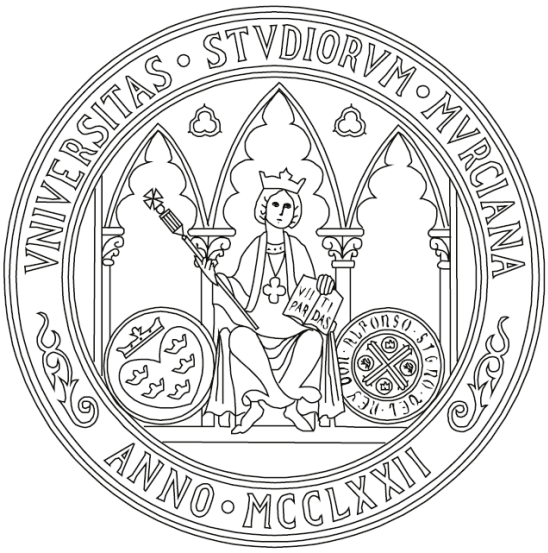

UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Neuroprotection in neurodegenerative processes associated with Parkinsonism and aging. Correlation between dopaminergic neuronal death and glial activation.

Neuroprotección en procesos neurodegenerativos asociados al Parkinsonismo y al envejecimiento. Correlación entre muerte neuronal dopaminérgica y activación glial.

Dña. Ana Luisa Gil Martínez

2019

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Dissertation

To obtain the Degree of Doctor at the University of Murcia and Maastricht University.

Memoria presentada para optar al Grado de Doctor por la Universidad de Murcia y la Universidad de Maastricht.

by/*por*

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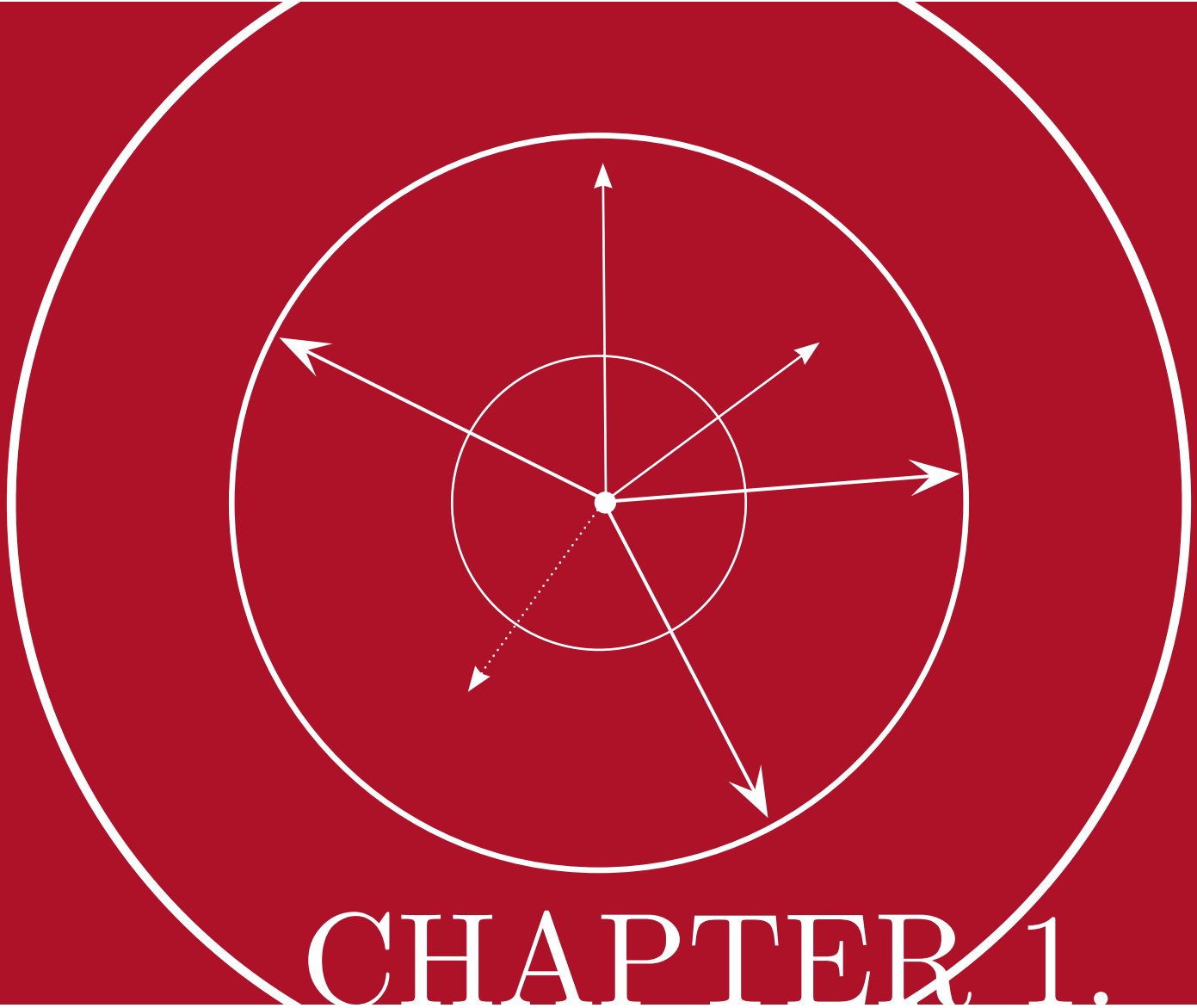
A mi familia.

A ti, siempre.

Contents



CHAPTER 1.

General Introduction

1.1. Reference frame

Parkinson's disease (PD) is a highly complex neurological disorder. Its main pathological feature is the presence of Lewy bodies, which consists of aggregated α-synuclein protein in dopaminergic neurons of the Substantia Nigra *pars compacta* (SNpc) (1).

1.1.1. Epidemiology

PD is the second most prevalent age-related neurodegenerative disorder in the world, after Alzheimer's disease, affecting between 100 to 200 people out of 100,000 people at the age between 65 and 70 years (2). Specifically, it is reported that it affects 1% of the population over 60 years of age, 2% over 70 years and 3% over 80-year-old., where men are 1.5 times more likely to have PD than women. As the aging population rate in our society increases, it is projected that approximately 9 million people will suffer from PD in the year 2030 in the 10 most populous countries. The impact cost of this projected data is estimated in billions of euros (3). For that reason, there is an immediate need to delay the development of PD by modifying the main risk factors and identifying the patients in the early stages of the disease for enrolment in clinical trials which are aimed to prevent the disease (4).

1.1.2. Etiology

Despite the efforts and advances in the knowledge of the cause of PD, its early development is mostly idiopathic and is still unknown. Epidemiologic studies in the population point out that aging is considered a major risk factor in the development of the disease (5). It has been linked in addition with complex interactions between the exposure to environmental (6) and, to a lesser extent, to genetic factors (7).

Data from epidemiological (8), animal and *in vitro* studies support the important impact of different prevalent environmental compounds on the development of PD. This negative effect is described for pesticides (rotenone, paraquat or organochloride compounds), polychlorinated biphenyls, solvents (trichloroethylene, TCE, and perchloroethylene, PERC), metals (iron, lead, manganese and mercury) and, other less evident risk factors such as infection or air pollution (9).

On the other hand, it has reported that genetic causes only accounts 10% of the diagnosed (7) PD cases when the genetic factor mainly contributes in the development of PD is due to a specific mutation that was observed into different PD genetic forms: autosomal dominant (PARK1 or PARK4), autosomal recessive (PARK2 or PARK6), X-linked inheritance (PARK12) and unclear (PARK10 and PARK16). It has also been described that alterations in these PD-related genes support a risk factor increasing the predisposition to develop the disease. Thus, it has been suggested that most of the sporadic PD cases are due to a cumulation of environmental and genetic factors (10).

It would be interesting in future research lines to focus on how are associated and involved environmental and genetic factors with common metabolic pathways in the aetiology of PD.

1.1.3. Symptomatology

The development of the clinical symptoms of PD are divided in different phases (11) (Figure 1.1). The first phase involves a long latent period with the progression of the neurodegeneration without motor symptoms (12). In the early stages of the disease, non-motor symptoms start to occur, and they can be distinguished as sleep disorders, autonomic dysfunction, psychiatric symptoms, pain, cognitive impairment, olfactory dysfunction and fatigue (13). Some of these non-motor symptoms can be explained because it is demonstrated that, apart from the nigrostriatal pathway, non-dopaminergic structures as the olfactory bulb or the gut myenteric plexus are affected in PD as well (14,15). Moreover, as the disease further progresses and 60-80% of the dopaminergic neurons in the SNpc have already been lost, motor symptoms begin to appear. In this line, the main motor alterations include stiffness, bradykinesia, muscular rigidity and postural instability. Thus, the pathological processes involved in PD results in a heterogeneous symptomatology which non-motor and motor alterations throughout the different phases of the development of the disease. For the design of therapeutic strategies, it is important to consider the intervention as there is a window-of-opportunity of several years before the manifestation of the clinical motor symptoms.

1.2. Statement of the problem

One of the main challenges in the research of PD is to describe the mechanisms underlying the progressive degeneration of dopaminergic neurons in the nigrostriatal pathway. In the last years, several investigations hallmarked that both the peripheral as well as the central nervous system (CNS) contribute to the pathogenesis (16). In addition, several pathways and molecular mechanisms are implicated, such as mitochondrial dysfunction, oxidative stress, calcium homeostasis and neuroinflammation (17).

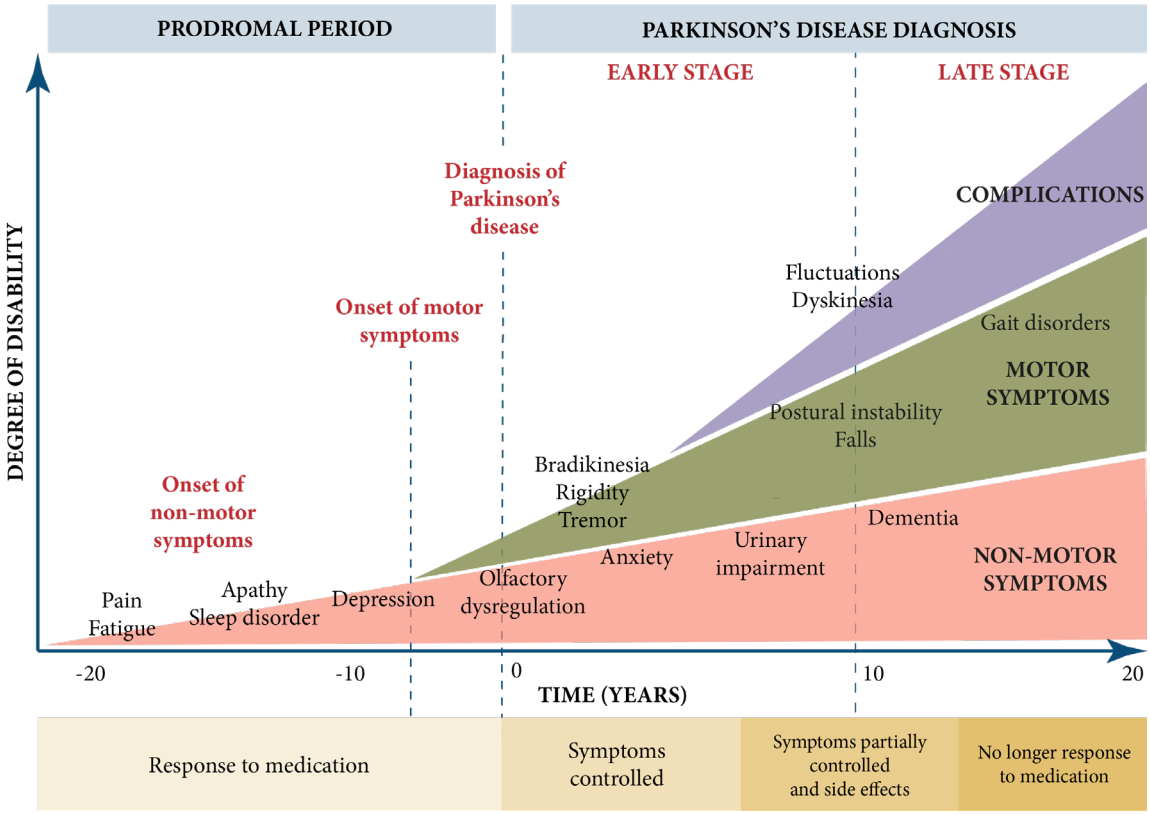


Figure 1.1. Clinical symptoms and course development of PD.

Specifically, different studies have demonstrated the involvement of neuroinflammation by persistent activated and uncontrolled glial response, which exacerbate neuronal cell death in the SNpc as observed in *post-mortem* brains from PD patients (18). Glial cells mediate the primary immune response in the CNS, which is initiated by microglial cells and, then, astrocytes amplified by reactive astrogliosis, in order to maintain brain homeostasis after an insult (19).

Microglia are the resident macrophages of the CNS which, under physiological conditions, co-exist in equilibrium between phenotype M1 or pro-inflammatory and, M2 or anti-inflammatory (20,21). In a healthy brain, there is a balance between microglia M1 and M2 in order to maintain the integrity of the CNS. However, apoptosis causes the migration of microglia cells from the M1 phenotype to the damaged area. It is reported that in PD, the progressive degeneration of dopaminergic neurons leads to an imbalance between M1/M2 that maintains the inflammatory phenomena, with an excessive production of pro-inflammatory cytokines and neurotrophic factors.

This uncontrolled neuroinflammatory microglia cells activate astrocytes, which begin a process known as reactive astrogliosis. During this process astrocytes secrete pro-inflammatory cytokines and reactive oxygen species (ROS). This will amplify

the inflammatory process affecting healthy neurons and generating a vicious cycle of neurodegeneration (21,22). Astrocytes, whose name refers to their star shape, are the most numerous cells of the CNS. They are specialized in providing support to neurons by regulating the ionic composition of the microenvironment, giving access to nutrients, growth factors or releasing neurotransmitters (23).

