Table 1.2 Loci involved in monogenic forms of Parkinson's disease

Adapted from (Klein & Westenberger 2012)

Locus	Gene	Protein	Inheritance
PARK1	α-syn	α-synuclein	AD
PARK2	PARK2	Parkin (Ubiquitin E3 ligase)	AR
PARK3	?	?	AD
PARK4	α-syn	α-synuclein	AD
PARK5	UCH-L1	Ubiquitin c terminal hydrolase	AD
PARK6	PINK-1	Pten-induced putative kinase 1	AR
PARK7	PARK7	DJ-1	AR
PARK8	LRRK2	Leucine-rich repeat kinase 2	AD
PARK9	ATP13A2	Lysosomal type 5 ATPase	AR
PARK10	?	?	Risk locus
PARK11	GIGYF2	GRB interacting GYF protein 2	AD
PARK12	?	?	X-linked
PARK13	HtrA2/Omi	HTRA serine peptidase 2	AD
PARK14	PLA2G6	Phospholipase A2	AR
PARK15	FBXO7	F-box only protein 7	AR
PARK16	?	?	Risk locus
PARK17	VPS35	Vacuolar protein sorting 35	AD
PARK18	EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	AD

(AD: autosomal dominant; AR: autosomal recessive)

Aforementioned PD genetics studies have provided valuable insights into the pathophysiology of PD sporadic forms at the molecular level (see 1.5).

1.4.2 Environmental hypothesis

For many years, idiopathic PD was considered to be caused mainly by environmental toxins (Di Monte 2003). Initial evidence came from findings showing that humans exposed to MPTP, a contaminant of a synthetic opiate, developed parkinsonism (Langston *et al.* 1983). Since then, numerous epidemiological studies were designed to assess a putative causality between environmental toxins and PD risk. Much attention has been devoted to heavy metals (e.g. manganese, copper, mercury, iron). However, inconclusive data emerged (Jankovic 2005, Lai *et al.* 2002). Yet, a statistically significant link between PD and farming, rural living and ingestion of well water, all likely surrogate markers for pesticide exposures, has been found (Hubble *et al.* 1993, Koller *et al.* 1990, Metzler 1982, Morano *et al.* 1994, Priyadarshi *et al.* 2000). Among these pesticides, paraquat and rotenone have been largely studied, particularly because they are all mitochondrial poisons, analogous to the prototypic parkinson-mimetic toxin MPTP. Since original work included in this Thesis (Chapters 2, 3 and 4) used MPTP-induced experimental PD models, cellular and molecular aspects of this toxin as well as some practical concerns regarding its manipulation and management will be presented.

1.4.2.1 MPTP

In 1982, young drug users developed a rapidly progressive parkinsonian syndrome that was traced to their intravenous use of a street preparation of 1-methyl-4-phenyl-4-propion-oxypiperidine (MPPP), an analog of the narcotic meperidine. MPTP, which was inadvertently produced during the illicit synthesis of MPPP in a basement laboratory, was the responsible neurotoxic contaminant (Langston *et al.* 1983). A complete history of these cases is presented in "The Case of the Frozen Addicts" (Langston & Palfreman 1995). In fact, in humans and non-human primates, MPTP can produce an irreversible and severe parkinsonian syndrome that replicates almost all PD features, including tremor, rigidity, slowness of movement, postural instability and even freezing. Neuropathological data arising mainly from primates show that MPTP administration damages the nigrostriatal dopaminergic pathway in a pattern similar to that seen in PD, with dopaminergic neurons enriched in neuromelanin being more susceptible to MPTP-induced degeneration (Hirsch *et al.* 1988, Przedborski *et al.* 2001). MPTP evokes glial reactivity in basal ganglia, further recapitulating PD (Barcia *et al.* 2004, McGeer *et al.* 2003). Yet,

two neuropathological features of PD have not been consistently reported in the MPTP monkey model: i) except for SNpc, pigmented nuclei such as locus coeruleus have been spared; ii) eosinophilic intraneuronal inclusions resembling Lewy bodies did not display the fully convincing morphological and immunocytochemical features, characteristics of the human inclusion bodies in PD (Forno *et al.* 1993, Forno *et al.* 1986). This is probably due to acute MPTP exposures that rapidly injure dopaminergic neurons.

Despite these neuropathologic shortcomings, the monkey MPTP model is still the gold standard for the assessment of novel strategies and agents for the treatment of PD symptoms. Nevertheless, largely because of technical, logistical, ethical and financial considerations, MPTP rodents models are typically used to explore the molecular mechanisms of dopaminergic neurodegeneration and subsequent therapeutic options (Dauer & Przedborski 2003).

1.4.2.1.1 MPTP metabolism

MPTP is a proneurotoxin with a complex multistep metabolism. After systemic administration, lipophilic MPTP freely crosses BBB within minutes (Markey *et al.* 1984). Once in the brain, MPTP is oxidized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by monoamine oxidase B (MAO-B), mainly in astrocytes (Brooks *et al.* 1989). Then, $MPDP^+$ is converted into the polar and active MPP^+ metabolite (probably by spontaneous oxidation) and released into the extracellular space. MPP^+ is a high-affinity substrate for the dopamine transporter DAT and concentrates mainly into DAergic terminals, where it initiates molecular events that ultimately result in dopaminergic cell death (Cochiolo *et al.* 2000, Herkenham *et al.* 1991). MPP^+ can move through several cellular compartments but mitochondria is the main target of MPP^+ cytotoxicity, impairing oxidative phosphorylation by inhibition of multienzyme complex I of the mitochondrial electron transport chain (see Figure 1.8). Alteration in energy metabolism (ATP contents) and generation of reactive oxygen species (ROS) peak within hours of MPTP administration, days before overt neuronal death (Jackson-Lewis *et al.* 1995). Therefore, these initial events are not likely to directly kill most cells but rather set into play downstream cellular events that ultimately kill most dopaminergic neurons (Dauer & Przedborski 2003). Indeed, dopaminergic neuronal damage is likely to result from compounds secondary to energy depletion [e.g. superoxide radicals, nitric oxide (NO)] that can be generated within and/or outside the cells, considering the great amount of neuron-glia cross-talk

underlying MPTP toxicokinetics (Cleeter *et al.* 1992, Przedborski *et al.* 1992). For instance, NO produced and released by glial cells can enter the cytosol of the neuron via simple membrane diffusion. At this point, superoxide radicals and NO can interact to form the very destructive oxidizing molecule peroxynitrite ($OONO^-$), which targets several number of neuronal components, including TH. Furthermore, glial cells directly contribute to the toxicity seen following MPTP administration through several mechanism, including the induction and modulation of inflammatory enzymes, cytokines and different molecules involved in oxidative stress [e.g. Hemoxygenase-1 (HO-1)] (Smeyne & Jackson-Lewis 2005, Yokoyama *et al.* 2008, Yokoyama *et al.* 2011).

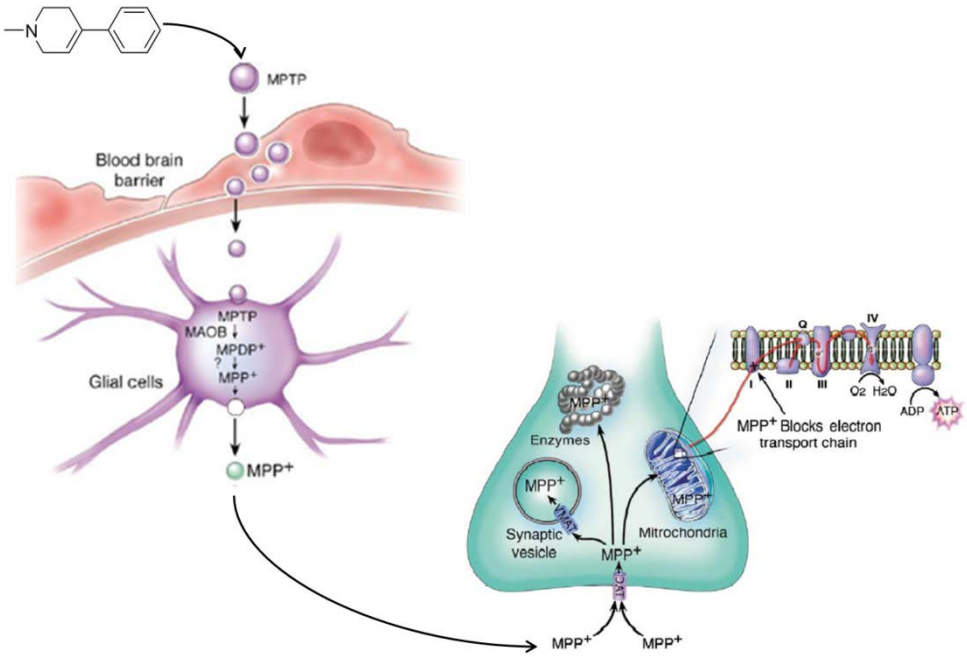

Figure 1.8 Schematic representation of MPTP metabolism and MPP+ intracellular pathways. MPTP crosses the blood-brain barrier and is converted into MPP^+ in glial cells. Once released intp extracellular space, MPP^+ is concentrated into dopaminergic neurons via the dopamine transporter (DAT) and can follow one of three routes: i) concentration into mitochondria through an active process (toxic); ii) interaction with cytosolic enzymes (toxic); iii) sequestration into synaptic vesicles via the vesicular monoamine transporters (VMAT; protective) (Adapted from Dauer & Przedborski 2003)

1.4.2.1.2 MPTP models

The administration of MPTP through a number of different routes (e.g. intraperitoneal, subcutaneous, intravenous) using distinct dosing regimens has led to the development of several distinct models, each characterized by some unique behavioral and/or biochemical features. Rodents are much less sensitive to MPTP than monkeys and much higher doses are required to produce significant damage (Przedborski *et al.* 2001). In mice, MPTP potency varies among

mouse strains although optimal reproducibility in MPTP neurotoxicity is obtained using male C57BL/6 mice weighting about 25-30g (Przedborski *et al.* 2001).The magnitude of nigrostriatal damage also depends on the dose and dosing schedule (Sonsalla & Heikkila 1986). Three MPTP protocols are widely used: i) the acute protocol generally involves 4 injections in one day at 2h intervals; ii) the subacute administration is a once daily injection over 5-8 days and iii) the chronic regimen which utilizes repeated treatments over 5 weeks and requires the co-administration of the adjuvant probenecid to retard the renal clearance of MPTP toxic metabolites. Overall, the MPTP-treated mouse models elicit a rapid loss of SNpc TH-immunoreactive neurons with little progressive loss of the nigrostriatal DA pathway. Nevertheless, the pattern of DA terminal loss in the striatum replicates that of PD (Meredith *et al.* 2008).

The use of MPTP in rats is far less studied. The main reason for this is that shortly after the discovery of parkinsonian syndrome in human and non-human-primates after systemic MPTP administration, no susceptibility of rats to MPTP was reported when the drug was administrated through similar routes (Giovanni *et al.* 1994a). However, rats injected with much higher doses of MPTP than those usually applied in mice, do exhibit significant dopaminergic neurodegeneration (Giovanni *et al.* 1994b). The apparent insensitivity of rats to MPTP toxicity may be due to the fact that rat brain capillaries contain exceptionally high levels of monoamine oxidase B (MAO-B), which constitutes an effective enzymatic blood-brain barrier to toxin systemic administration (Kalaria *et al.* 1987). This contention has been re-evaluated following the finding that direct nigral and/or intranasal infusion of MPTP causes a partial loss of dopaminergic neurons and depletion of striatal DA, resulting in sensorial and memory deficits with no major motor impairments, as is typically seen in PD early phase (Da Cunha *et al.* 2002, Da Cunha *et al.* 2001, Ferro *et al.* 2005, Prediger *et al.* 2006). These observations offer obvious experimental advantages, as a representative animal model of early preclinical stages is of utmost importance to evaluate experimental neuroprotective therapies (Prediger *et al.* 2011). Since the intranasal (i.n.) route bypasses the BBB, the olfactory pathway represents an interesting route for assessing the effects of MPTP on rats. For this reason, i.n. MPTP models in rodents have been proposed (Graff & Pollack 2005, Prediger *et al.* 2006, Rojo *et al.* 2006). Particularly, animals, when treated intranasally with a single bilateral MPTP infusion (0,1mg/nostril) (see Figure 1.9), suffer time-dependent olfactory, cognitive and emotional

impairments, with neither major motor deficits nor striatal DA depletion in early phases of intoxication, at least until 14 days post-treatment (Prediger *et al.* 2006). Moreover, pathogenic mechanisms involved in the i.n. MPTP administration include mitochondrial dysfunction, oxidative stress and apoptotic cell death mechanisms, processes that mimic some cellular events recognized within PD neurodegeneration (Moreira *et al.* 2010, Prediger *et al.* 2011, Prediger *et al.* 2010, Prediger *et al.* 2006, Prediger *et al.* 2009). Therefore, the current findings provide strong evidence that the i.n. MPTP administration represents a valuable rodent model showing biochemical and behavioral impairments similar to those observed in the early phase of PD when a moderate loss of the nigral DA neurons results in sensorial and memory deficits with no major motor impairments (Prediger *et al.* 2006).

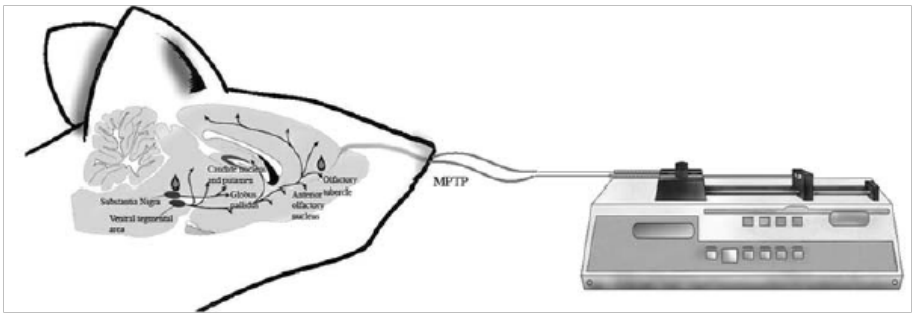

Figure 1.9 Schematic procedures of the intranasal administration of MPTP (1mg/nostril) in rats. Briefly, rats are lightly anaesthetized and a 10-mm piece of PE-50 tubing is inserted through the nostrils. The tubing is connected to a peristaltic pump set at a flow rate of 12.5µl/min to deliver a MPTP solution (20mg/ml) over 4 min (1mg/nostril). Control solution is sodium chloride 0,9%. Animals are given a 1-min interval to regain normal respiratory function and then this procedure is repeated with infusions administered through the contralateral nostrils (Adapted from Prediger *et al.* 2010)

1.4.2.1.3 MPTP safety procedures

The fact that sensitivity of MPTP closely follows the phylogenetic tree implies that higher doses are required to produce significant damage in rodents models (exception for the i.n. model) presenting a far greater hazardous situation to humans (Meredith *et al.* 2008, Prediger *et al.* 2006). Knowing where MPTP and its toxic metabolite (MPP^+) accumulate both inside and outside of the body of the injected animal following MPTP administration is therefore instrumental to the formulation of standard practices for the safe use of MPTP. The main route of MPTP excretion is the urine. MPTP is transformed essentially into ionized and non volatile metabolites (eg. MPP^+) by 12h-24h post-injection, which are well absorbed by the animal bedding. There is minimal risk from exposure due to airborne or vapor-borne forms of MPTP. The period of maximal risk of MPTP contamination is therefore from the moment of injection

to the time that MPTP or its metabolites are no longer found in the excreta of treated animals. Thus, safety procedures regarding i) personal protection, ii) MPTP storage and handling, iii) dedicated procedure room within animal room and cage changing and iv) decontamination, cleaning and disposal were preconized by Przedborski and colleagues and need to be strictly enforced at least within 3 days post-injection (Przedborski *et al.* 2001).

1.4.3 Lifestyle factors

Nutrition may influence neurodegeneration by affecting biological mechanisms underlying oxidative stress, including production of reactive oxygen or nitrogen species and/or levels of antioxidant defences (Ishihara & Brayne 2005). Previous studies have suggested that fruits and vegetables were protective for PD, due to their relatively high contents of antioxidantt nutrients such as vitamin C, A, E and carotenoids (Hellenbrand *et al.* 1996, Morens *et al.* 1996). Nevertheless, more recent studies, including a systematic review of nutritional risk factors of PD, found no consistent evidence linking dietary factors and antioxidant vitamins with the etiology of PD (Ishihara & Brayne 2005, Lai *et al.* 2002). Instead, an inverse association between PD risk and caffeine consumption was found (Costa *et al.* 2010, Ishihara & Brayne 2005). Similarly, a reduced risk of PD in cigarette smokers was consistently observed (Hernan *et al.* 2001, Li *et al.* 2015, Paganini-Hill 2001). Although this may be due to bias from selective mortality of smokers among people with undiagnosed PD, some proposed nicotine neuroprotective mechanisms involve its inhibitory action in MAO-B and its stimulatory action in nicotine acetylcholine receptors, leading to dopamine release and neuroprotection of nigrostriatal dopaminergic pathway (Fowler *et al.* 2003, Quik & Kulak 2002). In addition, it was recently found that nicotine protects these dopaminergic neurons against endoplasmic reticulum stress, via nicotinic receptors (Srinivasan *et al.* 2016). Conversely, preclinical and clinical data support an increase vulnerability of amphetamine-type stimulants users to develop PD (Callaghan *et al.* 2010, Callaghan *et al.* 2012, Curtin *et al.* 2015, Kousik *et al.* 2014, Kuehn 2011, Thrash *et al.* 2009).

Ageing, which is the strongest risk factor to neurologic disorders like idiopathic PD, is also highly correlated with the increase burden of metabolic diseases, such as diabetes and obesity (Alves *et al.* 2008, Song & Kim 2016). Noteworthy, oxidative stress and hyperglycemia

are known to evoke adverse neurophysiological phenomena, including hampered insulin signaling, synaptic dysfunction and neuronal loss (Song & Kim 2016). While this association is particularly well-studied in Alzheimer's disease (Mittal & Katare 2016, Morales-Corraliza *et al.* 2016, Xu *et al.* 2016), similar evidence starts to establish type II diabetes (T2D) as a risk factor for PD (Arvanitakis *et al.* 2004, Hu *et al.* 2007, Sun *et al.* 2012). For example, insulin resistance, hyperglycemia and protein glycation caused by T2D are putatively linked to nigrostriatal dopaminergic damage (Song & Kim 2016, Vicente Miranda *et al.* 2016). Moreover, peroxisome proliferator-activated receptor gamma (PPARγ) agonists, a class of antidiabetic drugs, have exhibited some protective effects in PD experimental models (Barbiero *et al.* 2014, Carta 2013, Lecca *et al.* 2015, Schintu *et al.* 2009), although further studies are still required (Cereda *et al.* 2011).

Besides, a link between infection and parkinsonism was firstly proposed during the influenza pandemic of the late 1910s, when it was observed that encephalitis lethargic often preceded parkinsonism, suggesting a possible infectious cause (Duvoisin *et al.* 1963). This was later corroborated by *in vivo* experiments with intranigral administration of lipopolysaccharide (LPS) endotoxin (a component of Gram-negative bacterial cell wall), which led to a primary loss of dopaminergic neurons in SN through microglia activation and inflammatory mechanisms (Duty & Jenner 2011, Sharma & Nehru 2015). Further evidence suggesting an infectious cause for Parkinsonism have arisen from reports suggesting that other infections (e.g. AIDS, cocksakie B, influenza A, herpes simplex, hepatitis C) can be associated with temporary parkinsonism, either acutely, or as long term complications (Mintz *et al.* 1996, Poser *et al.* 1969, Solbrig & Nashef 1993, Wu *et al.* 2015). Although many studies have been unable to identify infectious agents in PD, a number of works suggest that infections may play a role in idiopathic PD (Bu *et al.* 2015, Lai *et al.* 2002, Martyn 1997, Martyn & Osmond 1995, Takahashi & Yamada 1999, Yamada *et al.* 1996). Still far from being proven, the infective hypothesis is particularly aligned with the "Dual Hit" theory that was proposed by Hawkes and colleagues and is a corollary to Braak's hypothesis (2012). Authors claim that an unknown neurotropic pathogen (e.g. infectious, toxin) gains access to the brain through two major portals: the nose and the gut. In the case of inhaled materials, this is suggested to occur from the olfactory epithelium anterogradely to the olfactory tubercle, pirifrom, and entorhinal cortices. In the case of ingested materials, such attack would occur from the enteric plexus retrogradely via preganglionic vagal

fibers (Hawkes *et al.* 2009). Although olfactory and enteric means of access are unique in that they are both in close and constant contact with the potentially hostile outer environment, this theory experiences great difficulties to explain PD cases associated with early involvement of the spinal cord and peripheric autonomic nervous system, pointing to the likely heterogeneity of the etiology of the disease (Prediger *et al.* 2011).

Overall, current knowledge supports the long held notion that in most of PD cases, disease onset is probably triggered by a complex interplay of many genetic and non-genetic modest factors that present cumulative risk effects (Hernandez *et al.* 2016, Polito *et al.* 2016).

1.5 Pathogenic mechanisms – a complex cellular trait

Sporadic PD is probably caused by a complex interplay of many genetic variants interacting with nongenetic environmental risk factors that accumulate "stressfully" within ageing process (Blesa *et al.* 2015, Kannarkat *et al.* 2013, Schulz 2008). Despite the active quest for the ultimate PD cause, it is more likely that (i) mitochondrial dysfunction/oxidative stress (ii) impaired dopamine handling/oxidative stress (iii) proteolytic stress and (iv) neuroinflammation, are all intricately linked in a pathogenic feed-forward cycle that converges in a common pathological and clinical phenotype of PD (see Figure 1.10).

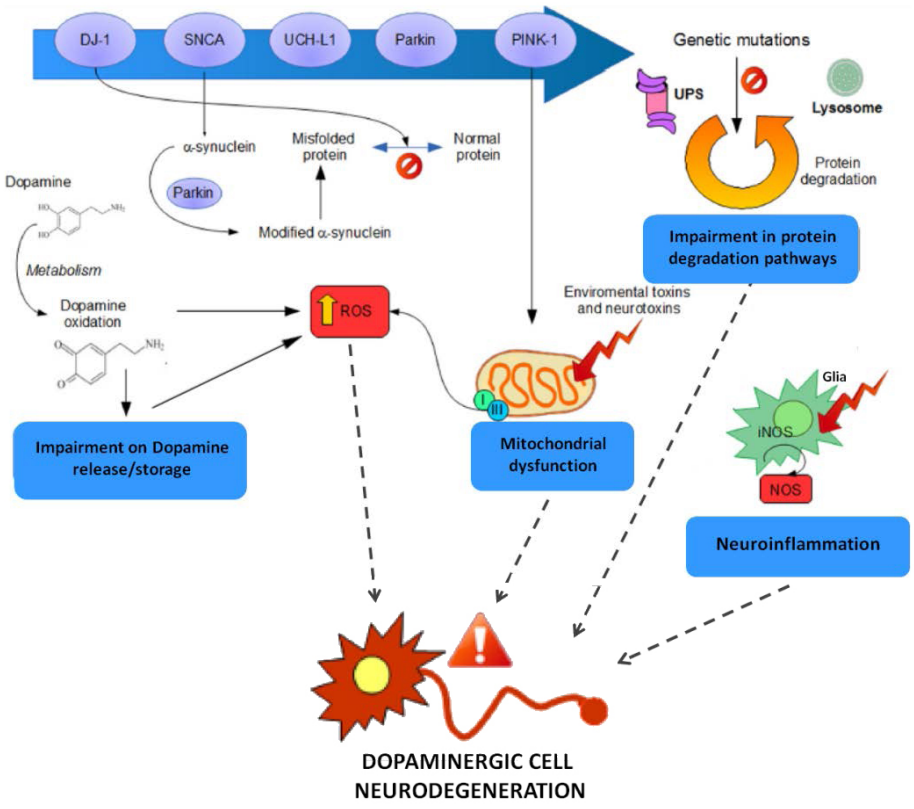

Figure 1.10 Suggested physiological processes related to pathogenesis of Parkinson's disease (PD). Mutations in PD-related genes or altered expression of these proteins result in mitochondria impairment, oxidative stress, protein misfolding and neuroinflammation. Also, dopamine metabolism may be oxidized to reactive dopamine quinones contributing to increased levels of reactive oxygen species (ROS), further contributing to dopaminergic cell neurodegeneration (Adapted from Blesa *et al.* 2015)

1.5.1 Mitochondrial dysfunction and oxidative stress

Mitochondria have long been considered as crucial organelles, primarily for their involvement in energy metabolism. However, it is has been recognized other critical roles in cellular function, namely as calcium buffer and transducer/effector in multiple cellular processes, including programmed cell death (Franco-Iborra *et al.* 2016). Mitochondrial deficiencies are thought to be implicated in several cellular deficits, the first being energy deprivation and ROS generation, and have been generically associated into the neurodegenerative process, namely PD (Perfeito *et al.* 2013).

The most compelling evidence of involvement of mitochondrial dysfunction in PD emerged following human parkinsonian syndrome elicited by accidental exposure to MPTP (see 1.4.2.1.). Aligned with this finding, biochemical studies showed altered mitochondrial complex I function in autopsied PD brains (Schapira *et al.* 1989), in patients platelets (Parker *et al.* 1989) and in lymphocytes (Barroso *et al.* 1993, Yoshino *et al.* 1992). Later, elegant *in vitro* approaches with cell lines engineered to contain mitochondria derived from platelets of PD patients definitely showed important mitochondrial deficits paralleling increased ROS production (Cassarino *et al.* 1997, Esteves *et al.* 2009, Swerdlow *et al.* 1996).

Several "PD genes" also provide evidence for the link between oxidative stress and mitochondrial dysfunction, although further detailed investigation at the molecular level is needed. Table 1.3 provides a list of PARK PD-related genes and subsequent pathological effects related to mitochondria (Moon & Paek 2015). For instance, both DJ-1 and PINK1 are individually indicated to protect cells from oxidative stress. Although the underlying mechanisms remain unclear, evidence suggests that oxidative damage due to decreased PINK1 stability results in overexpression of DJ-1 in mitochondria, which maintains steady-state levels of PINK1 through physical interaction. Loss of oxidative stress protection arising from DJ-1/PINK collaboration may culminate in PD (Tang *et al.* 2006).

Notably, mutations in other genes linked to familial forms of PD (e.g. α-syn, UCHL1, LRRK2) are also related to mitochondrial dysfunction with subsequent oxidative stress and dopaminergic cell damage (Blesa *et al.* 2015, Zuo & Motherwell 2013). Accordingly, brain tissue samples of *post mortem* PD patients showed increased levels of lipid peroxidation, protein oxidation, oxidative RNA and DNA damage in substantia nigra (Alam *et al.* 1997, Castellani *et al.* 1996, Dexter *et al.* 1989).

Table 1.3 Contributions of PARK-designated PD-associated genes

Adapted from (Moon & Paek 2015)

Symbol	Genes	Pathological effect of mitochondria
PARK1 PARK4	α-syn	• Abnormalities in mitochondrial morphology • Complex I activity ↓ • UPS & ALP dysfunction
PARK2	Parkin	• ETC enzyme activites↓ • Protein levels of several subunits of complexes I and IV ↓ • Mitochondrial integrity ↓ • UPS & ALP dysfunction
PARK5	UCHL1	• UPS dysfunction
PARK 6	PINK1	• ETC enzyme activities ↓ • ATP production ↓ • Mitochondrial fission ↓ • Disruption of mitochondrial morphology • ALP dysfunction
PARK7	DJ1	• Complex I and II activities ↓ • ATP production, O_2 consumption, and mitochondrial membrane potential ↓ • Defect in mitochondrial morphology • Defect in the assembly of complex I
PARK8	LRRK2	• ATP production and mitochondrial membrane potential ↓ • Defects in fission/fusion dynamics • ALP dysfunction
PARK9	ATP13A2	• ALP dysfunction
PARK13	HTRA2	• Abnormalities in mitochondrial morphology • ALP dysfunction

(UPS: ubiquitin–proteasomal system; ALP: autophagy lysosomal pathway; (ETC) electron transport chain)

In this respect, there are factors peculiar to nigrostriatal DAergic neurons that also contribute to oxidative stress in PD:

i) Defective DA handling, which may lead to excessive amounts of cytosolic DA that are not sequestered into vesicles. Spontaneous DA auto-oxidation produces DA-quinone species, superoxide anion (O_2^-), hydrogen peroxide and other ROS that can react unspecifically with many cellular components (e.g. α-syn) leading to a multitude of different products contributing to oxidative stress (Meiser *et al.* 2013);

ii) Progressive accumulation of intracellular iron in SNc neurons (and microglia). SNc iron levels are still relatively low early in the course of PD and SN perikaryal NM by binding transition metals (e.g. iron) can prevent Fenton-type $OH^{\bullet}$ radical generation thereby reducing the potential for oxidative stress. Later, NM's high affinity iron-binding sites become saturated and significant amounts of Fe^{2+} remain free in cytosol, being able to undergo Fenton reaction or

further react with DA-quinones to form neurotoxic species [e.g. 6-hydroxydopamine (6-OHDA)] (Alexander 2004, Meiser *et al.* 2013);

iii) Impairment of antioxidant defenses pathways within highly oxidative DAergic neuronal intracellular environment. In fact, a prominent role for leucine-zipper transcription factor Nrf2 that regulates the coordinated induction of the antioxidant response elements (ARE)-driven cascade (Nrf2/ARE signaling) has been proposed in PD pathogenesis (Ramsey *et al.* 2007). Authors showed a predominant Nrf2 nuclear localization in surviving PD nigral neurons, which may indicate that these neurons were able to delay death, but speculated that insufficient Nrf2 protection was in place in neurons that have been lost. Nrf2/ARE protector phenotype has also been demonstrated in the experimental MPTP mouse model, with a major focus in glial cells (Chen *et al.* 2009, Rojo *et al.* 2010). Loss of protective antioxidant mechanism is corroborated by the observation that reduced glutathione levels occur in SNpc of pre-symptomatic PD subjects, suggesting that oxidative damage occurs in early stages of PD degeneration (Bharath *et al.* 2002, Dexter *et al.* 1994).

Noteworthy, autopsied PD brains also revealed a generalized pattern of oxidative stress in extra-nigral regions including caudate nucleus, amygdala and frontal cortex (Dalfo & Ferrer 2008, Dalfo *et al.* 2005, Dexter *et al.* 1994). Some of these events are very likely to precede neuropathological hallmarks of PD degeneration (nigral neuron loss and LBs aggregates) as high levels of lipoxidative damage and accumulation of advanced glycation endproducts are present in early stages of parkinsonian neuropathology (Dalfo *et al.* 2005). Importantly, lipoxidized-α-syn was found in pre-clinical PD and preceded α-syn solubility aggregation and the formation of LBs (Dalfo & Ferrer 2008). Altogether, these observations give support to the idea that oxidatively-altered proteins are present in extra nigral regions (e.g. frontal cortex) in early stages of PD neurodegeneration and predate abnormal α-syn solubility modification and aggregation. It is also important to keep in mind that oxidative stress and mitochondrial impairment are not mutually exclusive events. Besides the obvious link between ROS and mitochondria, ROS has also a pivotal role in the regulation of other cellular processes linked to protein quality control systems - like the ubiquitin proteasome system and/or autophagy - which also play a key role in the appearance of PD deleterious events (Cook *et al.* 2012).

1.5.2 Proteolytic stress

It is not surprising that "proteolytic stress" has gained early support as one of the key underlying pathogenic mechanisms in PD as Lewy Bodies aggregate (composed mainly by α-syn associated with neurofilaments, cytoskeletal proteins, ubiquitin) are one of the neuropathological hallmarks of PD. Another concept which provides further support for this theory is that there is a good correlation with the aging process itself and the gradual increase in levels of damaged proteins that need to be cleared in PD (Keller *et al.* 2000, Zeng *et al.* 2005). But why are there neuronal proteostasis systems particularly vulnerable to the presence of misfolded/aggregated proteins? As postmitotic cells, neurons cannot dissipate protein aggregates simply by cell division, and the age-related decline of the degradation systems - ubiquitin-proteasome system and the autophagy-lysosomal system - further contributes to the vulnerability of neurons (Lim & Yue 2015). Protein misfolding and aggregation may also trigger molecular chaperones (e.g. heat shock proteins) in an attempt to assist in refolding or disaggregating oligomers (Morimoto 2011). However, this first line of defense is often hampered by local alterations in the concentration of chaperone proteins or activity of organelles responsible for protein folding (endoplasmic reticulum) (Yu *et al.* 2014). Familiar PD mutations (e.g. Parkin, UCH-L1), which are related to proteolytic stress, further emphasizes the contribution of protein quality control systems in PD pathogenesis.

Monomeric α-syn is a natively unfolded protein that is prone to misfolding, such as forming oligomeric and β-pleated sheet fibrils (Venda *et al.* 2010). This tendency is enhanced when the protein is in a mutant form (e.g. A30P, E46K and A53T) or present in excess levels, overwhelming proteastasis pathways. Although it was widely believed that insoluble inclusions were the main toxic species to dopaminergic cells, recent findings support the idea that α-synuclein oligomers conformers mediate toxicity while insoluble deposits, resistant to proteasomal recycling and/or autophagy, might reflect "detoxification" end-product structures (Bellucci *et al.* 2016, Del Tredici & Braak 2016, Winner *et al.* 2011). Several aberrant α-syn post-translational modifications (e.g. nitration, phosphorylation, glycation) contribute inequivocally to the toxic phenotype (Choi & Lim 2010, Giasson *et al.* 2000, Guerrero *et al.* 2013, Liu *et al.* 2011b, Shaikh & Nicholson 2008, Vicente Miranda *et al.* 2016). Although α-syn does not contain a sorting signal for extracellular release, soluble and aggregated α-syn were detected in

tissue culture medium and body fluids, such as brain interstitial fluid, plasma and CSF (Emmanouilidou *et al.* 2011, Hansson *et al.* 2014, Lee *et al.* 2012). In extracellular space, they trigger robust immune and inflammatory responses, ensuing protein aggregation and aggravating proteostasis. Hence, neuron-glia crosstalk is currently envisaged as an additional layer of the regulation of proteostasis (Lim & Yue 2015).

1.5.3 Immunity and neuroinflammation

Neuronal loss in PD is associated with chronic neuroinflammation, controlled mainly by resident glial cells (Barcia *et al.* 2003, Perry 2012). Glia activation has been found in both nigral and extra-nigral regions of PD patients (see 1.3.4.) and is also well documented in several animal paradigms of PD: MPTP (O'Callaghan *et al.* 2014, Yasuda *et al.* 2008), rotenone (Gao *et al.* 2002, Sherer *et al.* 2002) and 6-OHDA (Cicchetti *et al.* 2002) models, who also exhibit a pattern of inflammatory cytokines, which is particularly well-studied in nigrostriatal region (Whitton 2007). But what triggers neuroinflammation?

When dopamine containing neurons degenerate in the brain of PD patients, NM is detected outside of neurons and is often engulfed by innate immunity microglial cells (Hirsch *et al.* 2012). Other danger signals like calgranulin S100B, HMGB-1 and misfolded α-syn can also be recognized by pattern recognition receptors (PRR's) expressed in a variety of cell types resulting in proinflammatory profile (see 1.7). Innate immune system is also linked to an adaptive immune arm through its ability for antigen presentation, thus facilitating T-cell-mediated responses in the CNS. Modified α-syn epitopes, which do not normally occur under physiological conditions, may be taken as an endogenous pathological antigen, activating adaptive immune responses, lymphocytic infiltration and inflammation (Brochard *et al.* 2009). Altogether, immunogenic factors released upon insults have the potential to trigger a detrimental innate and adaptive immune response thereby amplifying the pathological process. These mechanisms not only imply a complex crosstalk between the central nervous system and the peripheral immune system but also interactions between the brain resident immune cells - microglial cells - and other brain parenchymal cells - neurons, astrocytes and endothelial cells (Mosley *et al.* 2012).