It is evident that inflammatory processes are implicated in the pathogenesis and progression of PD but the level to which they are involved is still unknown. Some authors support that inflammatory mechanisms precede neuronal degeneration while others defend that neuroinflammation appears as a consequence of the neuronal cell death (22,24). Thus, it is necessary to uncover the role of neuroinflammation on the onset of PD with the objective of reversing or slowing down the progression of the disease (25). As a consequence, in the last years a new concept has been implemented, called "Drug Repositioning" which consists of the re-use of drugs already approved by the EMA and FDA (26). This new therapy strategy is very attractive since it focuses on drugs with clinical safety data that could speed up their clinical use in PD patients and it could use to identify new molecular targets (26).

1.3. Thesis research approach

Based on the strengths and limitations of PD's research, the overall aim of this thesis was to investigate the role of neuroinflammation using an induced model of Parkinsonism in mice (**Figure 1.2**). To address this question, the research line was organized in two main experimental parts divided into the following chapters:

PART I, the question about the cause of the progression of the neurodegeneration in PD is approached since in the last years different studies have suggested that both brain and peripheral inflammation could play a key role. In the early stages of the disease, inflammation could be circumscribed to only one peripheral system as the gastrointestinal tract, in line with Braak's theory in which the disease could begin in the gut and α-syn aggregates spreads to the brain via the gut-brain axis (27). However, considering that midbrain dopaminergic neurons express the highest vulnerability to insults (as proposed by the "threshold theory" for PD (28)), in Chapter 3, it was explored if a systemic inflammation produced by a local injury circumscribed to the colon, by dextran sulfate sodium (DSS) administration in a Parkinsonism mouse model, is able to significantly enhance dopaminergic neurodegeneration and inflammation in the SNpc and in the striatum.

Regarding the significant link between inflammation and dopaminergic degeneration, PART II was based on "Drug Repositioning" which involves the re-use

of anti-inflammatory and anti-oxidant drugs. The main advantage of this therapeutic strategy is that the feasibility and safety studies in humans are already done. Considering this fact, in Chapter 4, a combination of an anti-inflammatory, HA-1077 (31,32), and an anti-oxidant, N-acetyl-cysteine, NAC (33-35) were used to study their possible synergistic beneficial effect in old Parkinsonian mice.

Following this line, the aim of Chapter 5 was to analyse the relationship between physical activity, consider a non-pharmacological strategy in PD (36), combined with an antioxidant, NAC (33-35), and its influence in reducing the inflammatory processes in parkinsonized animals treated subchronically with MPTP.

The final study presented in Chapter 6 takes a step away trying to clarify the role of MAPKs pathway, considered a molecular target of NAC, on the dopaminergic neuronal death and its association with neuroinflammatory mechanisms triggered after an acute intoxication regime of MPTP in old mice.

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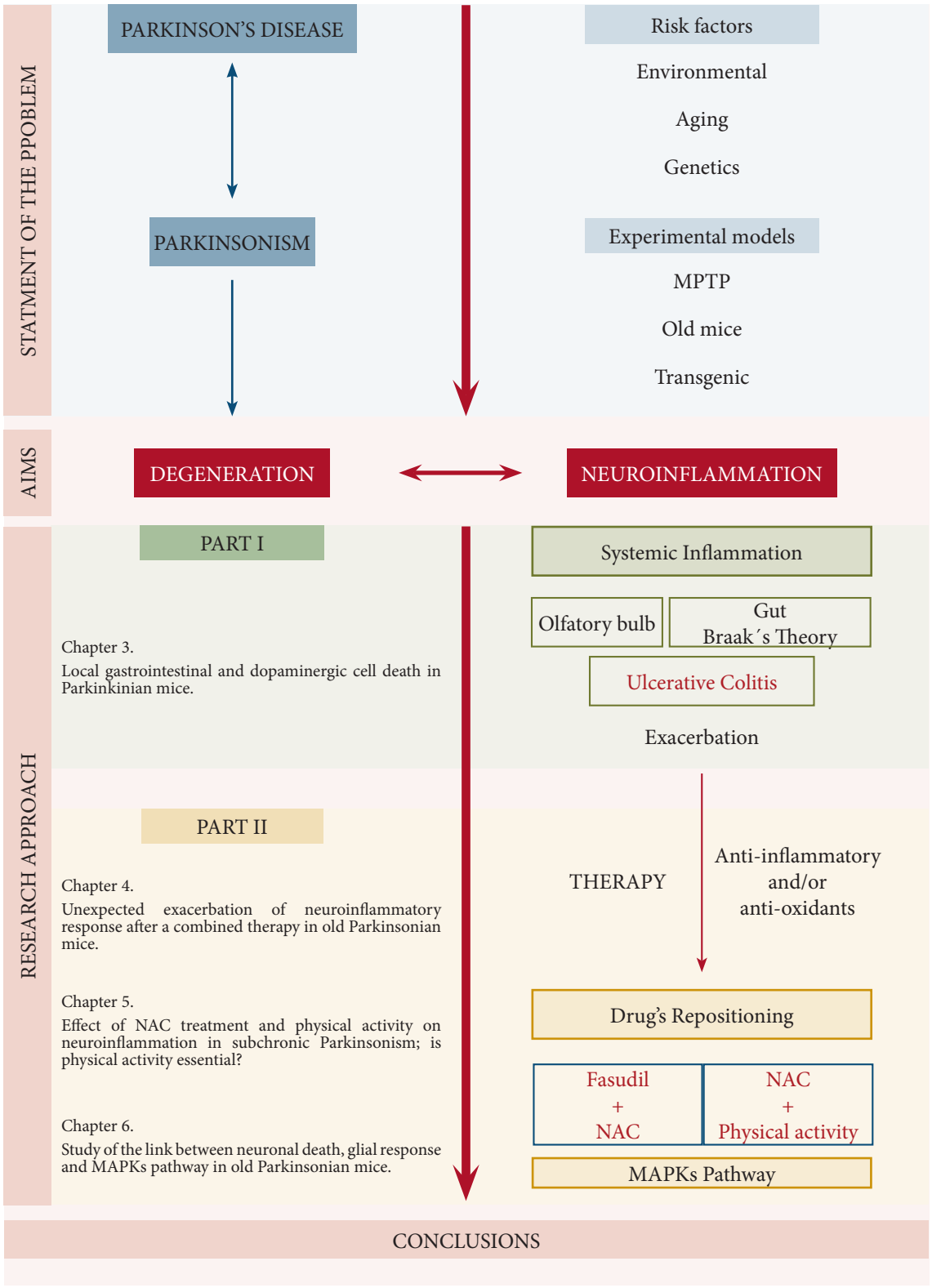

Figure 1.2. Thesis project scheme.

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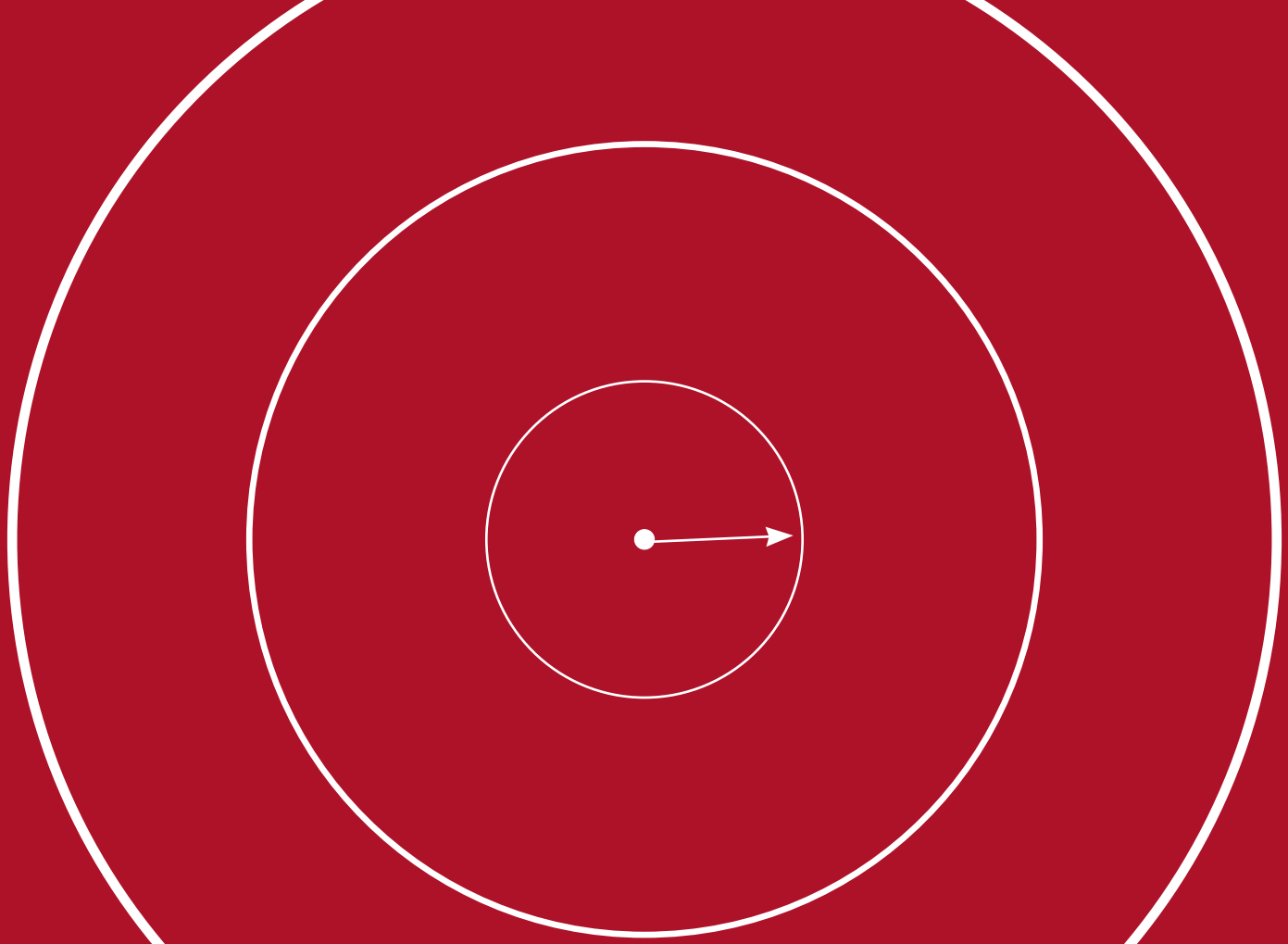

CHAPTER 2.

Unmasking the role of neuroinflammation in Parkinson's disease

Gil-Martinez AL, Cuenca L, Cano-Fernandez L, Sanchez-Rodrigo C, Estrada C, Fernandez-Villalba E, Steinbusch HWM , Herrero MT. *In preparation.*

2.1. Neuroinflammation in PD

2.1.1. General framework

In the molecular pathogenesis of Parkinon's Disease (PD) several pathways and mechanisms are involved such as calcium homeostasis, α-synuclein proteostasis, mitochondrial dysfunction, oxidative stress or neuroinflammation. Specifically, sustained inflammatory response is unlikely the first direct cause of this disease but emerging evidence from experimental, genetic and epidemiological reports have shown that it may contribute and perpetuate the initial neurodegenerative processes **(Table 2.1)** **(1)**.

The clearest starting point of the neuroinflammation-PD relationship arises from observations of activated and branched microglia in *post-mortem* tissue from PD patients. McGeer et al. first described the inflammatory component in the substantia nigra (SN) of PD brains showing reactive microglia expressing human leukocyte antigen - DR isotype (HLA)-DR and CD11b **(2)**. Subsequently, several research studies have reinforced this association by describing increased microglial activation with pro-inflammatory factors released in *post-mortem* brains **(3,4)**. At the same time, protoplasmic astrocytes **(5)** and, to a lesser extent, oligodendrocytes **(6,7)**, have been joined as important parameters in neuroinflammatory processes involved in neurodegenerative disorders as PD. In this sense, the development of experimental models of PD are crucial for the understanding of the role of the glial response. This could be the case of a neurotoxin called 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which produces a permanent Parkinsonian syndrome in human and in non-human primates. In 1982, it was described by Dr. Langston after observing that a group of drugs addicts presented similar symptoms to idiopathic PD. After several studies, it was deduced that the heroin that these Frozen Addicts had been consumed was intoxicated with MPTP **(8)**. Importantly, in 1999, three of the patients died and the *post-mortem* studies showed the depletion of dopaminergic (DA) neurons in the SN and active microglia expressing HLA-DR **(9)**, as McGeer's previously had described.

Moreover, it has been reported genetic evidence that support the importance of the involvement of neuroinflammation in PD. Several studies have identified that some PD-related genes are directly implicated in the progression of chronic PD by stimulating

inflammatory processes via microglia and astroglia response. For instance, leucine-rich repeat kinase 2 (LRRK2) (10,11) or Parkin (12,13) are highly expressed in microglial cells indicating a functional role in the immune system. Other reports demonstrated that inducing the deficiency of genes as PTEN induced putative kinase 1 (PINK1) (14) or DJ-1 (15) in mice triggers microglia vulnerability and increase cytokines release in response to brain injury. It is also importance to hallmark that the mutation in α-syn in PD induces microglia cells activation (16). In addition, these genes regulate different functions of glial cells. Mainly, DJ-1, PINK1 and Parkin regulates proliferation, glucose metabolism or mitochondrial function in astrocytes (17). In microglial cells, inflammation, surveillance or phagocytosis are regulated by DJ-1, PINK, LRRK2 and α-syn (reviewed in reference (18)).

Together with the experimental and genetic evidence, epidemiological studies based on the use of nonsteroidal anti-inflammatory drugs (NSAIDs) were performed to observe their effects on the onset of PD, but the data results in discrepant information. As it has been discussed, these could be because of the methodological differences between retrospective and prospective studies (19) as well as the starting time point and duration of the treatment. The idea is that NSAIDs have been proposed as a beneficial alternative in the primary and secondary prevention of PD (20,21) and, in fact, it is described that ibuprofen might have a slight protective effect on the development of PD (22). However, more scrupulous clinical studies based on the use of specific anti-inflammatory drugs are needed in order to clarify their involvement in the onset of PD (23).