While mild activation of microglia has apparent beneficial effects, chronic microglial activation in response to neuronal damage as is evident in PD, results in the death of otherwise viable cells (Gao & Hong 2008, Perry 2010). Moreover, activation of microglia by dying neurons may be both long-lived and self-perpetuating due to positive feedback coming from degenerating neurons. Thus, microglial activation and neuroinflammation are propagated to amplify neuronal degneration in PD, resulting in an accelerating feed forward cycle of inflammation and neuronal death (Collins *et al.* 2012). Accumulating evidence indicates that activation of distinct molecular effectors (e.g. NF-kB, brain angiotensin, NADPH oxidase, COX-2, iNOS) have a detrimental role in PD (Feng *et al.* 2002, Joglar *et al.* 2009, Kim *et al.* 2015, Ren *et al.* 2015, Sriram *et al.* 2002, Surace & Block 2012, Teismann 2012, Wu *et al.* 2003). Moreover, the apparent role of inflammation in PD pathogenesis led to the investigation of a broad spectrum of steroidal and non-steroidal anti-inflammatory drugs (SAID's and NSAID's, respectively), specific microglial inhibitors or anti-inflammatory cytokines in PD animal models (see Table 1.4). These studies not only have helped one to decipher the role of microglial activation in neuroinflammation in PD but also have indicated that inhibiting the specific processes involved in microglial activation may be a therapeutic avenue for PD. Nevertheless, epidemiological studies are still controversial to show a protective effect of NSAIDs in PD (Becker *et al.* 2011, Gagne & Power 2010, Manthripragada *et al.* 2011, Rees *et al.* 2011, Samii *et al.* 2009).

Far less studied is the potential role of astrocytes in PD neuroinflammation. In the MPTP mouse model, astrocytosis is a delayed response following nigrostriatal neuronal damage and is believed to be neuroprotective (Teismann & Schulz 2004). In fact, increased nigral activation of astrocytes in 6-OHDA model following infusion of interleukin-1β has been correlated with beneficial effects (Saura *et al.* 2003). Moreover, an active Wnt/β-catenin signaling in astrocytes of MPTP-injured midbrain was suggested to limit the degenerative process mainly by limiting the pro-inflammatory status. In brief, enriched Wnt1-like astrocytic proteins were found to send pro-survival signals to DAergic neurons, blocking GSK-3β-induced degradation of β-catenin, wich in turn facilitates cytoprotection/neurorepair (L'Episcopo *et al.* 2011a, L'Episcopo *et al.* 2011b).

Table 1.4 Anti-inflammatory agents in animal models of Parkinson's disease
Adapted from (Collins *et al.* 2012)

Agent	Mode of action	Species	PD model	Effects
Dexamethasone	SAID	Mouse	MPTP	1. Prevented striatal DA depletion 2. Protected DA neurons in SN
		Rat	LPS	1. Prevented striatal DA depletion 2. Protected DA neurons in SN
Acetylsalicylic acid	NSAID	Mouse	MPTP	Prevented striatal DA depletion
		Rat	6-OHDA	Prevented striatal DA depletion
Salicylic acid	NSAID	Mouse	MPTP	1. Attenuated akinesia and catalepsy 2. Prevented DA depletion and changes in DA turnover in nucleus caudate putamen
Ibuprofen	NSAID	Mouse	MPTP	Partially prevented striatal DA depletion
Indomethacin	NSAID	Mouse	MPTP	1. Prevented striatal DA depletion 2. Protected DA neurons in SN
Celecoxib	NSAID	Rat	6-OHDA	Reversed striatal DA neuronal fibres and nigral DA neuronal cell loss
Minocycline	Microglial activation inhibitor	Mouse	MPTP	1. Protected DA neurons in SN 2. Prevented DA depletion in striatum and nucleus accumbens
		Rat	6-OHDA	1. Reduced apomorphine-induced rotations 2. Protected DA neurons in SN
		Rat	LPS	Protected DA neurons in SN
Interleukine-10	Anti-inflammatory cytokine	Rat	LPS	Protected DA neurons in SN
		Rat	6-OHDA	1. Protected DA neurons in SN 2. Prevented striatal DA depletion 3. reduced apomorphine-induced rotation
Glatiramer acetate	Immunisation	Mouse	MPTP	1. Prevented striatal DA depletion 2. Protected DA neurons in SN
Vasoactive intestinal peptide	Anti-inflammatory molecule	Mouse	MPTP	1. Protected DA neurons in SN 2. Protected striatal DA fibers
Thalidomide	TNF inhibitor	Mouse	MPTP	1. Prevented striatal DA depletion 2. Protected DA neurons in SN
IKK inhibitor	Blockade of NF--κB signalling	Rat	LPS	Protected DA neurons in SN

(SAID: steroidal anti-inflammatory drug; NSAID: non-steroidal anti-inflammatory drug)

Nonetheless, exaggerated microglial pro-inflammatory status can impair astrocyte anti-inflammatory functions via inhibition of astrocytic Wnt1 expression and downregulation of anti-oxidant/anti-inflammatory cytoprotective proteins, thus abolishing DAergic neurorescue (L'Episcopo *et al.* 2014). A similar dual role for astrocyte protective/deleterious phenotype was also observed in lypopolysaccharide-induced damage of DAergic neurons: at low concentrations of LPS, the presence of astrocytes enhanced the survival of DAergic neurons while at high concentrations, astrocytes aggravated DAergic neuronal loss (Li *et al.* 2009). This also holds true

for astrocytic S100B protein: this is either a neurotrophin or an inflammatory cytokine, at low or high extracellular concentrations, respectively (Kato & Svensson 2015, Luo *et al.* 2010). Importantly, high levels of S100B were correlated with MPTP-DAergic degeneration (Sathe *et al.* 2012). Hence, inflammatory responses mounted by reactive astroglia in PD seem to be particularly dependent of neuron/microglia interactions and still require further elucidation.

Overall, the interest about the neuroinflammatory processes in PD has been growing during the last decade. While initially, it was considered as a simple consequence of neuronal degeneration, it has now become clear that neuroinflammation is involved in the progression of neurodegeneration even if it is not considered as a primary culprit of the disease (Hirsch *et al.* 2012).

1.6 Current treatments

Broadly, all therapeutic approaches can be considered as either disease modifying or symptomatic. Disease-modifying therapies are those able to delay, stop, or revert the progression of the neurodegenerative pathology, while symptomatic approaches are aimed to manage the disease symptoms (Valera & Masliah 2016). The prolonged preclinical phase in PD makes amenable for neuroprotective strategies. However, perhaps because of the poor understanding of PD etiology, there is to date no agent which has been proven in clinical trials to be neuroprotective. Yet, PD is unique in having several highly effective medications for suppressing its signs and symptoms, improving quality of life for many years (LeWitt & Fahn 2016).

Current treatments to manage motor symptoms are focused on restoring dopaminergic activity through a variety of mechanisms. Dopamine is predominantly ionized at physiological pH and is unable to cross BBB. The prodrug levodopa (L-DOPA; discovered in 1960s), when given exogenously, is able to cross BBB and is then metabolized by DOPA-descarboxilase to dopamine. This remains the gold-standard dopamine replacement therapy (Connolly & Lang 2014, DeMaagd & Philip 2015, Hornykiewicz 1998, Horstink *et al.* 2006a, Horstink *et al.* 2006b). To counter the extensive L-DOPA peripheral metabolism, this drug is administered in combination with carbidopa. This is a peripheral DOPA-decarboxylase inhibitor which allows greater passage of L-DOPA across BBB, enhancing its striatal availability. However, L-DOPA's clinical benefit is hampered by complex and variable pharmacokinetic and pharmacodynamic factors as the disease progresses. As a result, motor responses fluctuate and patients can experience periods of adequate symptom control ("on time"), considerable "off" time despite regular dosing, as well as involuntary movements which are referred to as dyskinesias (DeMaagd & Philip 2015, LeWitt & Fahn 2016). Other available pharmacotherapies include i) highly selective MAO-B inhibitors (e.g. selegiline, rasagiline) and COMT inhibitors (e.g. entacapone, tolcapone), aimed to hamper central and peripheral DA breakdown, respectively ii) dopamine agonists [(ergot derivatives (e.g. bromocriprine, pergolide) and non-ergot derivatives (e.g. apomorphine, pramipexole)] that activate DAergic receptors (mainly D2) and iii) anticholinergic drugs (e.g. biperiden) and amantadine (N-methyl-D-aspartate blocker) that act on postsynaptic receptors for other neurotransmitters in the striatum, an attempt to counteract

the imbalance that results from DAergic loss with subsequent increase in cholinergic and glutamatergic function (Connolly & Lang 2014)(see Figure 1.11).

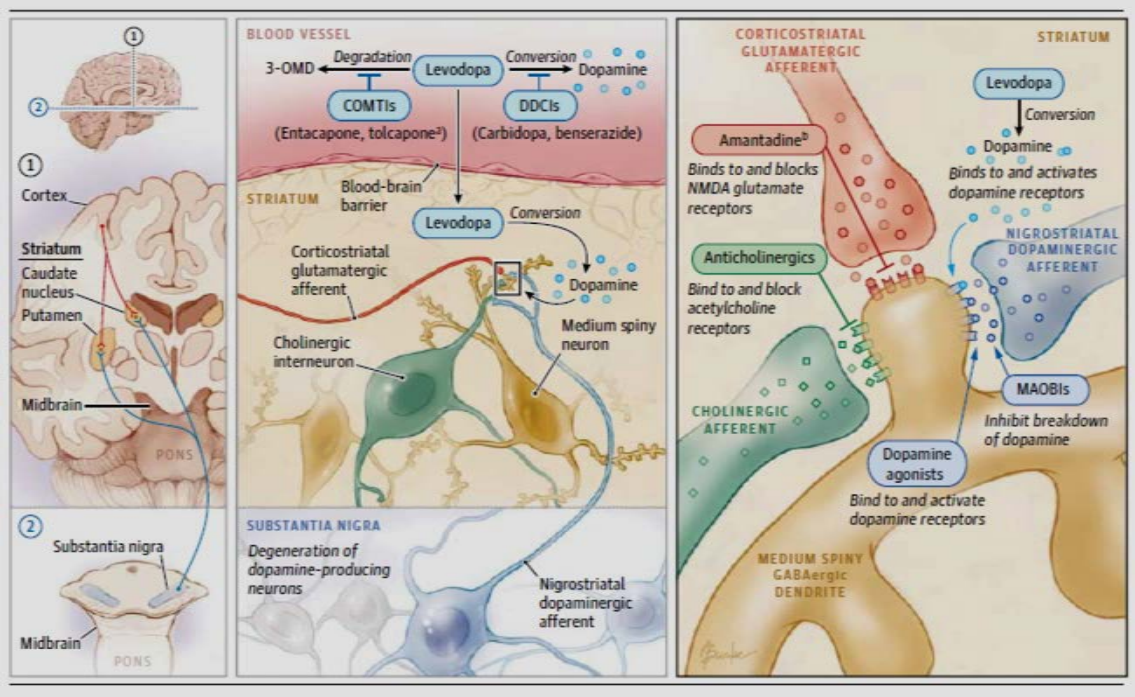

Figure 1.11 Available medications to treat motor symptoms of Parkinson's disease act on complex neurologic interactions in the striatum that affect motor activity. Dopaminergic afferents from the substantia nigra, glutamatergic afferents from the cerebral cortex and thalamus, and cholinergic striatal interneurons all converge to influence the activity of the striatal medium spiny GABAergic neurons (MSNs). Levodopa (L-DOPA) is transported from the peripheral circulation across the blood-brain barrier and is converted centrally to dopamine, replacing the lacking neurotransmitter in Parkinson disease. Outside the blood-brain barrier, in the peripheral circulation, dopamine decarboxylase inhibitors (DDCIs) block the conversion of L-DOPA to dopamine, and catechol-O-methyltransferase inhibitors (COMTIs) block its degradation to 3-O-methyldopa (3-OMD). At the striaal level, L-DOPA, dopamine agonists, and monoamine oxidase type B inhibitors (MAOBIs), all have dopaminergic effects. Anticholinergic drugs and amantadine block other postsynaptic receptors in the MSNs in striatum. NMDA stands for N-methyl-D-aspartate. a Tolcapone, unlike entacapone, is able to cross the blood-brain barrier and block degradation of levodopa and dopamine. b Amantadine has dopamine releasing effects in addition to affecting NMDA glutamate receptors (Taken from Connolly & Lang 2014)

Non-motor symptoms may originate from multiple causative processes associated with dopaminergic and non-dopaminergic circuits. NMS may be even secondary to motor symptoms medications, in the case of later PD stages (Connolly & Lang 2014, Vorovenci *et al.* 2016). Some pharmacological interventions have also been used for the symptomatic control of neuropsychiatric complications, including cholinesterase inhibitors (e.g. rivastigmine), atypical antipsychotics (e.g. clozapine) and tryciclic antidepressants (e.g. nortriptyline, desypramine) for demential, psychotic and depressive conditions respectively, to name just a few (Ferreira *et al.* 2013).

Ferreira and colleagues (2013) summarized the 2010 EFNS/MDS-ES evidence-based treatment recommendations for the management of early and late PD. It is worth mentioning

that non-pharmacological treatments are being increasingly explored due to the lack of effective pharmacological approaches with few side effects. These include functional neurosurgery [e.g. deep brain stimulation of the subthalamic nucleus (STN), posteroventral pallidum (GPi) and thalamus] and physical exercise (e.g. treadmill training, cued training, downhill walking, specific sensory attention-focused exercises), that have proved successful at reducing PD symptoms (Ferreira *et al.* 2013).

Notably, epidemiological and experimental studies support physical exercise (PE) as being beneficial with regards to motor and non-motor symptoms of PD (Aguiar *et al.* 2016, Dashtipour *et al.* 2015, Dibble *et al.* 2009, Goodwin *et al.* 2008, Mehrholz *et al.* 2015, Pothakos *et al.* 2009, Tuon *et al.* 2014). Both neuroprotective and neurorestorative effects were suggested to be triggered by PE in distinct experimental PD models. While neuroprotection to dopaminergic neurons has been principally attributed to an exercise-induced increase of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) or glial-derived neurotrophic factor (GDNF) (Cohen *et al.* 2003, Real *et al.* 2013), neurorestoration has been mainly related to modulation of dopaminergic (e.g. DA clearance reduction, restoration of DA-D2 receptors and dendritic spine loss reversion in striatal neurons) (Fisher *et al.* 2013, Smith *et al.* 2011, Toy *et al.* 2014) or glutamatergic neurotransmission (e.g. alterations in AMPA receptor and storage and release of glutamate in presynaptic terminals) (VanLeeuwen *et al.* 2010). Nevertheless, PE has more global effects on factors that influence general brain health (Petzinger *et al.* 2013). For instance, PE activates beneficial effects of peripheric immune system by mounting an effective anti-inflammatory status (e.g. increase levels of circulating cortisol, IL-10 and IL-1 receptor antagonist and decrease the expression of innate pattern-recognition receptors) which potentially affect brain function (Gleeson *et al.* 2011, Petzinger *et al.* 2013, Singhal *et al.* 2014). Hence, the putative impact of PE on central innate imune system should not be overlooked, particularly in neurodegenerative diseases with a strong neuroinflammatory background, such as PD (see 1.5.3). Nonetheless, this issue is in its infancy and represents an attractive and promising research avenue.

1.7 Brain innate immunity: a focus on Receptors for advanced glycation endproducts (RAGE) network

In this section, it will be provided an overview of brain innate immune system with a major focus on receptors for advanced glycation endproducts (RAGE) network, emphasizing in detail the recent advances on the involvement of RAGE in PD neurodegenerative process.

1.7.1 Brain innate immunity: an overview

Brain is isolated from the systemic circulation by BBB, a selectively permeable barrier that regulates the exchange of nutrients, waste and immune cells between blood and nervous tissue of brain parenchyma (Abbott *et al.* 2010). Further protective barriers are composed of specialized ciliated glia, the ependyma [which lines the ventricles preventing entry of pathogens from CSF into brain parenchyma] and meninges (Griffiths *et al.* 2007). Over the years, experiments demonstrated that tissue grafts, bacteria, viruses and vectors, all evaded immune recognition when delivered to brain parenchyma (Galea *et al.* 2007). These observations paved the way for the concept of **"brain immunologic privilege"**, which was firstly proposed by Billingham and Boswell (Billingham & Boswell 1953). Indeed, a few number of peripheral immune cells are detected in the CNS parenchyma, although resident microglia are found throughout the brain in the healthy state (Ransohoff & Brown 2012).

This dogma, which has prevailed for decades, has been challenged when foreign tissue grafts, Bacille Calmette-Guerin (BCG) and influenza virus were injected intracerebroventricularly (ICV). Such administrations resulted in tissue rejection, delayed-type hypersensitivity lesions in choroid plexus and humoral and cytotoxic T-cell responses, respectively (Matyszak & Perry 1996, Stevenson *et al.* 1997). Moreover, meningeal spaces were found to be highly populated by various immune cells, namely T cells, B cells and dendritic cells (Derecki *et al.* 2010, Kivisakk *et al.* 2009, Louveau *et al.* 2015). Thus, as far as adaptive immunity is concerned, immunologic privilege of the CNS is compartmentalized, being confined to brain parenchyma (Galea *et al.* 2007).

Immune system has evolved to virtually respond to any type of insult that threatens homeostasis (e.g. trauma, cellular and metabolic dysfunction, ischemia-reperfusion injury). Though clearly important in the periphery, its role in the CNS is indispensible regarding its limited regenerative capacity (Galea *et al.* 2007). Cellular "soldiers" of CNS innate immunity - resident phagocytic microglia, and to some extent mature astrocytes - sense danger/sterile insults and readily "sound the alarm" by producing chemokines and cytokines, mounting an inflammatory status (Chakraborty *et al.* 2010, Chen & Nunez 2010, Gadani *et al.* 2015). Under some circumstances, a stereotypic cascade of posterior recruitment of circulatory neutrophils (hours to days post-injury), monocytes and lymphocytes (days to weeks post-injury) to the site of injury occurs in order to facilitate vigorous inflammatory responses (Gadani *et al.* 2015, Ransohoff & Brown 2012).

But how do cells promptly discern between health and injury? **In other words, how do innate immune cells sound the alarm and initiate an immune response only when necessary?** (Gadani *et al.* 2015). The elegant "danger theory" formulated by P. Matzinger (2002) states that the ability of immune system for the classical recognition of pathogens may have an evolutionary origin in the detection of danger molecules on distressed cells within the organism itself (Matzinger 2002). Thus, damage-associated molecular patterns (DAMP's) can be either endogenously derived "alarmins" or exogenous "pathogen-associated molecular patterns" (PAMP's). This point of view is of particular interest for our understanding of immune responses in neurodegeneration in the absence of pathogenic triggers (Czirr & Wyss-Coray 2012, Sirisinha 2011). DAMP's encompass an extremely diverse class of molecules, ranging from bacterial lipids or peptides to endogenous proteins, nucleic acids, ATP or uric acid (Gadani *et al.* 2015). A large majority of endogenous DAMP's are normally sequestered intracellularly and are not exposed to the immune system under normal physiological conditions but are extensively released as a result of cell injury or death. In addition, a number of extracellular DAMP's have also been identified, generally misfolded proteins (e.g α-syn) or breakdown products of extracellular matrix (e.g. heparin sulfate, hyaluronan) (Mosley *et al.* 2012, Sirisinha 2011).

Immune system uses an elaborate network of immune sensors – pattern recognition receptors (PRR's) – that are not restricted to dedicated immune cells but are expressed by most cells including neurons (Czirr & Wyss-Coray 2012). These germline-encoded immune sensors

represent a large group of receptors recognizing a larger repertoire of potential ligands (Bianchi 2007). They can be broadly classified into five different classes: i) Toll-like receptors (TLR's); ii) nucleotide-binding leucine-rich repeat-containing receptors (NLRs); iii) retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs); iv) C-type lectins (CTLs) and v) absent-in-melanoma (AIM)-like receptors (ALRs). PRRs can be cell-associated receptors: found on the cell plasma membrane (e.g. TLR1,2,4,56 and CLRs), in association with membrane of endosome, endoplasmic reticulum or endolysosome (e.g. TLR 3,7,9) or intracellularly in the cytosol (e.g. RLRs and NLRs) (Bryant & Monie 2012, Sirisinha 2011). In addition, many secreted soluble PRRs have been identified and are often components of the complement system (e.g. C1q, mannose binding protein) that can be locally produced by CNS cells (Gasque *et al.* 2000, Gasque *et al.* 1995, Levi-Strauss & Mallat 1987). Soluble PRR's can interact with other innate receptors like TLR which, depending on situation and location, may be either beneficial or detrimental to the host. For instance, these "crosstalks" may substantially contribute to resolution of inflammation, promoting the elimination of damaged apoptotic cells and immune complexes. On the other hand, some crosstalks associated with bacterial infection (e.g. C5aR-TLR2) are known to impair macrophage function, possibly by interfering with the generation of nitric oxide (Sirisinha 2011).

Finally, the receptor for advanced glycation endproducts is an atypical PRR and was originally described as a receptor that recognizes glycosylated proteins and lipids in the forms of advanced glycation endproducts (AGEs). RAGE was later found to also detect DAMP's such as HMGB-1 and S100B (Gadani *et al.* 2015, Sirisinha 2011).

1.7.2 Receptor for advanced glycation endproducts (RAGE)

1.7.2.1 RAGE: From gene to protein

The human RAGE gene (termed AGER by the *HUGO* nomenclature) was cloned and characterized from mouse lung in 1992 (Neeper *et al.* 1992). It is encoded on the short arm of chromosome 6 (6p21.3) in the major histocompatibility complex III (MHC), a gene-rich region containing numerous genes with an inflammatory function including components of the complement system and various cytokines, such as TNF-α (Sugaya *et al.* 1994, Traherne 2008, Vissing *et al.* 1994). Studies have shown extensive genetic variability occurring within RAGE gene with more than 30 single nucleotide polymorphisms (SNPs) characterized, some of them potentially linked with pathological states (Daborg *et al.* 2010, Gao *et al.* 2014, Gomes *et al.* 2015, Kalea *et al.* 2011). Yet, additional mechanisms also account for transcriptomic diversity, including the following: i) the use of alternative promoters, namely NF-κB, NF-IL6, SP-1, AP-2 (Bianchi *et al.* 2010, Li & Schmidt 1997); ii) alternative poly(A) sites, known to affect mRNA turnover by the presence of stability elements in 3'UTR (Caballero *et al.* 2004) iii) miRNA regulation (Shi *et al.* 2008) and iv) alternative splicing, which represents the major mechanism to produce RAGE mRNA diversity (Kalea *et al.* 2011).

Alternative splicing (AS) offers flexibility to the transcriptome and proteome to help fine tune the protein expression in a cell-specific or tissue-specific manner in response to precise environmental or developmental cues. This evolutionary mechanism is widely used in the mammalian innate immune system: lacking antigen specific receptors, expresses a range of evolutionarily conserved pattern recognition receptors, such as RAGE (Yabas *et al.* 2016). Human RAGE gene is composed of a 5' flanking region that regulates its transcription, 11 exons and a short 3'UTR. The resulting transcribed mRNA is translated into a canonical full-length protein (commonly denominated flRAGE or mRAGE) of 404 amino acids with a molecular mass of ≈55kDa, consisting of an extracellular ligand-binding domain composed of three Ig-like domains (one Ig-V domain and two Ig-C- domains), a single transmembrane region and a highly-charged hydrophobic intracellular domain (Hudson *et al.* 2008a, Kalea *et al.* 2011, Neeper *et al.* 1992)(see Figure 1.12). Splice variants of RAGE result in changes that might affect the extracellular ligand-binding domain (commonly denominated as N-terminal truncated) as

well as the removal of the sequence encoding the transmembrane/cytosolic region, thus generating C-terminally truncated isoforms (Hudson *et al.* 2008a, Kalea *et al.* 2011, Lopez-Diez *et al.* 2013). One C-truncated isoform is produced by alternative inclusion of part of intron 9 and removal of exon 10, resulting in the production of endogenous secretory variant of RAGE (esRAGE) (Hudson *et al.* 2008a). Formation of secreted isoforms variants is a common phenomenon amongst membrane receptors, often acting as antagonist versions of their membrane bound version (Fry & Toker 2010, Muda *et al.* 2005, Scott *et al.* 2006). This is the case for esRAGE: it prevents RAGE signaling by acting as a "decoy" receptor. In addition, RAGE variants, who exhibit decreased ligand binding ability (N-terminally truncated) or lack cytosolic region (C-terminally truncated), may also contribute to the overall regulation of flRAGE cellular function.

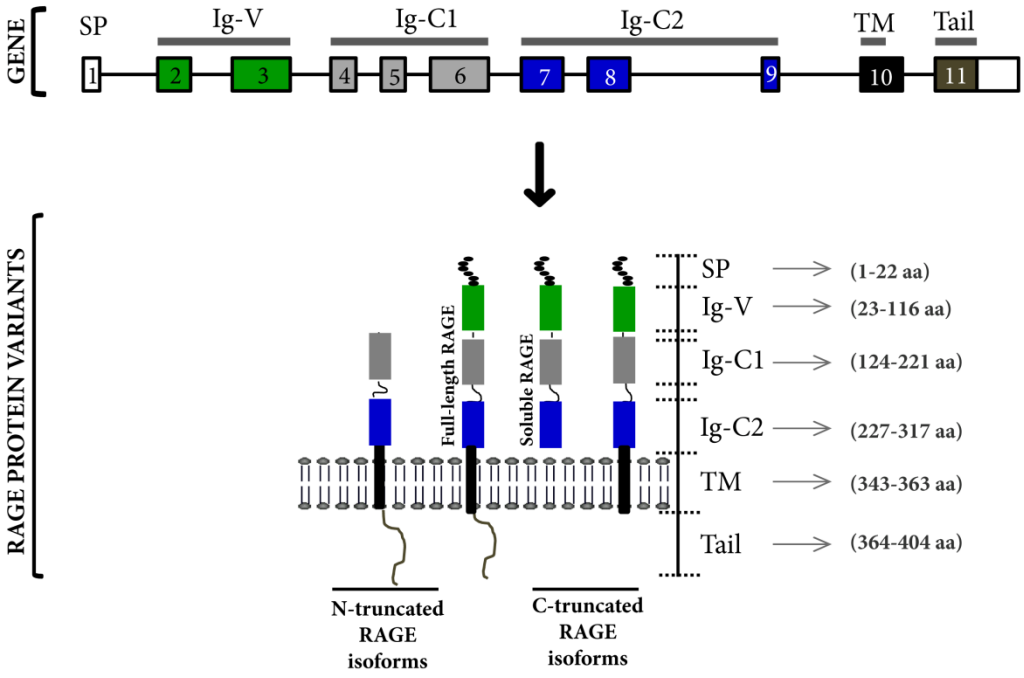

Figure 1.12 RAGE gene organization and prevalent RAGE protein isoforms. Color of the 11 exons correspond to the domains of RAGE for which they encode as shown in the RAGE protein schematic above, depicting the most common RAGE variants described to date (Ig, Immunoglobulin-like domain; TM, transmembrane domain)

In fact, RAGE was found to undergo extensive AS in a broad spectrum of mammalian species (including human and rodents) with distinct isoforms (>15 splice variants) distributed across tissues and species (Lopez-Diez *et al.* 2013). Among them, two totally conserved isoforms (flRAGE and N-truncated isoforms) and potentially soluble isoforms were found in all analyzed species. This represents a special evolution pattern at the mRNA level. Since the interplay

between signaling competent and decoy RAGE isoforms strongly modulate RAGE cellular function, elucidation of regulatory mechanisms that govern RAGE AS is mandatory. In this respect, G-rich cis-elements within exon 9 and heterogeneous nuclear ribonucleoprotein (hnRNPA1) and Transformer2b-1 (Tra2b-1) were found to be important modulators of RAGE splicing gene (Liu *et al.* 2015, Ohe *et al.* 2010). Noteworthy, a highly specialized regulation of RAGE AS seems to occur in human brain as esRAGE was found to be the predominant variant in brains of non-demented and neuropathology-free individuals. This asymmetric expression may be involved in maintaining brain homeostasis as the ability of secreted RAGE to prevent the aggregation of potential pathogenic RAGE ligands [e.g. amyloid-β petide (Aβ)] would be expected to not only reduce RAGE induced signal transduction but also provide a global benefit to the brain, possibly delaying or preventing the onset of proteinopathy-induced neurodegeneration (Ding & Keller 2005b). Remarkably, a novel C-truncated splice variant has been recently found to be prevalent in both human and mouse brain tissues, reinforcing the proposed endogenous mechanism to regulate brain RAGE signaling (Jules *et al.* 2013). It will be interesting to extend these findings in future studies.

Soluble RAGE isoforms can also be produced by constitutive or induced ectodomain shedding of flRAGE by metalloprotease 10 (ADAM10) metalloprotease 9 (MMP9) and/or γ-secretase (Braley *et al.* 2016, Galichet *et al.* 2008, Metz *et al.* 2012, Schmidt 2015). Both soluble RAGE isoforms (esRAGE and cleaved RAGE) circulate in human plasma and can be distinguished by commercially available immunoassays as esRAGE contains a unique span of 16 amino acids in the C-terminal region of molecule (Lanati *et al.* 2010, Schmidt 2015). Nevertheless, there is no specific method to accurately quantify the cleaved form of RAGE alone, implying that the most commonly used sRAGE immunoassays measures the total pool of soluble RAGE using antibodies that recognize both the spliced and cleaved forms of soluble RAGE (Kalea *et al.* 2011). Notably, genetic engineered soluble RAGE was proven to exert protection against adverse complications of inflammatory-related diseases where flRAGE and its ligands accumulate (Schmidt 2015, Yan *et al.* 2010). We will use sRAGE terminology to refer to all soluble isoforms regardless of their origin.

Finally, analysis of RAGE in tissues revealed cell specific regulation based on microenvironmental cues (Bierhaus *et al.* 2005, Bopp *et al.* 2008). For instance, RAGE

expression occurs both constitutively and inducibly, depending upon cell type and developmental stage. Whereas RAGE is constitutively expressed during embryonic stage supporting a potential role in development and tissue maturation (including brain), it is downregulated in adult life (lung and skin are exceptions). These data suggest the presence of negative regulatory elements within RAGE promoter, which are responsible for suppressing its expression thereby maintaining low RAGE levels under basal conditions (Hori *et al.* 1995, Li & Schmidt 1997).

1.7.2.2 RAGE: a pattern recognition receptor

One fascinating aspect of RAGE biology is its ability to recognize structurally diverse ligands. RAGE was originally identified as a receptor for advanced glycation endproducts (AGE's). This explains the choice of this receptor´s name. AGE's are products of non-enzymatic glycation, a posttranslational modification of proteins, lipids and other macromolecules that seem to play a major role in aging (Simm *et al.* 2015). The process of non-enzymatic glycation is known as the Maillard reaction, commonly divided into two reaction stages. The early-stage reaction starts with the covalent interaction of reducing sugars and by-products of glucose metabolism (e.g. methylglyoxal) with amino-containing groups (e.g. lysine and arginine residues), forming Schiff's bases that encompass early glycation products. In the late-stage, these products undergo a series of multistep reactions, the so-called Amadori rearrangement, forming final irreversible modifications – AGE's, such as N^{ε}-(carboxymethyl)lysine (CML), pentosidine, pyrraline, imidazolone (Kikuchi *et al.* 2003, Vicente Miranda *et al.* 2016). Protein glycation depends not only on the levels of glycation agents but also on several factors such as pH, temperature, and the half-life of each protein. Long-lived proteins often constitute preferential targets for glycation, allowing distinct cytotoxic phenomena: inter- and intraprotein linkages, accompanied by changes in biological activity and protein β-sheet aggregates formation. Furthermore, AGE-modified proteins may also induce reactive oxygen species and inflammatory cytokines via RAGE, thus entertaining a sustained tissue inflammation (see 1.7.2.3) (Kikuchi *et al.* 2003, Ott *et al.* 2014, Simm *et al.* 2015, Vicente Miranda *et al.* 2016). Notably, several proteins associated with neurodegenerative diseases are long-lived and glycation is suggested to take a big part on the onset and progression of some major pathologies

including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, familial amyloid polyneuropathy and prion disease (Salahuddin *et al.* 2014, Simm *et al.* 2015, Vicente Miranda *et al.* 2016).

Shortly after RAGE has been recognized as a receptor for AGE's, it became evident that a number of other ligands also interacted with the receptor. In addition to AGE's, RAGE also binds Aβ, S100 calgranulins family (calcium-binding polypeptides), DNA binding protein HMGB-1 (amphoterin), Mac-1 (CD11b/CD18), phosphatidylserine, prions, LPS, DNA and RNA. This broad repertoire of ligands with such structural diversity establishes RAGE as a pattern recognition receptor (Bierhaus *et al.* 2005, Ott *et al.* 2014, Sasaki *et al.* 2002, Tobon-Velasco *et al.* 2014). Hence, the next question arises: **How can this myriad of seemingly unrelated structures be recognized by one receptor?**

The molecular basis of RAGE activation by its diverse set of ligands has remained enigmatic as RAGE ligands exhibit different structure, size,and symmetry or even no symmetry, as in the case of glycated proteins or amyloid-β. The common factor linking these ligands is their tendency to oligomerize (Koch *et al.* 2010). Likewise, receptor oligomerization has been proposed as a general mechanism to explain ligand-RAGE three-dimensional interactions as well as RAGE signal transduction. In this respect, oligomerization seems to precede ligand binding to RAGE, similar to what have been shown for other class of receptors (e.g. Toll-like receptors) (Heldin 1995, Koch *et al.* 2010, Xie *et al.* 2008, Zong *et al.* 2010). In terms of RAGE structural features, it is well accepted that extracellular C1-C2 linker is fully flexible, in contrast with the stiff V-C1 linker. These features provide coupling between V and C1 domains which implies two structurally independent subunits - VC1 and C2 - within RAGE extracellular portion (Dattilo *et al.* 2007, Koch *et al.* 2010, Kupniewska-Kozak *et al.* 2010, Park *et al.* 2010, Sitkiewicz *et al.* 2013). Accordingly, dynamic light scattering and FRET studies suggested RAGE clustering by VC1 subunits (see Figure 1.13a). However, a new oligomerization interface between C1 and C2 was subsequently proposed. Albeit not fully elucidated, both dimer models were reconciled in a hypothetical RAGE tetramer who exhibits four VC1 domain arms capable of binding ligands of different sizes and symmetries, as S100B tetramer or Aβ oligomers (Sitkiewicz *et al.* 2013). Concurrently, transmembrane helix homo-interactions have also been proposed, implying that further structural studies are necessary to fully understand dynamic

mechanisms that govern RAGE-mediated ligand recognition and cell signaling (Yatime & Andersen 2013).

Majority of ligands bind to the V or C1 domains or both, with glycosylation of V domain strongly impacting ligand binding (e.g. HMGB-1, AGE's) (Leclerc *et al.* 2009, Xie *et al.* 2008). It is important to keep in mind that even though RAGE oligomerization preassembly is likely to facilitate ligand binding (thus increasing its efficiency in signaling), a tight regulation must occur in order to avoid nonspecific activation with non-functional consequences. For example, besides working as a "scavenger" receptor, sRAGE may also function as a direct inhibitor of flRAGE itself, by forming an heterocomplex with this RAGE variant (Koch *et al.* 2010) (see Figure 1.13b).

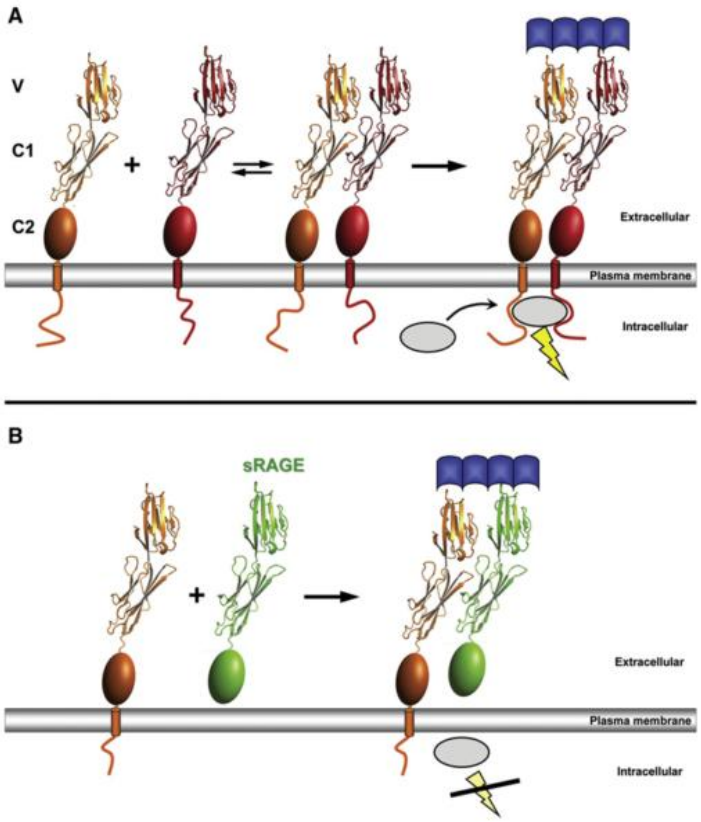

Figure 1.13 Models of RAGE activation (a) and inhibition by sRAGE (b). (a) Model in which RAGE preassembles in the plasma membrane. Ligand binding (blue squares) to RAGE stabilizes oligomers, which then can bind a signaling adaptor protein (gray sphere) to the cytoplasmic region of RAGE. (b) Diagram showing the action of sRAGE, in which interaction with intact RAGE to from a hetero-oligomer limits binding of intracellular adaptors and blocks signal transduction (Taken from Koch *et al.* 2010)

1.7.2.3. RAGE signaling

Concurrent with ligand recognition and binding, the requirement of receptor oligomerization has been also proposed as a general mechanism to explain signal transduction in RAGE. Clustering of receptor molecules increases the affinity/stability of multimeric ligand-receptor complexes, and drives the colocalization of RAGE cytoplasmic domains (Koch *et al.* 2010, Sitkiewicz *et al.* 2013). Although RAGE-dependent signal transduction is critically dependent of its short C-terminal cytoplasmic tail (ctRAGE) (Huttunen *et al.* 1999, Kierdorf & Fritz 2013, Rong *et al.* 2004), its sequence has no homology with any protein tyrosine or serine/threonine kinase motifs and does not have enzymatic activity (Borsi *et al.* 2012, Rai *et al.* 2012). Thus, RAGE needs to associate with adaptor proteins for intracellular signaling. In fact, several molecules have been identified so far: Diaphanous1 (mDia1), ERK, TIRAP, MyD88 and PRAK (Hudson *et al.* 2008b, Ishihara *et al.* 2003, Kim *et al.* 2016b, Rai *et al.* 2012, Sakaguchi *et al.* 2011). For instance, mDia1 mediates the effects of the small GTPases of Rho family on actin polymerization and cell motility, a process known to be triggered by S100B/RAGE axis within microglial migration and inflammatory chemokines production (Bianchi *et al.* 2011). Furthermore, mDia1 constitutively binds to ctRAGE without subsequent signaling. However, upon multimeric ligand-RAGE complexes formation, intracellular ctRAGE clusters which in turn increases local association of mDia1, restoring its activity and downstream signaling (Rai *et al.* 2012)(See Figure 1.14).

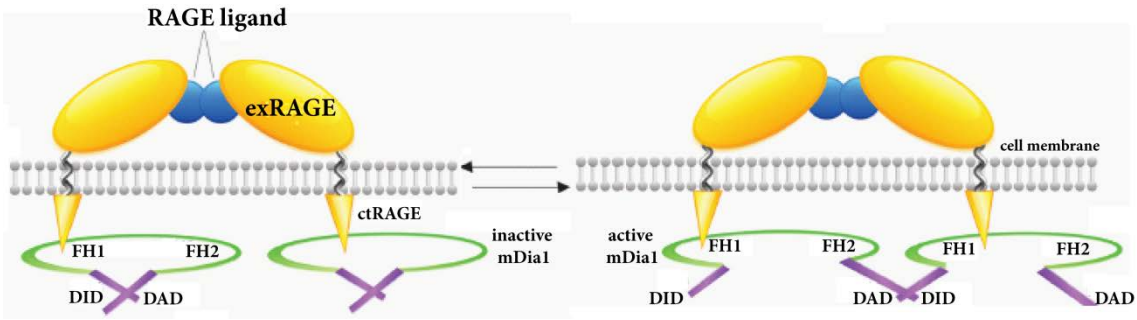

Figure 1.14 Model of RAGE-induced activation of mDia1. The formin homology (FH1 and FH2) domains of mDia1 are required for mDia1 activity. DID and DAD, N-terminal diaphanous inhibitory domain and C-terminal diaphanous autoregulatory domain of mDia1, respectively. mDia1 is autoinhibited due to the interaction between the regulatory diaphanous inhibitory and diaphanous autoregulatory domains, DID and DAD, respectively. RAGE can constitutively bind to mDia1 by using the ctRAGE-FH1 interaction. Clustering of mDia1 molecules due to extracellular multimeric RAGE (exRAGE) binding to a RAGE ligand results in intermolecular domain swapping and partial restoration of mDia1 activity (Adapted from Rai *et al.* 2012)

Regardless of the nature of the adaptor protein recruited by RAGE in different cell types, RAGE engages numerous ligands and is expressed on many cell types, including glia and neurons. Thus, it is not surprising that RAGE signaling is rather complex, influenced by a multitude of different factors and strongly dependent on intervening intracellular events such as the redox status, energy metabolism and cell-specific transcription and activity of definite genes (Kierdorf & Fritz 2013, Sorci *et al.* 2013). The initiation of RAGE signaling commonly occurs through production of ROS via NADPH oxidase (Daffu *et al.* 2013, Koulis *et al.* 2015, Ott *et al.* 2014, Wautier *et al.* 2001) and subsequent activation of various kinases, including Mitogen-activated protein kinases (MAPK's), such as extracellular signal-regulated kinases 1/2 (ERK1/2), stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), p38; phosphoinositide 3 kinases/Akt (PI3K/Akt); Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT); glycogen synthase kinase 3 beta (GSK3β); etc (Batkulwar *et al.* 2015, Kislinger *et al.* 1999, Kulkarni *et al.* 2013, Sorci *et al.* 2013).These kinases trigger several downstream signaling cascades often via activation of transcription factors including nuclear factor kappa B (NF-κB) and activator protein 1 (Ap1), thereby linking RAGE to a number of oxidative and inflammatory responses. Taking into account this signaling complexity, it is not surprising that RAGE appears to play an important role in the delicate balance between cell survival and cell death by influencing apoptosis and autophagy, depending on the intensity and/or duration of its activity (Kang *et al.* 2011, Rojas *et al.* 2010, Sorci *et al.* 2013)(see Figure 1.15).

Receptor internalization after ligands activation is another well-known mechanism to induce intracellular signaling in a plethora of receptors. Likewise, ligands/RAGE complexes (e.g. AGEs, S100B, Aβ) were found to be internalized in vesicular structures via lipid rafts, a process which is often caveolae-dependent (Andras *et al.* 2012, Perrone *et al.* 2008, Sevillano *et al.* 2009). Noteworthy, mobilization of RAGE vesicles from cytoplasmic internal stores allows an almost immediately increase of RAGE at cell surface without requiring *de novo* synthesis. This process was observed in endothelial cells exposed to a pro-inflammatory stimulus, where RAGE was presented at cell surface within a few minutes, acting as counter-receptor for leukocytes (Frommhold *et al.* 2011, Frommhold *et al.* 2010). By the other hand, receptor internalization after activation is also a mechanism to shut down signaling set in motion by active receptor/ligand complex. Further work dissecting RAGE endocytosis and recycling is needed as

dysregulation of this process is known to be involved in some pathological disorders (Kierdorf & Fritz 2013).

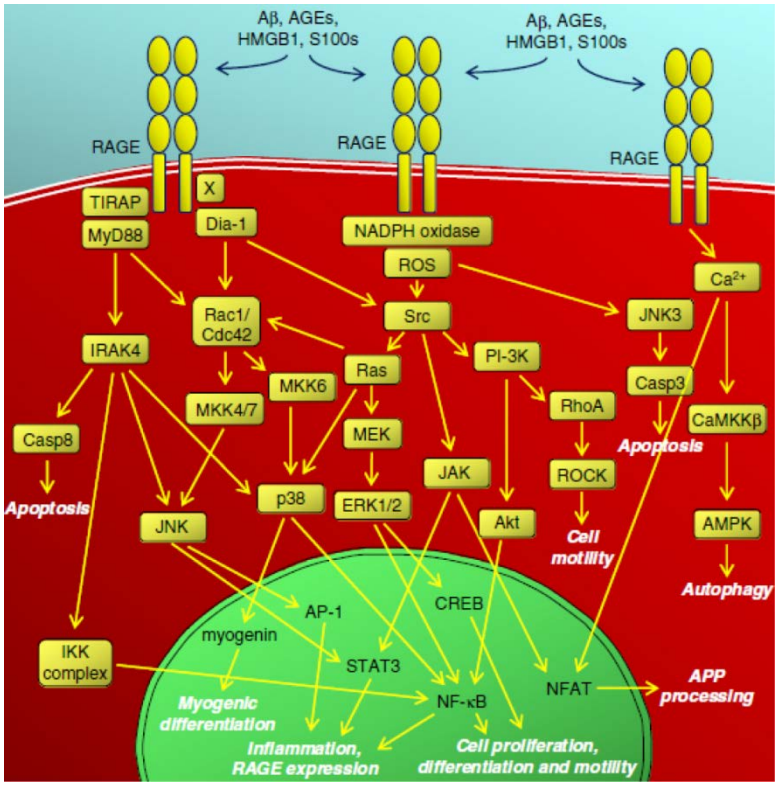

Figure 1.15 RAGE engagement by multiple ligands activates various signaling pathways in a cell-specific manner. Ligand binding causes RAGE oligomerization (or stabilization of RAGE oligomers) and association of the RAGE cytoplasmic domain with the adaptors Dia-1, TIRAP, MyD88 and/or as yet unidentified adaptors (X), which in turn activates signaling molecules impinging on transcription factors NF-κB, AP-1,CREB, STAT3, and/or myogenin. In doing so, RAGE regulates the inflammatory response and/or cell proliferation, survival, differentiation and motility in a cell-specific manner. RAGE signaling-dependent regulation of cell motility also occurs independently of its effects on gene transcription via a Dia-1/Src/Ras/PI3K/RhoA/ROCK and a Dia-1/Rac1/Cdc42/RhoA/ROCK pathway (Taken from Sorci *et al.* 2013)

Aside from the type and concentration of ligands and cell type, the presence of putative co-receptors adds an extra level of complexity to RAGE signaling. Similar to other PPR's, RAGE biology fits the innate immunity "non-specific" working model, where a wide array of receptors often cooperate and synergistically reinforce each other in the activation of transcriptional responses within host's first line of defense (Bianchi 2007). Curiously, the requirement of concomitant activation of at least two different PRR's, known as the "coincidence detection mechanism", obviates random activation thereby reducing the chance of false detection and the potential onset of autoimmunity. Thus, receptor diversity may be envisaged as a particular mechanism that facilitates a tailored and less "unspecific" response associated with complex DAMP's/PPR's interactions (Underhill 2007). This kind of collaboration also allows innate

immune system to i) rapidly scale up the response in the face of severe threats or ii) antagonize cell response upon chronic stimulation (Tan *et al.* 2014). One well-known example is the phenomenon of LPS tolerance, whereby cells stimulated chronically with LPS become refractive to subsequent TLR stimulation. Several mechanisms of negative regulation have been proposed, namely: microRNA's recruitment (Renzi *et al.* 2015), epigenetic modifications (Neagos *et al.* 2015), increased expression of negative regulators of TLR signaling (Piao *et al.* 2009, Xiong & Medvedev 2011), although consensus on this matter has not yet been reached. In this regard, findings from *in vitro* and *in vivo* studies show functional interactions between RAGE and some members of the TLR family (Mazarati *et al.* 2011, Nadatani *et al.* 2013, Sorci *et al.* 2011). RAGE/TLR's co-operation may regulate immune and inflammatory responses and increasing evidence supports their potential synergism: both PRR's share several common ligands including HMGB-1 (Yang *et al.* 2010), LPS (Yamamoto *et al.* 2011), β-sheet amyloid fibrils (Udan *et al.* 2008) or S100B (Sorci *et al.* 2011). Besides, RAGE also appears to recruit adaptor proteins involved in TLR's signaling transduction (e.g. TIRAP, MyD88) (Sakaguchi *et al.* 2011). Finally, RAGE/TLR2 and RAGE/TLR9 immunocomplexes were also demonstrated *in vitro* by immunoprecipitation and *in situ* proximity ligation assay (Sorci *et al.* 2011, Tian *et al.* 2007). Yet, the understanding of the mechanism of RAGE–TLR crosstalk, particularly at the receptor level, is extremely limited and important questions remain to be addressed (Ibrahim *et al.* 2013, van Beijnum *et al.* 2008), namely, the biology of this functional unit in neurodegenerative diseases, including PD.

1.7.2.4 RAGE: tissue homeostasis versus chronic degeneration

The fact that RAGE is a multi-ligand receptor expressed at basal levels in a range of physiological cell types corroborated the concept that no gene exists without any physiological function (Brett *et al.* 1993). In addition, limited inflammatory responses may be beneficial to the host when submitted to certain stresses (Alexiou *et al.* 2010). Therefore, short-lived inflammation may serve to foster repair and remodeling. This strengthens the initial assumption that RAGE plays a role in health. In this respect, ligands concentration seems to be an important factor in predicting the effect of RAGE on cellular homeostasis (Bierhaus & Nawroth 2009, Herold *et al.* 2007).

Many types of DAMP's are expressed in the healthy CNS and govern homeostatic functions. For instance, HMGB-1 is a mobile chromatin protein that acts as a DNA chaperone, facilitates nucleosome formation and participates in DNA transcription, replication and repair (Pandolfi *et al.* 2016). Another example is S100B, a member of Ca^{2+}-regulated proteins widely expressed in astrocytes and involved in a large number of cellular functions such as calcium homeostasis, cell growth and differentiation, dynamic of cytoskeleton or energy metabolism (Donato 2003). These intracellular molecules may achieve extracellular space at low concentrations, triggering trophic effects and repair after injury: nanomolar concentrations of S100B stimulates neurite outgrowth and enhanced neuronal survival during development or after injury (Saleh *et al.* 2013, Winningham-Major *et al.* 1989). Moreover, HMGB-1 released from reactive astrocytes promotes neural stem/progenitor cell proliferation upon injury (Li *et al.* 2014b). Noteworthy, these trophic events are RAGE-mediated (Huttunen *et al.* 2000). Other beneficial functions such as peripheric nerve outgrowth, muscle and cardiac regeneration have also been linked to DAMP/RAGE interactions (Riuzzi *et al.* 2012, Rong *et al.* 2004, Rossini *et al.* 2008).

Yet, such ligands that mediate repair upon acute injury are likely to sustain chronic tissue injury once massively released into the extracellular environment (Herold *et al.* 2007, Kono & Rock 2008). Thus, a Janus-faced role of DAMP molecules and their receptors seems to occur in homeostasis and tissue repair *versus* chronic inflammation. Notably, RAGE is a major receptor in homeostasis regulation, acting as a "master-switch" in the conversion of transient cellular activation into long-lasting cellular dysfunction (Bierhaus & Nawroth 2009, Chen & Nunez

2010, Sorci *et al.* 2013). Importantly, Herold and colleagues postulated that within acute stressed environments, uncomplicated forms of DAMP's are released into extracellular space and activate RAGE and other innate immune receptors, triggering repair mechanisms. Subsequent detoxification and removal of these ligands after the burst of release is a primal response to self-limited stresses, which are critical to return to homeostasis. However, environmental modulation (e.g. hyperglycemia, obesity, aging, chronic inflammation) supermodify those ligands toward multimeric forms that stabilize RAGE pre-assemblies and sustain downstream signaling, namely NF-κB activation (Herold *et al.* 2007). Since RAGE transcription is controlled by several proinflammatory transcription factors, including NF-κB, a positive autoregulatory loop evolves, culminating in RAGE inducible expression and subsequent sustainment of RAGE-mediated NF-κB activation (Bierhaus *et al.* 2005, Bierhaus & Nawroth 2009)(see Figure 1.16). Because increased receptor levels at the cell surface promotes preassembly, this explains the hyperactivation of RAGE signaling in the development of chronic inflammatory and neurodegenerative disorders (Basta 2008, Chavakis *et al.* 2004, Daffu *et al.* 2013, Stern *et al.* 2002, Tobon-Velasco *et al.* 2014).

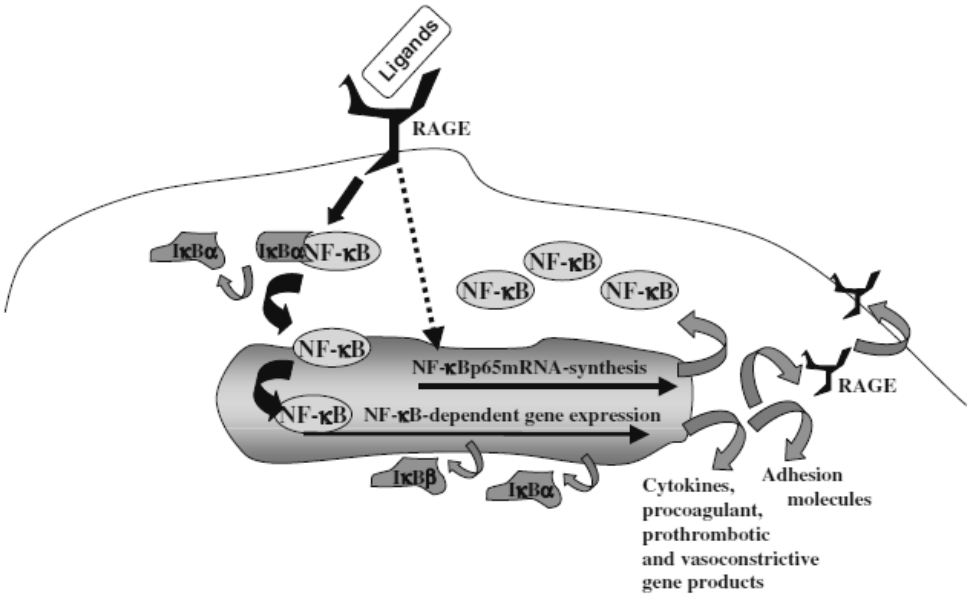

Figure 1.16 RAGE-dependent perpetuated NF-κB activation. RAGE-dependent NF-kB activation is mediated by initial degradation of inhibitory IkB-proteins (solid arrow) and subsequent translocation of NF-kB into the nucleus. This initial step is followed by the induction of NF-kBp65 synthesis (dashed arrow) in the presence of newly synthesized IkBα and IkBβ. De novo synthesis of NF-kBp65 thus provides a constantly growing pool of transcriptionally active NF-kBp65 which overrides IkB-dependent endogenous negative feedback mechanisms and might contribute to the sustained NF-kB activation observed in chronic disease (Taken from Bierhaus *et al.* 2005)

1.7.2.5 RAGE and neurodegenerative diseases: a focus on PD

The biology of RAGE and its ligands predict their up-regulation and increased accumulation in disease settings with a strong inflammatory background. Consistently, RAGE putative involvement has been documented in a great panoply of neurodegenerative conditions: Alzheimer's disease (Cai *et al.* 2016), amyotrophic lateral sclerosis (Juranek *et al.* 2015), Huntington's disease (Kim *et al.* 2015), Creutzfeld-Jakob disease (Sasaki *et al.* 2002), multiple sclerosis (Sternberg *et al.* 2016), diabetic neuropathy (Bierhaus *et al.* 2004), familial amyloid polyneuropathy (Sousa *et al.* 2001), Charcot neuroarthropathy (Juranek *et al.* 2013) and vasculitic neuropathy (Haslbeck *et al.* 2004).

As mentioned before (see 1.7.2.1-1.7.2.4), RAGE operates within a complicated biochemical network comprising i) a variety of ligands in distinct 3D-conformational complexities, ii) a panoply of receptor variants able to modulate the overall RAGE cellular signaling and iii) the recruitment of immune co-receptors aimed to amplify cellular outcomes. This "*modus operandis*" seems to cross the large majority of CNS proteinopathies where RAGE appears to be involved. When I started this PhD study in 2011, brain neuroinflammation was an accepted feature of PD degenerative process. The concept of a coordinated role of innate and adaptive immune systems in PD pathogenesis was becoming clear, with several studies reporting the recruitment of infiltrating T-cells into CNS tissues of PD patients and experimental models (Appel *et al.* 2010, Reynolds *et al.* 2009, Stone *et al.* 2009). While these studies started to support the importance of adaptive immunity in the regulation of PD-associated inflammation, a plethora of experimental data concomitantly underpinned the concept whereby misfolded CNS self-proteins may act as danger-associated molecular patterns (DAMP's), resulting in chronic activation of innate immune systems through PRR's (Frank *et al.* 2009, Golde & Miller 2009, Salminen *et al.* 2009, Scholtzova *et al.* 2009). Relevant to PD, post-translational modifications of α-syn by the action of AGE's were demonstrated both *in vitro* and *in vivo* (Choi & Lim 2010, Shaikh & Nicholson 2008). Moreover, AGE's and RAGE accumulation were also reported in *post-mortem* samples of early parkinsonism (Dalfo *et al.* 2005), advocating a putative deleterious role of RAGE network within PD neurodegenerative process that warranted further investigation. From there on, a growing wealth of evidence emerged, substantiating an operative RAGE network in Parkinson's disease pathology (see Table 1.5).

Table 1.5 Summary of evidence in the literature of immune RAGE network involvement in PD

LIGANDS	*Post-mortem* PD samples	Human PD patients	Experimental PD models
AGE's	Increased expression of AGE's in the substantia nigra, amygdala and frontal cortex in early stages of parkinsonian pathology *(Dalfo et al. 2005)*	————	***In vitro:*** •AGE's induce *in vitro* cross-linking of α-syn •AGE's accumulation precedes α-syn -positive intracellular inclusions in SH-SY5Y cell line treated with 5nM rotenone •Exogenous AGE's accelerate intracellular inclusion body formation in SH-SY5Y cell line treated with 5nM rotenone *(Shaikh & Nicholson 2008)*. ***In vivo:*** •Chronic MPTP (20mg/kg, i.p., 30 days) intoxication in C57BL/6 mice : - Co-localization of a-syn with CML and CEL in TH-positive dopaminergic neurons and CD11b-positive activated microglia (SNpc) - Oligomeric forms of a-syn modified with CML, CEL, pentosidine and pyrraline in MPTP brains (SNpc) *(Choi & Lim 2010)*
HMGB-1	Increased HMGB-1 protein levels in midbrain slices of parkinsonian pathology *(Santoro et al. 2016)*	Increased HMGB-1 levels in CSF and serum of parkinsonian pathology *(Santoro et al. 2016)*	***In vivo*** : •Sub-acute MPTP (30mg/kg, i.p., 5 days) intoxication in C57BL/6 mice: - Increased mRNA and protein levels in ventral midbrain at day 1 post-MPTP - HMGB-1 neutralizing antibody (200μg, i.p.) partly inhibited dopaminergic cell death and reduced the increase of RAGE (*Santoro et al. 2016*) •Intrastriatal 6-OHDA (20μg/4μl) administration in Sprague-Dawley rats: - HMGB-1 neutralizing antibody (1mg/kg) preserved dopaminergic neurons in SNpc, dopaminergic terminals in striatum, attenuated PD behavioral deficits, inhibited neuroinflammatory profile and BBB perturbation *(Sasaki et al. 2016)*
S100B	Increased S100B protein levels in midbrain slices of parkinsonian pathology *(Sathe et al. 2012)*	Increased S100B levels in CSF (but not serum) of parkinsonian pathology *(Sathe et al. 2012)*	***In vivo*** : •Brain-specific S100B transgenic mice developed features of Parkinson's Disease (Liu *et al.* 2011a) •Sub-acute MPTP (30mg/kg, i.p., 5 days) intoxication in C57BL/6 mice: -Increased S100B mRNA and protein levels in ventral midbrain at day 0 post-MPTP *(Sathe et al. 2012, Teismann et al. 2012)* -S100B KO mice were protected from insult, showing reduced microgliosis, decRAGE and TNF-α protein levels *(Sathe et al. 2012)*

(CML-N^{ε}-(carboxymethyl)lysine; CEL-N^{ε}-(carboxyethyl)lysine; 6-OHDA- 6-hydroxydopamine)

Table 1.5 Summary of evidence in the literature of brain RAGE network involvement in PD (cont.)

RECEPTORS	*Post-mortem* PD samples	Human PD patients	Experimental PD models
RAGE	Increased RAGE cellular expression in substantia nigra and frontal cortex of early parkinsonism cases *(Dalfo et al. 2005)* Basal RAGE protein levels in midbrain slices of early parkinsonism cases *(Sathe et al. 2012)*	_____	***In vivo*** : •Sub-acute MPTP (30mg/kg, i.p., 5 days) intoxication in C57BL/6 mice: -Increased mRNA and protein levels in ventral midbrain at day 2 post-MPTP *(Sathe et al. 2012, Teismann et al. 2012)* -RAGE KO mice were partially protected against MPTP injury *(Teismann et al. 2012)* • Rotenone intoxication (1,5mg/kg, s.c., 11 days) in adult Wistar rats: -Increased RAGE protein levels in striata 1 day post-rotenone *(Abdelsalam & Safar 2015)*
TLR 1,2,3,7	_____	_____	***In vitro:*** • BV2 microglia cell line exposed to α-syn: - Increased TLR2,3 and decreased TLR7 gene expression • Primary microglia cell cultures exposed to α-syn: - Increased TLR 1,2,3,7 gene expression - Decreased TLR4 gene expression *(Beraud et al. 2011)*
TLR 2, 4 and CD14	Increased TLR2 protein levels in SN and hippocampal CA1 subregion of LBD and PD patients (Doorn *et al.* 2014b) Increased TLR2/4 protein levels in caudate/putamen of PD patients *(Drouin-Ouellet et al. 2015)*	_____	***In vivo*** : • Single MPTP administration (30mg/kg, i.p.) intoxication in CD1 mice: -Increased mRNA and protein levels of TLR4 and CD14 in ventral midbrain 14 days post-MPTP *(Panaro et al. 2008)*
TLR 3,4,7,9	Increased TLR9 protein levels in striatum of PD patients *(Ros-Bernal et al. 2011)*	_____	***In vivo*** : • SN stereotaxic injection of poly(I:C), a TLR3 agonist in Sprague-Dawley rats: -Long-lasting inflammatory reaction in the SN and dorsolateral striatum - Altered expression of axonal and synaptic proteins in the SN and striatum - Increased vulnerability of nigral DA neuron loss to low-doses of 6-OHDA *(Deleidi et al. 2010)* ***In vivo*** : • Acute MPTP administration (20mg/kg, i.p., 4x, 2h interval) in C57BL/6 mice: -Increased mRNA levels of TLR3,4,7,9 in striatum and TLR 4,7,9 in ventral midbrain 1 day post-MPTP *(Ros-Bernal et al. 2011)*

(Poly(I:C) - Polyinosine-polycytidylic acid)

1.8. Aims and thesis outline

Recognizing and responding to brain insults is the cardinal function of brain innate immunity, a basic host defense mechanism that operates within CNS (Ransohoff & Brown 2012). Nevertheless, immunosenescence is a well-known feature of brain aging, culminating in a prolonged battle against accumulated threatens that provide a fertile ground for the development of chronic inflammatory conditions, as is the case of PD (Deleidi *et al.* 2015, Franceschi *et al.* 2007, Montecino-Rodriguez *et al.* 2013, Ransohoff & Brown 2012, Simm *et al.* 2015, Wyss-Coray & Mucke 2002). Consistently, it clearly emerges from Introduction of this thesis - **CHAPTER 1**- that an operative RAGE network is a key player in innate imune system and is involved in PD neurodegenerative process.

Even though RAGE is envisaged as a central player in homeostasis maintenance, maladaptive conditions culminate in perpetuated tissue dysfunction. This "friend or foe behavior" arising from RAGE biology is compatible with the overall innate immunity "double-edged sword" postulated by Wyss-Coray and Mucke some years ago (Sorci *et al.* 2013, Wyss-Coray & Mucke 2002). Hence, a major scientific challenge in present days relies on a better understanding of what governs the tipping of the balance between cytotoxic or neuroprotective action arising from innate immunity (Appel *et al.* 2010, Hutter-Saunders *et al.* 2011, Olson & Gendelman 2016). This also applies to RAGE network in PD neurodegeneration: harnessing RAGE network in PD settings requires a thorough understanding of its role in the neurodegenerative process in order to identify the best strategies aimed to facilitate beneficial RAGE outcomes and elicit therapeutic gain.

Biology of RAGE argues for environments where ligands accumulate are prone to sustained RAGE expression and perpetuated tissue dysfunction. Thus, the first goal of present study was to explore this concept in an *in vivo* experimental PD setting. To this end, we implemented a new chronic MPTP paradigm (20mg/kg, i.p., 2 i.d., 10 days) and assessed whether sustained ligands (S100B) and RAGE (signaling-competent RAGE isoform) accumulation would foster glial reactivity and dopaminergic axonopathy in striata of C57BL/6 mice – **CHAPTER 2**.

The presence of a large number of RAGE variants adds an extra layer of complexity to this issue: RAGE signaling results of the interplay between all these isoforms, some of them exhibiting antagonistic properties. Yet, vast majority of studies discuss RAGE in terms of being one single receptor. This is particularly the case of previous reports that while focusing on RAGE in PD degeneration (see table 1.5), shed considerable confusion to the RAGE network beneficial/noxious outcomes in PD. As a second endpoint, the regulation of RAGE protein variants was characterized at a molecular level (flRAGE, N- and C-truncated isoforms) in acute stressed striata. To achieve this goal, C57BL/6 mice were submitted to a well characterized acute MPTP paradigm (4x 20mg/kg, 2h apart, i.p.) and sacrificed 6 hours post-last MPTP administration. This time-point is translated into mild dopaminergic axonopathy, mimicking an early stage of PD striatal degeneration - **CHAPTER 3**.

Strong evidences link ligands, RAGE and TLR's to PD degenerative process (see Table 1.5). However, there is no report focusing on simultaneous analysis of such innate immune mediators within PD experimental settings. Hence, the next goal of this work was to perform a comprehensive transcriptomic approach of i) ligands (S100B, HMGB-1), ii) PRR's (RAGE, TLR 1-11), iii) adaptor molecules (mDia1, MyD88, TIRAP) and iv) effector molecules (TNF-α, NADPH oxidase) in frontal cortex, hippocampus and striatum (brain regions intimately related with PD non-motor symptons) of Wistar rats 12 days following i.n. MPTP administration, mimicking a pre-clinical PD stage (see 1.4.2.1.2). Moreover, based on epidemiological studies supporting physical exercise (PE) beneficial effects on non-motor PD symptomatology and also on the robust PE-peripheric immunomodulatory properties, a treadmill program was implemented. This approach aimed to explore the effects of PE on central immunomodulation of RAGE network and its putative correlation with PD non-motor symptoms improvement – **CHAPTER 4**.

Finally, an integrated conclusion with main results of this Thesis, studies limitations and potential areas for future research will be presented in **CHAPTER 5**.

Chapter 2

Presymptomatic MPTP mice show neurotrophic S100B/mRAGE striatal levels

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2.1 Abstract

Aims: Astrocytic S100B and receptor for advanced glycation endproducts (RAGE) have been implicated in Parkinson's disease (PD) pathogenesis through yet unclear mechanisms. This study attempted to characterize S100B/mRAGE (signaling isoform) axis in a dying-back dopaminergic (DAergic) axonopathy setting, which mimics an early event of PD pathology.

Methods: C57BL/6 mice were submitted to a chronic MPTP paradigm (20mg/kg i.p., 2 i.d-12h apart, 5 days/week for 2 weeks) and euthanized 7 days post-treatment to assess mRAGE cellular distribution and S100B/mRAGE density in striatum, after probing their locomotor activity (pole test and rota-rod). Dopaminergic status, oxidative stress and gliosis were also measured (HPLC-ED, WB, IHC).

Results: This MPTP regimen triggered increased oxidative stress (augmented HNE levels), gliosis (GS/Iba1 reactive morphology), loss of DAergic fibers (decreased tyrosine hydroxylase levels) and severe hypodopaminergia. Biochemical deficits were not translated into motor abnormalities, mimicking a pre-symptomatic PD period. Remarkably, striatal neurotrophic S100B/mRAGE levels and major neuronal mRAGE localization coexist with compensatory responses (3 fold increase in DA turnover), which are important to maintain normal motor function.

Conclusion: Our findings rule out the involvement of S100B/mRAGE axis in striatal reactive gliosis, DAergic axonopathy and warrant further exploration of its neurotrophic effects in a presymptomatic compensatory PD stage, which is a fundamental period for successful therapeutic strategies implementation.

Keywords: MPTP, pre-symptomatic, RAGE, S100B, striatum

2.2 Introduction

RAGE (Receptor for advanced glycation end products) is a multiligand receptor of the immunoglobulin superfamily long implicated in the sustainment of glial activation, oxidative stress and neurotoxicity (Angelo *et al.* 2014, Origlia *et al.* 2010, Origlia *et al.* 2014, Villarreal *et al.* 2014). RAGE is expressed in both full-length membrane-bound (mRAGE) and soluble (sRAGE) forms. Whereas mRAGE is the RAGE signaling isoform, sRAGE lacks the transmembrane domain and is a competitive inhibitor of mRAGE acting as a decoy for ligands (Kalea *et al.* 2011). Therefore, it is vital to dissect different RAGE isoforms as it is likely that subtle shifts in ratio of RAGE variants can significantly impact intracellular signaling.

Brain deleterious events have been linked to RAGE ligands (e.g. S100 proteins, amyloid peptide, HMGB-1) that get complex three-dimensional structures in oxidative settings (Herold *et al.* 2007). RAGE activation following accumulation of such ligands perpetuates neurodegeneration by triggering its own upregulation via a positive feedback loop (Bierhaus & Nawroth 2009). Nonetheless, a range of adult cell types shows low levels of RAGE expression underlying RAGE physiological function. Consistently, physiological levels of RAGE ligands may modulate brain plasticity and damage repair (Alexiou *et al.* 2010). RAGE pleiotropic effects seem to be strongly dependent on the cell type and the context (Sorci *et al.* 2013). For example, brain physiological levels of S100B tonically activate trophic RAGE signaling in neurons (Kleindienst *et al.* 2007). In contrast, S100B overproduction cause excessive neuronal RAGE stimulation that culminates in overproduction of ROS and neurotoxicity in injured brain tissue (Donato *et al.* 2009). Accordingly, S100B is envisaged as a double-edged sword because it could be a neurotrophin (Luo *et al.* 2010) or a damage-associated molecular protein (Kato & Svensson 2015), depending on its levels.

Increased expression of both S100B (Sathe *et al.* 2012) and RAGE (Dalfo *et al.* 2005) were reported in Parkinson's disease (PD) patients, suggesting that S100B/RAGE axis can be involved in PD neurodegenerative process. Moreover, RAGE was proposed to play a role in microglia-mediated dopaminergic degeneration in ventral midbrain of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice (Teismann *et al.* 2012), a gold-standard PD animal model (Langston & Irwin 1986). Therefore, we hypothesized that continuous accumulation of S100B and subsequent RAGE up-regulation would foster glial reactivity and

dopaminergic (DAergic) axonopathy in striata. Hence, we attempted to characterize the neurochemical, immunohistochemical and functional correlate of striatal S100B/mRAGE axis in a chronic MPTP mouse model of PD.

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2.3 Materials and methods

2.3.1 Animals and MPTP protocol

Male adult C57BL/6J mice (3 months old; 24–28 g; Charles River Laboratories, Barcelona, Spain) were housed four per cage, under controlled environmental conditions [12-h light/dark schedule at room temperature (RT) of 23±1 °C, with food and water supplied *ad libitum*]. All experiments were approved by the Institutional Animal Care and Use Committee from Faculty of Medicine, Coimbra University, and were performed following the European Community directive (2010/63/EU). The animal procedures were performed in strict accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press 1996). Herein we applied a new chronic MPTP paradigm (Figure 2.1). We considered this regimen since RAGE ligands accumulation occur preferentially in chronically stressed environments, which more closely mimic the progressive nature of PD (Bierhaus & Nawroth 2009).