Regardless of the use of NSAIDs, pathway analysis-based GWAS identified significant implication of genes involved in the "regulation of leucocyte/lymphocyte activity" and "cytokine-mediated signalling" that may confer an increased susceptibility to PD (24). In another study was found the high association signals at the HLA regions including HLA-DRA, HLA-DRB9, HLA-DRB1, HLA-DQA1, HLA-DRB9, and HLA-DPB2. These genes encoded proteins of the major histocompatibility complex which main roles are antigen presentation and immunity (25). More recently, since March 2018, there is an ongoing clinical trial with the objective of studying if the concentration and regional brain distribution of activated brain microglia/macrophages, using the PET ligand [18F]DPA-714, are increased in PD patients compared to controls (26).

This points towards an evident link between neuroinflammation and dopaminergic degeneration associated with PD but there are still many questions concerning the mechanism of action of glial cells, involving its morphological changes, functionality and activation, in both health and disease.

Table 2.1. Antibodies and protocols for the different techniques

Year	Evidence description	Ref.
1987	Astrocytes are involved in the induction of Parkinsonism by MPTP.	(27)
1988	Active and branched microglia expressing HLA-DR in PD patients' brains	(2)
1993	The scavenging of H2O2 by glutathione peroxidase contained in the glial cells produces a protective effect in the midbrains of PD patients.	(28)
1994	IL-1β, IL-6, EGF, TGF-α are increased.	(3,4)
1995	High brain levels of β2-microglobulin levels	(29)
	IL-1β and IL-6 are increased in the CSF of de novo PD and AD patients.	(30)
1998	Presence of IgG to ovalbumin modified by dopamine oxidation	(31)
1999	Active nerve cells degeneration after MPTP administration in humans related to the presence of gliosis and clustering of microglia	(9)
2000	Microgliosis is not accompanied by reactive astrocytosis.	(5)
	iNOS, lipocortin-1 and -2 is contained in amoeboid microglia.	(32)
2002	Increase levels of neurotrophic factors, such as BDNF, NT-3 and NGF, triggered by dopaminergic neuronal loss	(33)
	IL-1β T genotype is associated with idiopathic PD cases.	(34)
	Role of astrocytes in PD caused by parkin dysfunction.	(13)
2003	Activated microglia is highly distributed in brain areas with damaged neurons and neurites.	(35)
	Nonsteroideal anti-inflammatory drugs may delay or prevent the onset of PD.	(21)
	PINK1 is found in glial cytoplasmic inclusions.	(36)
2004	Glial response is associated with long-term neurodegeneration.	(37)
2005	Microglial activation is developed in the midbrain of PD patients at an early stage and it may be associated with apoptotic events.	(38)
	Ibuprofen may delay or prevent the onset of PD.	(22)
2006	PAR-1 is increased in astrocytes.	(39)
	Evidence from experimental studies suggest limited support for the for the hypothesis that the use of aspirin may reduce the risk of this disease.	(40)
2007	A cohort study does not support the hypothesis that NSAIDs might decrease the risk of Parkinson disease.	(41)
2012	LRRK2 inhibition attenuates microglia activation.	(11)
2013	GWAS study identified genes involved in the "regulation of leucocyte/lymphocyte activity" and "cytokine-mediated signalling".	(24)
2014	DJ-1-deficient microglia have reduced the risk of neuroinflammation in PD.	(15)

Note: Evidence from experimental studies (blue), genetic studies (yellow) and epidemiological studies (red).

2.2. Glial cells in PD

In the central nervous system (CNS), glial cells are CNS-resident immune cells that mainly mediate the innate immune response. The mechanism that underlies the crosstalk between microglia cells and astrocytes after an injury awakened the curiosity of scientists to relate it to degenerative processes.

2.2.1. Microglia

In 1919, Del Rio Hortega defined the "third element" (42,43) and demonstrated the reactive nature and phagocytic capacity of microglial cells (42). Although its observations were questioned, nowadays, microglia cells are highly described (44). Microglial cells are ubiquitous but not uniformly distributed. Thus, microglial cells represent 5-20% of the total glial cells population in the adult mouse brain (10% of the total glial cells in adult brains) and the most densely populated areas are the SN and the basal ganglia (45). Microglial cells are in constant activity extending and retracting their ramifications controlling the extracellular environment (46).

It is thought that the origin of microglial cells arises from monocytes (from bone marrow) as progenitors that can cross the wall of blood vessels into the fetal brain (47). At this stage, immature and amoeboid microglia cells are implicated in the selection of neurons. Although, it is described that there is practically no exchange between blood and brain parenchyma; some reports point out that monocytes cross the blood brain barrier (BBB) during adulthood (48,49). In the adult brains, these cells acquire a ramified morphology as a quiescent (not stationary) microglia with a dynamic surveillance activity (Figure 2.1) (46). In a recent study, it is demonstrated that *in vitro* microglia, from male rats, showed higher migration rates than microglia from female rats under normal and stimulated conditions because of an increase of mRNA levels of migratory genes (MCP1 and RANTES). On the other hand, microglia cells from female rats have higher phagocytic activity in both scenarios (50).

In healthy brains or early stages of the disease, the activation of microglial cells can be beneficial to host by polarization of the anti-inflammatory phenotype (or M2). Microglia of M2 phenotype produce a wide variety of cytokines such as IL-4, IL-13, IL-10 and TGF-β. However, as the disease progresses, it has been shown that there will be a misbalance towards a pro-inflammatory phenotype (or M1) (51). Both damaged neurons and activated astrocytes release a cocktail of high levels of ATP, adenosine and cytokines that stimulate microglial cells (52). M1 is characterized by the increase production of tumour necrosis factor α (TNF-α), IL-6 and IL-12. In addition, it has also been described that microglia cells are a robust source of oxidative stress producing superoxide (SOD) and reactive oxygen species (ROS) (53) contributing to the amplification of the inflammatory

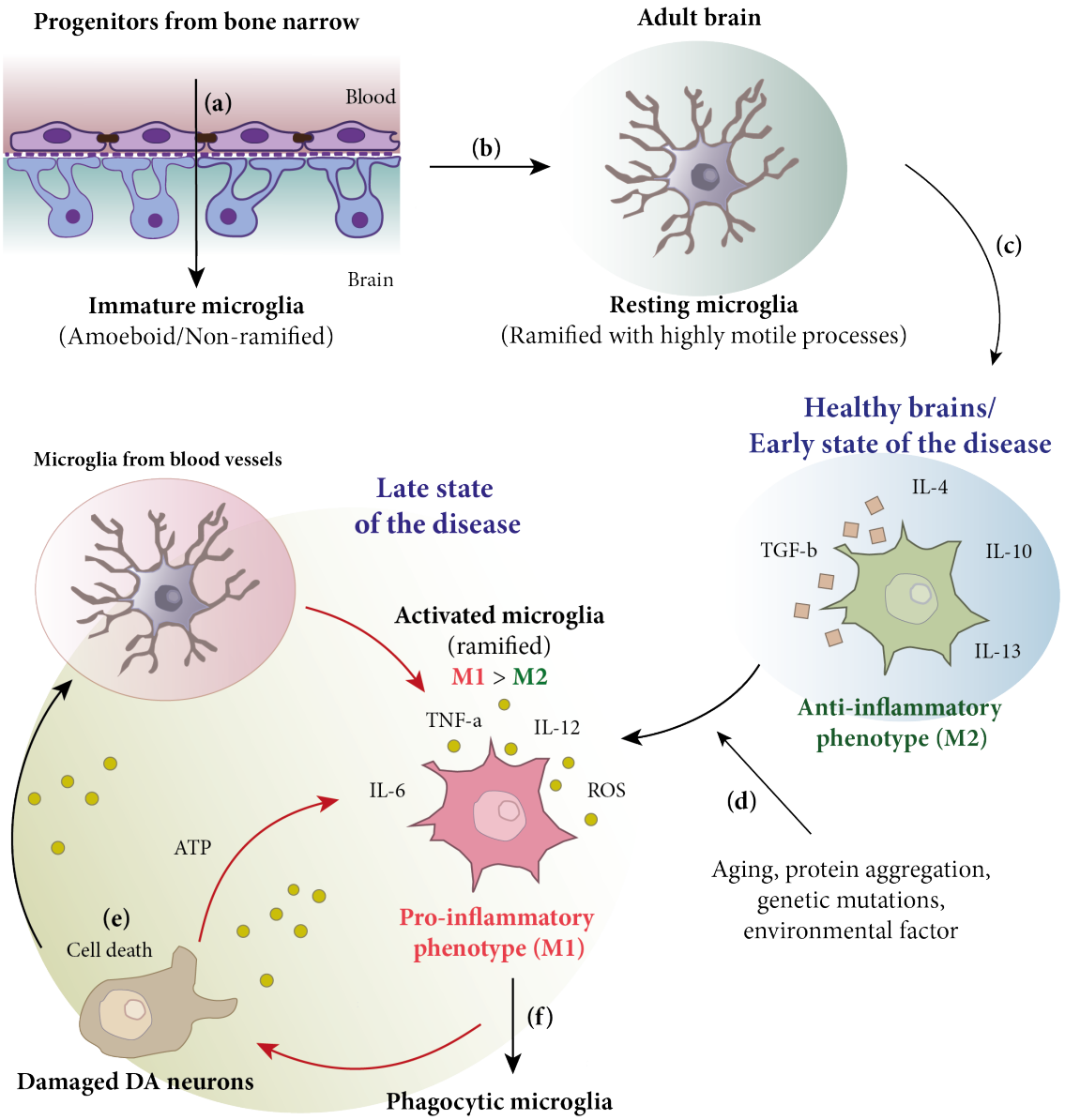

Figure 2.1. Representation of microglia processes during embryonic development and health and pathology condition in adult brain. (a) Progenitors from bone narrow cross the BBB into the brain to become immature microglia. (b) In the adult brain, resting microglia have a ramified morphology with highly motile processes. (c) In healthy brains or in the early state of the disease, M2 phenotype have a protective role. (d) Several stimuli as aging, protein aggregation, genetic mutations or environmental factor transform microglia into M1 phenotype. (e) As the disease progresses, the number of damaged DA neurons is increased enhancing microglial activation and stimulating the migration of microglia from blood vessels to the damaged area. (f) Finally, microglia transform into phagocytes.

response in brain and exacerbating neurodegeneration (51). Finally, activated microglia can become phagocytic to clear tissue debris or damaged neurons (54). Taken together, M1 microglial cells release pro-inflammatory mediators enhancing the degeneration of neurons causing a vicious circle of neuronal damage (55,56).

2.2.2. Astroglia

In 1856, Rudolf Virchow defined "neuroglia" as connective tissue which embed nerve cells (57). In 1893, Mihály Lenhossék added the concept of "astroglia" to his observations of star-shaped cells (58). From that moment, with the advance of immunohistochemical staining methods and microscopic techniques, its morphology and functionality could be described (59).

Astrocytes constitute the most abundant glial cells in the adult brain (60) and, represent a heterogeneous population organized in strategic positions in relation to neurons (61). Depending on their morphological characteristics and their anatomical distribution, they can be divided into two subtypes: (i) protoplasmic, mostly located in the gray matter and characterized by numerous fine processes, and (ii) fibrous, found in the white matter with long fiber-like processes (62). To date, important functions have been related to astrocytes including formation of the BBB, involvement in the tripartite synapse or regulation of water and ion homeostasis, making them crucial for maintaining neuronal health. Furthermore, they are especially interesting for participating in neuroinflammatory processes related to a specific damage or to a degenerative state. In this line, since their discovery 100 years ago, the number of research articles published about their function in disease has increased from 12 in 1990 to 1034 in 2017 (63).

Specifically, some evidence are emerging describing how astrocytes are involved in dopaminergic neuronal degeneration (17). In general, astrocytes respond to injury and disease in the CNS through a process called reactive astrogliosis (64). This mechanism has been reported to be a complex process which includes potential changes from cellular and molecular to gene level, which can have an impact both positive and negative on the surrounding neuronal and non-neuronal cells (64). These alterations in astrocytes depend on the severity of the insult entailing morphological changes such as hypertrophy in moderate states or proliferation and scar formation in severe conditions (60) . Particularly, in the most cases of PD and related syndromes, reactive astrogliosis has been described as mild or moderate in autopsies of the SN from PD patients (5). Expression of glial fibrillary acid protein (GFAP) is widely used as a marker for immunohistochemical studies of astrocytes and for the identification of reactive astrogliosis since its isolation form in old demyelinated plaques from multiple sclerosis patients (65). Briefly, the process implicates the migration of astrocytes to the damaged area adopting the main morphological and functional features of astrogliosis. Once they get to the site of injury, they wall it off while they secrete pro-inflammatory and neurotrophic factors that may stimulate microglial cells (66,67). It has been described that this mechanism follows dopaminergic cell death in SN in PD patients (28,68) and in parkinsonian monkeys (37). Halliday et al. point out that glia plays a fundamental role in an early period of tissue damage. Thus, the accumulation of a-syn in astrocytes stimulates the activation and recruitment of phagocytic microglia

that selectively damages dopaminergic neurons (69). Surprisingly, in experimental models in rodents, it has been suggesting that it follows microglial activation in SN and striatum (70,71). These conflicted data lead to the question of how and why glia cells adopt different mechanisms between species after dopaminergic insult.

Altogether suggest that astrocytes have a detrimental, active and direct effect on the regulation of neuronal survival that can be translated to different neurodegenerative disorders (59,61,72–74). In this context, the crucial roles of astrocytes constitute an interesting development with promising novel therapeutic strategies for the pathogenesis of neurodegenerative diseases (75).

2.3. PD Experimental Models in Rodent

2.3.1. The utopia of an experimental model for PD?

Animal models are essentials to identify molecular targets needed to design new therapeutic strategies. To validate a new experimental model, it has to respond to the medications already used to alleviate or to exacerbate the characteristic symptoms described in the clinical pathophysiology of the disease. Specifically, PD is a multisystem neurodegenerative disorder with both motor as well as non-motor symptoms. Ideally, an experimental model of PD should collect the clinical pathological framework observed in patients: α-synuclein aggregation, gastrointestinal dysfunction, neuronal death, motor disability and depression (Figure 2.2). Unlikely, it is difficult to replicate all the pathological features of the human disease in one experimental model. In this line, PD experimental models described for rodent offer a wide spectrum of possibilities to address specific questions (76).

PD models in rodents have been widely reported to elucidate the pathological mechanisms of dopaminergic neuronal death. The experimental models currently used can be classified into three groups based on the induction method of the specific pathogenic mechanism: genetic, pharmacological and neurotoxic (76,77).

It has been reported that the genetic relevance of PD incidence is significantly lower than its environmental causes at aged population (78). Thus, the exposure to highly used pesticides, such as rotenone and paraquat, has been considered as important risk factors for PD (79). In addition, neurotoxic-based models of PD are interesting in order to overcome deficiencies in sustained dopaminergic neuronal depletion that characterizes pharmacological models (80). Among the different neurotoxins, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are highlighted for their reliability and reproducibility.

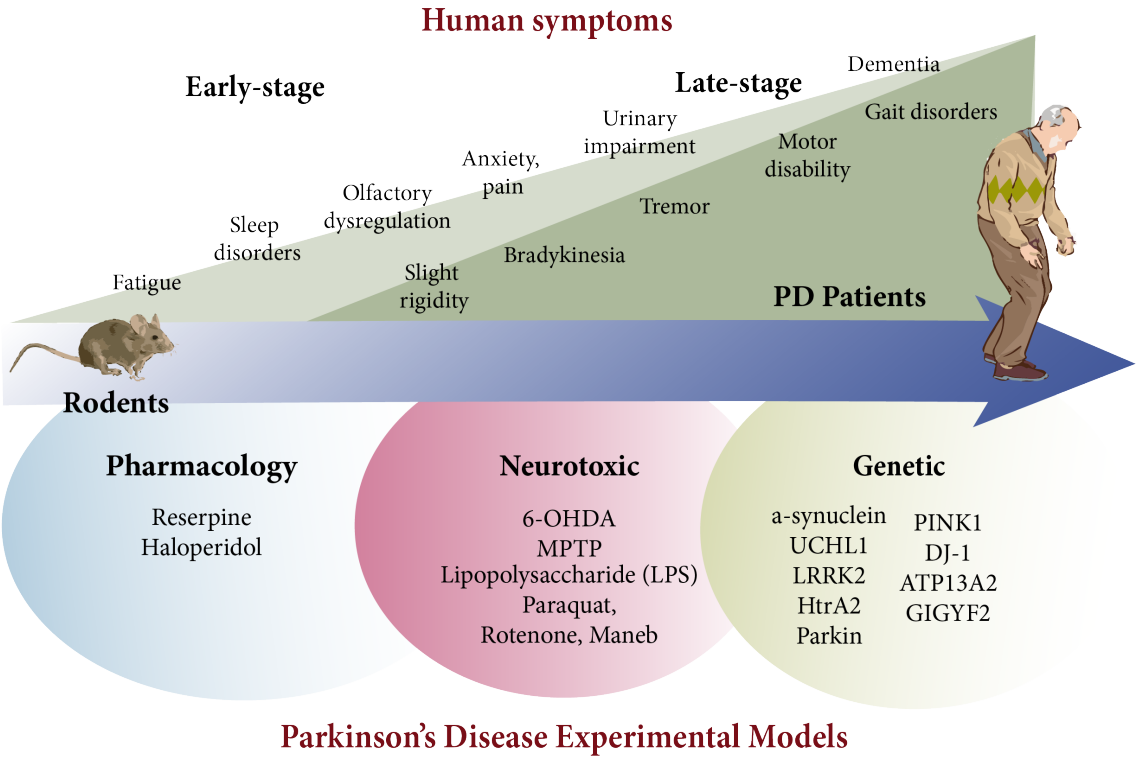

Figure 2.2. Representation of the relationship between PD human symptoms and PD experimental models.

Both 6-OHDA and MPTP have important characteristics as experimental models suitable for investigations focused on neuroprotection. It is well-defined that a therapeutic strategy is "neuroprotective" if acts reducing the process of neuronal depletion. Since their respective discoveries, the use and description of these experimental models has increased considerably in the published studies from 1 in 1963 to 293 in 2018 publications about 6-OHDA; and, from 7 in 1983 to 334 publications in 2018 about MPTP **(Figure 2.3)**. In this sense, neurotoxin based-models are interesting to use for studies with this purpose **(76)**.

2.3.2. Description of neuroinflammation in 6-OHDA-induced Parkinsonism model

6-OHDA was used as PD experimental model in 1968 by Ungerstedt **(81)**. Its mechanism of action is based on its structure, which is similar to dopamine but with a hydroxyl group on the six prime carbon allowing it to specifically kill DA and norepinephrine (NE) neurons. In the brain, it gets inside the DA and NE neurons through the respective transporters DAT and NET **(82,83)**. Once transferred into neurons, 6-OHDA induces cell death via oxidative stress inhibiting mitochondrial respiration **(84)** and, in part, through the stimulation of inflammatory processes **(85)**. Over the years, it has become

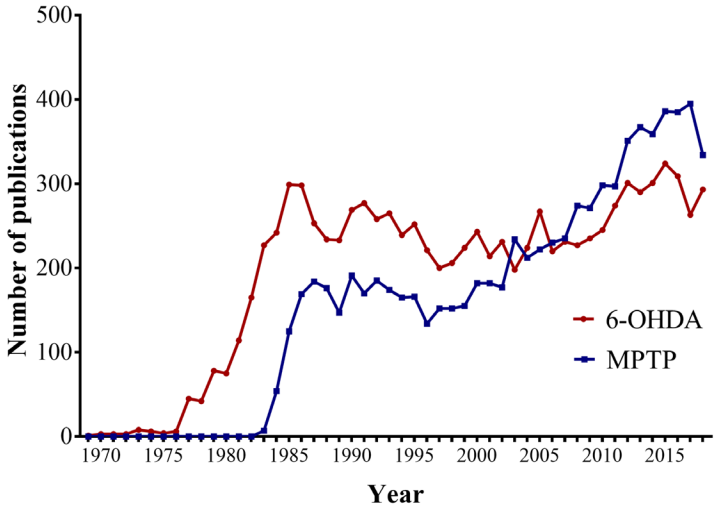

Figure 2.3. Graphs related to the number of published studies about 6-OHDA and MPTP from their respective descoveries in PubMed-NCBI.

the most widely used experimental model because it mimics both early and late stages of the neurodegeneration. As it does not cross the BBB, it has to be locally injected directly into: SNpc, medial forebrain bundle (MFB) or striatum (83). The most used administration is via unilateral injection into the MFB rat. Although, its main limitation is that it cannot be used to study the mechanisms of protein aggregation (α-synuclein inclusions) and it produces acute and unilateral non-progressive neurodegeneration.

As in PD patients whose motor symptoms appear once the loss of dopaminergic neurons is 30% in SNpc and 50% of striatal DA; the intrastriatal injection of 6-OHDA produces a heterogeneous dopaminergic depletion pattern having to reach a threshold of dopaminergic loss to trigger the onset of motor symptoms (86). In addition, 6-OHDA offers another pathological feature of PD, i.e. the glia-mediated response. Microglial activation was observed using *in vivo* positron emission tomography (PET) imaging in rats after unilateral intrastriatal administration of 6-OHDA (87). Later, it was supported by different studies that pointed out an increase of pro-inflammatory mediators (85). However, the pattern of inflammation followed after 6-OHDA lesion is difficult to establish because published results generate some inconsistencies, since it depends on both timing of administration and region of the injection. For instance, one report described that when 6-OHDA is administrated by bilateral intrastriatal injection, microglial response is significantly increased in SN, striatum and hippocampus after 7 days (88). However, in other study, it has been described that the microglial response seems to be more significant in the striatum than in the SN, both 7 and 28 days after injection (89). Depino et al. also previously observed this glial activation pattern indicating non-astrocytic activation in the SNpc but a microglial and an astroglial response in the striatum (90). In addition,

it was reported an increase of microglial and astroglial activation in both lesioned and unlesioned striatum (91).

On the other hand, results from studies based on unilateral 6-OHDA injections in the MFB generate mixed data concerning glial activation. In this sense, it had been described that microglial activation is a secondary phenomenon associated with dopaminergic cell death (92) while other study showed that microglial activation precedes DA neuronal death. Consequently, neurons degeneration may be phagocytosed in an early stage by phagocytic microglia cells which are stimulated by apoptosis signals (93).

Taking all the information together it can be appreciated a promising relationship between neurodegeneration caused by the injection of 6-OHDA with related neuroinflammatory processes. Nevertheless, there are still many questions to be resolved regarding the role of glial response in this model based on a neurotoxin analogous to DA.

2.3.3. Modeling MPTP-intoxicated mice by neuroinflammatory insights

Since the discovery that MPTP could be used as a Parkinsonism-inducing neurotoxin, our understanding of the cause and course of PD has improved (8). MPTP is a byproduct in the synthesis of a meperidine analog whose MPP+ metabolite selectively destroys neurons in the SN, which results in an acute and irreversible human Parkinsonism (94,95). One of the major features of this neurotoxin is its lipophilic property that allows easily crossing the BBB. Once in the brain, MPTP is converted to the intermediate species MPDP+ (1-methyl-4-phenyl-2,3-dihydropyridinium) by the astrocytic monoamine oxidase B (MAO-B) and sequentially oxidized to the active toxic compound MPP+ (1-methyl-4-phenylpyridinium) (96,97). MPP+ is released into the extracellular space from astrocytes through the organic cation transporter 3 (27). To gain access to neurons, it depends on the plasma membrane because of its polar molecule properties. MPP+ has high affinity for the plasma membrane dopamine transporter, as well as for serotonin and norepinephrine transporters (98). In dopaminergic neurons, MPP+ accumulates in synaptosomal vesicles or concentrates in mitochondria inducing neurotoxicity primarily by inhibiting complex I of the mitochondrial electron transport chain (99,100), which results in ATP depletion (101) and an increase in ROS production (102), followed by neuronal death.

It has been described that MPTP has a toxic effect in a variety of species from invertebrates such as C. elegans, zebrafish to rodents and non-human primates. However, the MPTP mouse model has been the most commonly used model for elucidating neuronal death and inflammation through the nigrostriatal pathway in PD (103,104). In this context, rats have been excluded from the modelling of PD because they are exceptionally resistant to MPTP (without MAO-B). Stereotaxic injection of MPP+ or

intranasal administration of MPTP are the alternatives options used to model PD in rats.

As an experimental model, its main disadvantages are the large variability in behavioral and biochemical impairments depending on the mouse strain, age, gender or body weight, it does not present α-synuclein inclusions and it is a non-progressive model. The inbred mouse strain C57BL/6 is sensitive to MPTP intoxication due to its high MAO-B activity. On the other hand, its main advantages are that it causes PD motor impairments, it does not require intracerebral injections and it can be combined with genetic models.

2.3.4. Neuroinflammatory processes triggered by MPTP intoxication

From the point of view of neuroinflammatory processes, the MPTP-based model has provided new insights that highlight the importance of the glial response in the development of dopaminergic degeneration (105). However, it must be taken into account that the published studies cover different MPTP administration regimes that may affect the obtaining of different data (106). In this sense, regimen can be divided mainly into: (i) acute, (ii) sub-acute or sub-chronic and (iii) chronic (Table 2.2). All the degenerative events and associated inflammatory responses depend on the regimen, which involves doses (number of injections, concentration and interval time); and, days of administration.

In general, acute administration regimens in young mice (9-12 weeks old) are the most widely used because MPTP has a fast toxicokinetics with non-progressive effect (76). Thus, after the exposure to MPTP, the nigrostriatal pathway is firstly affected. Specifically, it has been reported that dopaminergic striatal innervations are more sensitive to MPTP insult than dopaminergic neurons in the SNpc. Furthermore, glial processes begin with the activation of microglia cells detected in the striatum 90 min after the last injection of MPTP (119). Then, 12h after the administration of MPTP, active microglia

Table 2.2. MPTP intoxication protocols most used depending on the administration regimen.

Regimen	Doses	Time of administration	References
Acute	(4 x 5-15 mg/kg, 1-2 h)	1 day	(127,128)[2,*]
	(4 x 15-20 mg/kg, 2 h)	1 day	(129-132)[1,2,*]
	(<4 x 20-25 mg/kg, 2 h)	1 day	(123,124)[2,*]
Sub-acute	(1 x 10-20 mg/kg, 24 h)	4-5 days	(132)[2,*]
	(2 x 15-25 mg/kg, 6-12 h)	2 days	(125)[2,*]
	(1 x 20-30 mg/kg, 24 h)	4-5 days	(133)[1,*] (134,135)[2,*] (136)[1,*,**]
Chronic	(1 x 20-30 mg/kg, 56 h)	1-3 months	(136)[2,**], (137)[2,*]

[1]Free base MPTP dose.
[2]1 mg of MPTP-HCl equal to 0.826 mg of free base MPTP (118).
*i.p. / **s.c.