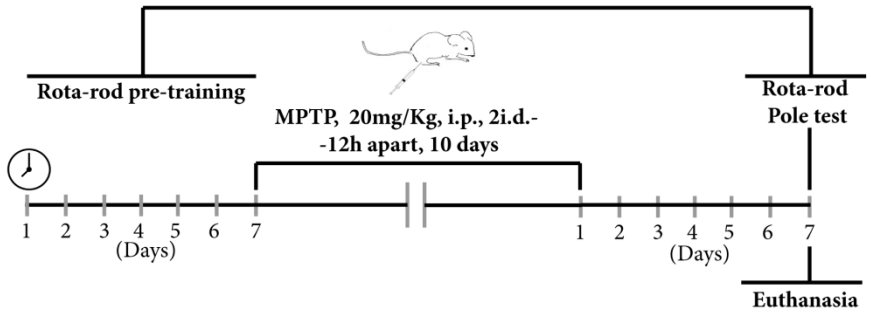

Figure 2.1 Experimental design including MPTP administration protocol, locomotor behaviour assessment and euthanasia

Briefly, animals were injected intraperitoneally (i.p.) with 20mg/kg of MPTP hydrochloride in saline solution (Sigma-Aldrich, St. Louis, MO, USA; MPTP, n=8) or with saline solution (0.9% NaCl; SAL, n=8), twice daily 12h-apart for 10 days, and a pause time of 2 consecutive days at the end of the first 5 days of administration. Technical recommendations of MPTP safety handling were strictly enforced (Przedborski *et al.* 2001). All animals survived this dosing regimen and none showed weight reductions. Sacrifice of both saline (n=4) and MPTP-treated mice (n=4) were performed seven days after last administration by decapitation, following motor function assessment. Brains were rapidly dissected and immediately frozen in

liquid nitrogen and stored at -80ºC until protein levels assessment and dopamine (DA) and metabolites quantification. Remaining animals (SAL; n=4 and MPTP; n=4) were deeply anesthetized with pentobarbital and transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed and post-fixed for 24 h in 4% PFA and then dehydrated in 30% sucrose in 0.1 M PBS for 24 h for subsequent immunohistochemistry analysis. All chemicals (ultrapure and pro analysis quality) were purchased from Sigma-Aldrich and Merck AG (Darmstadt, Germany).

2.3.2 Locomotor assessment: rota-rod and pole tests

Animals [SAL (n=8) and MPTP (n=8)] were submitted to rota-rod and pole tests at day 7 post-treatment. These tests have been successfully used to probe motor performance and coordination following DA system lesions in MPTP-treated mice (Luchtman *et al.* 2009, Ogawa *et al.* 1985). All tests were carried out between 9:00 and 17:00 h in a sound-attenuated room under low-intensity light (12 lx) and were scored by the same person in an observation room where the mice had been habituated for at least 1 h before the beginning of the tests.

2.3.2.1 Rota-rod test

Motor coordination and balance were evaluated using the rota-rod test. The rota-rod apparatus (Letica Scientific Instruments, LE 8200 model) consisted of a rotating spindle (3 cm of diameter distancing 15 cm of base) with 5 individual compartments distancing 3 cm so that 5 animals could be simultaneously tested. Animals were initially trained to maintain themselves on the rotating rod at 15 rotations per minute (RPM) for 120 seconds (habituation phase), prior to the trial. This assured that all the animals reached an analogous baseline. An increasing speed protocol was implemented herein, since the sensitivity to motor disability has been shown to improve when animals are forced to move at increasingly faster speeds (Monville *et al.* 2006). Briefly, animals were placed on the rotating rod and tested using an increasing rotating speed (from 10 to 22 rpm) for 240 sec. The time that mice remained on the rota-rod was recorded over 3 trials with an inter-trial interval of approximately 5 minutes. Data are presented as the mean time on the rotating bar over 3 test trials.

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2.3.2.2 Pole test

Pole test was performed to determine the degree of bradykinesia and slight modifications to a previous protocol were implemented (Ogawa *et al.* 1985). A metal pole (50 cm high and 8 mm diameter), doubly wrapped with cloth tape to prevent slipping and whose base was positioned in the home cages was used. Mice were placed head upward on the top of the pole. Thereafter, time taken to orientate the body completely downwards (Tturn) and to land on all four paws on the floor were recorded (TLA) with a cutoff of 60 seconds. The test consisted of 3 trials (20 minute interval between each) and trial average was used for statistical analysis.

2.3.3 Quantification of dopamine and metabolites by HPLC-ED

Right striata were sonicated in ice-cold 0.2 M perchloric acid, centrifuged (15.500×*g*, 7 min, 4 °C) and supernatants were used to determine the content of dopamine (DA) and metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by high-performance liquid chromatography with an electrochemical detector (HPLC-ED), as previously described (Pereira *et al.* 2012, Silva *et al.* 2014). The pellet was resuspended in 1 M NaOH and stored at -80 °C for protein quantification by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, IL, USA). Concentration of DA and its metabolites were determined by comparison with peak areas of standards and expressed in nanogram per mg of protein.

2.3.4 Western blot analysis

Left striata were used for total protein extracts as previously described (Silva *et al.* 2014) and total protein concentration was determined by the BCA protein assay (Pierce Biotechnology, IL, USA). Equal amounts of protein were separated using 8-15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Madrid, Spain) and blocked by 5% defatted milk for 2 h (Silva *et al.* 2014). Membranes were incubated overnight at 4 °C with the following primary antibodies: mouse anti-TH (1:1,000; MAB318, Millipore), rabbit anti-HNE (1:500; 393207, Calbiochem), mouse anti-GFAP (1:1,000; IF03L, Calbiochem), rabbit anti-S100B (1:500; JBC1771181, Millipore) and rabbit anti-RAGE raised against the C-terminal domain

recognizing specifically the full-length membrane bound isoform (1:500; Ab3611, Abcam). Membranes were then incubated with alkaline phosphatase-conjugated IgG secondary antibodies (mouse or rabbit 1:10,000; GE Healthcare, USA). Finally, membranes were visualized on Thyphoon FLA 9000 (GE Healthcare) and analysed using Image Quant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). Results were normalized against internal controls β-actin (1:1,000; A5316, Sigma-Aldrich) or GAPDH (1:1,000; MAB374, Millipore) and then expressed as percentage of control.

2.3.5 Immunohistochemistry

Striatum anatomical limits were identified using a mouse brain atlas (AP, +1.32 to +0.5 mm). Striatal coronal sections of 40 μm thickness were collected from cryostat (Leica CM3050S, Nussloch, Germany) in 0.1 M PBS and used for free-floating immunohistochemistry. Briefly, slices were washed twice with 0.1 M PBS, blocked with 0.25% Triton X-100 and 5% normal fetal bovine serum (FBS) in 0.1 M PBS for 1 h at RT, and then incubated for 24 h at 4 °C with the following primary antibodies: rabbit anti-RAGE (1:200; Ab3160, Abcam), mouse anti-GFAP (1:1,000; IF03L, Calbiochem), mouse anti-glutamine synthetase (GS; 1:500; MAB302, Millipore), goat anti-ionized calcium-binding adapter molecule 1 (Iba1; 1:200, Ab5076, Abcam), mouse anti-NeuN (1:500; MAB377, Millipore) or mouse anti-TH (1:500; MAB318, Millipore). Sections were then rinsed with 0.1 M PBS (3 x 10 min) and incubated with 4´,6-diamidino-2-phenylindole (DAPI; 1:5,000; D1306, Invitrogen) for nuclear staining and the secondary fluorescent antibodies for 2 h at room. Finally, sections were rinsed with 0.1 M PBS (3 x 10 min) and mounted with glycergel (Dako mounting medium). Samples were imaged using a Confocal laser-scanning microscope (LSM 710 Meta, Carl Zeiss Gottingen, Germany).

2.3.6 Statistical analysis

Differences in locomotor and biochemical data were compared using unpaired Student's t-test. The accepted level of significance for the tests was $p<0.05$. Data are expressed as means ± S.E.M. and analyses were performed using GraphPad Prism 5.0 software for Windows.

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2.4 Results

2.4.1 Mice chronically exposed to MPTP maintain normal motor function regardless severe disruption of dopaminergic nerve terminals

MPTP-treated animals displayed motor performances similar to control animals in both tests (p>0.05, Figure 2.2a,b). Nonetheless, MPTP imposed a severe disturbance of striatal dopaminergic system as demonstrated by a significant reduction in TH (~68% of control, p<0.01), DA and its metabolites levels (~11%, ~40% and ~21% of control, respectively; p<0.001) and a significant increase in DA turnover (DOPAC+HVA/DA ratio, ~300% of control; p<0.05) 7 days after the toxic insult (Figure 2.2c-g).

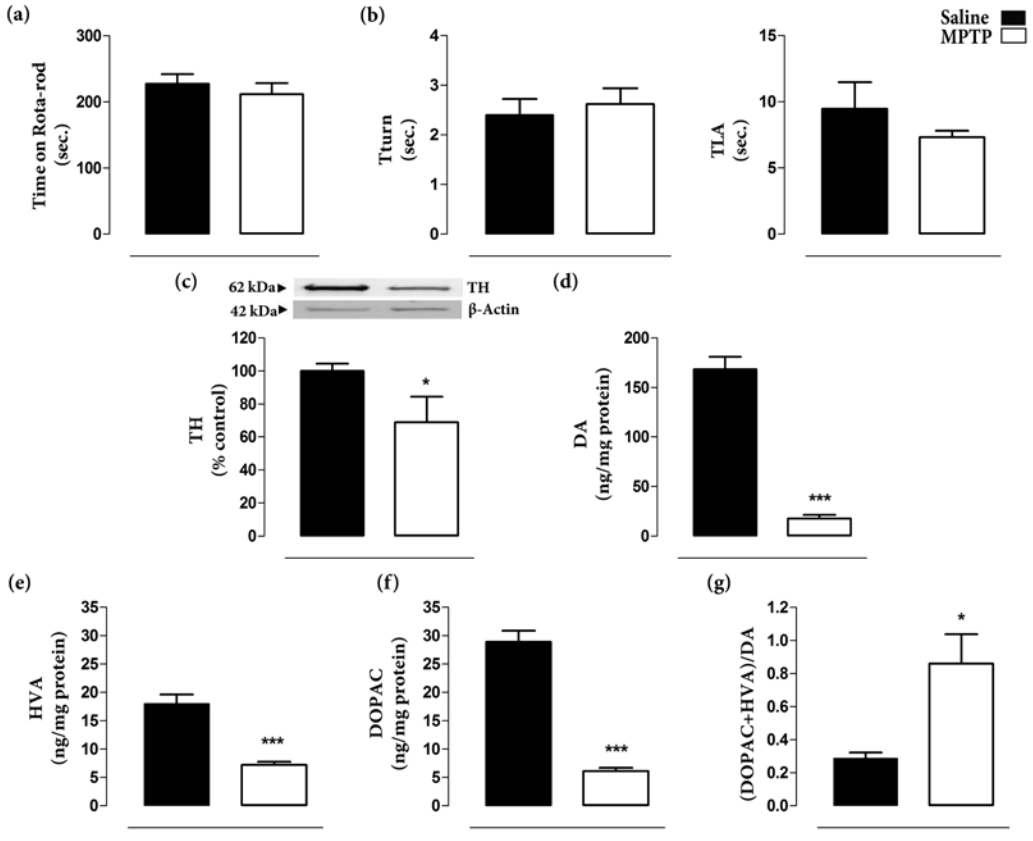

Figure 2.2 Effect of MPTP chronic treatment on motor skills and striatal dopamine status 7 days post-MPTP. MPTP mice showed normal motor function, exhibiting similar performances of control animals in a) time spent on rota-rod and b) time until they turned completely downward (Tturn) and landed floor (TLA) in pole test. Normal motor function occurred in the presence of striatal dopaminergic disruption MPTP-driven, as shown by reduced c) TH, d) DA, e) HVA and f) DOPAC tissue levels and a significant increase in e) (DOPAC+HVA)/DA, which reflects dopamine turnover. Representative Western blot of TH protein levels quantification, normalized with β-actin and expressed as mean ± S.E.M. DA and metabolites were assessed by HPLC-ED and expressed as ng/mg protein. Results are representative of 4 animals per group *p<0.05, ***p<0.001 using unpaired Student's t-test for comparison with control

2.4.2 Striata of chronically MPTP-treated mice show an oxidant and gliotic profile

Chronic MPTP treatment triggered an accumulation of phospholipid peroxidation 4-hydroxy-2-nonenal (HNE) adducts (≈75 and 200 kDa) in striata when compared with control animals (122 and 125% of control, respectively, $p<0.05$), 7 days post-dosing (Figure 2.3).

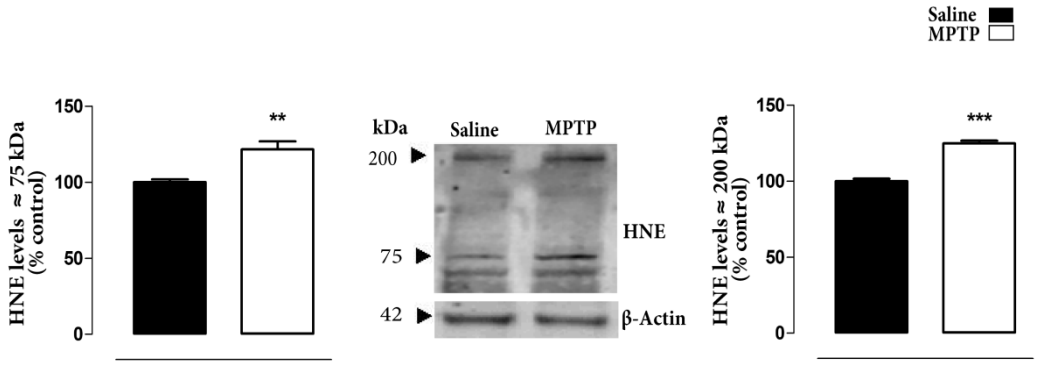

Figure 2.3 Effect of chronic MPTP treatment on striatal oxidative status. MPTP triggered an oxidant setting confirmed by the accumulation of HNE protein adducts (75 and 200kDa) in intoxicated animals. Representative Western blot of HNE protein levels quantification, normalized with β-actin and expressed as mean ± S.E.M. Results are representative of 4 animals per group $^{**}p<0.01$, $^{***}p<0.001$ using unpaired Student's t-test for comparison with control

This oxidant setting paralleled a robust gliotic profile. In fact, a significant increase in glial fibrillary acidic protein (GFAP) levels (175%, $p<0.05$; Figure 2.4a) and profound astrocytic morphological changes comprising enlarged cell bodies and increased ramified processes were observed upon MPTP, as highlighted by GFAP and glutamine synthetase (GS) immunostaining (Figure 2.4b,c). Reactive profile of microglia cells, classically described to respond prior to astrogliosis in the acute phase of MPTP insult (Kohutnicka *et al.* 1998), is less robust than the astrocytic one. Nevertheless, actin-crosslinking ionized calcium binding adaptor molecule 1 (Iba-1) immunostaining showed a reduced complexity of microglia shape with shortened and thicker processes branching off from soma in striata of MPTP-treated mice when compared to control mice (Figure 2.4d).

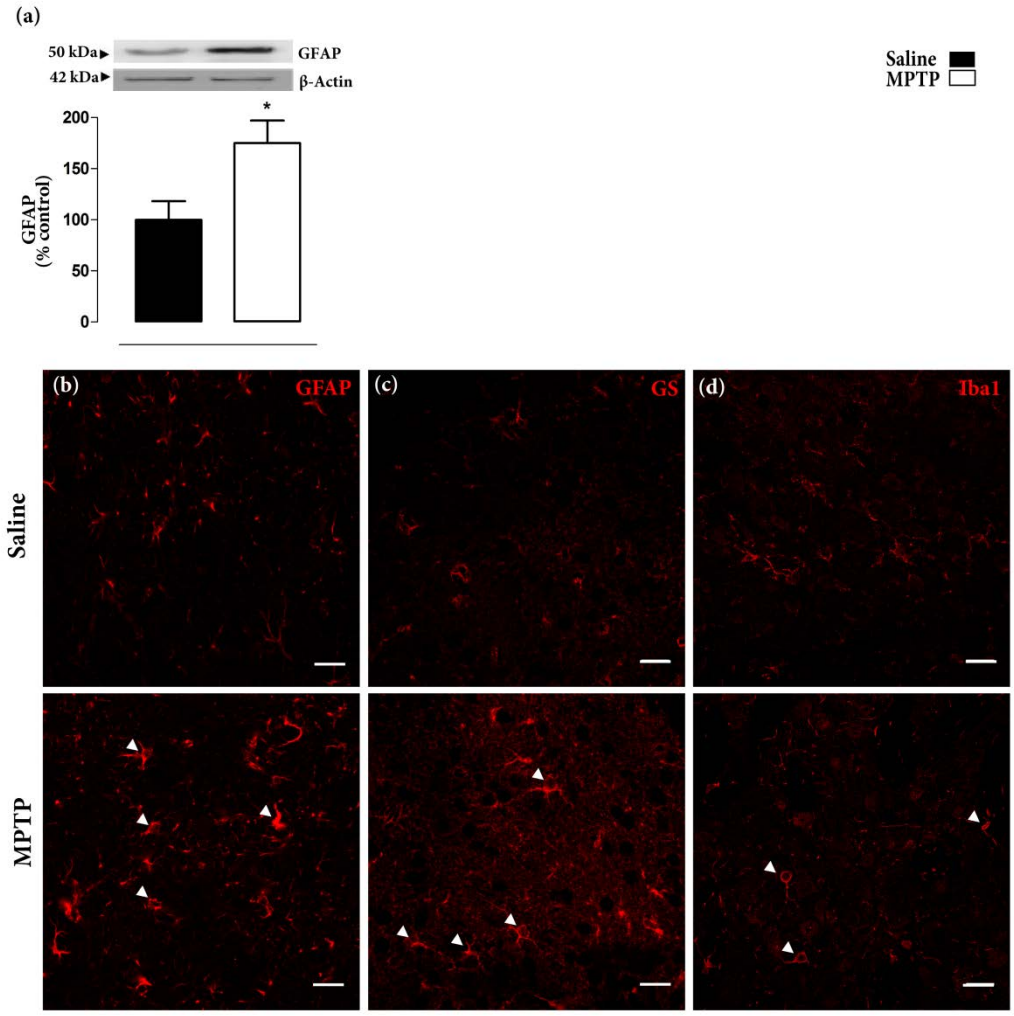

Figure 2.4 Effect of chronic MPTP treatment on glial reactivity. MPTP triggered a gliotic profile, typified by increased GFAP levels (a) and enlarged astrocytic cell bodies with increased ramified processes (b,c- GFAP and GS representative immunofluorescence confocal images, respectively) and shortened and thicker microglia processes (d-Iba-1 immunofluorescence) in MPTP-animals when compared to control animals (arrows in MPTP; representative confocal images). Representative Western blot of GFAP protein levels quantification, normalized with β-actin and expressed as mean ± S.E.M. Results are representative of 4-5 animals per group *p<0.05 using unpaired Student's t-test for comparison with control. Scale bar: 20μm

2.4.3 Mice chronically exposed to MPTP display striatal physiological S100B levels and basal neuronal mRAGE expression

Impact of chronic MPTP on S100B/mRAGE protein levels and mRAGE cellular localization was assessed by Western blot and double-labeling immunohistochemistry. Both S100B and mRAGE remained at physiological levels (107 and 101% of control, respectively, p>0.05; Figure 2.5 a,b) regardless the oxidant and gliotic profile MPTP-driven. mRAGE was predominantly found in striatal neurons from saline and MPTP mice and showed a punctuated distribution mostly localized in nuclei and perikarya of nearly all neuron-specific protein (NeuN)-positive cells (Figure 2.5c). mRAGE immunofluorescence was further detected in somas

of both resting and reactive astrocytes (Figure 2.5d). mRAGE immunofluorescence does not seem to be localized in microglia cells (Figure 2.5e) nor in DAergic axons (Figure 2.5f) in basal conditions, a distribution pattern that remained unchanged after MPTP treatment.

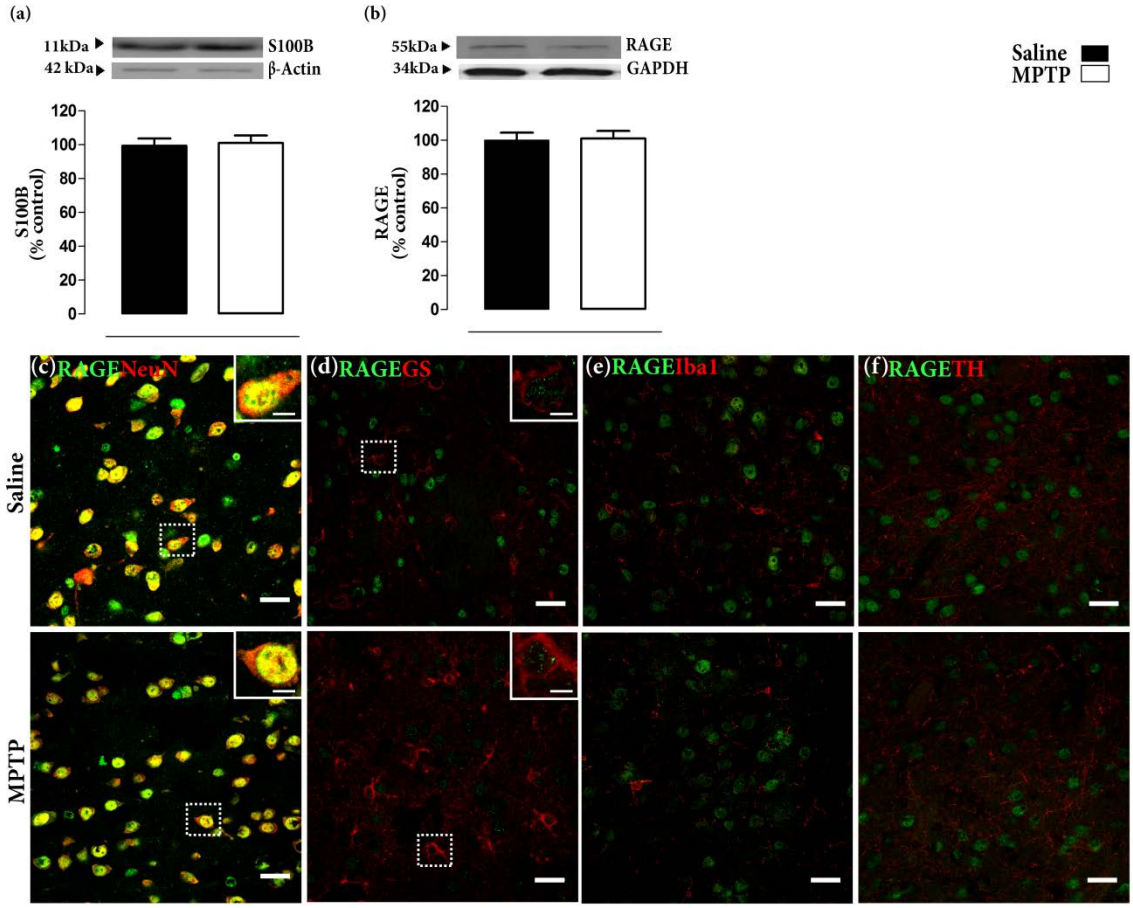

Figure 2.5 Effect of chronic MPTP treatment on S100B/RAGE axis. Physiological levels of both a) S100B and b) mRAGE proteins were observed in striatal total homogenates of MPTP animals. Representative confocal images of double immunofluorescent staining for RAGE (green) and neuronal marker NeuN, astrocytic GS, microglial Iba1 or TH- positive fibers (red) in striata of saline and MPTP mice. mRAGE showed a punctate distribution mostly localized in nuclei and perikarya of striatal NeuN-positive cells (co-labeling in yellow) in both saline and MPTP animals (c). RAGE immunostaining was also found in soma of GS-positive cells in both saline- and MPTP mice (d), regardless of the astrocytic reactive status. RAGE does not seem to be localized in Iba1-positive cells (e) nor in TH-positive fibers from both saline or MPTP striata (f). Representative Western blot of S100B and mRAGE protein levels quantification, normalized with β-actin/GAPDH and expressed as mean ± S.E.M. Results are representative of 4-5 animals per group. Scale bar: 20μm

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2.5 Discussion

Nearly all of the available treatments for PD progression are symptomatic in nature and do not appear to slow or reverse its natural course. Novel therapeutic strategies for PD are therefore critical. This prompted us to characterize striatal S100B/mRAGE axis in a chronic MPTP mouse model of PD. Chronic MPTP protocols provide a sustained oxidative setting that favor accumulation of RAGE ligands in striatum, a brain area comprising early events in PD-related pathology (Cheng *et al.* 2010). Our results clearly demonstrate that this MPTP model triggered a severe DAergic disruption as shown by depletion of striatal DA and its metabolites DOPAC and HVA. These findings are aligned with DAergic toxicity reported for other chronic and acute MPTP mice models (Pain *et al.* 2013). DA depletion surmounts the range of decrease in TH suggesting that DAergic dysfunction is more prominent than DAergic terminal degeneration in this MPTP regimen.

Most of these biochemical events occur over several years prior to diagnosis of typical PD motor symptoms (Kozina *et al.* 2014). Multifactorial mechanisms occurring at cortico-basal ganglia-thalamo loop are suggested to display key compensatory roles for motor function until striatal DAergic deficits reaches a critical threshold (Ugrumov *et al.* 2011). MPTP experimental models revolve around a controversial correlation between motor deficits and striatal DA levels. In fact, motor disabilities are transient and identified shortly after treatment, sometimes when mice are still intoxicated by MPTP (Kim *et al.* 2013, Meredith & Rademacher 2011). Not surprisingly, our chronic MPTP model displayed normal motor function 7 days post-treatment, in spite of profound striatal hypodopaminergia. Increased DA turnover is one of the striatal compensatory mechanisms engaged in maintaining the minimum concentration of DA required for normal motor function in PD (Blesa *et al.* 2012, Luchtman *et al.* 2009, Nandhagopal *et al.* 2011). Consistently, we report a 3-fold increase in DA to metabolite ratio in MPTP-treated mice, suggesting that such striatal compensatory actions are recruited in this experimental setting and contribute to maintain motor skills. Overall, this MPTP model presents biochemical and locomotor similarities with presymptomatic PD stage, fundamental for successful implementation of strategies aimed at altering the progressive evolution of PD (Kozina *et al.* 2014).

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Evidences arising from both PD patients (Dalfo *et al.* 2005) and experimental PD models (Przedborski & Ischiropoulos 2005) suggest oxidative stress as a key player in PD pathology. This was observed in our model by strong HNE-immunoreactive bands in MPTP treated animals, probably reflecting distinct protein adducts arising from lipoxidative damage, as previously reported in early stages of human parkinsonism (Dalfo *et al.* 2005). Glial cells also contribute to PD progression with release of oxidative stress mediators involved in inflammatory processes (Halliday & Stevens 2011, O'Callaghan *et al.* 2008, Yasuda *et al.* 2008). Consistently, our immunohistochemical data clearly showed a sustained gliotic profile in MPTP-treated animals, lending additional credence to the protocol used herein. Since environments laden with oxidative stress are prone to accumulation of RAGE ligands (Herold *et al.* 2007), we investigated the likely accumulation of ligands/mRAGE in MPTP-chronically stressed striata. Remarkably, we found normal density of astrocytic ligand S100B and mRAGE in spite of an oxidative and gliotic setting.

Identity of RAGE-bearing cells also dictates the outcome of RAGE biology (Alexiou *et al.* 2010). Our results clearly showed a prominent neuronal mRAGE localization as RAGE is present virtually in all NeuN-positive cells in both saline and MPTP-treated striata. This finding is consistent with RAGE being distributed in 98% of striatal spiny projection neurons and in all interneurons both in WT and in Huntington's disease (HD) animal model (Anzilotti *et al.* 2012). Importantly, a robust neuronal localization for RAGE was already described in nigral DAergic neurons following MPTP (Teismann *et al.* 2012). Nevertheless, RAGE immunofluorescence did not extend to striatal TH-positive axons, which was further confirmed by our findings. Regarding glial cells, evenly distribution of mRAGE in soma of astrocytes was unchanged following astrogliosis in striata from MPTP-mice. Therefore, even though RAGE is envisaged as a central player in glial reactivity (Origlia *et al.* 2010, Origlia *et al.* 2014, Villarreal *et al.* 2014), our observations rule out a chief role for S100B/RAGE axis in sustaining striatal reactive gliosis. Our data further exclude a direct involvement of RAGE in DAergic axonopathy under the present experimental setting.

Previous works have demonstrated an early upregulation of striatal S100B contents returning to basal levels at 7 days, in an acute MPTP model (Muramatsu *et al.* 2003, Yasuda *et al.* 2008). These findings are aligned with normal S100B reported here 7 days post-chronic MPTP model. However, initial increase of S100B reported by former authors suggests earlier

changes in ligands/RAGE levels are likely to occur in striatum in the acute phase of MPTP toxic insult. Notably, a long-lasting repression of S100B and RAGE gene expression after a transient increase was also reported in mesencephalon of MPTP-treated mice (Teismann *et al.* 2012). S100B and RAGE gene are both under complex transcriptional regulation as negative regulatory elements within promoters are responsible of suppressing their expression, maintaining low levels of protein (Donato *et al.* 2009, Riehl *et al.* 2009). Altogether, these findings imply the existence of active compensatory processes tightly regulating S100B/RAGE axis toward homeostasis within striatonigral injury.

RAGE upregulation in chronically injured tissues led to a large number of studies focusing on RAGE-mediated neurotoxicity. For instance, a link between increased neuronal RAGE staining and neuronal death in preclinical and clinical HD settings is already established in striatum (Anzilotti *et al.* 2012, Ma & Nicholson 2004). Nevertheless, RAGE's protective effects in homeostasis and repair after injury has been recently highlighted, substantiating the pleiotropism of this receptor (Saleh *et al.* 2013, Sorci *et al.* 2013). In this chronic MPTP model, basal levels of S100B, endowed with neurotrophic activity (Donato *et al.* 2009, Leclerc *et al.* 2009), and normal neuronal RAGE density are more likely to be engaged in striatal neuroprotective mechanisms rather than in toxic events. For example, S100B/RAGE axis was recently linked to dendritic rearborization in cortical neurons following an excitotoxic glutamatergic insult (Villarreal *et al.* 2011). Curiously, dendritic remodeling in striatal spiny projection neurons was correlated with mice locomotor spontaneous recovery 7 days post-acute MPTP (Kim *et al.* 2013). Therefore, RAGE prevalent neuronal expression in striatum after MPTP exposure warrants future studies to explore whether S100B/RAGE axis is playing a role in presymptomatic compensatory responses in PD neuropathology.

Nevertheless, increased levels of RAGE and ligands reported in PD patients (Dalfo *et al.* 2005, Sathe *et al.* 2012) necessarily imply failure of these overwhelmed repair-driven pathways in the course of human PD neuropathology. The next imperative question is as follows: which RAGE isoform are we talking about when addressing RAGE axis? In fact, RAGE signaling is dependent upon the interplay between the membrane-bound isoform (mRAGE, which induces cellular signaling) and the soluble one (sRAGE, which is able to scavenge RAGE ligands thereby antagonizing mRAGE signaling) (Bierhaus & Nawroth 2009, Herold *et al.* 2007, Kalea *et al.* 2011). In fact, RAGE ligands may either accumulate in PD due to upregulated biosynthesis or

impaired clearance systems (e.g. sRAGE). Unfortunately, most of previous studies used antibodies, which are incapable of distinguishing both RAGE isoforms, thus neglecting this RAGE complexity.

We newly characterize the biology of mRAGE isoform and its astrocytic S100B ligand in striatum of experimental PD. Our findings rule out the involvement of S100B/mRAGE axis in striatal gliosis and DAergic axonopathy, early events of PD neuropathology. Instead, neurotrophic S100B/mRAGE protein levels observed herein warrant further exploration of its putative neuroprotective role in a pre-symptomatic compensatory PD stage, a fundamental period for successful therapeutic strategies implementation.

Chapter 3

Regulation of striatal astrocytic RAGE variants in an early stage of experimental Parkinson's disease

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3.1 Abstract

Convincing evidence indicates that advanced glycation endproducts and danger-associated protein S100B play a role in Parkinson's disease (PD). These agents operate through receptor for advanced glycation endproducts (RAGE), which displays distinct isoforms playing protective/deleterious effects. However, nature of RAGE variants has been overlooked in PD studies. Hence, we attempted to characterize RAGE regulation in early stages of PD striatal pathology. A neurotoxin-based rodent model of PD was used in the present study, through administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to C57BL/6 mice. Animals were euthanized 6 hours post-MPTP to assess S100B/RAGE contents (RT-qPCR, ELISA,WB) and RAGE isoforms density (WB) and cellular distribution (IHC). Dopaminergic and gliotic status were also mapped (HPLC-ED, WB, IHC). At this preliminary stage of MPTP-induced PD in mice, RAGE inhibitory isoforms were increased whereas full-length RAGE was not affected. This putative cytoprotective RAGE phenotype paired an inflammatory and pro-oxidant setting fuelling DAergic denervation. Increased RAGE inhibitory variants occur in astrocytes showing higher S100B density but not overt signs of hypertrophy or NF-κB activation, a canonical effector of RAGE. These findings expand our understanding of the toxic effect of MPTP on striatum and offer first *in vivo* evidence of RAGE being a responder in early stages of astrogliosis dynamics, supporting a protective rather tissue-destructive phenotype of RAGE in initial phase of PD degeneration. These data lay the groundwork for future studies on the relevance of astrocytic RAGE in DAergic neuroprotection strategies.

Keywords: Astrocytes; MPTP; S100B; Striatum; RAGE isoforms

3.2 Introduction

The receptor for advanced glycation endproducts (RAGE, also known as AGER) is a pattern recognition receptor with a broad repertoire of ligands [e.g. advanced glycation end products (AGE's), S100/calgranulins, amphoterin (HMGB-1), amyloid fibrils] that share the propensity to accumulate and aggregate during aging and development of neurodegenerative diseases including Parkinson´s Disease (PD) (Alexiou *et al.* 2010, Schaf *et al.* 2005, Dalfo *et al.* 2005). Importantly, accumulation of ligands within pathological states fosters RAGE overexpression with important downstream consequences, namely NF-κB activation. Apart from being an important effector by which RAGE exerts effect on gene transcription, NF-κB also controls RAGE expression. This feed-forward loop may perpetuate inflammation, culminating in long-term tissue dysfunction (Bierhaus & Nawroth 2009, Rojas *et al.* 2010). Yet, healthy adult brain expresses low levels of ligands, which interact with RAGE to mediate trophic effects in defined physiological processes (Leclerc *et al.* 2009, Donato *et al.* 2009). Accordingly, ongoing research has been focused on RAGE potential role in tissue homeostasis and repair/regeneration after injury (Meneghini *et al.* 2013; Sorci *et al.* 2013). Thus, RAGE is likely to represent one main factor intervening in the fine regulation of the balance between homeostasis and disease (Sorci *et al.* 2013, Herold *et al.* 2007).

RAGE is a complex alternative spliced gene resulting in variants that exhibit decreased ligand binding ability (N-terminally truncated isoforms) or lack transmembranar/cytosolic region (C-terminally truncated isoforms), thus representing signaling-incompetent isoforms (Ding & Keller 2005a, Hudson *et al.* 2008a). One of the C-truncated variants (sRAGE) is soluble and interacts with ligands in extracellular compartment, working as "decoy" receptors. In contrast, full-length RAGE (flRAGE, also known as mRAGE) consists of an N-terminal extracellular portion, C-terminal region and a transmembrane-spanning domain. Therefore, RAGE signaling is not only dependent upon ligand identity and concentration, cell type and putative co-receptors but also upon the interplay between signaling competent and decoy RAGE isoforms. In fact, sRAGE dictates the amount of ligands which encounters flRAGE (Ding & Keller 2005a). By maintaining the levels of ligands within a physiological range, sRAGE tightly regulates a low-grade of ligand/RAGE trophic signaling, thereby preventing flRAGE over-expression and subsequent noxious events. Aforementioned reasons support the traditional

view of sRAGE and flRAGE having potential cytoprotective/tissue-destructive properties , respectively (Rojas *et al.* 2010, Yan *et al.* 2010).