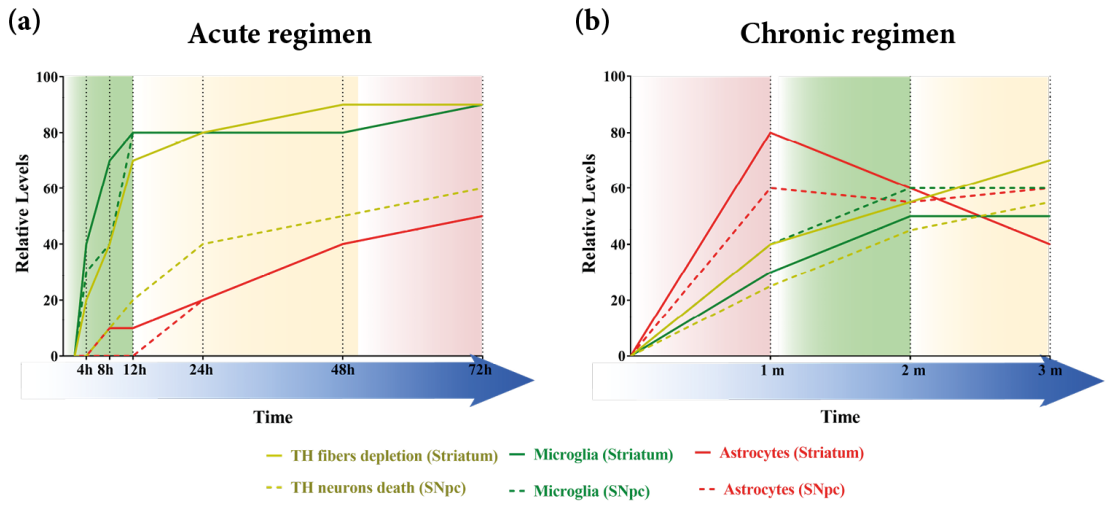

Figure 2.4. Comparative representations of changes in glial response and neuronal death in both **(a)** acute and **(b)** chronic MPTP regimens (Data based on the study from Muñoz-Manchado *et al.* **(117)**).

cells are present in both the striatum and the SNpc although the hydropyridine or the metabolite product from MPTP is cleared from the brain **(120,121)**, preceding neuronal death and, after 24h, striatal depletion still decreasing along with the increase of microglial activation **(104)**. Microglial cells reach their maximum activation peak 48h after the last MPTP injection **(104)**. Astrocytes identified by GFAP+ expression do not change until 3 days after MPTP intoxication **(122)** and, it has been reported that they can be activated till 90 days after the last injection **(105)**. Astrocytes have a delayed response mediating long-term inflammatory mechanisms related to degenerative processes **(70)** **(Figure 2.4.a)**.

In the other hand, regimes known as sub-acute and chronic are, indeed, serial acute insults over days or weeks. In a recent study, chronic regimen was stablished from 1 to 3 months of MPTP administration **(Table 2.2)**. It was demonstrated a progressive and stable neurodegeneration of striatal depletion and dopaminergic cell loss in the SNpc throughout the 3 months. Regarding glial activation, they showed a significant increase of active Iba-1+ cells both in the striatum and in the SNpc. On the other hand, they reported that GFAP+ reached a peak of astroglial response 5 and 15 days of MPTP that progressively and slightly decreases, although staying higher that the control group. In SNpc, GFAP+ cells are significantly increase since the first month **(117)** **(Figure 2.4.b)**.

2.4. Therapeutics strategies

Idiopathic PD is a heterogeneous disorder that involves both motor and non-motor complications. This feature makes research for an effective therapy a complicated

challenge (Table 2.3). Currently, the problem is that the commonly used therapies (pharmacological, surgical and supportive) only treat the symptoms without disease-modifying effect (123). Specifically, current pharmacological treatments for PD are dopamine receptors such us MAO-B inhibitors and COMT inhibitors. Levodopa-based therapy is usually used in the early stage of the motor symptoms development (5-10 years after the disease is diagnosed) alleviating the characteristic motor symptoms of this phase (bradykinesia, rigidity and tremor). However, as the disease progresses, the medications response decrease and the appearance of side effects become significant. It is also described that these treatments have limited effect on non-motor symptoms as cognition and depression, which implicates complications in the most advanced stages of the disease encouraging the increase of morbidity. This fact makes currently therapeutic approaches in PD limited to treat effectively motor symptoms in the early stages and to address insufficient functional deficiencies in later stages, aside from failing to stop the progression of the disease.

Thus, it is an urgent necessity accelerate the process of identifying new treatments for PD. During the last years, a new therapeutic strategy has been suggested entitled "drug repositioning" (124). It is an attractive option since it is focuses on drugs whose clinical safety data have been approved. In fact, the re-use of treatments in some therapeutic areas has provided strategic and important advances in the understanding and identifying new pharmacological targets. The success rates of the use of the repositioning of drug which have already passed the safety studies in Phase I can approach 30%, which represents a huge improvement and advantage compared to traditional forms of drug discovery that it is less than 10%.

Table 2.3. The key questions for the design of an effective therapy for PD.

Questions	Answers	Future directions
What is the main problem?	Current treatments for PD remain symptomatic.	Design a therapy with disease-modifying effect to prevent the progression.
What is the target?	- Motor symptoms: tremor, rigidity, bradykinesia, postural instability... - Non-motor symptoms: depression, anxiety, sleep disturbance, pain, fatigue...	(i)Therapy that prevents the manifestation. (ii) Combined treatment for both type of symptoms. (iii) Therapy that slows down the progression.
What must be considered?	Time of onset, duration of the disease, type of disease, age and social situation	(i) Specific therapies with short- and long-term efficacy. (ii) Personalized treatments
What should be avoided?	Side effects	Improve the quality of life of PD patients.
What should be maintained?	Independence in the ability to carry out daily routine situations	Enhance supportive therapy as education, nutrition or exercise.

In this line, the study on the relationship between neurodegenerative and inflammatory processes may be interesting for the design of new therapeutic strategies (125). In the last years, different anti-inflammatory drugs have been analyzed in both epidemiological and experimental studies (including cell culture and animal models) (124). The data obtained from epidemiological studies are conflicted and not clarify if the use of NSAIDs could delay or prevent the onset of PE. In this line, the paradox in the use of anti-inflammatories, as treatment for PD, arises when it takes into account the following situations proposed by Patrick L. and Edith G. McGeer: (i) if inflammation is combating the disease, this type of treatment makes us more vulnerable to degeneration?; (ii) if anti-inflammatory drugs only help to remove debris, is there any effect?; and, (iii) if microglial cells have autotoxic actions, when patients consume anti-inflammatories drugs, is the degeneration of the disease slowing down? (66).

It is evident that more studies are needed to determine if these drugs can be used as promising treatment in PD. To date, only works carried out with experimental models offer us more conclusive results regarding the use of anti-inflammatories or anti-oxidants drugs with an immunomodulatory effect (reviewed in (126)).

At this point, we wonder why the results obtained from both experimental and epidemiological studies are so discordant. Regardless of the differences in the regulation of degenerative processes between species (human versus rodent), we believe that one of the reasons lies in the fact that most studies are carried out in young/adult animals. This consideration implies obviating the deleterious mechanisms that underlie aging. From the point of view of neuroinflammation, a concept called "neuro-inflammaging" defines a state in aged and PD brains with basal levels of chronic inflammation with different and complex changes in the activity of microglia and astrocytes (127). This feature together with all physiological events occur in aging may result in a different response to pharmacological treatments (128).

2.5. Concluding remarks

Taking everything together, the beginning of the neuroinflammation process is a fundamental piece to understand the cause of the progression of degeneration processes in PD. Thus, unmasking the roles of glial cells in the CNS under pathological conditions could bring us an important point of view of the environment where dopaminergic neuronal cell death occur. The tools available for study, such as experimental models, are essential to recreate that situation. Considering both, the advantages and disadvantages of 6-OHDA and MPTP model, the most important features make them very interesting to study the effect of different anti-inflammatory drugs in dopaminergic degeneration and neuroinflammation. For this reason, the study of strategies based on the effect of

glial response could delay progression and not only treat the symptoms, being closer to a therapy with disease-modifying effect

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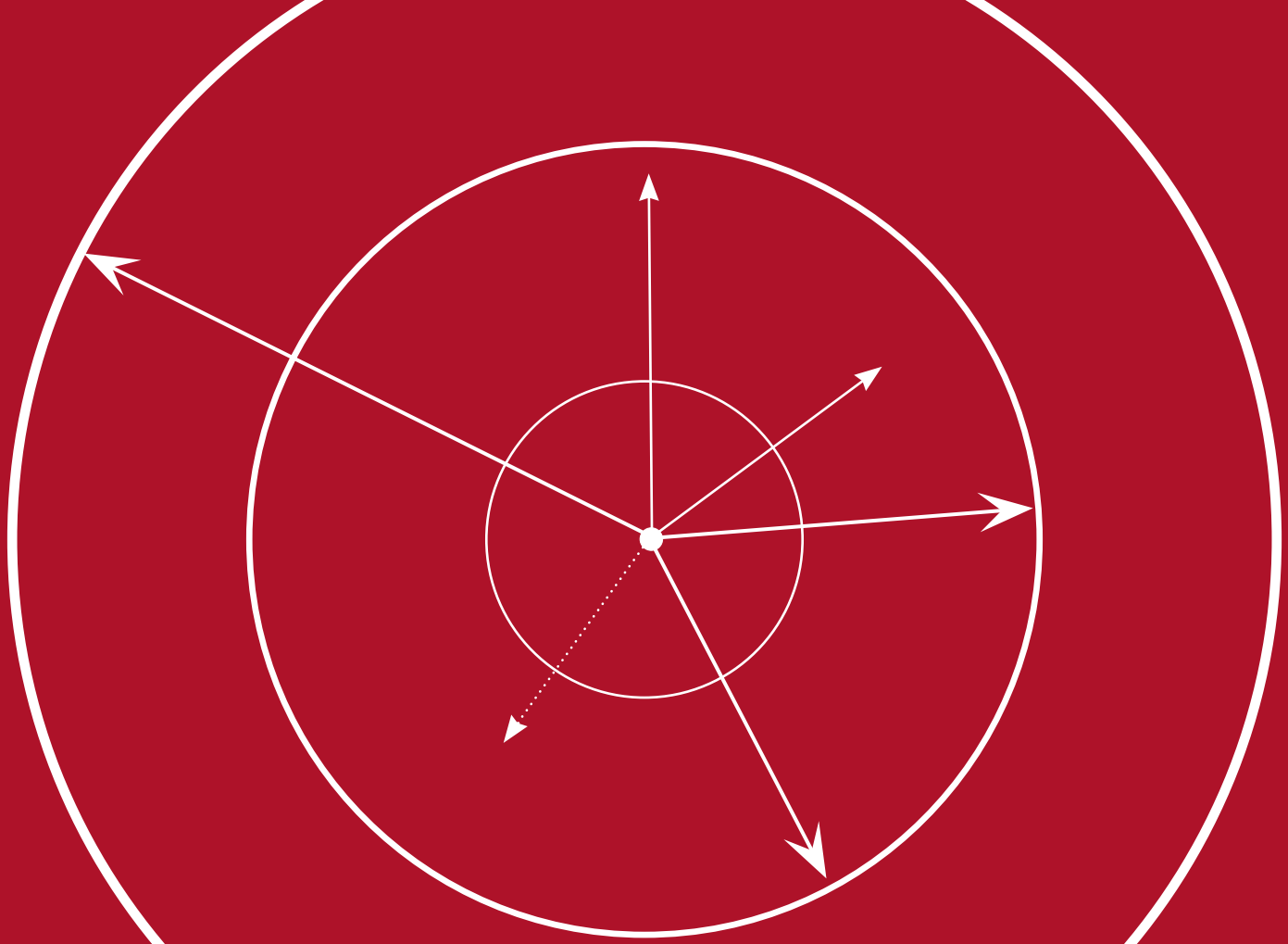
PART I



CHAPTER 3.

Local gastrointestinal injury exacerbates inflammation and dopaminergic cell death in Parkinsonian mice

Gil-Martínez AL[1,2], Estrada C[1,2], Cuenca L[1,2], Cano JA[1], Valiente M[1], Martínez-Cáceres CM[3], Fernández-Villalba E[1,2], Herrero MT[4,5]. **Neurotoxicity Research.** 2019 Feb. 22. doi: 10.1007/s12640-019-0010-z.