Accumulation of RAGE ligands (e.g. S100B, AGE's, HMGB-1) was described in PD patients and in experimental PD models (Dalfo *et al.* 2005, Sathe *et al.* 2012, Teismann *et al.* 2012, Choi & Lim 2010). Nevertheless, conflicting findings on RAGE biology subsist in PD studies: while RAGE upregulation paralleled AGE's accumulation in patients in early stages of PD (Dalfo *et al.* 2005), basal RAGE levels and S100B upregulation coexisted in a distinct cohort of PD's patients within stage 2 of Hoehn and Yahr scale (Sathe *et al.* 2012). RAGE upregulation was also reported in sub-acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone-mice models of PD (Teismann *et al.* 2012, Abdelsalam & Safar 2015). However, exact nature of RAGE variants was neglected in these pre-clinical and clinical studies. Noteworthy, we recently reported striatal neurotrophic levels of S100B and its signaling competent RAGE isoform in a chronic MPTP model (Viana *et al.* 2016). Therefore, it is vital to understand the overall balance of RAGE isoforms in physiological and pathological conditions since subtle shifts in the ratio of flRAGE and of RAGE inhibitory forms may significantly impact RAGE signaling (Bierhaus & Nawroth 2009, Ding & Keller 2005a). Hence, we attempted to characterize RAGE protein variants regulation in striatum of an acute MPTP mouse model to better understand how RAGE functions in early stages of PD degeneration.

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3.3 Materials and methods

3.3.1 Animals and MPTP protocol

Male adult C57BL/6J mice (3 months old; 24–28 g; Charles River Laboratories, Barcelona, Spain) were housed four per cage, under controlled environmental conditions [12-h light/dark schedule at 23±1 °C, with food and water supplied ad libitum]. All experiments were approved by the Institutional Animal Care and Use Committee from Faculty of Medicine, Coimbra University and were performed following the European Community directive (2010/63/EU) and in compliance with ARRIVE guidelines. Animal procedures were in strict accordance with "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy Press 1996).

Animals were injected intraperitoneally (i.p.) with four doses of 20mg/kg of MPTP hydrochloride in saline solution (Sigma-Aldrich, St. Louis, MO, USA; MPTP, n=14) or with saline solution (0.9% NaCl; SAL, n=14) every 2 hours, according to MPTP acute paradigm and were sacrificed six hours following last MPTP administration (see Figure 3.1).

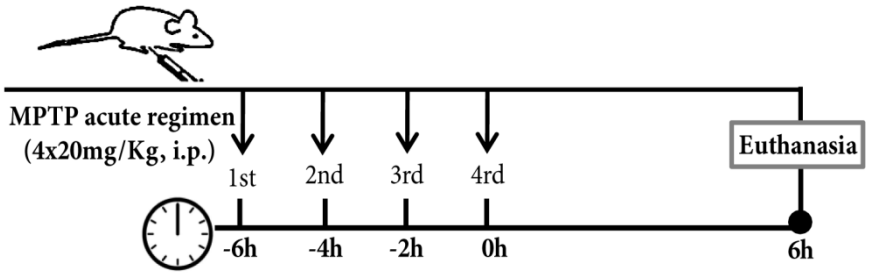

Figure 3.1 Experimental MPTP protocol design

This acute MPTP paradigm is highly accepted in the field and allows an accurate time-dependent analysis of neuroadaptive changes occurring in the striatum (Jackson-Lewis *et al.* 1995; Mandir *et al.* 1999; Panicker *et al.* 2015; Peng and Andersen 2011). We chose a time-point that corresponds to the onset of the active phase of MPTP-induced dopaminergic neurodegeneration, providing valuable insights into mechanisms of DAergic neurodegeneration in PD (Pattarini *et al.* 2008; Przedborski et al, 1996; Wu *et al.* 2003). First set of animals (n=10 SAL/MPTP) were sacrificed by decapitation and brains were dissected and immediately frozen in liquid nitrogen and stored at -80ºC. Left hemisphere of striata from 5 animals of each experimental group were stored in RNA later™ solution (Ambion, Austin, TX, USA) for RNA

extraction and RT-qPCR analysis and right hemisphere of striata were processed for ELISA analysis. Striata from another cohort of animals (n=5 SAL/MPTP) were processed for HPLC-ED (left hemisphere) and WB (right hemisphere) analysis. Second set of animals (n=4 SAL/MPTP) were deeply anesthetized with pentobarbital and transcardially perfused with 0.1 M phosphate buffer saline pH 7.4 (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed, post-fixed for 24 h in 4% PFA and dehydrated in 30% sucrose in 0.1 M PBS for 24 h for subsequent immunohistochemistry (IHC) analysis. All chemicals (ultrapure and pro analysis quality) were purchased from Sigma-Aldrich and MerckAG (Darmstadt, Germany) and technical recommendations of MPTP safety handling were strictly enforced (Przedborski *et al.* 2001).

3.3.2 Quantification of dopamine and metabolites by HPLC-ED

Striatal contents of dopamine (DA) and metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by high-performance liquid chromatography with an electrochemical detector (HPLC-ED), as previously described (Pereira *et al.* 2012, Silva *et al.* 2014, Viana *et al.* 2016). Concentration of DA and its metabolites were determined by comparison with peak areas of standards and expressed in nanograms per mg of protein.

3.3.3 RT-qPCR gene expression

RNA was isolated from striata according to protocol from RecoverAll™ Total Nucleic Acid isolation kit (AM1975, Alfagene). Total amounts of RNA extracted, RNA integrity (RIN, RNA Integrity Number) and purity (A260/A280) were measured by RNA Nano Chip® kit in Agilent 2100 Bioanalyzer (2100 expert software, Agilent Technologies, Walbronn, Germany) and ND-1000 spectrophotometer (NanoDrop Technologies), respectively. RNA was reverse transcribed with Transcriptor Universal cDNA Master (Roche Diagnostics, Mannheim, Germany): one microgram of total RNA was mixed with a 5× Transcriptor Universal Reaction Buffer and 20x Transcriptor Universal Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany) in a total reaction volume of 20µl. Reactions were carried out in a thermocycler Eppendorf vapo.protect with the following thermal profile: 5 min at 25 °C, 10 min at 55 °C and 5 min at 85 °C. Gene expression was performed by Real Time quantitative Polymerase chain

Reaction (RT-qPCR) using LightCycler 480 II system (Roche Diagnostics, Mannheim, Germany). RT-qPCR amplification of S100B, RAGE and endogenous controls 18SrRNA, Ywhaz and β-actin used optimized primers from Real time ready catalog assays (Cat. No 315571, 31148, 300236, 307906 and 317883, respectively - Roche Diagnostics, Mannheim, Germany) and LightCycler® 480 Probes Master 2x (Roche Diagnostics, Mannheim, Germany), according to manufacturer's instructions. The primer mouse sequences used were as follows: RAGE, 5'-GTCAGCATCAGGGTCACAGA-3' (forward) and 5'-AAGGCCAGGGCTAGCGTA-3' (reverse); S100B, 5'-AACAACGAGCTCTCTCACTTCC-3' (forward) and 5'-CTCCATCACTTTGTCCACCA-3' (reverse); 18SrRNA, 5'-GCAATTATTCCCCATGAACG-3' (forward) and 5'-GGGACTTAATCAACGCAAGC-3' (reverse); β-actin, 5'-CTAAGGCCAACCGTGAAAAG-3' (forward) and 5'-ACCAGAGGCATACAGGGACA-3' (reverse); Ywhaz, 5'-CTTCCTGCAGCCAGAAGC-3' (forward) and 5'-GGGTTTCCTCCAATCACTAGC-3' (reverse). Non-template control reactions were performed for each gene, in order to assure no unspecific amplification. RT-qPCR results were analyzed with qbase+ software (Biogazelle, Gent, Belgium). The relative expression ratio of each of the target gene was computed based on its real-time PCR efficiencies (E) and the crossing point difference (ΔCq) for an unknown sample versus a control ($E^{\Delta Cq}$ method). Results were obtained in NRQ (normalized relative quantities) and then converted to percentage using control group as reference.

3.3.4 Protein content analysis

Total striatal protein extracts and western blot (WB) analysis were performed as previously described (Silva *et al.* 2014, Viana *et al.* 2016). Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). Primary antibodies (listed in Table 3.1) and alkaline phosphatase-conjugated IgG secondary antibodies (mouse, rabbit or goat 1:10,000; GE Healthcare, USA) were used. Membranes were visualized on Thyphoon FLA 9000 (GE Healthcare) and analysed using Image Quant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). Results were normalized against internal controls β-actin/GAPDH and then expressed as percentage of control.

Table 3.1 Primary antibodies used for western blot and immunohistochemistry

Antigen	Company	Catalog number	Host	Dilution
Tyrosine hydroxilase (TH)	Merck Millipore	MAB318	Mouse	WB: — IHC: 1:500
Hydroxynonenal (HNE)	Merck Millipore	393207	Rabbit	WB: 1:1000 IHC: —
S100 calcium binding protein B (S100B)	Merck Millipore	JBC1771181	Rabbit	WB: 1:500 IHC: 1:1000
Ionized calcium-binding adapter 1 (Iba1)	Wako	019-19741	Rabbit	WB: 1:500 IHC: 1:1000
Tumor necrosis factor α (TNF-α)	Abcam	Ab6671	Rabbit	WB: 1:500 IHC: —
RAGE (C-terminus)	Abcam	Ab3611	Rabbit	WB: 1:1000 IHC: 1:500
RAGE (N-terminus)	Sta Cruz Biotech	N16	Goat	WB: 1:400 IHC: 1:200
Glial fibrillary acidic protein (GFAP)	Merck Millipore	IF03L	Mouse	WB: 1:1000 IHC: 1:500
Neuronal nuclei (NeuN)	Merck Millipore	MAB377	Mouse	WB: — IHC: 1:1000
NF-kB p65 (p65)	Abcam	Ab16502	Rabbit	WB: — IHC: 1:1000
β-actin	Sigma-Aldrich	A5316	Mouse	WB: 1:1000 IHC: —
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Merck Millipore	MAB374	Mouse	WB: 1:1000 IHC: —

Right striata were sonicated in 100 μl 0.1M PBS for ELISA methodology. An equal volume of Cell lysis buffer 2 (R&D Systems, catalog #895347) was added and tissues were lysed at room temperature (RT) for 30min with gentle agitation. Aliquots of lysates were removed for protein determination (BIO-RAD protein assay kit) and assayed for RAGE according to protocol from Mouse RAGE Immunoassay Quantikine® ELISA (R&D systems, #MRG00). This assay uses an antibody raised against RAGE extracellular portion, detecting flRAGE/C-truncated RAGE isoforms but not N-truncated isoforms. Results were normalized for total amounts of protein and converted to percentage with saline group as reference.

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3.3.5 Immunohistochemistry

Striatum anatomical limits (AP, +1.32 to +0.5 mm) were identified using a mouse brain atlas (Paxinos & Franklin 2004). Striatal coronal sections of 40 μm thickness were collected from cryostat (Leica CM3050S, Nussloch, Germany) in antifreezing solution and used for free-floating immunohistochemistry, as previously described (Viana *et al.* 2016). Primary antibodies are listed in Table 3.1 and the following appropriate secondary antibodies were used: donkey anti-rabbit Alexa Fluor 594, anti-goat Alexa Fluor 488 or anti-mouse Alexa Fluor 647 antibodies (all 1:200 and all from Invitrogen). Nuclei were visualized after 4´,6-diamidino-2-phenylindole (DAPI; 1:5,000; D1306, Invitrogen) staining. Cytosolic/nuclear location of NF-κB p65subunit, a readout of NF-κB activation, was assessed by immunofluorescence, as previously performed in rat striatum by Wang *et al.* (2013). Samples were imaged using a confocal laser-scanning microscope (LSM 710 Meta, Carl Zeiss Gottingen, Germany).

3.3.6 Statistical analysis

Differences between experimental groups were compared using unpaired Student's t-test. The accepted level of significance for the tests was $p<0.05$. Data is depicted as mean values ± S.E.M. and analyses were performed using GraphPad Prism 5.0 software for Windows.

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3.4 Results

3.4.1 Striatal dopaminergic derangement and glial reactivity occur early after MPTP

TH immunostaining revealed a decrease in TH-immunopositive fibers in agreement with a reduction of DA (p<0.05), DOPAC and HVA (p<0.001) and DA turnover (p<0.01) detected using HPLC-ED in striatum 6 hours following last MPTP injection (Figure 3.2a and Table 3.2, respectively). DAergic disruption is accompanied by an oxidative setting, demonstrated by high levels of 4-hydroxy-2-nonenal (HNE) lipid peroxidation marker (p<0.05) (Figure 3.2b).

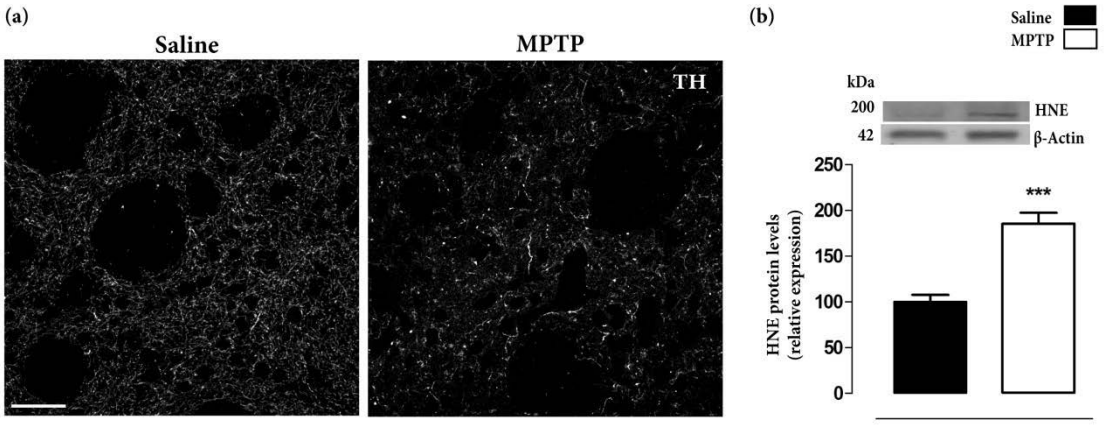

Figure 3.2 MPTP (4x20mg/kg) induces dopaminergic impairments and an oxidant setting. Representative confocal images of dopaminergic fibers (TH) in saline and MPTP groups (a. left and right, respectively; Scale bar=100 μm) b. Representative Western blot of HNE-protein adducts quantification, normalized with β-actin and expressed as % mean of control ± S.E.M. Results are representative of five animals per group ***p<0.001 using unpaired Student's t-test for comparison with control

Table 3.2 Effect of MPTP (4x20mg/kg) on striatal catecholamines

	Saline	**MPTP**
	Mean ± S.E.M.	Mean ± S.E.M.
Dopamine	130.00 ± 9.45	80.34 ± 21.85*
DOPAC	30.77 ± 3.10	4.02 ± 0.67***
HVA	13.65 ± 1.16	4.26 ± 0.51***
(DOPAC+HVA)/DA	0.37 ± 0.04	0.14 ± 0.05**

Values are mean ± SEM for 5 mice per group and expressed as ng/mg protein.
*p<0.05, ** p <0.01 and *** p <0.001 using unpaired Student's t-test for comparison with control

Regarding glia status, strong S100B$^+$-astrocytes were found evenly distributed in striata of MPTP-treated mice, consistent with S100B mRNA/protein contents upregulation (p<0.01 and p<0.05, respectively; Figure 3.3a-c). This is suggestive of acute astrocytic reactivity.

Moreover, a trend to increased Iba1 protein levels upon MPTP (Figure 3.3d,e) is also suggestive of progressive microglia activation. Upregulation of pro-inflammatory TNF-α cytokine (p<0.05, Figure 3.3f) further corroborated glial reactivity in the acute phase of MPTP-injury.

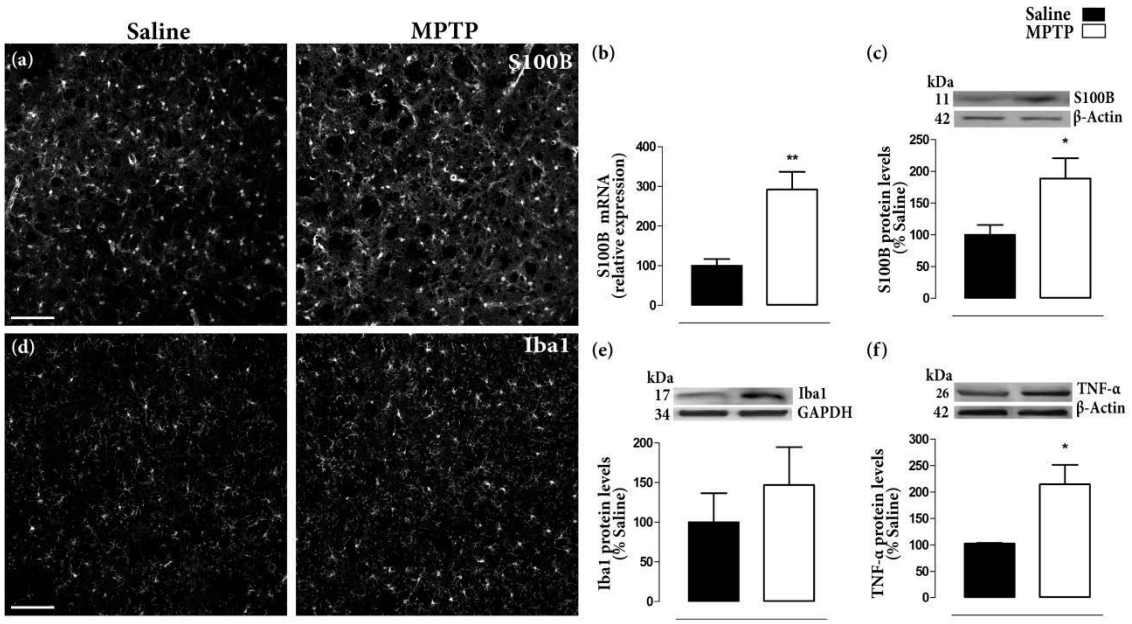

Figure 3.3 MPTP (4x20mg/kg) induces gliosis a. Representative confocal images of S100B$^+$-astrocytes in saline and MPTP groups (left and right, respectively; Scale bar=100 μm) b. mRNA expression of S100B, expressed as % mean NRQ of control ± S.E.M. c. Representative Western blot and quantification of S100B protein, normalized with β-actin and expressed as % mean of control ±S.E.M. d. Representative confocal images of Iba1-positive microglia in saline and MPTP groups (left and right, respectively; Scale bar=100 μm) g. Representative Western blot and quantification of Iba1 protein, normalized with GAPDH and expressed as % mean of control ±S.E.M. e. Representative Western blot and quantification of TNF-α protein, normalized with β-actin and expressed as % mean of control ±S.E.M. Results are representative of 4-5 animals per group *p<0.05 and ** p <0.01 using unpaired Student's t-test for comparison with control

3.4.2 Early increase of inhibitory RAGE variants after MPTP

RAGE mRNA and total protein contents were quantified by RT-qPCR and ELISA. A robust increase in RAGE gene transcription (p<0.01) and total protein contents (p<0.01) were observed 6 hours following MPTP insult (Figure 3.4a,b).

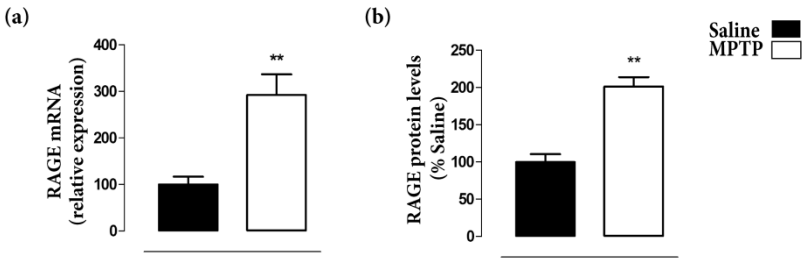

Figure 3.4 MPTP (4x20mg/kg) upregulates RAGE a. mRNA expression of RAGE, expressed as % mean NRQ of control ±S.E.M. b. RAGE protein quantification by ELISA immunoassay, normalized for total amounts of protein and expressed as % mean of control ± S.E.M. Results are representative of five animals per group **p<0.01 using unpaired Student's t-test for comparison with control

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We then investigated RAGE density as full-length, N- and C-terminally truncated isoforms (WB) to further dissect the complex proteomic of RAGE variants (Figure 3.5a). Use of an antibody raised against C-terminal domain of RAGE (anti-C-terminal RAGE Ab) revealed normal levels of RAGE immunoreactivity at ≈ 50, 37 and 25 kDa, which probably reflect monomeric flRAGE, pre-glycosylated flRAGE/N-truncated isoforms and a proteolytic product of RAGE, respectively [Figure 3.5b (right), $p>0.05$]. These observations indicate that MPTP does not impact signaling competent RAGE isoform. Nevertheless, an increased immunoreactivity in bands around 40/45kDa [Figure 3.5b (left), $p<0.05$] were found when an antibody raised against N-terminal RAGE (anti-N-terminal RAGE Ab) was used. These bands likely represent inhibitory variants (membrane-bound C-truncated or soluble RAGE isoforms). Immunoreactivity at ≈ 50kDa (probably corresponding to flRAGE) remained unchanged after treatment ($p>0.05$).

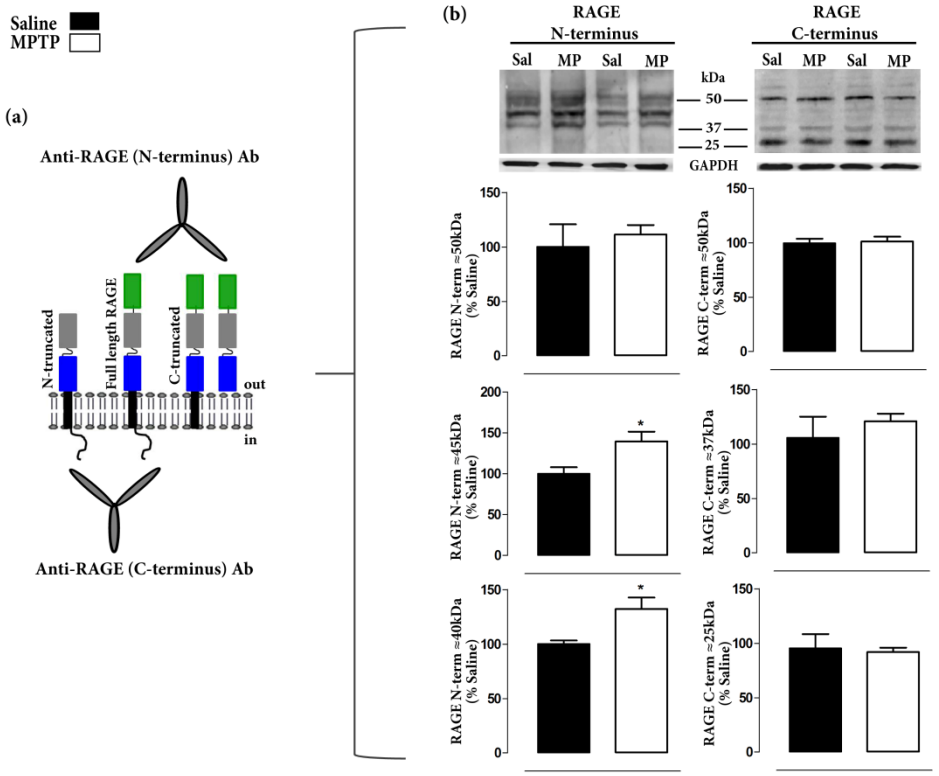

Figure 3.5 MPTP (4x20mg/kg) modulates RAGE towards increased inhibitory isoforms. a.Schematic representation of most prevalent RAGE isoforms [full-length RAGE (flRAGE), N-terminally and C-terminally truncated RAGE)] showing differential recognition by antibodies used in present study. Polyclonal anti-RAGE N-terminal Ab (raised against recombinant fragment corresponding to RAGE extracellular domain) recognizes both flRAGE and C-terminally truncated RAGE but lacks reactivity against N-terminally truncated RAGE. Polyclonal anti-RAGE C-terminal (raised against synthetic peptide corresponding to a.a. 362-380 of cytosolic tail) recognizes both flRAGE and N-terminally truncated RAGE but lacks reactivity against C-terminally truncated RAGE. b. Representative Western blot and quantification of RAGE proteins with polyclonal goat anti-RAGE N-terminal antibody (left) and polyclonal rabbit anti-RAGE C-terminal (right). Results were normalized with GAPDH and expressed as % mean of control ± S.E.M. Results are representative of 5 animals per group *$p<0.05$ using unpaired Student's t-test for comparison with control

RAGE proteomic analysis was also extended to RAGE variants cellular and subcellular localization (IHC). RAGE was predominantly found in cytosol and to some extent in neuropil in majority of cells, when labeling was performed with anti-N-terminal RAGE Ab (Figure 3.6a). In contrast, large number of cells displayed strong RAGE immunoreactivity in nucleus and a faint cytoplasmatic immunoreactivity when anti-C-terminal RAGE Ab was used (Figure 3.6b). These distinct staining patterns were supported by the weak co-localization profile obtained when both anti-RAGE antibodies were used (Figure 3.6c-e). However, there is a different set of cells showing N-RAGE immunolabeling homogeneously across soma displaying faint C-RAGE labeling (arrows in Figure 3.6a-c, MPTP condition). In line with WB analysis, we found increased N-terminal RAGE immunoreactivity whereas C-terminal RAGE immunostaining remained apparently unchanged upon MPTP (Figure 3.6a,b). This increase in N-RAGE density seems to be localized in the second set of cells.

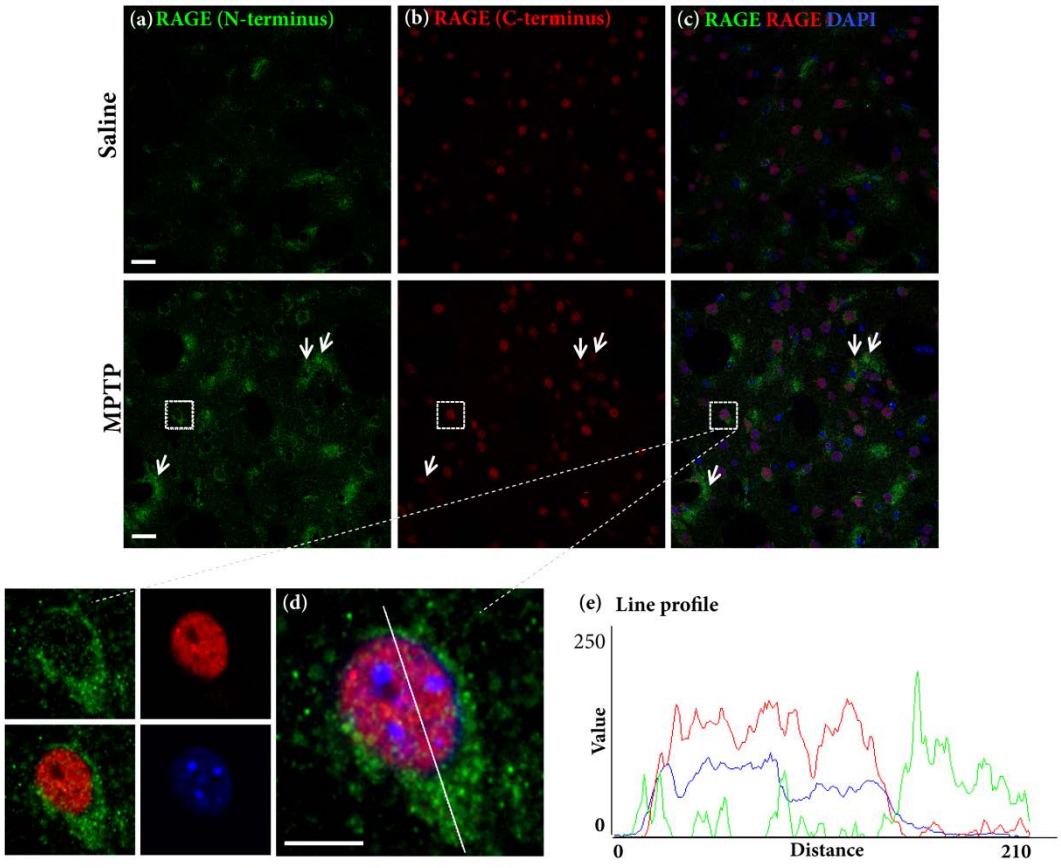

Figure 3.6 Representative confocal images of immunolabeling for N-terminal (green) or C-terminal portion (red) of RAGE and DAPI staining (blue) in saline and MPTP groups. Arrows in 3a-c show increased N-RAGE immunostaining spanning across soma of cells that display a weak C-terminal RAGE immunoreactivity (Scale bar=40 μm) d. Image magnification of a N-and C-terminus RAGE-positive cell in MPTP group (Scale bar=5 μm) and two-dimensional graph of pixels intensities (y-axis) along a line within the image (X-axis). Results are representative of 4 animals per group

3.4.3 Reactive but non-hypertrophic astrocytes showed an increase of inhibitory RAGE variants at an early time-point after MPTP

Since RAGE upregulation occurs upon ligands accumulation (Bierhaus & Nawroth 2009), we hypothesized that the increased inhibitory RAGE isoforms occurred mainly in astrocytes given previous findings of MPTP-striatal S100B upregulation. Double immunofluorescence staining for N-terminal RAGE and S100B showed S100B-enriched astrocytes displaying increased RAGE immunoreactivity in soma and astrocytic processes (Figure 3.7a-d).

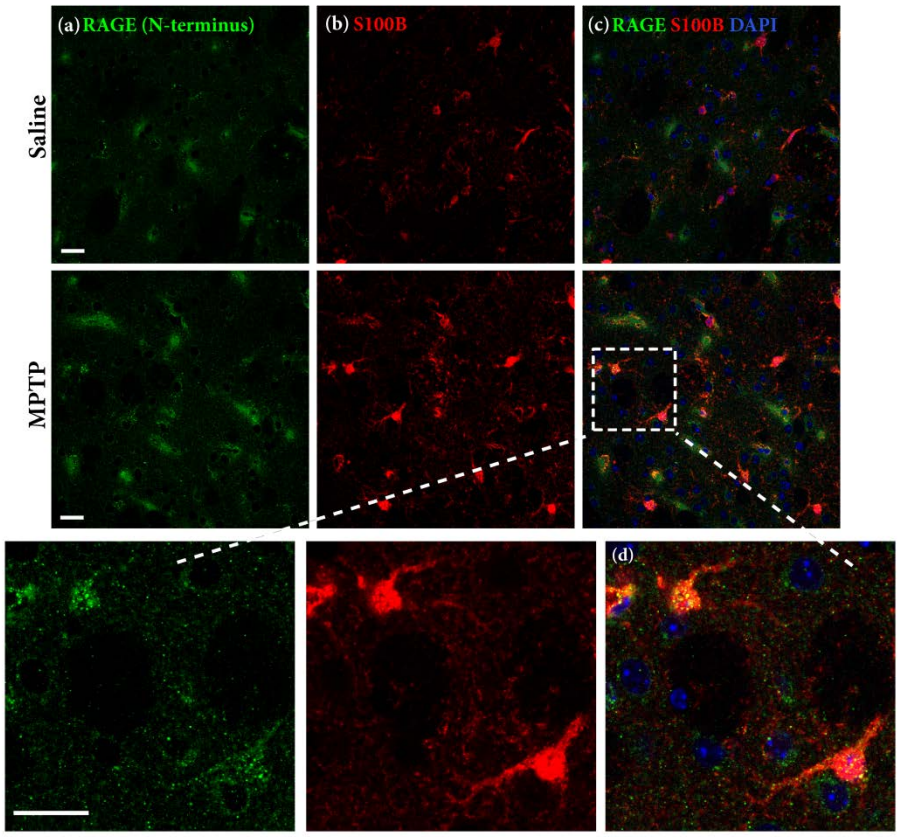

Figure 3.7 MPTP (4x20mg/kg) induces inhibitory RAGE variants accumulation in S100B^{+}-enriched astrocytes. a-c. Representative confocal images of immunolabeling for N-terminal portion of RAGE (green), S100B (red) and DAPI staining (blue) in saline and MPTP groups (a-c, above and below respectively; Scale bar=40 μm). d. Image magnification of S100B/RAGE positive astrocytes in MPTP group (Scale bar=20 μm) showing enhanced RAGE labeling in astrocytic soma and stem processes. Results are representative of 4 animals per group

Astrocytes having enhanced S100B and RAGE levels failed to show obvious signs of hypertrophy of cell bodies and stem processes, as GFAP protein levels and astrocytic morphologic features clearly demonstrate (Figure 3.8a,b). S100B/RAGE astrocytic upregulation led us to investigate activation of NF-κB, the recognized RAGE downstream signal transducer. p65 NF-κB subunit was mainly located in cytosol of both astrocytes and other non-GFAP

positive cells (Figure 3.8b), ruling out NF-κB activation in this phase of MPTP striatal dysfunction.

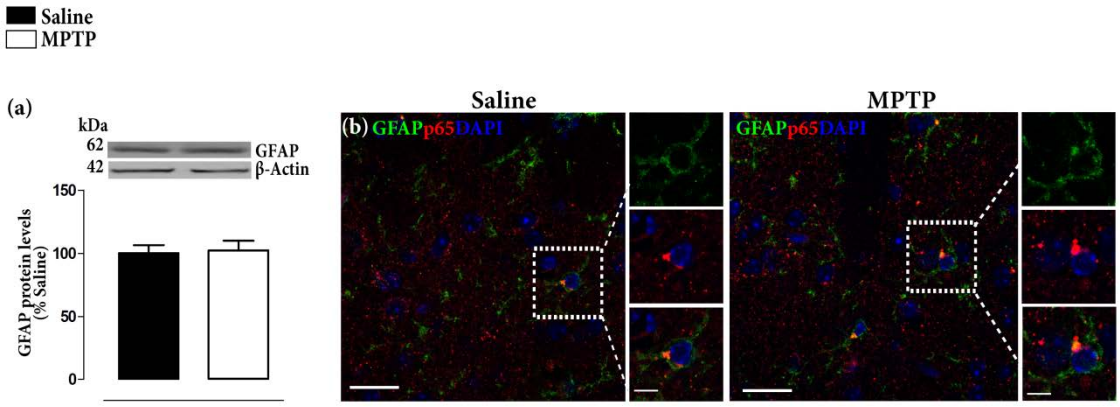

Figure 3.8 MPTP (4x20mg/kg) induces inhibitory RAGE variants accumulation in reactive non-hypertrophic astrocytes. a. Representative Western blot of GFAP protein levels quantification, normalized with β-actin and expressed as mean ± S.E.M. b. Representative confocal images of immunolabeling for GFAP (green), p65 NF-κB sbunit (red) and DAPI staining (blue) in saline and MPTP groups (b, left and right respectively). Results are representative of 4-5 animals per group. $p>0.05$ using unpaired Student's t-test for comparison with control. Scale bar: 20μm and 5 μm for magnified images

We further investigated if additional cell types related to DAergic neurodegeneration did also accumulate RAGE antagonistic isoforms. RAGE constitutive expression was predominantly found in neurons and to a lesser extent in microglia cells (Figure 3.9a-f), as demonstrated by double immunofluorescence staining against RAGE and neuron specific nuclear protein NeuN (neuronal marker) or Iba1 (microglia marker). This expression pattern remained unchanged following MPTP treatment. Furthermore, RAGE was neither present in healthy dopaminergic axons nor induced in degenerating fibers upon MPTP-treatment (Figure 3.9g-i), as shown by double immunofluorescence staining against RAGE and TH (DAergic axonal marker).

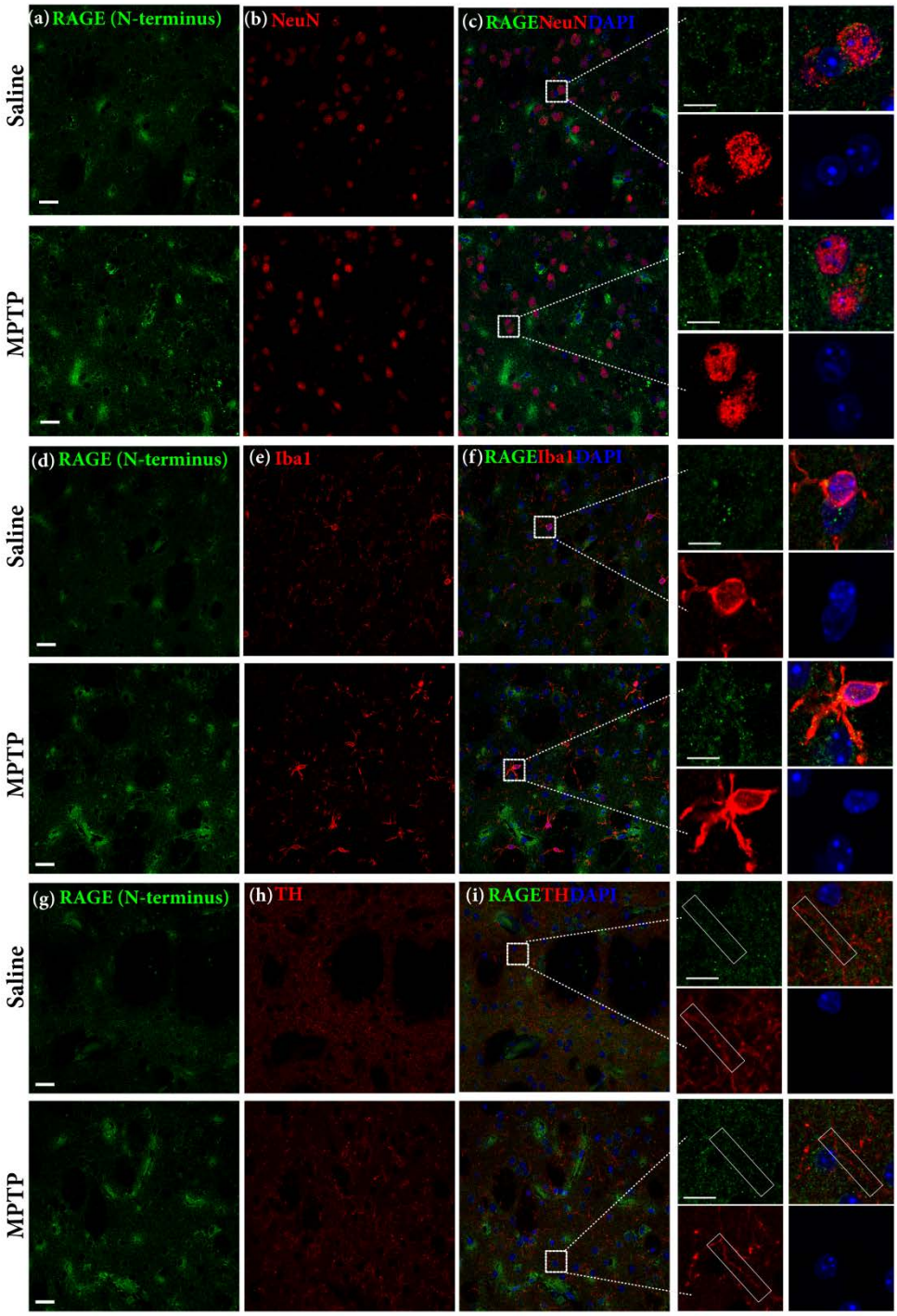

Figure 3.9 MPTP (4x20mg/kg) does not induce changes in RAGE density in neurons, microglia or dopaminergic fibers. a-c. Representative confocal images of immunolabeling for N-terminal portion of RAGE (green), neuronal marker NeuN (red) and DAPI staining (blue) in saline and MPTP groups (above and below, respectively; Scale bar=40 μm and 5 μm for magnified images). d-f. Representative confocal images of immunolabeling for N-terminal portion of RAGE (green), microglia marker Iba1 (red) and DAPI staining (blue) in saline and MPTP groups (above and below, respectively; Scale bar=40 μm and 5 μm for magnified images). g-i. Representative confocal images of immunolabeling for N-terminal portion of RAGE (green), DAergic fibers marker TH (red) and DAPI staining (blue) in saline and MPTP groups [above and below, respectively; Scale bar=40 μm and 5 μm for magnified images (DAergic fibers are signalized by a rectangle)]. Results are representative of 4 animals per group

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3.5 Discussion

Exposure of animals to MPTP closely recapitulates biochemical and phenotypic features of PD (Prediger *et al.* 2006). This proneurotoxin is processed in astrocytes into MPP^+ active metabolite which is then uptaken mostly by dopamine transporter (DAT) in striatal DAergic terminals, causing mitochondrial complex-I inhibition (Smeyne and Jackson-Lewis 2005). Alterations in energy metabolism and generation of reactive oxygen species (ROS) peak within hours of MPTP administration, days before overt retrograde neuronal death (Jackson-Lewis *et al.* 1995). These initial striatal events are not likely to directly kill most cells but rather set into play downstream cellular events that ultimately lead to DAergic neuronal death (Dauer and Przedborski 2003). Therefore, understanding how MPTP insults striatum helps to dissect cellular and molecular processes that take place in early stages of the PD degenerative process.

Our data unequivocally shows a reduction of TH^+ area underlining hypodopaminergia in striatum as early as 6 hours following last MPTP administration, which confirms previous findings from Himeda and colleagues (Himeda *et al.* 2006). A dynamic progression towards glia activation and inflammation accompanies MPTP-neuronal injury (Yasuda *et al.* 2008, Hunot & Hirsch 2003, Muramatsu *et al.* 2003). Accordingly, striatal TNF-α and S100B upregulation are reported herein. This scenario of neuroinflammation and increased local concentration of ligands warranted investigation of likelihood of RAGE up-regulation. As expected, we found increased RAGE transcription and total protein contents as early as 6 hours following MPTP.

RAGE displays great proteomic diversity, thus imposing protective/deleterious effects on cellular function. Nevertheless, previous studies addressed RAGE as a single receptor, casting a shadow on RAGE role in PD neurodegeneration. To accurately interpret RAGE dynamics, we dissected the impact of MPTP on striatal RAGE variants using two distinct anti-RAGE antibodies raised against extra- and intracellular epitopes. We observed RAGE immunoreactivity in a broad spectrum of molecular weight patterns, likely to reflect distinct RAGE isoforms. This is in line with previous studies showing simultaneous existence of 50 kDa, flRAGE isoform and 40-45 kDa RAGE variants (Greco *et al.* 2012, Lizotte *et al.* 2007). Notably, inhibitory C-truncated RAGE isoforms were increased whereas basal flRAGE levels were observed upon injury. The impact of MPTP on RAGE biology was further analyzed by investigating RAGE subcellular distribution. In basal conditions, we found RAGE

immunoreactivity homogeneously distributed in cellular cytoplasm with a moderate staining of striatal neuropil when an antibody raised against extracellular portion of receptor was used, coincident to what has been previously described by Anzilloti and colleagues (2012). Nevertheless, a very dense nuclear staining was observed when an antibody raised against RAGE cytosolic tail was employed, in agreement with other reports focused on RAGE expression in rat cortical neurons using an anti-C-terminal RAGE antibody (Greco *et al.* 2012, Li *et al.* 2014a). Furthermore, there was weak co-localization profile obtained when both antibodies raised against N- and C-terminal portions of RAGE were simultaneously used. Collectively, these observations are suggestive of different RAGE isoforms existing in different subcellular compartments. Both alternative splicing (Lopez-Diez *et al.* 2013) and constitutive proteolysis (Galichet *et al.* 2008) can contribute to this phenomenon.

MPTP evoked increased N-RAGE immunostaining homogeneously across soma of cells showing faint C-terminal labeling. Thus, we tested the hypothesis whereby RAGE upregulation was specifically occurring in astrocytes, taking into account accumulation of its astrocytic ligand S100B in MPTP mice. In fact, RAGE immunostaining almost overlapped S100B expression pattern in MPTP mice, with a strong co-localization observed in astrocytic soma and stem processes. It is therefore likely that increased levels of astrocytic S100B ligand upregulate antagonistic RAGE variants. This could be a compensatory attempt to modulate S100B autocrine functions and/or govern extracellular paracrine effects. This assumption is in line with the inverse correlation between enriched $S100B^{+}$-astrocytes and S100B cerebrospinal fluid contents following MPTP found by Yasuda and colleagues (2008), suggesting the recruitment of regulatory mechanisms aimed to keep extracellular S100B within a homeostatic range in early stages of PD pathogenesis. One cannot rule out the possible contribution of endothelial cells to increased N-RAGE immunostaining. In fact, endothelial cells are functionally associated with astrocytes in the neurovascular unit of blood-brain barrier (BBB), and MPTP imposes a noxious impact on BBB function (Chung *et al.* 2013, Coelho-Santos *et al.* 2015).

Noteworthy, astrocytic S100B/RAGE variants accumulation occurred with no signs of astrocytic hypertrophy as shown by normal GFAP levels. This is consistent with GFAP reaching maximum levels no sooner than 3 days following MPTP (Ookubo *et al.* 2009). S100B takes a great part in the process of astrogliosis including a chief role in cytoskeleton dynamics and astrocytic cell shape and migration (Donato *et al.* 2009, Sorci *et al.* 2010). Increased density of

C-truncated RAGE isoforms in soma and astrocytic processes suggest that these signaling-incompetent RAGE isoforms may be mediating S100B intracellular effects through undisclosed transducing mechanisms that do not require NF-κB activation. This is consistent with previous reports that showed S100B-induced intracellular effects were independent of RAGE signaling and NF-κB activation, but dependent on the density of RAGE extracellular domain (Adami *et al.* 2004). Importantly, RAGE ablation reduced the number of striatal GFAP-positive cells upon MPTP intoxication, substantiating a role for RAGE in astrocytic reactivity (Teismann *et al.* 2012). On the other hand, S100B is also likely to activate other core immune receptors, namely Toll-like receptors (TLR's), which are also expressed in astrocytes (Marinelli *et al.* 2015). In fact, TLR's most likely play a role in PD because a strong upregulation of TLR's was observed in acute phase of MPTP injury and in *post-mortem* PD striatal homogenates (Noelker *et al.* 2013, Ros-Bernal *et al.* 2011). Therefore, it is tempting to hypothesize that S100B/TLR's/RAGE functional tripod is engaged in the complex regulation of danger-induced inflammation and astrocytic reactivity in MPTP striatal injury, as described in other noxious conditions (Sorci *et al.* 2011, van Beijnum *et al.* 2008, Zurolo *et al.* 2011). Notably, a long-lasting repression of S100B and RAGE transcription occurs after a transient increase in striatonigral pathway upon MPTP injury, corroborating the thesis of a tight regulated immune mechanism aimed to restrain the initial inflammatory response (Teismann *et al.* 2012). Increased inhibitory RAGE isoforms and basal NF-κB activity reported here corroborate those observations, ruling out an active feed-forward loop between RAGE/NF-κB at early stages of PD striatal dysfunction. Rather, it suggests the activation of distinct transcription factors underlying higher rates of RAGE transcription (e.g. SP1, AP2) and S100B-intracellular signaling (Bierhaus & Nawroth 2009, Bianchi *et al.* 2010).

Due to the role of astrocytes in MPTP bioactivation, these cells would be expected to be targets of MPP^+ cytotoxicity (Di Monte *et al.* 1992). However, astrocytic degeneration does not appear to be an obvious neuropathological feature of MPTP exposure. Instead, striatal astrocytes display enhanced protective capacity against oxidative stress by showing increased antioxidant enzymatic activities (e.g. SOD and HO-1) after MPTP exposure when compared to mesencephalic and cortical astrocytes (Fernandez-Gonzalez *et al.* 2000, Wong *et al.* 1999). Additionally, increased antioxidant mechanisms linked to astrocytic proliferation and detoxification of ROS seem to facilitate protection against oxidative damage in non-SN brain

regions such as striatum in experimental and human PD patients (Mythri *et al.* 2011, Chen *et al.* 2009). Thus, a better understanding of mechanisms behind oxidative stress dynamics may have potential therapeutic implication in PD. Importantly, high levels of S100B confer astrocytic protection to oxidative cellular stress (Villarreal *et al.* 2014). Therefore, striatal S100B transient up-regulation after MPTP may also be engaged in such antioxidative defense mechanisms. We further hypothesize that increased antagonistic RAGE variants, traditionally endowed with a protective profile, is very likely to represent an astrocytic beneficial adaptive change to oxidative insult. Several lines of evidence support this hypothesis. First, elevated striatal transcription of RAGE immune receptor timely follows a coordinated response to oxidative stress comprising the activation of immediate early genes in striatum at early stages of MPTP toxicity. Repression of NF-κB activation seen herein is envisioned as one of these early responses (Pattarini *et al.* 2008). Second, inhibitory antagonistic RAGE variants prevent ligands-flRAGE interaction and downstream ROS generation (Daffu *et al.* 2013). Third, generation of inhibitory RAGE variants was suggested to be a key component of the body's antioxidant defense system in other important oxidative challenges, namely in demyelinating diseases or in pre-diabetic conditions (Qin *et al.* 2008, Huang *et al.* 2015). Fourth, ablation of RAGE gene (and therefore loss of protective outcomes arising from inhibitory RAGE isoforms) confer a very weak protection against MPTP toxicity (Teismann *et al.* 2012).

The picture emerging from the combination of our results and data from literature suggests a model in which RAGE/co-receptors/ligands carry out an initial adaptive repair strategy that switches into deleterious events further feeding PD dopaminergic dysfunction (Herold *et al.* 2007; Sathe *et al.* 2012; Teismann *et al.* 2012). Soon after acute MPTP insult, increased astrocytic inhibitory RAGE variants is likely to reflect a self protective mechanism in an attempt to maintain extracellular S100B within a tight range, avoiding perpetuation of flRAGE/TLR's signaling and subsequent oxidative stress, inflammation and long-term dopaminergic degeneration (Daffu *et al.* 2013). Nevertheless, accumulation of ligands consistently reported in PD animal models and PD patients implies the loss of such protective phenotype as PD evolves. In fact, ligands may acquire more complex three-dimensional structures which chronically activate flRAGE/co-receptors with persistent NF-κB activation, resulting in a vicious cycle of glial autocrine and paracrine amplification of inflammation that may culminate in DAergic axonopathy and ultimately, retrograde neuronal death.

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Overall, our findings offer first *in vivo* evidence of selective upregulation of inhibitory RAGE variants in early stages of MPTP-induced astrogliosis dynamics. Although we acknowledge that this acute MPTP model gives only an approximation to the human pathology, it generated data that has practical implications including identifying a putative therapeutic target (RAGE-astrocytes duo) in a time-window that facilitates interventions aiming to deterrent the progression of the dopaminergic degeneration which is a PD hallmark. Future studies are warranted to elucidate mechanisms underlying the loss of RAGE primal protective response to stress. This may offer novel pharmacological approaches in the field of astrocyte-neuron crosstalk within DAergic neurorescue/neuroprotection.

Chapter 4

Physical exercise regulates RAGE network and improves cognitive and emotional deficits in experimental Parkinson's disease

(*Original article submitted*)

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4.1 Abstract

Parkinson's disease (PD) prodromic stages comprise non-motor symptoms (NMS) that critically compromise patient's quality of life. Dysregulated innate immunity (hallmark of overt PD) has been associated with emotional and cognitive impairments, common PD prodromic features. Physical exercise (PE) is a non-pharmacological intervention with the potential to improve PD emotional/cognitive dysfunctions. Noteworthy, studies focused on the capacity of PE to modulate the intricate network of receptors for advanced glycation endproducts (RAGE), Toll-like receptors (TLR's) and ligands are emerging. Accordingly, we hypothesized that PE could improves PD cognitive/emotional deficits by regulating neuroimmune RAGE network. Herein, adult Wistar rats subjected to long-term mild-treadmill (and untrained) were administered intranasally with 1-methyl-4-phenyl-tetrahydropyridine (MPTP), a suitable model for the study of NMS of PD. Twelve days post-MPTP, the animals were sacrificed for RAGE-network neuroimmune transcriptomic analysis (RT-qPCR) in frontal cortex, hippocampus and striatum after probing their behavioral performances. Untrained-MPTP animals displayed habit learning disruption and anhedonic-like behavior without gross motor impairments (assessed by cued-version of water-maze, splash and open-field tests, respectively). A selective suppression of RAGE and neuroimmune-related genes was observed in frontal cortex of untrained-MPTP and trained-saline animals, the latter showing a more robust effect. PE protected against MPTP-induced cognitive and emotional deficits since trained-MPTP animals displayed normal behavioral performances. This behavioral phenotype was accompanied by a major recovery of RAGE-network immunosupression and a significant up-regulation of neuroprotective PD-related DJ-1 gene. Overall, these findings provide new evidence of the PE efficacy to improve NMS of PD and that this response can be associated with its modulatory role on neuroimmune RAGE network in frontal cortex.

Keywords: Physical exercise, Innate immunity; RAGE; TLR's; intranasal MPTP; Frontal cortex

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4.2 Introduction

Parkinson's disease (PD) is classically diagnosed by the onset of clinical motor symptoms underlying progressive degeneration of dopaminergic neurons in the nigrostriatal pathway (Tolosa *et al.* 2007). Increasing evidence highlight a myriad of non-motor symptoms (NMS) comprising executive dysfunction, mild cognitive impairments and mood and affective disorders (e.g. apathy, anxiety, depression) that often appear at the pre-motor phase of PD (Tolosa *et al.* 2007, Varanese *et al.* 2011, Pfeiffer 2016). Early recognition and management of NMS are relevant to improve daily patient's well-being and to reduce social economic burden. Thus, clinical approaches to manage NMS in PD patients are among the top ten list priorities in PD research strategy recommendations (Deane *et al.* 2014). It is worth stressing that pharmacological management of cognitive and emotional impairments in PD remain suboptimal (Pfeiffer 2016). This emphasizes the importance of non-pharmacological strategies to alleviate PD non-motor manifestations. In this regard, studies suggest physical exercise (PE) as a suitable therapy to halt or slow down motor as well as non-motor features of PD (Cruise *et al.* 2011, Tanaka *et al.* 2009). However, controversies revolve around the neuroprotective or neurorestorative properties of exercise in PD experimental animals models, namely in those using the gold-standard 1-methyl-4-phenyl-tetrahydropyridine (MPTP) neurotoxin (Al-Jarrah *et al.* 2007, Fisher *et al.* 2004). These authors focused on the impact of PE on motor performance and overlooked neurobiological processes that govern cognitive and emotional impairments of PD. To date, few studies have demonstrated the positive effects of PE on cognitive and emotional deficits in experimental PD (Aguiar *et al.* 2016, Gorton *et al.* 2010, Klein *et al.* 2016). Experimental data extending previous observations is therefore mandatory.

It is widely accepted that neuroimmune system plays a key role in PD as well as in other CNS disorders encompassing sickness-like behavior, anhedonia and cognitive dysfunction symptomatology (Dantzer *et al.* 2008, Mazarati *et al.* 2011, Rong *et al.* 2010). There is bold evidence of dysregulation of danger associated molecular patterns (DAMP's) and related pattern recognition receptors (PRR's) – Receptor for advanced glycation endproducts (RAGE) and Toll-like receptors (TLR's) – in human and experimental PD subjects (Ros-Bernal *et al.* 2011, Sathe *et al.* 2012, Viana *et al.* 2016). PRR's are among the first responders to CNS injury and activation of these receptors on both immune (e.g. microglia) and non-immune cells (e.g. neurons)

triggers a central innate immune response (Czirr & Wyss-Coray 2012). A tight balance between rapid resolution *versus* overactive immune responses and amplification of injury is utmost critical in CNS because of its poor regenerative capacity. RAGE and its cross-talk with TLR's play a major role in the fine tuning of repair/injury balance (Herold *et al.* 2007). For example, functional interactions between RAGE and TLR's contributed to the restraining of danger-induced inflammation (Sorci *et al.* 2011). However, accumulation of ligands foster signaling pathways amplification through DAMP's/RAGE/TLR's functional tripods, triggering dysregulation of immune response (Mazarati *et al.* 2011).

The immune response to PE has been in the spotlight (Eyre *et al.* 2013, Gleeson *et al.* 2011). Particularly, the number of studies on the aptitude of PE for modulating immune RAGE network in physiological and pathological settings is rising: first, treadmill exercise training suppressed RAGE axis activation and conferred vascular protection in aged rats (Gu *et al.* 2014); second, exercise training reduced expression of TLR's on monocytes and macrophages in control subjects as well as in cerebral ischemic rats (Ma *et al.* 2013, Neubauer *et al.* 2013); last and most important, treadmill training reduced striatal S100B contents (a RAGE/TLR's ligand) in a MPTP mouse model of PD (Al-Jarrah & Jamous 2011). Hence, we hypothesized that PE would be able to alleviate PD cognitive/emotional deficits and to regulate neuroimmune RAGE network. Therefore, rats submitted to a long-term forced mild treadmill exercise were infused intranasally (i.n.) with MPTP to address this issue. This well-characterized MPTP model elicits a time-course of cognitive, emotional and motor impairments conceivably analogous to those observed during different stages of human PD (Prediger *et al.* 2006, Prediger *et al.* 2009, Prediger *et al.* 2012). Prediger and collaborators have previously demonstrated that cognitive and emotional impairments predate locomotor activity disruption that occurs no sooner than 14 days after i.n. MPTP treatment (Prediger *et al.* 2006, Prediger *et al.* 2009, Prediger *et al.* 2012, Castro *et al.* 2013). Herein we characterize emotional and cognitive behaviors and their neurochemical and neuroimmune correlates in striatum, frontal cortex and hippocampus in a time-window prior to appearance of gross motor impairments (12 days post i.n. MPTP).

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4.3 Material and methods

4.3.1 Animals and MPTP protocol

Male Wistar rats (Charles River Laboratories, 10 weeks old at the onset of the experiment) were housed two per cage in standard makrolon cages (49 cm in length; 34 cm in width; 16 cm in depth) and maintained under controlled environmental conditions (12-h light/dark schedule at 23±1 °C, 60±5% humidity, with food and water supplied *ad libitum*). Experimental protocol was approved by Ethics Committee of University of Coimbra (Approval ID: ORBEA_55_2014/0406) and was carried out according to the European Community Directive (2010/63/EU) and in compliance with ARRIVE guidelines. Animals were randomly allocated to four groups and the total body weight was balanced before starting the experiment: sedentary-saline, sedentary-MPTP, treadmill-saline and treadmill-MPTP (n=11/each experimental group).

MPTP HCl (Sigma-Aldrich, St. Louis, MO, USA) was administered by i.n. route in sedentary- and 30 days-trained rats, according to the procedure described by Prediger *et al.* (2012). Briefly, rats were lightly anaesthetized with isoflurane 0.96% (0.75 CAM; Abbott, CA, UK) using a vaporizer system (SurgiVet Inc., WI, USA) and a 10-mm piece of PE-50 tubing was inserted through the nostrils. The tubing was connected to a peristaltic pump set at a flow rate of 12.5 µl/min. The MPTP HCl was dissolved in 0.9% NaCl (saline) at a concentration of 20 mg/ml, after which it was infused for 4 min (1 mg/nostril). The control solution consisted of saline. Animals were given a 1-min interval to regain normal respiratory function and then this procedure was repeated with infusions administered through the contralateral nostrils. MPTP was handled in accordance with the safety guidelines (Przedborski *et al.* 2001).

4.3.2 Treadmill training procedure

Brain beneficial effects of exercise are intimately related with intensity and duration of PE paradigms. In fact, long-term mild rather than acute intense protocols seem to be associated with positive effects (Bayod *et al.* 2011, Inoue *et al.* 2015). Although acute activation of sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis is triggered by exercise training to maintain or restore homeostasis, chronic or prolonged activation of such

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stress responses can adversely affect animal well-being and confound interpretation of the outcome variables of interest, particularly when immune system is envisaged (Hau & Schapiro 2011). For these reasons, we implemented a long-term mild treadmill running paradigm, which is a standard form of aerobic exercise aimed to keep exercise intensity below blood lactate threshold (LT, approximately 20 m/min) and thus being considered as a "stress-free" mild exercise (Inoue *et al.* 2015). Treadmill training implemented herein (see Table 4.1) was adapted from a protocol described by Inoue and colleagues (2015) that lacks stress-related features, namely hypercorticosteremia, adrenal hypertrophy or thymus atrophy.

Table 4.1 Treadmill training protocol

Week 1	
Day	**Duration/Speed**
1	**0-15 min:** 7,2m/min **15-35 min:** 12m/min **35-40 min:** 7,2m/min
2	**0-15 min:** 7,2m/min **15-35 min:** 12m/min **35-40 min:** 7,2m/min
3	**0-15 min:** 7,2m/min **15-35 min:** 15m/min **35-40 min:** 7,2m/min
4	**0-15 min:** 7,2m/min **15-35 min:** 15m/min **35-40 min:** 7,2m/min
5	**0-15 min:** 7,2m/min **15-35 min:** 15m/min **35-40 min:** 7,2m/min
6	**Rest**
7	**Rest**

Week 2	
Day	**Duration/Speed**
8	**0-15 min:** 7,2m/min **15-35 min:** 15m/min **35-40 min:** 7,2m/min
9	**0-15 min:** 7,2m/min **15-35 min:** 15m/min **35-40 min:** 7,2m/min
10	**0-15 min:** 9m/min **15-55 min:** 15m/min **55-60 min:** 9m/min
11	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
12	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
13	**Rest**
14	**Rest**

Week 3	
Day	**Duration/Speed**
15	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
16	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
17	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
18	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
19	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
20	**Rest**
21	**Rest**

Week 4	
Day	**Duration/Speed**
22	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
23	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
24	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
25	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
26	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
27	**Rest**
28	**Rest**

Week 5	
Day	**Duration/Speed**
29	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
30	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
31	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
32	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
33	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
34	**Rest**
35	**Rest**

Week 6	
Day	**Duration/Speed**
36	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
37	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
38	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
39	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
40	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
41	**0-15 min:** 9m/min **15-55 min:** 18m/min **55-60 min:** 9m/min
42	**Euthanasia**

.

Two treadmills were used, each consisting of 5 parallel runways (53 x 10 x 15 cm, LE8710RTS model, Panlab/Harvard Apparatus, Barcelona, Spain) without inclination. Rats were subjected to the continuous running training for 60 min/day, 5 times/week, for 6 weeks including the habituation period. Rats were handled on treadmill apparatus (30 min at 0 cm/s), once a day for two consecutive days, to become familiar with the apparatus and to minimize possible novelty-induced stress. Then, exercise training began gently for gradual adaptation to the intensity and duration of running during 10 days. The intensity of treadmill protocol increased from the eleventh day onward, and was maintained until the end of the experiment. Daily running time included a warm-up phase to prepare rats for running at the set speed (18 m/min, < LT). Training sessions were conducted in the colony room, 5 days per week, between 8 and 12 h (or between 14-18 h on days of behavioral assessment, post-behavioral tests). Aversive stimuli (e.g. electrical shock) were not used to motivate the animals to exercise. Alternatively, when animals stopped running, they were gently pushed by hand for a few seconds to resume running. This type of running behavior disappeared as rats became familiar with this activity. All rats maintained a forward position on the treadmill for at least 75% of the running period. Untrained-rats remained in a stationary treadmill (0 cm/s) for the same number of sessions and the same amount of time. Body weight of rats was measured every 8 days until the end of experiment to monitor putative stress induced by treadmill running.

4.3.3 Behavioral analysis

The animals were submitted to a battery of behavioral tests (Figure 4.1) that included open-field, splash test and the cued version of water maze task, during a period of 6–10 days after the i.n. administration of MPTP. This time-window was chosen based on our previous studies using the i.n. MPTP model (Prediger *et al.* 2006, Prediger *et al.* 2009, Prediger *et al.* 2012, Castro *et al.* 2013) and aimed to characterize non-motor behavior profile. All tests were scored by the same rater in an observation room where the rats had been habituated for at least 1 h before the beginning of the tests. Behavior was monitored through a video camera positioned above the apparatuses and the images were later analyzed with the ANY Maze video tracking (Stoelting Co., Wood Dale, IL, USA) by an experienced experimenter who was unaware of the experimental group of the animals tested.

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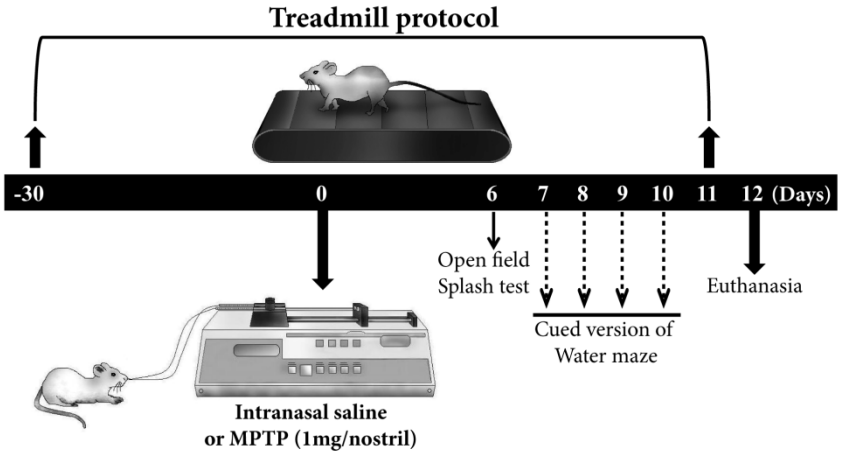

Figure 4.1 Experimental design. Time course of the treadmill training, behavioral tests and euthanasia.

4.3.3.1 Open-field

Animals were placed for 5 min in an open-field arena to evaluate the impact of MPTP on spontaneous locomotor activity. The apparatus was made of wood covered with impermeable Formica and had a gray floor of 100 x 100 cm and transparent walls, 40 cm high. Each rat was placed in the center of the apparatus and the total distance traveled (m) and the average speed (m/s), indicators of spontaneous locomotor activity, were recorded. The apparatus was cleaned with 10% ethanol after each animal exposure (Castro *et al.* 2013).

4.3.3.2 Cued-version of water maze

Animals were submitted to a cued version of the water maze to measure striatal-dependent habit learning, as previously reported (Prediger *et al.* 2006, Prediger *et al.* 2012). Tests were performed in a circular swimming pool made of black painted fiberglass, 1.2 m internal diameter, 0.8 m high, which was filled with water maintained at 25 ºC to a depth of 0.6 m. Starting points for the animals were marked on the outside of the pool as north (N), south (S), east (E) and west (W). Four distant visual cues (55 x 55 cm) were placed on the walls of the water maze room. They were all positioned with the lower edge 30 cm above the upper edge of the water tank, and the position of each symbol marked the midpoint of the perimeter of a quadrant (circle=NE quadrant, square=SE quadrant, cross=SW quadrant and diamond=NW quadrant), in the standard setting. The apparatus was located in a room with indirect incandescent illumination. In this protocol, the position of the transparent Plexiglas escape

platform (10 x 10 cm) was cued by a 7-cm diameter white ball attached to the top of the platform and protruding above the water. This task consisted of four training days, four consecutive trials per day, during which the animals were left in the tank facing the wall, then being allowed to swim freely to the submerged platform placed in the center of one of the four imaginary quadrants of the tank. The initial position in which the animal was left in the tank was one of the four vertices of the imaginary quadrants of the tank, and this was varied among trials in a pseudo-random way. This implies that the position of the platform was always changed in each trial of the day. If a rat did not find the platform during a period of 60 s, it was gently guided to it. After the animal had escaped to the platform, it was allowed to remain on it for 10 s and was then removed from the tank for 20 s before being placed in the next random initial position.

4.3.3.3 Splash test

To evaluate the impact of MPTP on emotional/motivational behaviors paralleling some anhedonic symptoms such as apathetic behavior, dorsal coats of rats were vaporized with 10% sucrose solution in its home cage. This test evaluates conflicting motivations between the fear due to spraying and the drive to groom dirty coat. Latency (idle time between spray and initiation of grooming), time spent grooming (defined as cleaning of the fur by licking or scratching) and number of groomings were recorded for a period of 5 min, as previously described (Matheus *et al.* 2015, Yalcin *et al.* 2008).

4.3.4 Tissue collection and processing

Animals were euthanized 12 days after MPTP administration by decapitation under isoflurane anesthesia and discrete brain regions (frontal cortex, hippocampus and striata) were dissected on ice. These brain regions were chosen because they are intimately related with NMS of PD. Brain regions from 5 animals were immediately frozen in liquid nitrogen and stored at -80 ºC. The remaining brain material (n=6) were immersed in RNA later™ solution (Ambion, Austin, TX, USA) and then immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction and gene expression analysis. Animals were submitted to treadmill protocol until the day before sacrifice to avoid physiological adaptation to training interruption.

4.3.5 RT-qPCR gene expression

To evaluate the effect of MPTP and PE on RAGE-network neuroimmunomodulation, we performed a comprehensive transcriptomic approach aimed to address the following parameters: ligands (S100B, HMGB-1) and PRR's (RAGE, TLR 1-11) and its adaptor molecules (e.g. MyD88, TIRAP, mDia1) which act as important messengers to activate downstream transcription factors (e.g. NF-κB) and effector molecules including cytokines (e.g. TNF-α) and phagocytic oxidase enzymatic complex (e.g. NADPH oxidase p22-subunit). Glucocorticoid receptor (GR) and DJ-1 genes expression, readouts of corticosterone and oxidative stress, were also measured (Figure 4.2).

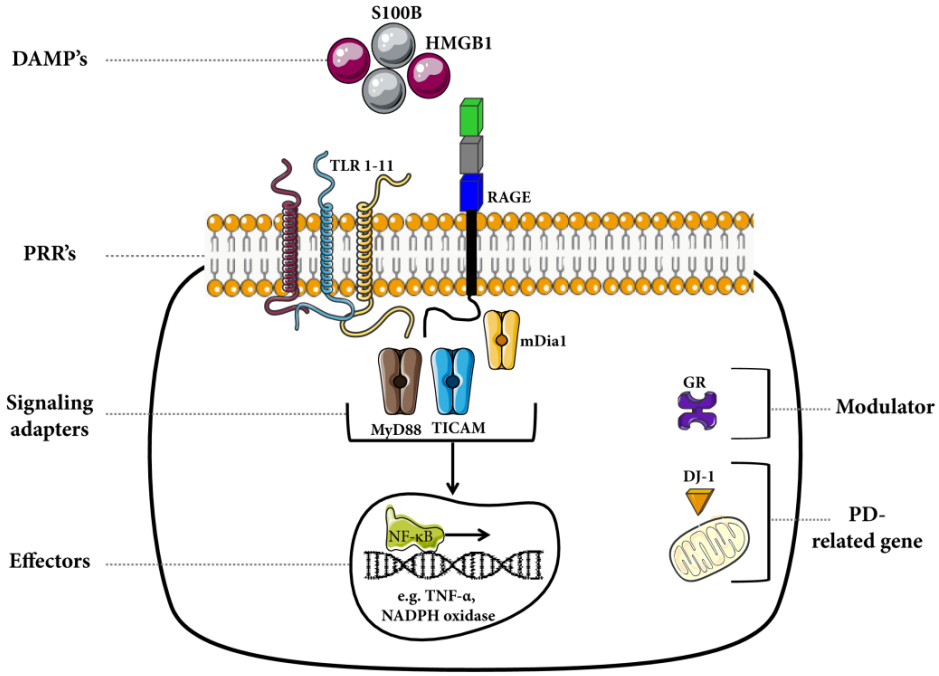

Figure 4.2 RAGE-network neuroimmune gene transcriptomic assay. This pre-plated panel was designed to assess mRNA levels of RAGE neuroimmune-related genes: danger associated molecular patterns (DAMP's) HMGB-1 and S100B, which are recognized by pattern recognition receptors (PRR's), namely RAGE and TLR1-11. These receptors recruit intracellular adapters (e.g. MyD88, TIRAP, mDia1) to evoke intracellular signaling, activating NF-κB transcription factor and subsequent target genes (e.g. TNF-α, NADPH oxidase). Glucocorticoid receptors (GR) and DJ-1 related PD gene were also assessed

Expression profile of neuroimmune-related genes were assayed with a pre-plated 384-well gene expression panel designed by Roche (Configuration number 100079184, Roche Diagnostics, Mannheim, Germany; Primer sequences in Table 4.2).