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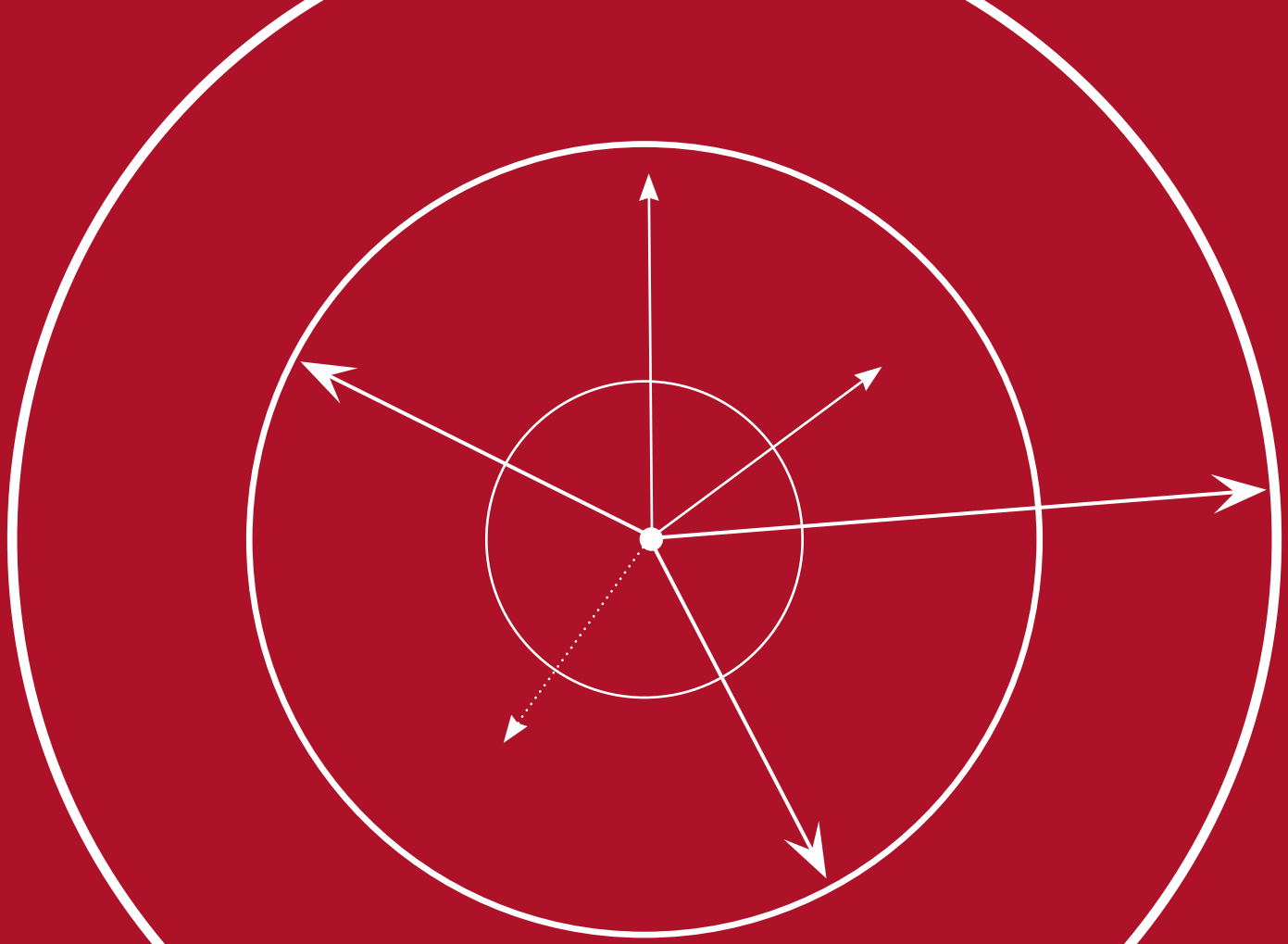
Abstract

The cause of progressive degeneration in Parkinson's disease is not clear, although, in the last years, different studies have suggested that both brain and peripheral inflammation could play a key role in the progression of this disorder. In our study, we aimed to analyze the effect of an acute inflammation confined to the colon on dopaminergic neuronal death and glial response in mice intoxicated with MPTP. The results obtained show a very significant decrease of dopaminergic neurons in the SNpc as well as a significant decrease of dopaminergic fibers in the striatum of the MPTP+DSS-treated group compared with the control animals. In addition, there was a significant exacerbation of microglial and astrocytes activation in MPTP+DSS animals compared with the control group. This data suggests that a specific gastrointestinal injury, which induces a systemic inflammatory response, is able to exacerbate cell death mechanisms of the remaining dopaminergic neurons and then contributes to the persistent progression of the disease. These results leave open new lines of research on the role of exclusive colonic inflammation and the progression of nigrostriatal dopaminergic degeneration.

Keywords

Neurodegeneration; Parkinson's disease; Systemic inflammation; Ulcerative colitis

PART II



CHAPTER 4.

Unexpected exacerbation of neuroinflammatory response after a combined therapy in old Parkinsonian mice

Gil-Martínez AL[1,2], Cuenca L[1,2], Estrada C[1,2], Sánchez-Rodrigo C[1,2], Fernández-Villalba E[1,2], Herrero MT[1,2]. **Frontiers in Cellular Neuroscience.** 2018 Nov 30;12:451. doi: 10.3389/fncel.2018.00451. eCollection 2018.

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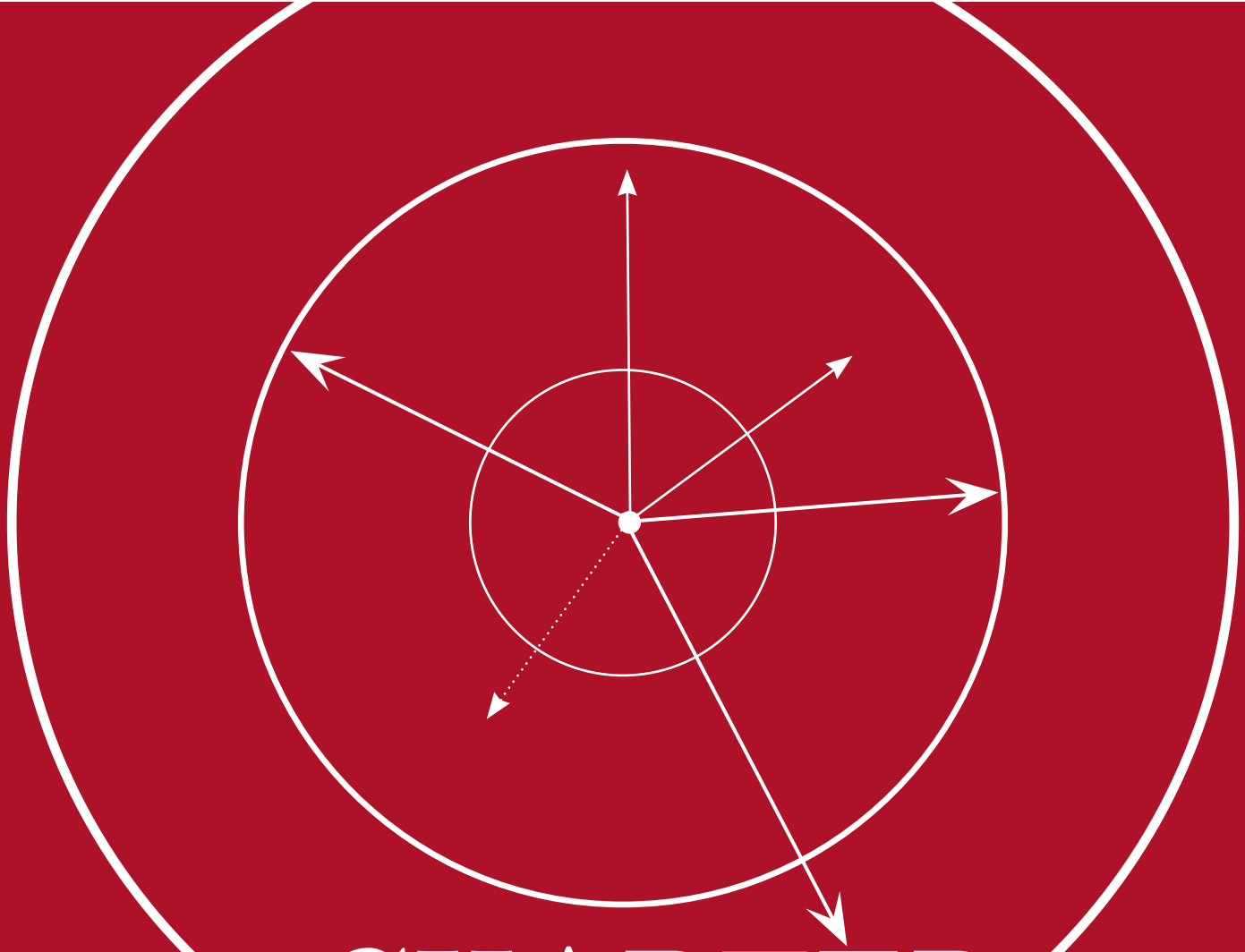
Abstract

The design of therapeutic strategies that focus on the repositioning of anti-inflammatory and antioxidant drugs are a great bet to slow down the progression of neurodegenerative disorders. Despite the fact that Parkinson's disease (PD) is an age-related pathology, almost all experimental studies are carried out in young animals. Here, we evaluated the possible neuroprotective effect of the combination of the antioxidant N-acetylcysteine (NAC) and the anti-inflammatory HA-1077 in aged 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice (C57BL/6 mice, 20 months old), whose individual treatment has been shown to have neuroprotective effects in this Parkinsonism model. Interestingly, NAC+HA-1077-based treatment produced a significant increase in dopaminergic neuronal death accompanied by an increase in microglial and astroglial activation in the Substantia Nigra pars compacta (SNpc) and striatum of old-Parkinsonian mice compared to their control group. The astroglial response was also explored by co-immunostaining for GFAP and S100b together with p-JNK and it was found to be particularly exacerbated in the MPTP+NAC+HA-1077 group. The unexpected toxic effects found in the combined use of NAC and HA-1077 in old-Parkinsonian mice highlight the importance of taking into account that in elderly Parkinsonian patients the combination of some drugs (most of them used for other different age-related alterations) can have side effects that may result in the exacerbation of the neurodegenerative process.

Keywords

Parkinsonism; Aging; Drug-Repositioning; Glia; Neuroinflammation; Oxidative Stress



CHAPTER 5.

Effect of NAC treatment and physical activity on neuroinflammation in subchronic Parkinsonism; is physical activity essential?

Gil-Martínez AL[1,2], Cuenca L[1,2], Sánchez C[1,2], Estrada C[1,2], Fernández-Villalba E[1,2], Herrero MT[3,4]. **Journal of Neuroinflammation**. 2018 Nov 26;15(1):328. doi: 10.1186/s12974-018-1357-4.

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Abstract

BACKGROUND: Neuroprotective strategies are becoming relevant to slow down dopaminergic cell death and inflammatory processes related to the progressive neurodegeneration in Parkinson's disease (PD). Interestingly, among others, physical activity (PA) or anti-oxidant agents (such as N-acetyl-L-cysteine, NAC) are common therapeutic strategies. Therefore, this study aims to analyze if there is a synergistic effect of physical activity along with NAC treatment on dopaminergic degeneration and neuroinflammatory response in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism model after subchronic intoxication.

METHODS: To ascertain this possibility, 48 8-week-old male mice (C57BL/6 strain) were used. Twenty four of them were placed individually in cages where voluntary physical activity was automatically monitored during 30 days and were divided into groups: (i) control; (ii) NAC; (iii) MPTP, and (iv) MPTP+NAC. The other 24 mice were divided into the same four groups but without physical activity.

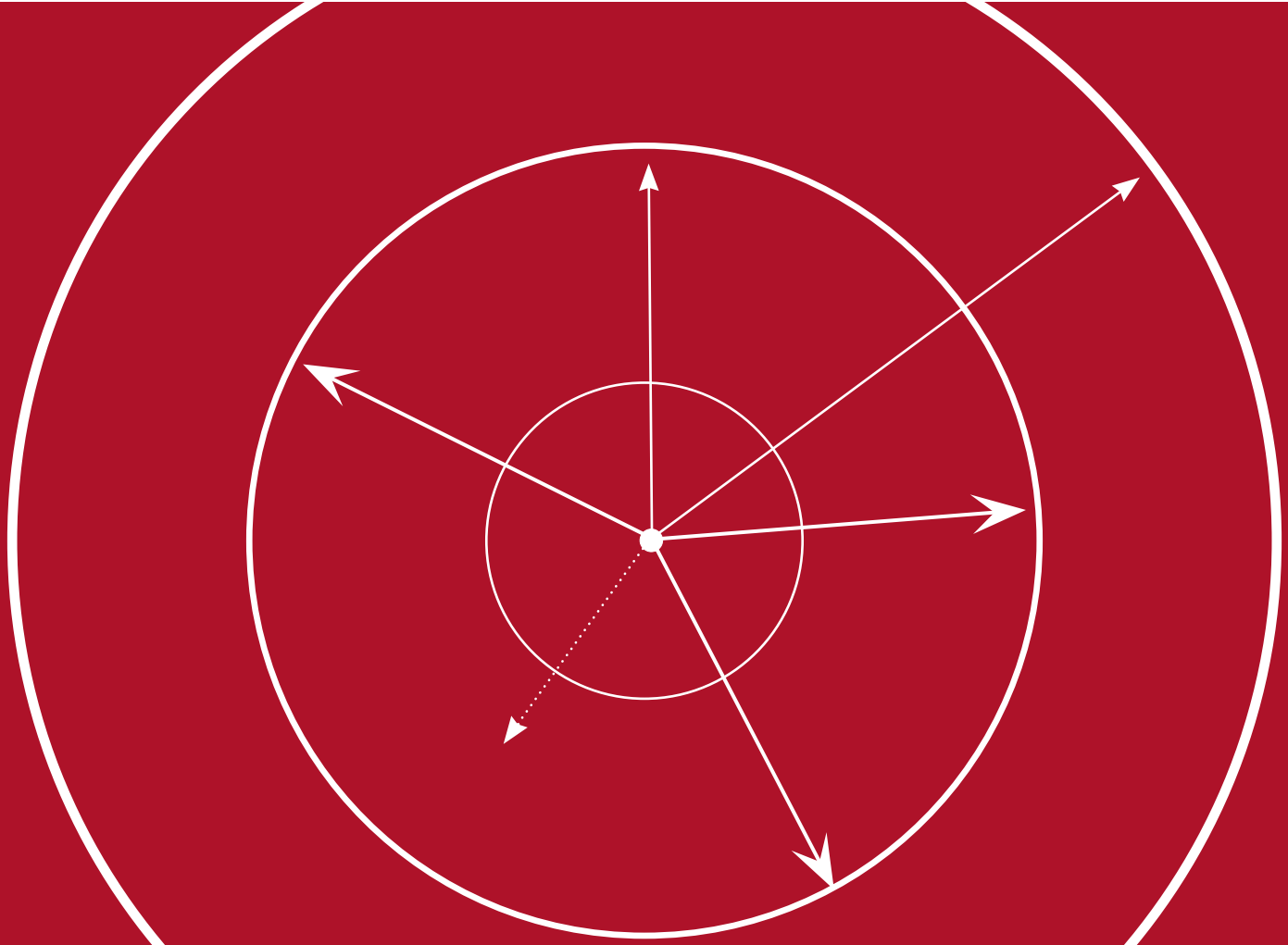
RESULTS: The data collected during the treatment period showed that there was an overall increase in the total running distance in all groups under physical activity, including Parkinsonian animals. However, the monitoring data per day showed that the activity routine by MPTP and MPTP+NAC groups was disrupted by alterations in the circardian rhythm because of MPTP intoxication. Results from post-mortem studies in the substantia nigra pars compacta (SNpc) showed significant decrease in the number of TH+ cells in all MPTP groups. Moreover, TH+ expression in the striatum was significantly decreased in all MPTP groups. Thus, PA + NAC treatment do not protect dopaminergic neurons against a subchronic intoxication of MPTP. Regarding glial response, the results obtained from microglial analysis do not show significant increase in the number of Iba-1+ cell in MPTP+NAC and MPTP+PA + NAC. In the striatum, a significant decrease

is observed only in the MPTP+NAC group compared with that of the MPTP group. The microglial results are reinforced by those obtained from the analysis of astroglial response, in which a decrease in the expression of GFAP+ cells are observed in MPTP+NAC and MPTP+PA + NAC compared with MPTP groups both in the SNpc and in the striatum. Finally, from the study of the astroglial response by the co-localization of GFAP/S100b, we described some expression patterns observed based on the severity of the damage produced by the MPTP intoxication in the different treated groups.