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Table 4.2 Primer sequences used in RT-qPCR analysis

ASSAY ID	GENE	FORWARD_SEQUENCE	REVERSE_SEQUENCE
506129	**S100B**	AAGGGCCACTGAGATGTGTC	CTAGGCACCAGCAGGTCAG
506140	**HMGB-1**	AACCTGATGCAGCGAAAAAG	CGTCGTCTTCCTCTTCCTTC
503530	**RAGE**	CCTGGGTACTGGTTCTTGCT	ATCCGGGCTGTGATGTTC
506092	**TLR1**	CCTGCCCATATGCAAAGAGT	AACCGCTCAATCCCAGAAA
505424	**TLR2**	CAGATGGCCAGAGGACTCA	CAAAGAGCTTGCAGCATCC
506075	**TLR3**	TGTCATCAAATCCACTTAAAGAGTTT	GGTTCAGTTGGGCATTGTTC
500605	**TLR4**	GGATGATGCCTCTCTTGCAT	TGATCCATGCATTGGTAGGTAA
500833	**TLR5**	GTACGGTTGTCACCACATCG	TCCCCAGCTCCAGTAGCA
500916	**TLR6**	CACAGGGCCAGGAACATC	AGACAAAAGCATGGAACTGGA
506131	**TLR7**	TCCAGACTCCTTCCATAGGC	CCCCAGTAGAATAGGTACACAGTTG
506148	**TLR8**	TCGGCAGAGGATCTGTAAGAG	AAACAAGTTTTCCGCTTTGG
506094	**TLR9**	GACTGCAACTGGCTGTTCCT	GATGTTGGAACGGGGTTCT
506141	**TLR10**	GCACTGAGAAAAGCTATAAGTCCA	ATTATGGCACCACCCATTCT
506138	**TLR11**	CCAGAAGGACTCTTTAGCACAAA	CCACGGGGTATTTCACAGAG
500764	**MyD88**	CCGTGAGGATATACTGTATGAACTG	TTTCTGCTGGTTGCGTATGT
506144	**Ticam-2**	CTCTGGCTGGAGCAGGTC	CGCTAGGATGCTTTGTGGTAT
506088	**mDia1**	TTTGCCAAACTTACCCTTGC	CACCTTCTTGATCCTTCTTGGT
500911	**p105-NF-κB subunit**	ACTGCTCAGGCCCACTTG	TGTCATTATCTCGGAGCTCATCT
502875	**TNFα**	GCCGATTTGCCATTTCATAC	AAGGGCTCTTGATGGCAGA
503279	**p22-NADPH subunit**	TGCCAGTGTGATCTACCTGCT	CTTGGGTTTAGGCTCAATGG
506136	**DJ-1**	CCGCTGCTCAGGAATCTC	TTGGGGAACTGTTTATTCTACTAGC
500529	**Glucocorticoid receptor**	AAAGCTTCTGGACTCCATGC	TCAATACTCATGGTCTTATCCAAAAA
502300	**18S rRNA**	ATCCATTGGAGGGCAAGTCT	AACTGCAGCAACTTTAATATACGC
503799	**GAPDH**	CATCGTGGAAGGGCTCAT	CGCCACAGCTTTCCAGAG
502311	**Ywhaz**	CTACCGCTACTTGGCTGAGG	TTCTTGGTATGCTTGCTGTGA

RNA extraction, evaluation of RNA integrity and purity and cDNA synthesis were performed as previously described (Viana *et al.* 2016). Gene expression was performed by Real Time quantitative Polymerase chain Reaction (RT-qPCR) using LightCycler 480 II system (Roche Diagnostics, Mannheim, Germany): one microgram of total cDNA was mixed with 2x LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany) and Water (PCR grade Roche Diagnostics, Mannheim, Germany), in a total reaction volume of 10 µl, according to manufacturer's instructions. Lightcycler® 480 II system experimental protocol consisted of 1 cycle of pre-incubation (95ºC, 10 min.), followed by 45 cycles of amplification (Thermal profile: 95ºC, 10 sec; 60ºC, 30 sec; 72ºC, 1 sec) and 1 cycle of cooling (40ºC, 30 sec). Non-template

control reactions were performed for each gene, in order to assure no unspecific amplification. Tyrosine 3-monooxygenase (Ywhaz), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and18S rRNA sequence (18S rRNA) were used as endogenous reference (ER) genes to normalize RNA sample variation. RT-qPCR results were analyzed with qbase$^{PLUS\ 3.0}$ software with implemented geNorm^{PLUS} algorithm (Biogazelle, Gent, Belgium) for evaluation of ER genes expression stability. This software provides the two most stable reference genes or a combination of multiple stable genes by calculating a control gene stability measure M (average expression stability) and a coefficient of variation (CV) based on the geometric mean of candidate endogenous reference genes. The relative expression ratio of each of the target gene was computed based on its real-time PCR efficiencies (E) and the crossing point difference (ΔCq) for an unknown sample versus a control ($E^{\Delta Cq}$ method). In order to compare samples from different runs to each other, an inter-run calibration step was introduced by measuring three samples (called inter-run calibrators – IRCs) in each run, together with samples that are different across the runs. Results were obtained in CNRQ (calibrated normalized relative quantities) and then converted to fold changes using control group (Sedentary saline animals) as reference.

4.3.6 Statistical analysis

Shapiro Wilk test was used to analyze data normality. Data from behavioral experiments and rats body weight were compared with two-way analysis of variance (ANOVA) with repeated measures (body weight and cued version of water maze) and Newman-Keuls post-hoc and expressed as mean ± S.E.M. For gene expression analysis, we pre-established that 20% of variability on gene expression fold changes could be explained by natural inter-individual biological variance. Hence, only fold changes > 1.2 or < 0.8 in the estimated population mean expression were considered to have biological meaning and were submitted to further statistical analysis (Mann-Whitney test). Data was expressed resorting to a non-parametric confidence interval obtained by a bootstrapping method with 1000 samples. The 95% confidence interval was defined taking the 2.5 and 97.5 percentiles from the distribution of the means values originated in the bootstrapping algorithm. The null hypothesis was rejected at 0.05 significance level. Analyses were performed with the GraphPad Prism® v5.0 (Graphpad Software, La Jolla, CA, USA), IBM® SPSS®v20 and Matlab™ R14 (Mathworks).

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4.4 Results

4.4.1 The effect of PE and MPTP on animals' body weight and stress-related parameters

Since chronic exposure to environmental stressors may impact nearly all the body's homeostatic mechanisms including body weight control (Hau & Schapiro 2011), body weight and clinical signs of animal well-being were monitored throughout the entire experiment. Figure 4.3 shows that animals' body weight significantly increased over time [$F(15,200)=2.13$, $p<0.01$)], according to the normal growth rate curve of Wistar rats provided by supplier. This effect is independent of both MPTP and PE [$F(3,200)=1.55$, $p>0.05$)]. Other stress-related parameters were monitored, including decreased spontaneous activity, porphyrin rings around the eyes, disturbed pelage as ruffled fur, abnormal gait or posture, but were also not observed.

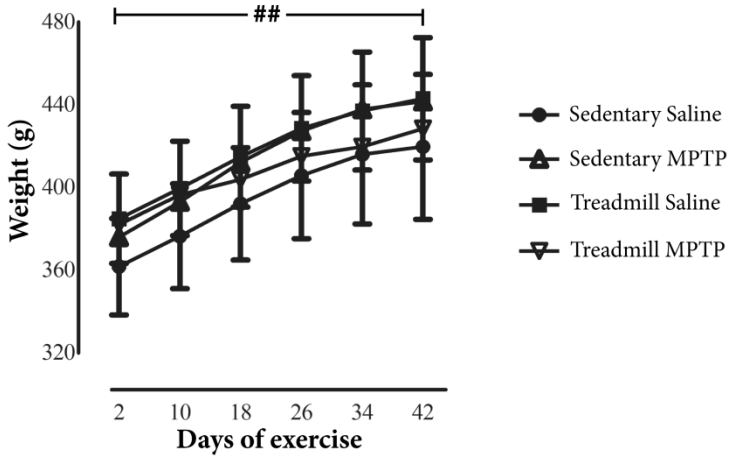

Figure 4.3 Body weight variation. Two-way Anova (repeated measures) shows that animals body weight (11 animals/experimental group) significantly increased over time [$F(15,200)=2,13$, $p<0.01$)], an effect independent of MPTP or physical exercise [$F(3,200)=1,55$, $p>0.05$)]. Results are expressed as mean ± S.E.M. ##$p<0.01$ when comparing day 2 with day 42 in each experimental group

4.4.2 The effect of PE on cognitive and emotional behavior in MPTP-treated rats

Open field test, cued version of water maze test and splash test were performed to investigate putative changes in behavioral features in our experimental setting. Two-way ANOVA analysis indicated no significant effects of MPTP or treadmill-training on spontaneous locomotor activity, evaluated by distance travelled in open field arena [sedentary:

F(1,39)=0.054, p>0.05; treadmill: F(1,39)=0.295, p>0.05), Figure 4.4a]. We then focused on NMS that occur early in PD. In this regard, we found distinct cognitive and emotional performances between groups. Figure 4.4b shows that all animals learned the cued-version of water maze task, improving the mean escape latency throughout training sessions. However, while sedentary-MPTP animals spent more time finding the platform in all trials [F(1,39)=61.499 p<0.001, Figure 4.4b], exercise boosted memory of rats by alleviating MPTP-induced cognitive impairment, with a significant exercise X MPTP interaction observed in Two-way ANOVA with repeated measures [F(3,117)=0.596, p<0.05, Figure 4.4b). Intranasal MPTP also induced emotional/motivational impairments, assessed by the splash test. A decreased time spent grooming [F(1,39)=3.895, p<0.05, Figure 4.4c] and a trend to increased latency and decreased number of grooming (Figure 4.4d,e) were observed in sedentary MPTP-treated animals. Noticeably, treadmill training restored normal grooming time in MPTP-rats, as shown by a significant Treadmill X MPTP interaction in grooming time observed in Two-way ANOVA [F(1,39) = 1.308, p<0.05, Figure 4.4c].

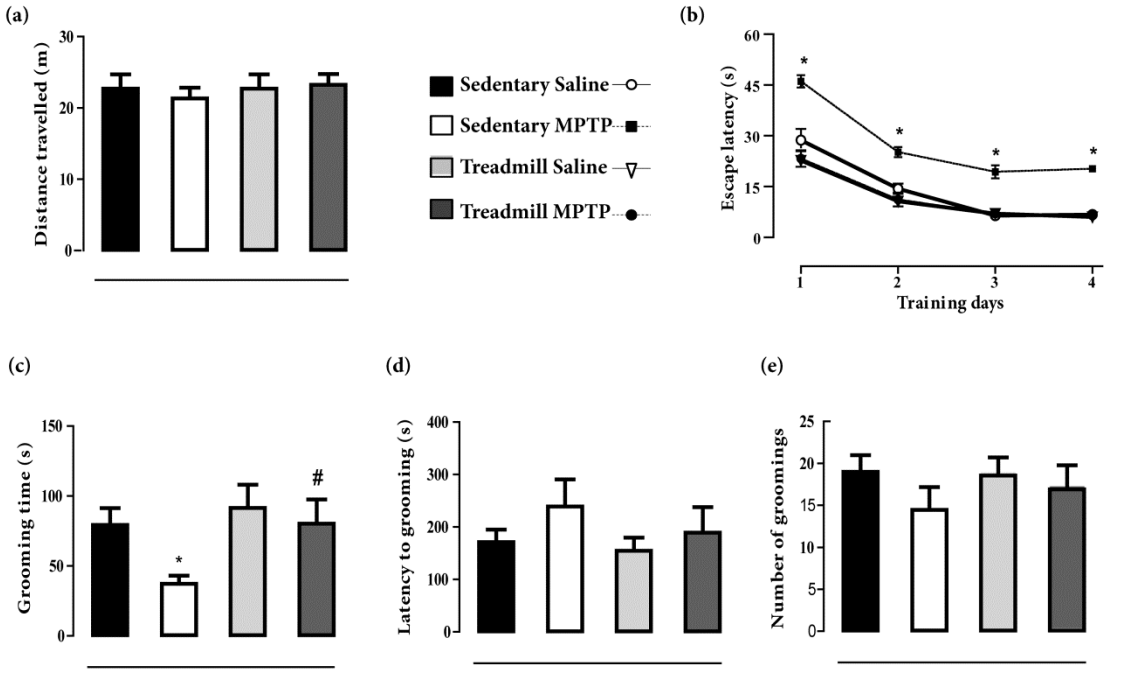

Figure 4.4 Effect of MPTP and treadmill-training on behavioral tests. Experimental groups show similar spontaneous locomotor activity, assessed by Open field test (a). Yet, treadmill training alleviated procedural memory impairments displayed by i.n. MPTP-treated animals in the Cued-Version of Water-maze (b). Intranasal MPTP had a negative impact on emotional/motivational behavior, estimated by a significant decrease on the time spent grooming on the Splash test (c), without changing the latency or number of grooming(s) (d,e, respectively). Remarkably, treadmill training reversed emotional/motivational deficits, as evidenced by the normal performance of treadmill MPTP animals on grooming time on the splash test. Tests were performed in 11 animals/experimental group. Results are expressed as mean ± S.E.M. *p<0.05 compared with sedentary saline and #p<0.05 compared with sedentary MPTP (Two-way ANOVA)

4.4.3 The effect of PE on neuroimmmune transcriptomic profile in MPTP-treated rats

We then interrogated the effect of MPTP and PE on the expression of RAGE neuroimmune-related genes in frontal cortex, hippocampus and striatum. Considering that underestimation of inter-individual biological variance often leads to the identification of "noisy genes" which are falsely identified as differentially expressed (statistically significant) but lacking biological meaning, we established gene expression variations > 20% to predict any biological outcome (Cheng *et al.* 2012, Storey *et al.* 2007). Heat map diagrams (Figure 4.5 a-c) clearly show that both MPTP (SM) or Treadmill-training (TS) conditions exert a regional effect on RAGE-network transcriptomic profile, with significant effects exclusively observed in frontal cortex. These effects are broadly linked with neuroimmune gene repression. However, glucocorticoid receptor (GR) expression was unchanged in all experimental conditions (Figure 4.5 a-c).

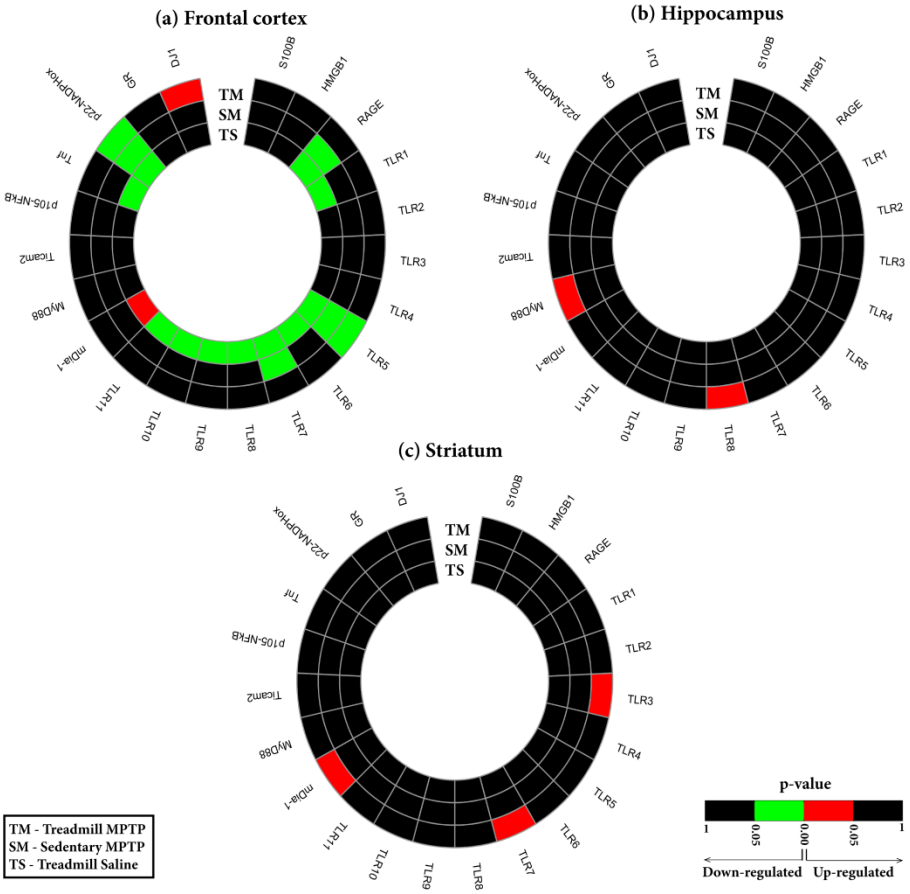

Figure 4.5 Heat maps diagrams showing neuroimmune transcriptomic profile with an absolute fold-change > 20% in frontal cortex (a), hippocampus (b) and striatum (c) in all experimental groups. Calibrated normalized relative quantities of each analyzed gene were used for the analysis (n=4-6 samples/each experimental group) using untrained-saline group as reference. The color scale illustrates the level of significance achieved for the fold of variation observed in each targeted gene. Red indicates up-regulated genes and green indicates down-regulated genes as compared with reference (dark), analyzed using Mann-Whitney test

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Importantly, a distinct transcriptomic profile is observed when both interventions are combined (TM). Cortical repression of RAGE and large majority of neuroimmune-related genes is no longer corroborated by statistical evidence in the Treadmill-MPTP subjects (Figure 4.5a:TM group). In contrast, hippocampi and striata regions which have been spared from MPTP and treadmill-training neuroimmune-modulatory effects show a significant up-regulation of TLR3,7 and mDia1 (striata) and TLR8 and MyD88 (hippocampi) genes in Treadmill-MPTP rats (Figure 4.5b,c:TM group).

It is noteworthy that neuroimmune-modulatory properties of MPTP and treadmill-training observed in frontal cortex display distinct magnitudes, as underlined in radar chart presentation (Figure 4.6a). While MPTP elicited a significant decrease in RAGE, TLR5,7 and NADPH oxidase p22-subunit gene expression (Figure 4.6b, $p<0.05$), PE triggered a more robust immunosuppressive profile with a strong repression of RAGE, large majority of PRR's (TLR1,5-11) and effectors TNF-α and p22-NADPH oxidase subunit gene expression (Figure 4.6c, $p<0.05$). This pattern was not followed by the signaling adaptor mDia-1, who showed a significant up-regulation (Figure 4.6c, $p<0.05$). Recovery of RAGE and large majority of neuroimmune-related genes expression in MPTP-treadmill training animals (exceptions are TLR5 and NADPH p22-NADPH oxidase) and DJ-1 over-expression is further depicted in Figure 4.6d.

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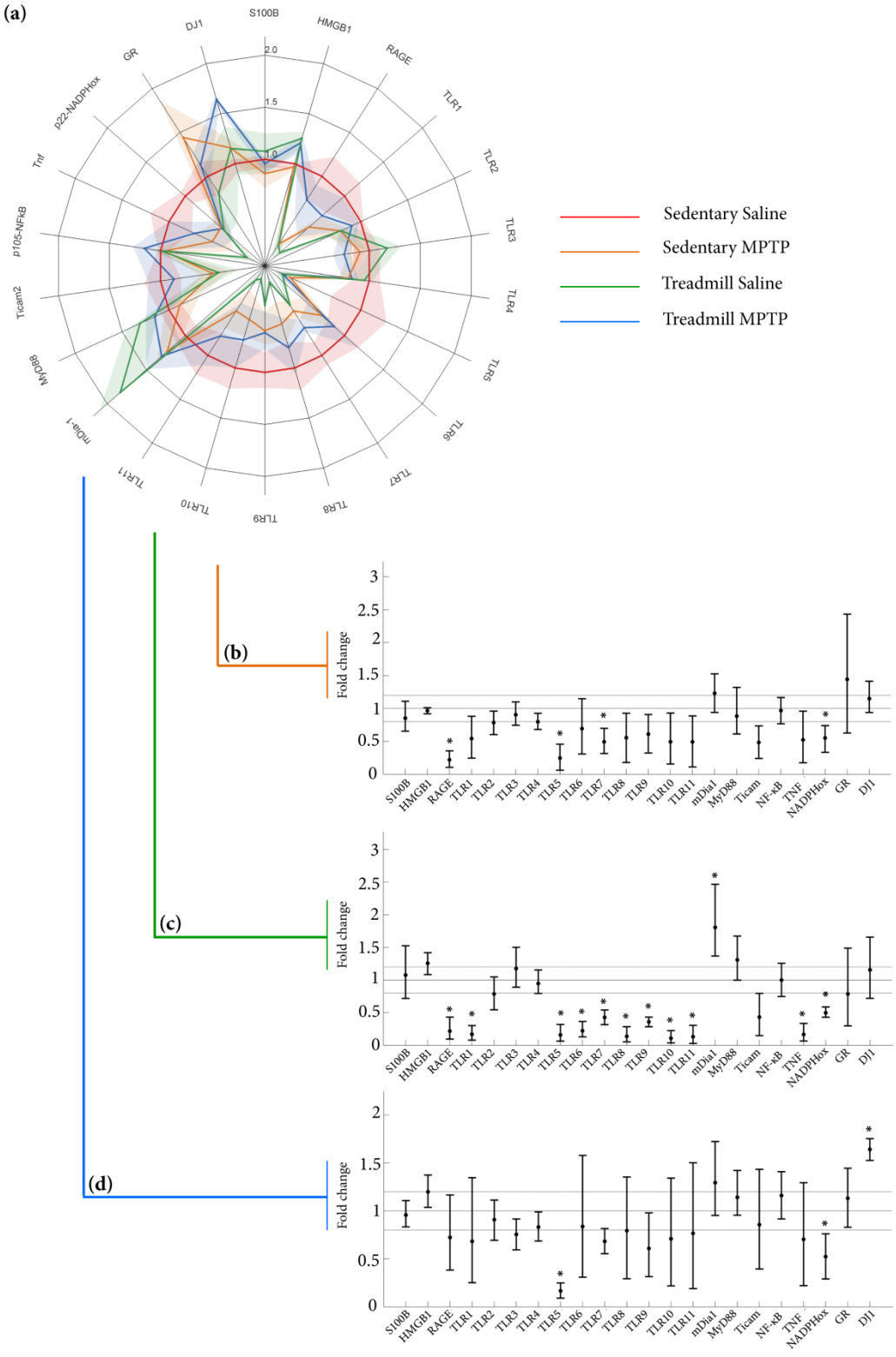

Fig. 4.6 Radar chart presentation of RAGE-neuroimmune transcriptomic profile in frontal cortex (a) displaying the mean value and confidence bands for each gene (n=4-6 samples/each experimental group). Each radial axis in the chart represents the fold of variation in relation to sedentary saline group (control, depicted at red). Scatter plots depicting the fold changes of each analyzed gene in sedentary MPTP (b), treadmill saline (c) and treadmill MPTP (d) conditions. Calibrated normalized relative quantities of each analyzed gene were used for the analysis (n=4-6 samples/each experimental group) using sedentary saline group as reference. Results are expressed as mean ± 95% confidence interval and were analyzed using Mann-Whitney test. *P<0.05 compared with sedentary saline experimental group

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4.5 Discussion

Treadmill training may halt or slow down non-motor symptoms in early stages of experimental Parkinson's disease

The first purpose of this study was to provide experimental evidence supporting the cognitive- and emotional-enhancing effects of PE in a preclinical animal model of PD, analogous to what is generally accepted in other cognitive and neuropsychiatric disorders (Eyre *et al.* 2013, Gleeson *et al.* 2011). To this end, we used the i.n. MPTP model of PD that has been extensively used by Prediger and collaborators (Prediger *et al.* 2006, Prediger *et al.* 2009, Prediger *et al.* 2012, Castro *et al.* 2013). Twelve days following MPTP insult, untrained MPTP-treated rats did not present gross motor alterations but scored worse than untrained-saline group in the cued version of water maze and in splash test. These behavioral deficits were previously reported in rats submitted to i.n. MPTP and are aligned with striatal dependent habit learning disruption and emotional/motivational deficits such as apathetic behavior, that reflects inefficient cognitive strategies in early stages of PD (Prediger *et al.* 2006, Prediger *et al.* 2009, Varanese *et al.* 2011, Prediger *et al.* 2012, Castro *et al.* 2013).

To address potential PE-positive effects in NMS of experimental PD, we implemented a treadmill training 30 days prior to MPTP lesion that persisted for eleven days post-insult, thereby including both neuroprotective and neurorehabilitive effects. MPTP-treated rats that were assigned to treadmill displayed restored cognitive and emotional functions, as shown by normal performances scored in cued version of water maze and in splash test. These observations support and extend previous studies suggesting PE as a valuable approach to improve NMS in experimental PD (Aguiar *et al.* 2016, Gorton *et al.* 2010, Klein *et al.* 2016).

Repression of neuroimmune RAGE network transcriptomics occurs in frontal cortex – is this mechanism associated with PD early compensation?

There is a growing body of evidence showing that PE has modulatory properties on RAGE and TLR's networks in distinct pathological settings (Al-Jarrah & Jamous 2011, Gu *et al.* 2014, Ma *et al.* 2013). Therefore, we hypothesized similar mechanism would co-occur with PE-beneficial effects on MPTP-cognitive and emotional impairments. Hence, we went through a comprehensive transcriptomic approach of neuroimmune RAGE network comprising ligands,

PRR's, signaling adapters and key-effectors of innate immune response in frontal cortex, hippocampus and striatum. Surprisingly, both experimental variables (MPTP and PE) elicited a selective repression of RAGE-neuroimmune network transcriptomics in frontal cortex.

Regarding MPTP, we first questioned whether this regional effect was related with any special feature of MPTP kinetics within frontal cortex. This proneurotoxin is converted into its active metabolite (MPP^+) in astrocytes and subsequently released into extracellular space to be essentially up-taken by dopamine transporter (DAT) in dopaminergic neurons. Although not fully elucidated, organic cationic transporters (OCT's) seem to play a role in astrocytic MPP^+ extrusion (Cui *et al.* 2009). Nevertheless, low density of OCT's (1,2,3) in frontal cortex and the absence of OCT3 in rat cortical astrocytes seem to exclude a preferential impact of MPTP in frontal cortex (Cui *et al.* 2009, Gasser *et al.* 2009). In addition, MPP^+ reaches almost all brain areas after i.n. MPTP administration and remains particularly concentrated in some brain regions (e.g. basal ganglia, hippocampus, ventral mesenchepalon) but not in frontal cortex, in C57BL/6 mice (Kadar *et al.* 2014). Additionally, lower density of DAT in frontal cortex as compared with striatum may further contribute to ruling out a putative MPP^+ cortical preferential accumulation (Sesack *et al.* 1998).

Although we currently ignore the mechanisms that govern this selective gene effect, our findings are aligned with Storvik and colleagues work (2010) who observed a specific regional transcriptomic profile in prefrontal cortex in early stages of progressive MPTP monkey model. These authors suggest that PD transcriptomic changes may be related with compensatory mechanisms occurring in prefrontal cortex that delay the onset of motor abnormalities and/or prodromal PD manifestations (Storvik *et al.* 2010). Regarding prodromal PD manifestations, we highlight that mild cognitive dysfunctions and anhedonic and apathetic-like behavior - typical prodromic PD features - are related with activation (and not repression) of neuroimmune system and inherent pro-inflammatory profile (Eyre *et al.* 2013, Dantzer *et al.* 2008). Thus, down-regulation of RAGE, TLR5,7 and NADPH oxidase p22-subunit upon MPTP seen herein is probably linked with repression of cytokine and oxidative signaling pathways that become chronically activated in overt PD, and could be interpreted as a compensatory cortical mechanism (Anantharam *et al.* 2007, Mosley *et al.* 2012). In fact, considering that prefrontal cortex receives massive projections from basal ganglia (Storvik *et al.* 2010), ongoing striatal alterations (highlighted by striatal-dependent habit learning perturbations) could be detected by

frontal cortex and trigger immune cortical alterations. Nonetheless, this frontal cortical adaptive immunosuppression was not sufficient to prevent behavioral deficits in our PD model. Therefore, other brain mechanisms and brain areas (e.g. hippocampus) engaged in cognitive and emotion regulation are likely to be strongly impacted by MPTP, thus contributing to the phenotypic deficits observed herein.

One of the most impressive findings of the present work is the selective and robust immunosuppression in frontal cortex of PE animals: repression of RAGE, large majority of PRR's and downstream effector molecules are particularly noteworthy. Trained-saline animals didn't show any abnormal behavioral feature**.** This suggests that neuroimmune gene repression is not sufficient to trigger cognitive or emotional behavioral deficits**.** Given that physical exercise regulates glucocorticoids signaling which is intimately related with immune suppression (Coutinho & Chapman 2011), we asked whether any particular adaptative changes in frontal cortical GR gene expression could underlie immunosupression seen herein. There were no significant changes in GR gene expression in any brain region from any experimental group. Moreover, chronic treadmill protocols are reported to maintain normal plasmatic corticosterone levels (Bayod *et al.* 2011, Inoue *et al.* 2015). Therefore, glucocorticoids are not likely responsible for immunosuppression in our experimental context. Although one currently ignores the causes underpinning PE-regional immunomodulatory effect, benefits from aerobic exercise in functional integrity of frontal cortical areas are widely reported (Harada *et al.* 2004, Kemppainen *et al.* 2005, Voss *et al.* 2010).

Perturbations of nigro-striatum-thalamus-cortical circuits that interconnect striatum with prefrontal cortex are critically related to PD executive processes derangements and with apathy which is recapitulated in this i.n. MPTP model (Tanaka *et al.* 2009, Varanese *et al.* 2011). Noticeably, PE was shown to improve cognition in PD patients by ameliorating frontal lobe based executive function (Cruise *et al.* 2011). Herein, we report restored cognitive and emotional/motivational performances of trained-MPTP animals paralleling frontal cortical adaptative neuroimmune transcriptomic changes linked with restoration of RAGE expression and with positive management of oxidative stress responses (e.g. repressed and enhanced NADPH oxidase p22-subunit and DJ-1 expression, respectively). In terms of neuroprotective PD-related DJ-1 gene up-regulation, we highlight that DJ-1 is a redox-sensitive protein that dampens mitochondrial oxidative stress, regulates anti-apoptotic, antioxidative gene expression

and glial inflammatory responses, to name just a few actions. In fact, DJ-1 point mutations are linked to PARK7 autosomal-recessive PD, where DJ-1 protein loss of function effectively deprive cells of its inherent cytoprotective and antioxidant signaling (Milani *et al.* 2013).

Even though a proper understanding of innate immune system within brain parenchyma is still in its infancy, frontal cortex is the brain region more extensively studied in CNS pathologies linked with innate immunity dysregulation (e.g. major depressive disorder, schizophrenia, addiction) (Kim *et al.* 2016a, Pandey *et al.* 2014, Vetreno *et al.* 2013). Importantly, this brain region showed higher responsiveness (when compared with brain hippocampus and striatum) upon lipopolysaccharide-induced innate immune activation (Noh *et al.* 2014). Similarly, our data suggests that frontal cortical region is a very immunosensitive region in terms of neuroimmune-RAGE network when submitted to chemical or physical stressors (MPTP and PE, respectively). Whatever the cause is, selective repression of RAGE and related-neuroimmune genes upon MPTP lends further support to the notion that active changes occur outside basal ganglia in the prodromal PD phase. Moreover, present report newly identify frontal cortical neuroimmune RAGE-network as a neural substrate of PE in both physiological and experimental PD-settings.

Neuroimmune RAGE network transcriptomic adaptation: an hormetic response towards increased brain tolerance?

Albeit chronic proinflammatory innate glial response is a PD hallmark (Mosley *et al.* 2012), a time-dependent kinetics of PRR's expression and downstream effectors seems to occur in experimental PD: while MPTP up-regulates RAGE, TLR's expression and subsequent downstream production of pro-inflammatory cytokines (e.g. TNF-α) in the acute phase of MPTP intoxication (Ros-Bernal *et al.* 2011, Sathe *et al.* 2012, Viana *et al.* 2016, O'Callaghan *et al.* 2008), a subsequent prominent long-lasting repression of RAGE transcription was found in mesencephalon of MPTP-treated mice (Teismann *et al.* 2012). This is consistent with the repression of RAGE and related neuroimmune genes observed in frontal cortex of untrained-MPTP animals 12 days post-insult. As mentioned before, such PRR's gene expression adaptation upon MPTP is likely to be engaged in immunological feedback mechanisms aimed to self-limit chronic inflammation. This is further supported by normal inflammatory profile observed in distinct brain regions 21 days after i.n. MPTP (Castro *et al.* 2013). This biphasic

phenomenon could be envisioned as 'hormesis': an universal adaptive stress-response driven by sub-lethal doses of a stressor, resulting in favorable biological adaptations aimed to enhance defense, repair and protection against subsequent stressful and deleterious events (Calabrese 2016, Mollereau *et al.* 2016). Such highly conserved process declines with age and age-related chronic disorders such as PD ensue (Della-Morte *et al.* 2013). In fact, great efforts are being made to restore the hormetic-priming that is lost during aging. For example, physical exercise is one of the few successful approaches (Calabrese 2016, Voss *et al.* 2010).

Remarkably, the notion of hormesis underlies much of what is known about how exercise conditions the body to improve tolerance to stressors (Bayod *et al.* 2011, Radak *et al.* 2008). In this regard, a large number of studies broadly recognize PE ability to prime innate immune cellular activity towards a peripheral anti-inflammatory profile (Gleeson *et al.* 2011, Gleeson *et al.* 2006, Lancaster *et al.* 2005, Neubauer *et al.* 2013, Ostrowski *et al.* 1999). These authors showed a similar biphasic inflammatory response: an initial inflammatory response triggered by distinct stressors exercise-driven (e.g. thermal, metabolic, oxidative, mechanical) is promptly balanced by increased cytokine inhibitors (IL-1ra, sTNF-r1), anti-inflammatory cytokines (IL-10), potent immune negative regulators of TLR's (e.g. IRAK3) and repressed TLR's cell surface expression that restrict the magnitude and duration of inflammation at both transcriptional and translational level. This anti-inflammatory profile, combined with other physiological adaptations, underpins the protective nature of regular exercise against the development of various chronic diseases (Gleeson *et al.* 2011). Although experimental data focused on the impact of PE on brain innate immunity is extremely scarce, inhibition of receptors and/or effectors of inflammatory pathways are suggested to be a common mechanism by which different preconditioning (PC) paradigms (e.g. exercise, ischemic and endotoxin-induced PC) improve brain tolerance to stressors (Garcia-Bonilla *et al.* 2014, Kariko *et al.* 2004, Ma *et al.* 2013, Cadet & Krasnova 2009). Likewise, we postulate that the preconditioning stimulus (treadmill-training) employed in the present study was able to recruit immune feedback inhibitors aimed to accelerate homeostasis restoration when a second insult (i.n. MPTP) was imposed. PE post-conditioning isolated effects were not evaluated in present study. Nevertheless, PE putative restorative properties likely cooperated with PE pre-conditioning hormetic priming effects. It is tempting to suggest that this immunomodulatory action of PE contributes to its cognitive and emotional enhancing properties.

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Finally, a clear explanation for the distinct neuroimmune profiles observed in striatum (TLR3,7 and mDia1 up-regulation) and hippocampus (TLR8, MyD88 up-regulation) in trained-MPTP animals is lacking and difficult to provide. To begin with, one should consider that differences in cytoarchitecture and organization between three studied brain regions could be held responsible for distinct immunological responses. Moreover, an intricate dysregulation of distinct neuroimmune mediators with opposing effects was observed in different brain regions at the same stages of disease progression in *post-mortem* PD samples, revealing a new scenario that is even more complex than formerly envisaged (Garcia-Esparcia *et al.* 2014).

Concluding remarks

To our knowledge, this is the first report dedicated to a comprehensive transcriptomic profile of RAGE ligands, TLR's co-receptors and downstream related-molecules in distinct brain regions in the context of PE-potential benefits in PD. In fact, we newly demonstrated a similar pattern of transcriptomic response (down-regulation) between RAGE, some TLR's and downstream effectors, which suggests an integrated immune response. Although this seemingly PE-immunological hormetic priming mechanism could be related to recovered emotional/cognitive performances in trained-MPTP animals, other PE-triggered brain mechanisms could also be instrumental in our experimental setting: e.g. enhanced hippocampal neurogenesis, increased neurotrophic factors, modulation of striatal monoaminergic neurotransmission and correction of fronto-striatal connectivity (Aguiar *et al.* 2016, Inoue *et al.* 2015, Klein *et al.* 2016, Voss *et al.* 2010). Besides, exercise increases brain blood flow, which may influence the vasculature of CNS through angiogenesis. Hence, a more effective delivery of peripheric signaling molecules (e.g. insulin-like growth factor-1) may also contribute to PE-beneficial effects observed herein (Petzinger *et al.* 2013, Nishijima *et al.* 2016). Nevertheless, our findings reflect an important advancement in the research field as experimental data on the impact of PE on brain immune mediators is extremely scarce in physiological contexts, being almost ignored in PD pathology. Hence, we suggest that modulation of central neuroimmune system by PE is a new and promising avenue to be explored in PD. Additional research is warranted to further study the mechanism and the functional consequences of RAGE and TLR's adaptation in frontal cortex associated with physical exercise, particularly in early stages of PD.