CONCLUSIONS: These results suggest that the combination of physical activity with an anti-oxidant agent does not have a synergistic neuroprotective effect in the nigrostriatal pathway. Our results show a potential positive effect, only due to NAC treatment, on the neuroinflammatory response after subchronic MPTP intoxication. Thus, physical activity is not essential, under these conditions. However, we believe that physical activity, used for therapeutic purposes, has a beneficial long-term effect. In this line, these results open the door to design longer studies to demonstrate its promising effect as neuroprotective strategy.

Keywords

Astrocytes; Microglia; Neuroinflammation; Oxidative Stress; Parkinsonism; Physical Activity; S100b



CHAPTER 6.

Study of the link between neuronal death, glial response and MAPKs pathway in old Parkinsonian mice

Gil-Martinez AL[1,2], Cuenca L[1,2], Cano-Fernandez L[1,2], Sanchez-Rodrigo C[1,2], Estrada C[1,2], Fernandez-Villalba E[1,2], Steinbusch HWM[3], Herrero MT[1,2]. *In preparation.*

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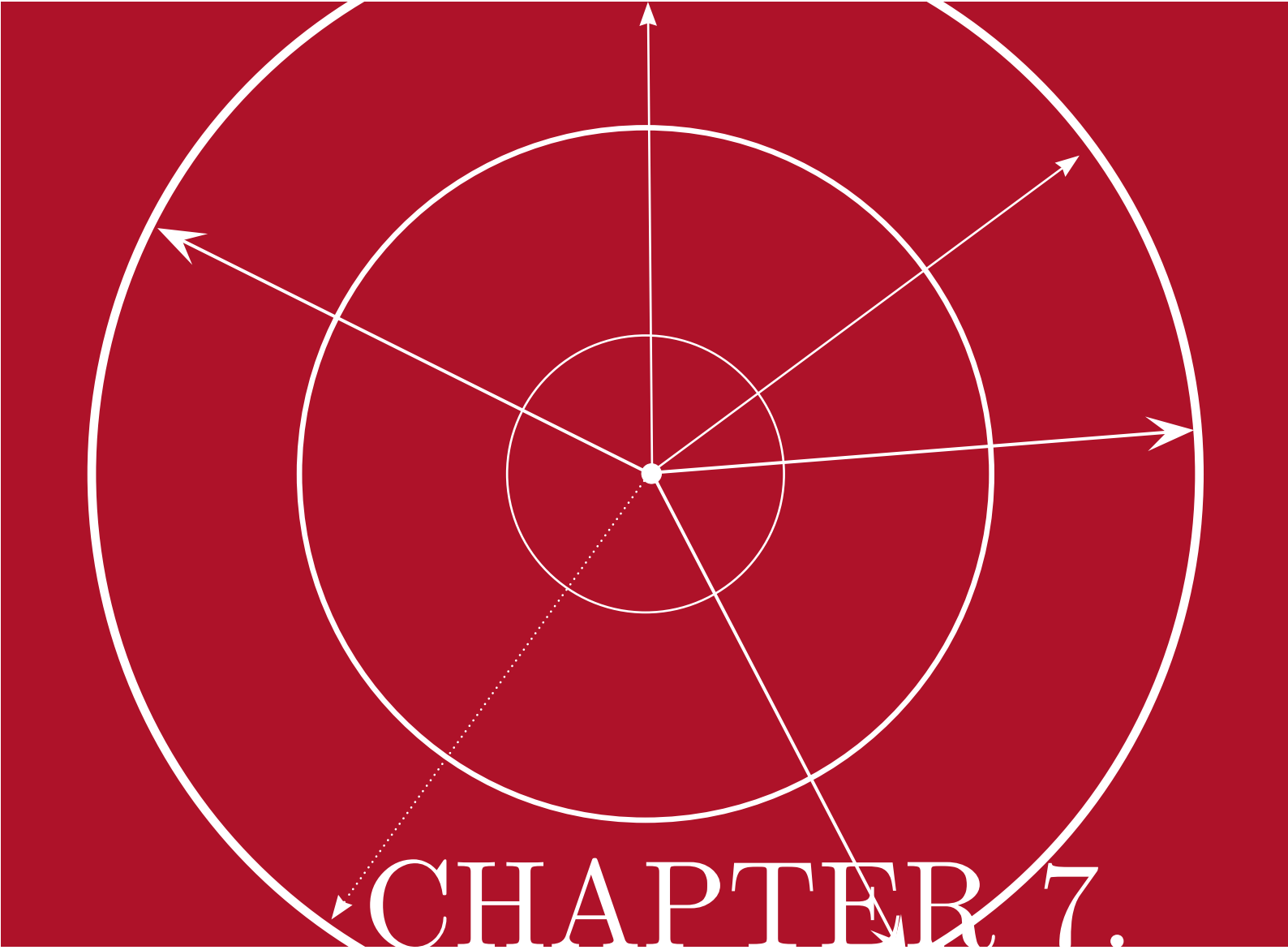
Abstract

In order to establish new therapeutic strategies that slow down neuronal death, one of the great challenges of the present is to describe which are the conditions that perpetuate degeneration and neuroinflammation associate with Parkinson's disease (PD). Thus, mitogen-activated protein kinases (MAPKs) has been implicated in the development of PD. To further define the mechanism of MAPKs expression with the glial response and neuronal death, Parkinsonism was induced in old mice and sacrifice was carried out at different time points (4h, 8h, 24h and 48h) after MPTP injections. The results revealed that neuronal death decreases as glial response increases in the nigrostriatal pathway. Moreover, p-ERK levels decrease while p-p38 expression increases. The importance of these data lies in the possibility of elucidating the underlying mechanisms of neurodegenerative processes to provide knowledge for the search for solutions that slow down the progression of PD.

Keywords

MAPKs, Parkinsonism, Aging, Neuroinflammation, Neurodegeneration



CHAPTER 7.

Conclusions

7.1. Key findings

"A question that turns on your thoughts, that connects your ideas, that projects a world full of possible answers."

This thesis contributes to the investigation of one of the most complex question to answer in the research of Parkinson's disease: what is the main cause of the progression of dopaminergic neurodegeneration? Among all the possible answers, this dissertation is focused on describing the relationship between dopaminergic neuronal cell death and glial cell activation.

To address this issue, Chapter 1 commences with the exposition of the reference frame of the disease followed by the description of the main challenges in the research of PD. Right from the beginning, the involvement of persistent neuroinflammatory mechanisms in the onset of PD is suggested. The clinical frame of the disease is provided with a special focus on the multiple factors that can trigger and maintain dopaminergic degeneration. Once the complex nature of PD is stated, it becomes clear that, among several pathways and molecular mechanism, neuroinflammation have an important contribution to the pathogenesis of the disease.

After this preface, in Chapter 2, the contribution in relation to the involvement of glial cells in Parkinson's disease is reviewed. Starting with a timeline of evidence from experimental, genetic and epidemiological studies that point out a direct association between neuroinflammation and dopaminergic degeneration in PD. From this, it is described the dynamic changes of microglia cells and astrocytes within the central nervous system under health and pathological conditions and, it is highlighted the crucial cross-talk between glial cell and neurons. In this sense, most of the contributions come from experimental studies based on the use of animal models. To this, it is questioned if we are facing an impossible scenario because this kind of disorders, such as Parkinson's disease, are multisystem affected which make it unlikely the fact that an experimental model could collect the entire human clinical frame. Nevertheless, experimental models, specifically in rodent, offer a wide range of possibilities. To study the mechanisms focused on neuroinflammation and neuroprotection, those models based on neurotoxins are

reported to be more suitable. In this chapter, the MPTP-intoxicated based model is widely described as all the studies included within this thesis rely on it. At this point, the potential deleterious implication of inflammatory processes in PD leads to the following question: could therapies based on anti-inflammatory drugs delay the progression of PD? It seems that the answer, so far, is not entirely clear because the results in both experimental and epidemiological investigations are discordant. That is why there is an urgent necessity to gather information in studies that clarify this question. In that way, the present dissertation attempts to elucidate the effects of common drugs on Parkinsonism and to explore the possible molecular targets that are triggered to get an approach of a better understanding.

Following up this exposition of the status of the research in PD; in Chapter 3, it is aimed whether a systemic inflammation, due to a local injury in the colon by the administration of DSS, is able to exacerbate the neuroinflammation produced by MPTP intoxication. The data obtained support that gut inflammation and the subsequent systemic inflammation may lead to nigrostriatal inflammatory changes (both in microglial and astroglial response) that increase dopaminergic neuron vulnerability in the SNpc. Moreover, the loss of dopaminergic neurons in mice treated with DSS alone is particularly interesting and more in-depth studies would be needed to describe the mechanisms that are involved.

In this sense, it is important to highlight one of the most commented observations of this study since the loss of dopaminergic neurons in the nigra does not lead to significant loss of DA terminals in the striatum and rotarod performance. Based on these appreciations and the published literature, the remaining neurons in the nigra could be able to compensate for the decrease of TH expression in the terminals from the neurons, which have disappeared by compensatory mechanisms. In line with this, DSS intoxicated animals did not show a significant increase in motor dysfunction because the clinical effects occur when the loss of dopamine is high enough in the striatum. Taking all together, the main observation of this chapter is based on the larger functional reserve of dopamine neurons in the midbrain and how the administration of a toxic, producing a systemic inflammation, could enhance the degeneration of dopaminergic neurons previously affected by the MPTP intoxication, to reach the functional threshold in which Parkinsonism motor symptoms occur.

Once described the relationship between neurodegenerative and inflammatory processes in MPTP mice, the studies included in Part II are focused on the administration of combinations of common drugs based on “Drug Repositioning”.

In Chapter 4, it is tested if an anti-inflammatory drug, HA-1077, and an anti-oxidant agent, NAC, can block the neurotoxicity observed in the nigrostriatal pathway in old parkinsonian mice. Strikingly, it was observed that the individual drugs, but

particularly NAC, exert a neuroprotective effect in old parkinsonian mice, while the combination of these drugs resulted in the exacerbation of neurodegeneration via the inflammatory response. Based on the outcomes showed, it was further analyzed a possible metabolic pathway that could be affected by the combined treatment. Thus, for being one of the most well-known targets of NAC, JNK expression, belonging to the MAPKinases protein family, was analyzed. The data showed an increase of the phosphorylated form in all parkinsonian groups, except for those animals treated with NAC. The significance of this finding suggests an interesting line of research to expand, by which blocking the expression of this protein, neuroprotective effects are observed in parkinsonian mice. Overall, this chapter highlighted the possible deleterious effect when combining common drugs in the treatment for elderly Parkinsonian patients. In this sense, the importance to increase the studies using old animals is crucial to understand the processes related to aging since elderly brains are more vulnerable. Considerably, future perspectives should attempt to overcome the side effects produced by common drugs by the support of personalized therapy.

In light of the previous results, in Chapter 5, we examined the effects of NAC in combination with physical activity on dopaminergic degeneration and neuroinflammatory response in an MPTP-induced Parkinsonism model after subchronic intoxication. Although the main objective was to observe a synergistic positive action, the results reported that the principal beneficial effect was only due to the NAC administration and, physical activity, under these conditions, was not essential. The key findings were in line with the observations made in the previous chapter because the data not only reinforced the neuroprotective role of NAC but also added a description of its beneficial effect on Parkinsonian adult mice under a sub-chronic regime. On a side note, it is obliquely highlighted some considerations regarding immunofluorescence analysis. Based on the dual immunolabeling for GFAP and S100b, it was described different expression profiles that suggest changes in the cellular location of these proteins depending on the severity of the injury. This data is especially interesting to identify astrocytes in different states of activation and, therefore, to recognize the degree of severity of the disease.

Finally, in Chapter 6, it is converged the most fundamental aspects previously highlighted. It is a more in-depth study that aimed to examine over time the relation between dopaminergic cell death, astroglial response and MAPKinase in old mice after an acute intoxication of MPTP. Thus, it is shown the primary events triggered after the MPTP intoxication. The main observation of this study is, under this regime of intoxication, that events related to neurodegeneration and astroglial response begin to be significant after 48h after the last injection of MPTP. In the same way, the expression of phospho-p38 starts to be significantly detected.

7.2. Limitations and strenghs

"I was taugh that the way of progress was neither swift nor easy". Marie Curie

This thesis is a collection of projects with a common denominator; the interrogation about the involvement of neuroinflammation in age-related disorder, like Parkinson's disease. A major strong aspect of the studies presented here is the consistency throughout the results. That is, all studies point to the crucial effect of neuroinflammation on the dopaminergic neuronal death.

However, there are some general as well as specific limitations inherent in the biological research, and the studies presented in this thesis are also subjected to some of them. One of the most controversial limitations lies in the data coming from the use of experimental models. Generally, it is difficult to work with a large and homogeneous sample population since the funds are not enough, the regulation for animal experimentation is increasingly restrictive and the biological system respond differently depending on variables, which sometimes the researchers do not even control. As a consequence, the moment to statistically analyse and interpret the results becomes a real headache.