Chapter 5

Main findings and general discussion

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Development of a disease-modifying treatment to slow the relentless downward trajectory of neurodegeneration represents the most important unmet medical need in Parkinson's disease (Brundin & Wyse 2015, Zhang *et al.* 2016). Yet, the cloudy understanding of disease pathogenesis and etiology have been deterring the appearance of innovative neuroprotective/regenerative therapies. Recent studies have indicated that receptors for advanced glycation endproducts (RAGE) play an important role in the development of PD neurodegeneration. Moreover, RAGE has been envisaged has a promising therapeutic target in distinct neurodegenerative conditions (Galasko *et al.* 2014, Juranek *et al.* 2016, Teismann *et al.* 2012). However, RAGE biology is complex with both protective/deleterious outcomes arising from RAGE signaling, which is largely dependent upon the: i) activating ligand, ii) identity of RAGE-bearing cells; iii) ratio between RAGE isoforms; iv) recruitment of co-receptors (e.g. TLR's) and v) presence of short-limited versus long-lasting noxious environments. Hence, achieving the optimal balance in therapeutic targeting of RAGE relies on the identification of strategies to retain primal RAGE responses to stress, while derailing deleterious RAGE amplification pathways in mal-adaptive conditions. The broad aims of this Thesis were to provide a global characterization of RAGE biology within PD neurodegenerative process. This chapter firstly summarizes the main findings of present work. Secondly, it provides an overview of study strengths and limitations. Last part focuses on the implications of present findings for therapeutic PD innovative strategies and identifies gaps for further research.

5.1. Main findings

Whatever insult initially provokes neurodegeneration, mitochondrial dysfunction and the consequent burst of oxidative stress have a central role in PD pathogenesis. Thus, mitochondrial toxins-based models have received most attention. Among the used neurotoxins, only MPTP is clearly linked to a form of human parkinsonism, thus being the most widely studied model (Dauer & Przedborski 2003). The first endpoint herein was the following: we aimed to characterize RAGE biology within MPTP-striatal dysfunction, an early event of dopaminergic neurodegenerative process that classically characterizes PD (Przedborski *et al.* 1996). We have used two distinct regimens [acute versus chronic models (chapter 2 and 3, respectively)] to further dissect RAGE dynamics in environments that were submitted to both short-limited

versus long-lasting insults, thereby critically regulating the protective/deleterious RAGE outcomes (Bierhaus *et al.* 2005, Herold *et al.* 2007). Among the great panoply of RAGE ligands, we focused our attention in S100B regarding strong evidences on its involvement in human PD neurodegenerative process and also in MPTP-models (Liu *et al.* 2011a, Sathe *et al.* 2012).

We first hypothesized that environments offering a sustained oxidative setting (Chronic MPTP regimen – Chapter 2) would elicit a feed-forward loop between RAGE ligands accumulation and signaling competent RAGE isoform (flRAGE) up-regulation, fostering striatal glial reactivity and dopaminergic axonopathy. Instead, we provide experimental evidence about neurotrophic S100B levels and basal flRAGE density paralleling striatal dopaminergic compensatory responses (increased dopamine turnover) underlying normal motor function. These findings ruled out a deleterious role of flRAGE in sustaining striatal reactive gliosis and DAergic axonopathy in a pre-motor phase of experimental PD. Rather, they suggest the existence of active compensatory processes tightly regulating S100B/RAGE axis towards homeostasis within striatal injury (see Figure 5.1).

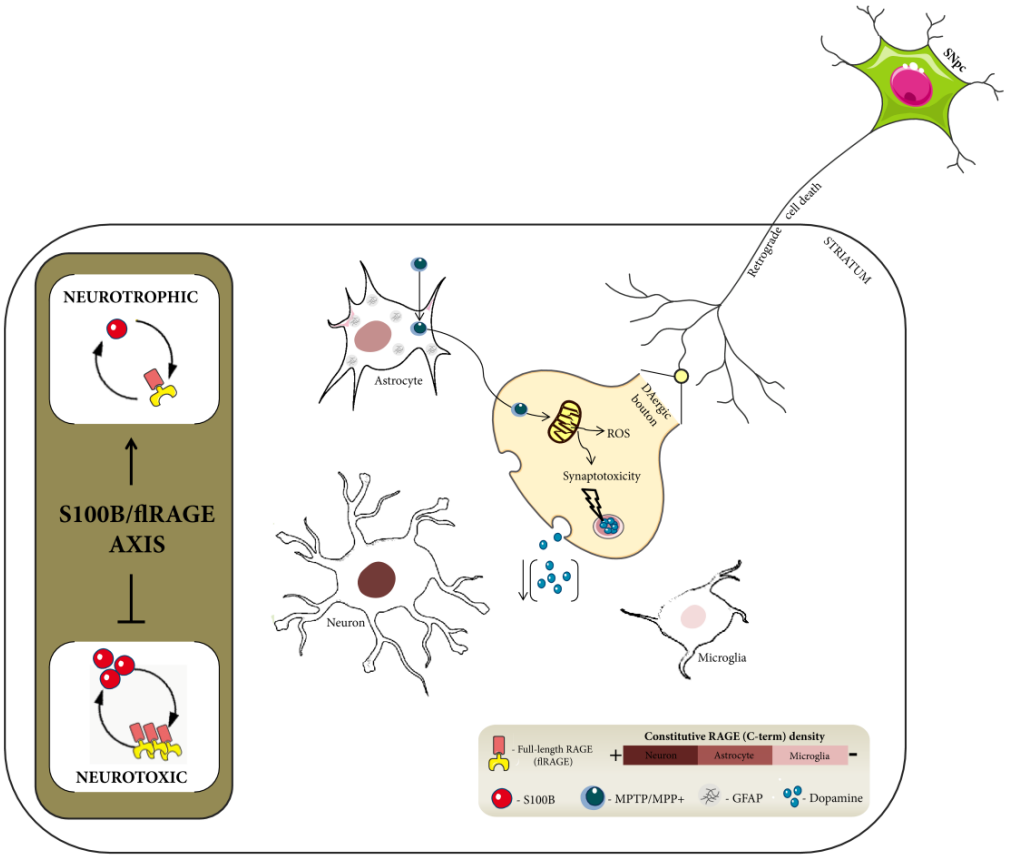

Figure 5.1 We report basal levels of S100B, known to be neurotrophic, along with normal flRAGE contents in striata after chronic MPTP insult. These findings rule out the activation of feed-forward loops between ligands and RAGE up-regulation underpinning sustained reactive gliosis and DAergic axonopathy in this chronic MPTP model

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Our second experimental approach aimed to characterize RAGE biology in the acute phase of MPTP insult (Acute MPTP model – Chapter 3), which is coincident with the onset of cellular and molecular events that ultimately lead to dopaminergic axonopathy and cell death. Following our previous findings about striatal seemingly neurotrophic S100B/flRAGE levels in a chronic MPTP setting, we wanted to dissect the overall balance of RAGE isoforms since subtle shifts in the ratio between signaling competent flRAGE and RAGE inhibitory forms [C-truncated isoforms (e.g. sRAGE)] may determine which role RAGE will be playing: protective or deleterious. Noteworthy, we found increased levels of inhibitory RAGE variants, traditionally endowed with a cytoprotective phenotype (Ding & Keller 2005a), while flRAGE contents were normal in this preliminary stage of MPTP insult. Increased inhibitory RAGE variants occurred in enriched S100B-astrocytes prior to overt astrogliosis (see Figure 5.2).

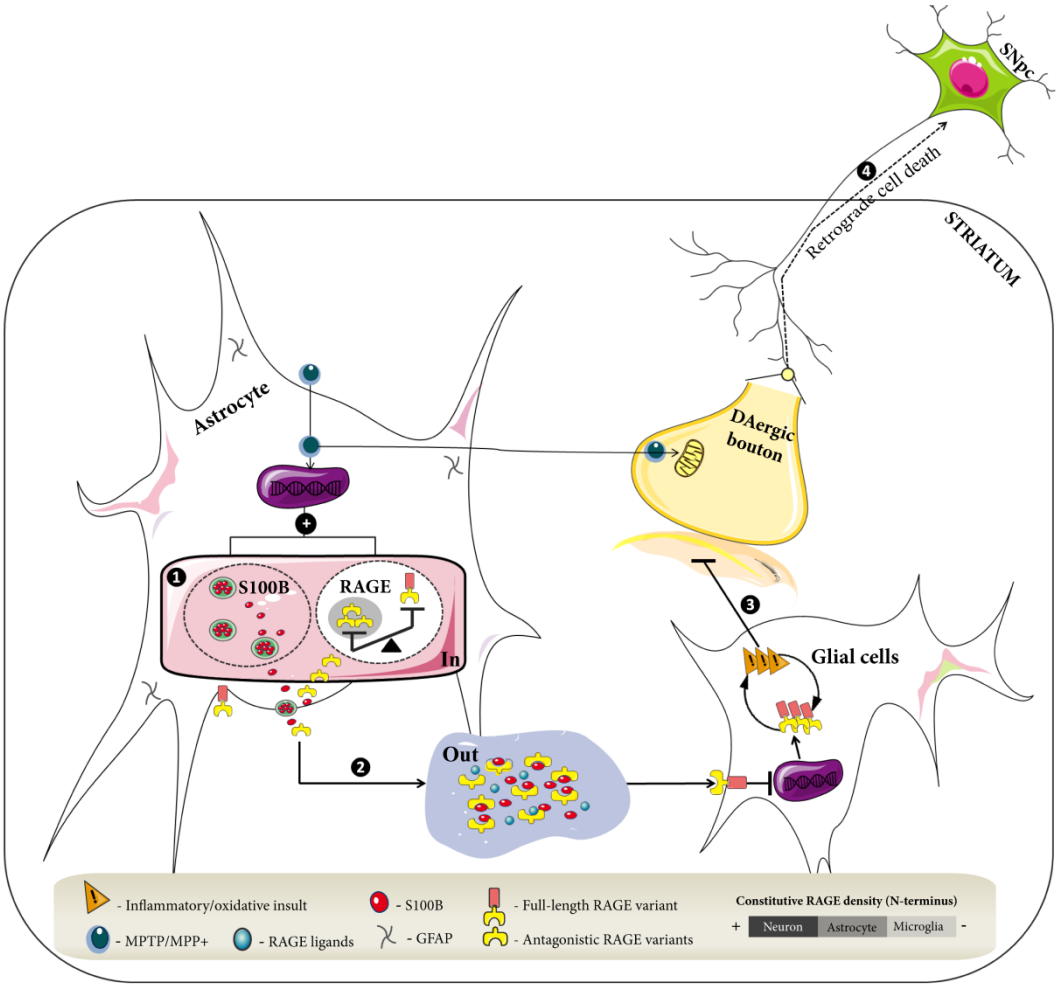

Figure 5.2 We report increased antagonistic RAGE variants paralleling S100B up-regulation in early stages of MPTP-induced astrogliosis dynamics prior to astrocytes hypertrophy (1). We propose that selective RAGE regulation reflects a self-protective mechanism to maintain low levels of RAGE ligands (2), preventing long-term inflammation and oxidative stress arising from sustained ligands/flRAGE activation (3). Understanding loss of RAGE protective response to stress may provide new therapeutic options to halt or slow down dopaminergic axonopathy and, ultimately, neuronal death (4). Astrocyte-RAGE dynamic duo may be a putative therapeutic target in PD that needs to be further explored

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Even though striatal dopaminergic lesion is critically involved in PD motor symptomatology, the degeneration of other neurotransmitter systems (e.g. NE/5-HT) and brain areas during long prodromal PD phase is beyond question (Prediger *et al.* 2011). Thus, the long-standing research focus in nigrostriatal dopaminergic pathway in PD needs to open up into new areas centered in the early stages of PD neurodegeneration associated with non-motor symptoms (Golde *et al.* 2013, Pfeiffer 2016). In the third experimental part of this Thesis (Chapter 4), we aimed to get a global understanding of RAGE biology operating within a central innate immunity network through a comprehensive transcriptomic analysis of ligands, putative co-receptors (TLR's) and intracellular effectors in a pre-motor phase of disease. To this end, we implemented the well-characterized single i.n. MPTP model, known to elicit time-dependent impairments in cognitive, emotional and motor functions similar to the sequence of analogous changes seen in PD (Prediger *et al.* 2011). In cognitive/emotionally impaired MPTP-animals, suppression of RAGE and other core components of innate immunity (e.g. TLR5,7, NADPH oxidase p22-subunit) were found in frontal cortex. This strongly suggests that this parkinsonian neurotoxin triggered a regional orchestrated immune response, which is seemingly engaged in counter-regulatory mechanisms preventing exacerbated innate inflammation and tissue injury. This selective regional transcriptomic profile in frontal cortex of intranasal MPTP intoxication also supports the thesis of active compensatory changes occurring outside basal ganglia in early stages of PD degeneration (Storvik *et al.* 2010).

On the other hand, benefits of physical exercise (PE) in PD NMS are attractive. Among the multitude of CNS mechanisms modulated by PE, the immunomodulatory effects of exercise has garnered a lot of research attention (Gleeson *et al.* 2011). As a second endpoint, we took advantage of this third experimental paradigm to test the hypothesis whereby PE can regulate neuroimmune RAGE network. One of the most impressive findings of present work relies on the robust immunosuppressive profile elicited by long-term treadmill-protocol in frontal cortex, with the repression of RAGE gene transcription, large majority of TLR's and downstream effectors. This finding newly identifies cortical neuroimmune RAGE-network as a substrate of PE in physiological settings. Remarkably, a recovery of RAGE and almost all related neuroimmune genes expression and a significant up-regulation of neuroprotective PD-related gene DJ-1 was accompanied by normal cognitive and emotional/motivational phenotypes in trained-MPTP subjects. Overall, these findings provide new evidence of the PE efficacy to

improve NMS of PD and that this response can be associated with its modulatory role on neuroimmune RAGE network in frontal cortex (see Figure 5.3).

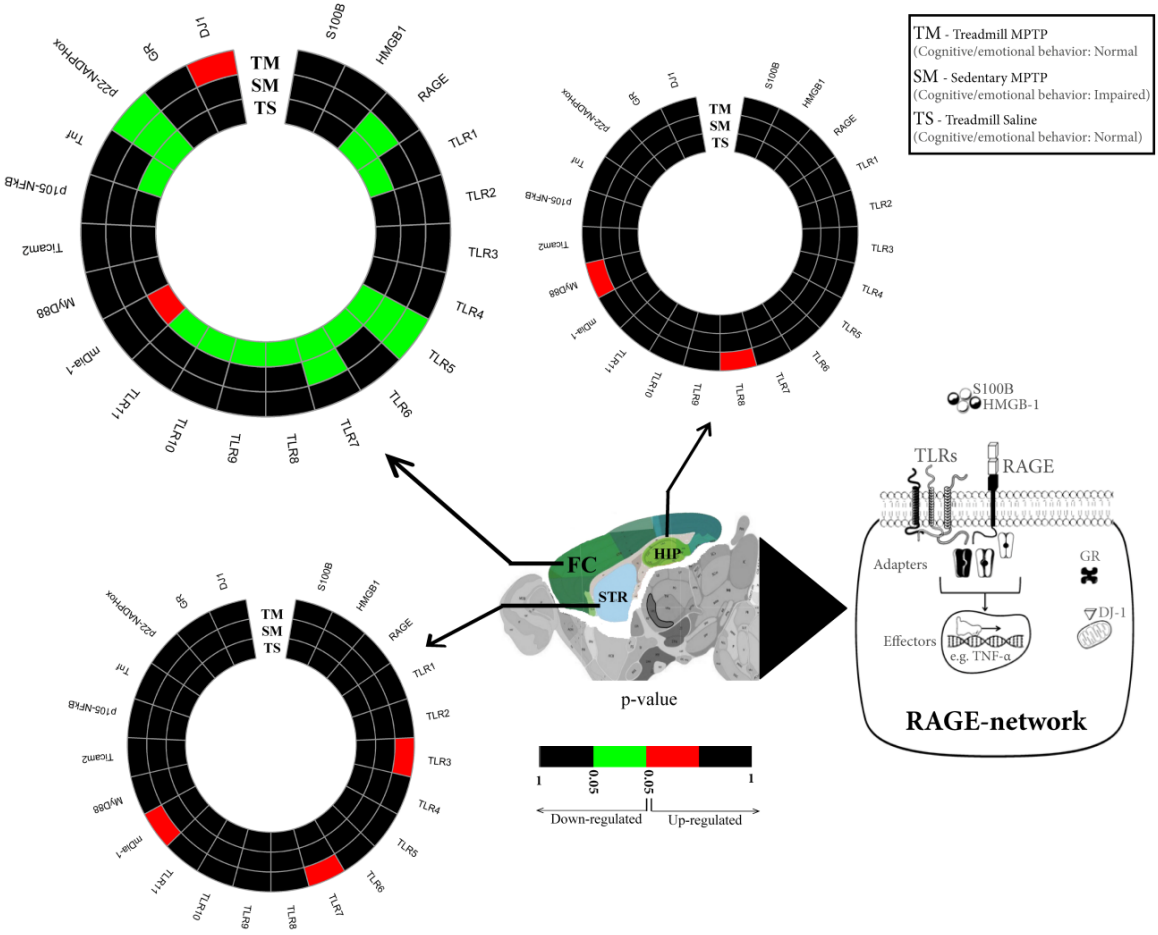

Figure 5.3. We report a regional suppression of neuroimmune RAGE-network in frontal cortex upon chemical and physical stressors (MPTP and physical exercise, respectively). Noticeably, a major recovery of RAGE-network immunosupression and a significant up-regulation of neuroprotective PD-related DJ-1 gene were observed in trained-MPTP rats. We propose that the coordinated expression pattern of RAGE, innate immune partners and downstream effectors is an orchestrated regulatory mechanism. Importantly, immunological restoration driven by PE in frontal cortex of MPTP-rats was paralleled by emotional/cognitive behavioral recovery. Therefore, an in-depth understanding of underlying mechanisms and functional consequences of this immunomodulatory effect is a promising avenue to be explored in PD (FC - Frontal cortex, STR - Striatum, HIP – Hippocampus)

Overall, findings from all experimental paradigms presented herein are convergent and strongly suggest that RAGE has a protective rather tissue-destructive phenotype in early stages of PD neurodegeneration.

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5.2. Study strengths and limitations

5.2.1. Strengths

✓ Even though inflammation is a well-recognized vector of PD pathogenesis, brain innate immunity including RAGE aspects is far from being well-characterized. In fact, there was no information available regarding the multitude of RAGE isoforms (signaling competent *versus* decoy RAGE isoforms) in PD. *This is the first study focused on a comprehensive picture of RAGE biology in different PD experimental settings across species (mouse and rat).*

✓ Furthermore, this work attempted to shed light into areas that are beyond locomotor impairments associated with nigral degeneration in advanced phases of PD (Prediger *et al.* 2011). In the current clinical paradigm, active efforts are being made to identify individuals at the prodromal PD stage. This strategy is valuable because it provides clues about possible etiologic and pathogenic factors, facilitating the design of clinical trials to assess therapeutic agents aimed to earlier time points of the disease (Golde *et al.* 2013, Olanow & Obeso 2012, Salat *et al.* 2016). *The growing recognition of the importance of prodromal PD phase research led us to deepen key aspects of RAGE biology in extra-nigral pathology (i.e. MPTP-striatal axonopathy and MPTP cortical/hippocampi disturbances) in a pre-manifest phase of classical motor impairments.*

5.2.2. Limitations

✓ Although MPTP has emerged unquestionably as a popular tool for inducing a model of PD in a variety of animal species, MPTP rodent models have drawbacks. Among them, intraneuronal inclusions immunoreactive for both α-synuclein and ubiquitin – reminiscent of Lewy bodies - have not been convincingly observed in rodents, in opposition to what occur in MPTP-injected monkeys (Przedborski *et al.* 2001, Tieu 2011). Misfolded/aggregated α-syn is envisaged as an endogenous DAMP able to trigger detrimental immune responses that amplify PD pathological process (Mosley *et al.* 2012). Thus, addressing immune RAGE-network dynamics upon α-syn danger signaling is warranted. Alternative MPTP regimens employing

longer expositions to the toxin and/or genetic PD models characterized by α-syn overexpression are more likely to be successful experimental approaches.

✓ A special evolution pattern of RAGE gene alternative splicing seems to occur within mammals (human, mouse and rat included) for two conserved RAGE variants (flRAGE and N-truncated RAGE); however, *most splice variants of RAGE display a significant tissue and species specificity* (Lopez-Diez *et al.* 2013). Moreover, mice do not display measurable plasma levels of soluble RAGE as rats and humans do (Forbes *et al.* 2005, Lu *et al.* 2011, Yan *et al.* 2010). Thus, RAGE isoforms regulation upon MPTP lesion reported herein needs to be further validated by distinct disease models in order to better inform about their potential translation into clinics.

✓ Whereas ageing remains the most compelling PD risk factor, the impact of age in PD preclinical studies has been largely overlooked (Bourdenx *et al.* 2015). This item is particularly interesting in the context of brain innate immunity since ageing is a triggering factor of innate immunity dysregulation within PD neurodegeneration (Czirr & Wyss-Coray 2012, Deleidi *et al.* 2015, Northrop & Yamamoto 2011, Su & Federoff 2014). For this reason, *it would have been interesting to further dissect RAGE biology in MPTP-intoxicated aged subjects.* However one should bear in mind that aged C57BL/6 mice display increased sensitivity to MPTP and would probably increase lethality rate within 48 hours post-injection (Filipov *et al.* 2009, Przedborski *et al.* 2001).

5.3. General discussion and future perspectives

The biology of RAGE frequently coincides with settings in which its ligands accumulate, especially in proinflammatory and oxidative environments such as Parkinson's disease (Dalfo *et al.* 2005, Santoro *et al.* 2016, Sathe *et al.* 2012). In such cases, RAGE inhibitory mechanisms seems to become overwhelmed, triggering signaling-competent RAGE isoform up-regulation thus fueling sustained deleterious signaling (Bierhaus *et al.* 2005). Present work suggests that active regulatory mechanisms influence RAGE-network operating in glial-neuronal interconnections towards homeostasis maintenance in early stages of experimental PD. Hence, the identification of mechanisms involved in the loss of initial RAGE neuroprotective/neurorepair responses to stress as the pathology evolves, is challenging. Firstly, a promise attempt may be the characterization of RAGE isoforms differential expression in distinct brain regions affected by PD neurodegeneration. For example, an asymmetric expression of RAGE isoforms towards a 4 fold increase in soluble RAGE was found in hippocampus of non-demented and neuropathology-free individuals. This raises the possibility that brain utilizes alternative splicing as a mean of selectively altering RAGE expression in order to cope with specific ligands building up in brain. Conversely, a loss of soluble RAGE variants expression may play a causal role in neurodegeneration (Ding & Keller 2005b). Noticeably, lower levels of inhibitory RAGE variants (secreted and signaling-deficient RAGE) were recently reported in multiple brain regions of AD subjects (Zhu & Ding 2015). *Whether these seemingly cytoprotective RAGE regulatory mechanisms seen in early stages of striatal MPTP lesion are also lost in PD neurodegeneration needs to be further explored.* Besides alternative splicing, a second mechanism that also accounts for sRAGE synthesis is constitutive/inducible regulated intramembrane proteolysis (RIP) through the action of distinct metalloproteases, namely ADAM10 (Braley *et al.* 2016, Galichet *et al.* 2008). Ensuing research led to the recognition of RIP as a conserved signaling mechanism where membrane proteins are cleaved within their transmembrane domain to release extracellular fragments able to function at a new location and a cytosolic signaling fragment that can enter the nucleus to control gene transcription (Chen *et al.* 2015, Groot & Vooijs 2012). This is in line with subcellular compartmentalization of RAGE C-terminal portion in nucleus (predictive of gene transcription regulation) and N-terminal RAGE fragments in cytosol/neuropil (predictive of its release to function as ligands scavenger)

observed in present study. Future *in vivo* studies dissecting subcellular RAGE location with higher resolution techniques (e.g. immunoelectron microscopy) as well as *in vitro* experiments dissecting the molecular aspects of RAGE variants regulation in experimental PD settings are warranted.

Astrocytes are good candidates for these *in vitro* approaches. Indeed, the presence of increased cytoprotective RAGE variants in astrocytes upon MPTP fits with their active secretome profile of trophic molecules (e.g. antioxidants, gliotransmitters) that globally influence neuronal survival and synaptic plasticity. In fact, one of the key roles of astrocytes is providing neuronal metabolic support (Ben Haim *et al.* 2015). Thus, understanding how and which astrocytic metabolic pathways may be impacting RAGE variants regulation in PD, seems fundamental. For example, a recent study reports insulin-protective effects in MPP^+-treated C6 glial cells (Ramalingam & Kim 2016). Noteworthy, insulin was shown to enhance RAGE proteolysis through its effect on ADAM10 activity (Lee *et al.* 2015). *Since insulin resistance, hyperglycemia, protein glycation and RAGE-noxious signaling - common features of Type 2 Diabetes (T2D) - are suggested to play a role in PD pathogenesis (Song & Kim 2016, Teismann et al. 2012, Vicente Miranda et al. 2016), a more in-depth understanding of such mechanisms may also help to contribute to the open debate of whether T2D is a risk factor for PD* (Arvanitakis *et al.* 2004, Cereda *et al.* 2011, Hu *et al.* 2007, Sun *et al.* 2012). Metabolomic profiling of plasma, CSF and/or urine from PD subjects (preferentially from early stages of disease) may give further insights into this currently under-researched area. Moreover, measurement of RAGE soluble isoforms in these body fluids may also help to establish a putative correlation between sRAGE levels and disease staging, analogous to what has been reported for other metabolic and inflammatory disorders. However, the predictive value of sRAGE as *bona fide* biomarker in these pathological conditions is still questionable (Schmidt 2015, Yan *et al.* 2010).

A persistent RAGE down-regulation has been previously observed in mesenchepalon of MPTP-treated mice after an initial rise (Teismann *et al.* 2012). *Present work further expands this observations in terms of MPTP models, brain regions and innate immune sensors.* In fact, we newly report the following: i) basal flRAGE levels parallel normal contents of astrocytic S100B neurotrophin in chronic insulted striata; ii) increased RAGE inhibitory RAGE variants co-ocurred with high levels of astrocytic S100B cytokine in acute insulted striata and iii) a coordinated frontal cortical long-lasting repression of RAGE and innate immune partners (i.e.

TLR's) in the i.n. MPTP model. Globally, our results suggest the existence of concerted counter-regulatory mechanisms that control immune sensors to restrain immune reaction and neuroinflammation. Based on biochemical peculiar properties governing RAGE expression and signaling, the suggested thesis about RAGE functioning as a "master-switch" between transient cellular activation into long-lasting cellular dysfunction (Bierhaus & Nawroth 2009, Chen & Nunez 2010, Sorci *et al.* 2013) brings the following question: *Is RAGE the maestro of this orchestrated immune response?* Unfortunately, our present knowledge is still insufficient to provide a suitable response as a large number of missing answers subsist. First, we have overlooked the complete characterization of RAGE isoforms in our chronic and i.n. MPTP experimental paradigms. Hence, we ignore whether an imbalanced expression of RAGE variants toward cytoprotective/deleterious phenotypes occurs in these experimental settings, analogous to what we have observed in the acute MPTP paradigm. Similarly, we missed whether immune transcriptomic repression upon i.n. MPTP is effectively translated into decreased protein levels and which cell types are potentially involved. In this regard, immune sensors (i.e. RAGE and TLR's) are not restricted to glial cells but are expressed by most cell types of brain parenchyma, including neurons (Greco *et al.* 2012, Kato *et al.* 2016, Liu *et al.* 2014). Among the great panoply of future approaches to dissect RAGE intricate immune network in PD, we believe that studies focused on neuronal self-immune responses as neurodegeneration evolve are mandatory. In fact, little is known at this point about how neurons sense danger signals and activate intracellular defenses mechanisms and/or alert neighboring cells (Czirr & Wyss-Coray 2012). We postulate that neuronal RAGE may be involved in neuroprotective/neurorepair signaling pathways (e.g. autophagic pathways activation in response to damaged mitochondria and/or intraneuronal protein misfolding; dendritic structure remodeling) in PD initial stages. Neuronal-glial interactions are likely to support neuronal trophic events that are RAGE-mediated, preventing ligands accumulation through astrocytic cell-specific up-regulation of RAGE inhibitory isoforms. This is consistent with the presumed protective role of astrocytic reactivity in initial stages of PD neurodegeneration (Ben Haim *et al.* 2015, L'Episcopo *et al.* 2014, Rappold & Tieu 2010, Teismann & Schulz 2004). Frontal cortical co-ordinated immune sensors repression (i.e. RAGE and TLR's) reinforces the thesis of an attempt of the system to repress chronic innate inflammation in early stages of PD neurodegeneration. However, accumulated injury may lead to massive release of DAMP's from injured/dying neurons (e.g.

HMGB-1; misfolded α-syn) and/or glia (e.g. S100B), overwhelming counter-regulatory immune mechanisms towards chronic activation of immune sensors and sustained glia innate inflammation (e.g. microglia) and neuronal pro-injury pathways.

Considering the extensive impact of deregulated immune over-activation in central and peripheric nervous systems in PD (Su & Federoff 2014), identification of mechanisms that govern neuroimmune modulation is of great translational relevance. An important role of small non-coding microRNA's (miRNA) in brain innate immune regulation have been extensively reported (Iyer *et al.* 2012, Lee *et al.* 2016, Lukiw *et al.* 2011, Saba *et al.* 2012). Importantly, altered miRNA expression was found in brains from human and experimental PD (Ghanbari *et al.* 2016, Hoss *et al.* 2016, Jiang *et al.* 2016, Kong *et al.* 2015) and was envisaged as an emerging preclinical PD pharmacological target with great therapeutic potential (More & Choi 2016). Thus, *new perspectives may arise from future studies addressing miRNA's role on central innate immune dysregulation in experimental PD paradigms.*

Interestingly, miRNAs respond to aerobic exercise in distinct tissues, including brain (Cui *et al.* 2016, Denham *et al.* 2014). Noteworthy, gathered data is supportive of a role for miRNAs in regulation of immune pathways during pre-conditioning paradigms (Garcia-Bonilla *et al.* 2014). Treadmill-training protocol implemented 30 days prior to intranasal MPTP administration could also illustrate a pre-conditioning paradigm associated with immune regulation. Thus, exploring this putative correlation is required. Again, we have missed the characterization of RAGE isoforms upon PE. Taking into account PE ability to decrease striatal S100B levels upon MPTP (Al-Jarrah & Jamous 2011), it is also tempting to speculate that PE may elicit an asymmetric ratio in brain RAGE isoforms levels, similarly to what has been described at periphery in type 2 diabetic patients (Choi *et al.* 2012). *Overall, a more complete understanding of PE aptitude to regulate brain RAGE variants at protein and cellular level is therefore appealing in the context of healthy ageing and experimental PD.*

Accumulation of age-related genetic damage and by-products of ongoing oxidative damage (e.g. AGE's) (Garinis *et al.* 2008, Hindle 2010, Reynaert *et al.* 2016) combined with age-dependent inactivation of hormetic-stress responses (Calabrese 2016) underpin the onset of brain neurodegeneration, including sporadic PD. We believe that it will be of utmost importance to dissect the impact of ageing in brain RAGE variants regulation. In this regard, Geroldi and colleagues (2006) reported that highly elevated soluble RAGE levels correlated with

extreme longevity (centenarians) in healthy human subjects (Geroldi *et al.* 2006). Although we acknowledge that future studies are needed to corroborate this observation, this is especially interesting given the potential impact of an asymmetric ratio between sRAGE/flRAGE variants on brain tolerance/susceptibility to stressors. *We hypothesize that mechanisms underlying increased cytoprotective RAGE variants observed in our MPTP-intoxicated young rodent models may become dysregulated with age.* Such dysregulation, in combination with other triggering mechanisms, may contribute to PD neurodegenerative process. In this regard, the interplay between innate and adaptive immunity in PD pathobiology has become a major focus (Amor & Woodroofe 2014, Deleidi *et al.* 2015, Mosley *et al.* 2012, Perry 2012). Several lines of evidence support the existence of a bidirectional communication between the peripheral immune system and brain mainly through immune molecules including cytokines, chemokines and acute phase proteins (Deleidi *et al.* 2015). Importantly, aging brain experiences a diminished BBB function, alters the basal recruitment of systemic immune cells and fosters central innate immune dysregulation towards a constitutive pro-inflammatory environment (inflamm-aging), mechanisms contributing to immunosenescence (Gemechu & Bentivoglio 2012, Shaw *et al.* 2010). Such immunosenesence features including BBB dysfunction have already been observed in human and experimental PD (Brochard *et al.* 2009, Grozdanov *et al.* 2014, Saunders *et al.* 2012). On the other hand, anti-inflammatory properties of physical exercise are partially mediated through serum changes in adipokines (e.g. leptin), miokines (e.g. IL-6, IL-10) and peptide hormone ghrelin (Gleeson *et al.* 2011, Markofski *et al.* 2014). Future studies may be conducted to further explore the senescent interplay between innate and peripheric immune systems as well as the putative neurotrophic effects of orexigenic/anorexigenic peptides (ghrelin and leptin, respectively) arising from PE modulation in PD (Andrews *et al.* 2009, Mueller *et al.* 2015).

Finally, looking across the spectrum of various neurodegenerative diseases allows one to identify more commonalties than differences among degenerative cascades downstream to specific triggering factors of CNS proteinopathies. It has been suggested that exploring similarities between these disorders may provide critical insights into the development of novel therapeutic strategies that might work even in later disease stages and possibly in multiple disorders (Golde *et al.* 2013). Noteworthy, RAGE is involved in the most prevalent age-related neurodegenerative diseases. It is worthwile mentioning that secreted RAGE isoforms, besides

preventing chronic flRAGE activation by acting as decoy receptors, also provide a global benefit to the brain, preventing or delaying the aggregation phenomenon that characterizes proteinopathies in general (Ding & Keller 2005a). However, the effects of RAGE (dys)regulation in mouse models of neurodegenerative diseases have been scarce and mainly focused on probing the effects of genetic deletion/modification of RAGE (Arancio *et al.* 2004, Deane *et al.* 2012, Teismann *et al.* 2012). At a clinical level one should highlight that a RAGE antagonist (Azeliragon®, vTV therapeutics) is being evaluated for mild AD. In fact, it has been granted Fast Track designation by the United States Food and Drug Administration and has successfully completed an End of Phase 2 meeting. A phase 3 trial, the STEADFAST Study, under a Special Protocol Assessment (SPA) is ongoing (http://vtvtherapeutics.com/pipeline/azeliragon). So far, there are two relevant outcomes: 1-acute, reversible, concentration dependent cognitive worsening were reported and originated stopping the use of the higher dose; 2- a lower dose demonstrated greater efficacy for mild AD than patients with moderate AD. Conceptually, one would think that an antagonist would also non-selectively prevent the protective action of the soluble RAGE forms. In fact, *we highlight that RAGE -/- mice exhibited only a partial protection against MPTP intoxication* (Teismann *et al.* 2012). Likewise, in late diabetic complications, protection from development of the pathological features was more profound in wild-type mice treated with sRAGE than in RAGE−/− mice. In diabetic neuropathy for example, diabetic RAGE−/− mice were only partly protected from a loss of pain perception, while administration of sRAGE to diabetic wild-type mice completely restored pain perception (Bierhaus *et al.* 2004).

Moreover, accumulation of danger signals (e.g. S100B, HMGB-1), that act via RAGE and other PPR's (e.g. TLR's), suggests that the use of an active scavenger instead of RAGE blockade is theoretically a more effective mechanism to thwart RAGE-related diseases, namely PD. In fact, sRAGE administration was recently proven to have beneficial effects in an animal model of amyotrophic lateral sclerosis (Juranek *et al.* 2016). *Although challenging, the potential therapeutics of sRAGE modulatory strategies deserves to be further exploited in the context of other brain proteinopathies, namely in PD.*

Chapter 6

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