In the present dissertation, the results have been analysed statistically with caution since we handled a small to moderate sample size. However, it is important to be aware that the reduced sample size implies different problems, since, sometimes, it makes difficult to obtain enough power to detect changes with a significant p-value. This means that as the power decreases, the probability of finding true effects also decreases. In addition, another compromised problem is the reproducibility of the results. Surprisingly, this is exemplified in this thesis by the results from the administration of a combined treatment of drugs, which individually has been related to positive results in young parkinsonian mice. However, we obtained a clear and an unexpected exacerbation of dopaminergic degeneration together with an activation of the glial response in old parkinsonian mice. To this, the heterogeneity within and between experimental groups in this kind of studies is a characteristic that it must be kept in mind from the beginning of the research until the interpretation of the results. Despite these implicit limitations, the results from these studies have been discussed and rigorously interpreted, taking into account all these considerations.

Other concern throughout the studies collected in this thesis constitutes the disadvantages subjected to experimental models (reviewed in Chapter 2). The major limitation is not to gather all the pathological features of the disease. For instance, in Chapter 3, we were asked about the effect of the intoxication of MPTP and DSS on

the accumulations of a-synuclein. However, it is highly described that it is not observed retrograde transport of a-syn depositions after an acute regimen of MPTP intoxication (1). In addition, in an attempt to study motor performed by open-field and rotarod test, we found it extremely complex to obtain homogeneous results. These difficulties are supported by other previous studies that showed that under an acute administration of MPTP, motor symptoms are hardly exerted (2–5). A way to overcome these limitations, is the development of experimental models combining different kind of them (for example, genetic and neurotoxic based models) to address a greater range of aspects of the disease.

It is also important to be aware of the extent limitations of the methodology used to investigate. The main techniques used throughout the thesis have been immunohistochemistry/immunofluorescence (Chapters 3, 4 and 5) and western-blot (Chapter 4 and 6) for the detection of different markers. Specifically, despite being a simple technique, immunohistochemistry entails different particularities that depend on many factors (6). Especially, it is important to optimize and adapt the protocols for each antibody so that false positives are avoided or to get the maximum signal based on the right dilution and/or incubation time. The interpretation of the data must be done with carefulness. In this thesis, the use of immunohistochemistry has been based on the foundations and procedures of stereology. Thus, all the quantifications have been performed by a systemic, random and unbiased selection of the brain areas. A way to strengthen and empower results from these methodologies is to perform studies regarding the advances in microarray and sequencing techniques to generate data that offers a wider vision.

Finally, it is part of my responsibility to highlight one of the most important limitations that the scientific system is facing: the publication bias. The pressure that researchers are subjected to link one grant to another one has turned out to be a race to get the maximum number of publications. In addition, the "obsession" of high impact journals to publish only positive results, only complicates the situation. However, this fact will also be an advantage for the young researchers, since they will have more opportunities to be more cited. All this has led to a loophole of publication bias. In fact, I have experienced this situation during the publication process of the papers concerning Chapters 4 and 5. It have been especially difficult to get these papers accepted because they mainly show unexpected results. This kind of data should be more valued because it would save time and resources, especially if the study is based on animal experimentation. Definitely, the lack of interest of the scientific community in negative results is one of the most important challenges that the scientific/academic system needs to face.

7.3. Future perspectives

"When we thought we had all the answers, suddenly, all the questions changed". Mario Bennedeti

The key findings described in each chapter together with the limitations previously exposed leave the door open to answer more questions. The future challenges are aimed to innovate and to improve the methodology to obtain results close to the cause and development of the disease. From the first to the last chapter, "time" has been a crucial aspect when designing the experiments. The molecular mechanisms are constantly changing and describing them through a temporary window is fundamental to the diagnosis and the treatment in order to slow down or reverse the progression of Parkinson's disease. Therefore, since these studies have been carried out under an acute regime in order to evaluate the immediate response of the treatments; it would be interesting to study the long-term effects.

Another decisive characteristic has been the use of old animals. To this, it is necessary to expand the studies also to describe possible differences in gender. With the exception of some reports, it is hardly known if there are differences related to glial response males and females or if there is a different response to treatments. In this sense, and as it has been mentioned, the design and the implementation of personalized medicine, that takes into account age or gender could benefit the progression and development of effective therapeutic strategies for complex diseases such as PD.

Finally, there is a rapidly evolving field whose techniques and methods are crucial for the understanding and the modelling of different processes in the brain. Computational neuroscience supposes an essential tool based on mathematical modelling to describe the vast complexity of neurobiological systems and their numerous interactions. The development of integrative genomics, transcriptomics, and epigenomics approaches offer an innovative and advanced point of view. The future perspectives related to these studies are promising to generate and to exchange big datasets.

7.4. References

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SUMMARY

Summary

Different shreds of evidence have been point out that the neuroinflammatory processes have a key role in the initial state and progression of Parkinson's disease. These mechanisms are mainly regulated by glial cells from which microglial cells and astrocytes stand out. These cells are the main components involve when neuroinflammation processes are triggered after a dopaminergic insult. In fact, one of the considerations that deserve attention is the study of the cross talk between glial cells and dopaminergic neurons in both health and pathological conditions since is still unclear. In this line, experimental models based on the induction of Parkinsonism by a neurotoxin, such as 6-OHDA or MPTP, are very useful to elucidate these mechanisms in order to find new molecular targets. To this, therapeutic strategies based on anti-inflammatory drugs are studied in detail in order to design new ones more effective that can reduce or avoid the side effects produced by the current treatments commonly used in Parkinson's disease. Thus, the research focus on the effect of glial response, mediated by microglial cells and astrocytes, on the dopaminergic neurodegeneration could offer new insights to delay the progression of Parkinson's disease by a therapy with disease-modifying effect. With this in mind, the work presented in this thesis examines and explores the involvement of neuroinflammatory processes in the dopaminergic neurodegeneration produced by the induction of Parkinsonism by the administration of MPTP in both young and old mice.

First, Chapter 1 familiarizes the reader with the pathology and pathogenesis of Parkinson's disease and draws the attention to the main problem to be addressed in this work and how it has been approached. After this brief introduction, Chapter 2 presents a thorough overview of the role of neuroinflammation in Parkinson's disease. This report unfolds the timeline of the main contributions, from experimental, genetic and epidemiological studies, that involve inflammatory processes as crucial in the development of

the disease. Finally, it is exposed the main challenges that the research focus on therapeutic strategies to treat PD patients has to overcome. As an alternative, it is suggested the different advantages that the "drug repositioning" offers to avoid possible side effects, since the compounds have already passed the safety studies in Phase I, and to accelerate the identification of new pharmacological targets.

The first experimental part of this thesis, Chapter 3, starts off with an investigation about the effect of systemic inflammation in the neurodegeneration and glial activation in Parkinsonian mice. The hypothesis of this work is inspired by the literature that suggests that both brain and peripherally inflammation could play a key role in the progression of this disorder. In the study, a model of ulcerative-colitis induced by the administration of DSS was carefully combined with an experimental model of Parkinsonism induced by MPTP intoxication. Thus, this chapter aimed to analyze the effect of a systemic inflammation triggered by a local injury confined to the colon on the dopaminergic neuronal death and glial response in parkinsonian mice. The study was performed on 22 four-months male mice (C57BL/6 strain) distributed into four groups: (a) Control, mice drinking tap water; (b) DSS, mice drinking 2-2.5% DSS; (c) MPTP, mice drinking tap water and receiving MPTP injections; and (d) MPTP+DSS, mice drinking 2-2.5% DSS and receiving MPTP injections. The development of the ulcerative-colitis was carefully monitored over the 8 days of the intoxication. The *in vivo* results presented clear clinical features of ulcerative colitis that were confirmed by the histopathological analysis of colon sections. On the other hand, motor behaviour was evaluated by the rotarod test. The data showed significantly lower performance in motor coordination in animals that were MPTP-injected compared to the control groups. Afterwards, *post-mortem* studies of the brain sections showed a very significant decrease of dopaminergic neurons in the SNpc. We also observed a significant decrease of dopaminergic fibers in the striatum of the MPTP+DSS-treated group compared with control animals. In addition, there was a significant exacerbation of microglial and astroglial activation in the MPTP+DSS animals compared to the untreated group. Overall, the data indicated that a specific gastrointestinal injury, which induces a systemic inflammatory response, is able to exacerbate cell death mechanisms of the remaining dopaminergic neurons and then, contributes to the persistent progression of the disease. These results open new lines of research about the role of exclusive colonic inflammation and the progression of nigrostriatal dopaminergic degeneration.

The design of therapeutic strategies focus on "drug repositioning", the

re-use of anti-inflammatory and anti-oxidant drugs are a great bet to slow down the progression of neurodegenerative disorders. In Chapter 4, it was evaluated the possible neuroprotective effect of the combination of two different common drugs: (i) the N-acetylcysteine (NAC), a glutathione precursor and JNK inhibitor with anti-oxidant actions, and (ii) HA-1077, a ROCKinase inhibitor and microglia polarizer. Along with this, it was taking into account the fact that despite that Parkinson's disease is an age-related pathology, almost all experimental studies are carried out in young animals. Consequently, it was studied the effect of the combination of NAC and HA-1077 on the neurodegeneration and glial response in aged 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. 64 twenty-weeks-old male mice (C57BL/6 strain) were used in this study and they were randomly divided into two main groups: 24 of them were non-MPTP mice and the other 40 animals were acutely intoxicated with MPTP. At the same time, the animals were divided into four equal subgroups according to the different treatments: i) Control (untreated); ii) MPTP+NAC; iii) MPTP+HA-1077; iv) MPTP+NAC+HA-1077. Interestingly, the *post-mortem* studies showed that NAC+HA-1077-based treatment produced a significant increase in the degeneration of the dopaminergic striatal terminals. This event was accompanied by an increase in microglial and astroglial activation in the SNpc and in the striatum of old-Parkinsonian mice compared to their control groups. To these results, it was added further analysis of the astroglial response by the co-immunostaining of GFAP and S100b together with p-JNK and the quantification of the expression of JNK, as possible disrupted metabolic pathway. From there, it was observed that JNK and p-JNK expression was particularly exacerbated in all MPTP groups while the levels remained decreasing in MPTP+NAC treated mice. On the whole, the unexpected toxic effects, found after the combined administration of NAC and HA-1077 in old-Parkinsonian mice, highlight the importance of taking into account that in elderly Parkinsonian patients the combination of some drugs (most of them used for other different age-related alterations) can have side effects that may result in the exacerbation of the neurodegenerative process. Therefore, in order to overcome this situation, it is important to support the research focused on the development of more personalized therapeutic strategies.

Chapter 5 covers a similar line of research as the previous chapter. Thus, according to the neuroprotective effects obtained by the administration of NAC, this study aimed to analyse if there was a synergistic positive effect of NAC along voluntary physical activity (PA) on dopaminergic neurodegeneration and glial response in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism model after sub-chronic intoxication. Among other strategies, physical activity is described as a non-pharmacological strategy that takes part

in different brain functions, especially, by inflammatory modifying effects. To ascertain this possibility, 48 eight-week-old male mice (C57BL/6 strain) were used. Twenty four of them were housed individually where voluntary physical activity was automatically monitored during 30 days and animals were divided into four groups: (i) control; (ii) NAC; (iii) MPTP, and (iv) MPTP+NAC. The other 24 mice were divided into the same four groups but without physical activity. The data collected during the treatment period showed that there was an overall increase in the total running distance in all groups under physical activity, including Parkinsonian animals. However, the monitoring data per day showed that the activity routine by MPTP and MPTP+NAC groups was disrupted by alterations in the circadian rhythm because of MPTP intoxication. Moreover, the results from *post-mortem* studies in the substantia nigra pars compacta (SNpc) showed a significant decrease in the number of TH+ cells in all MPTP groups. TH+ expression in the striatum was significantly decreased in all MPTP groups. Thus, the combined treatment does not overprotect dopaminergic neurons against a subchronic intoxication of MPTP. Regarding glial response, the results obtained from microglial analysis do not show a significant increase in the number of Iba-1+ cell in MPTP+NAC and MPTP+PA+NAC. In the striatum, a significant decrease is observed only in the MPTP+NAC group compared to the MPTP group. The microglial results are reinforced by those obtained from the analysis of astroglial activation, in which a decrease in the expression of GFAP+ cells are observed in MPTP+NAC and MPTP+PA+NAC compared with MPTP groups both in the SNpc and in the striatum. Finally, from the study of the astroglial response by the co-localization of GFAP/S100b, it was described different expression profiles regarding the cellular location of these proteins observed depending on the degree of severity produced by the MPTP intoxication in the different treated groups. To end, this study indicates that the combination of physical activity with an anti-oxidant agent does not have a synergistic neuroprotective effect in the nigrostriatal pathway. Our results show a potential positive effect, only due to NAC treatment, on the neuroinflammatory response after subchronic MPTP intoxication. Thus, physical activity is not essential, under these conditions. However, we believe that physical activity, used for therapeutic purposes, has a beneficial long-term effect but more studies are needed to confirm it. In this line, these results open the door to design longer studies to demonstrate its promising effect as a neuroprotective strategy.

The final experimental part of this thesis is presented in Chapter 6, which delves into the changes, over time, of the events related to the dopaminergic degeneration, the astroglial response and the expression of the MAPKinases. As it has been highlighted throughout the chapter of this dissertation, it is important to establish new therapeutic strategies that slow down the dopaminergic neuronal

death at the right time and, importantly, that consider the deleterious processes associated with aging. In this line, mitogen-activated protein kinases (MAPKs) has been pointed out as one of the main metabolic pathways involved in the regulation of inflammation and, as a consequence, it has been related to different neurodegenerative diseases. To further define the relationship between MAPKs expression with the glial response and the dopaminergic neurodegeneration, 40 twenty-weeks-old mice (C57BL/6 strain) were randomly distributed into the control group (untreated mice) and the MPTP group. Animals were sacrificed at different time points after the last injection of MPTP: 4h, 8h, 24h and 48h. The results showed that both processes related to neurodegeneration and astroglial response started to increase significantly in comparison to untreated animals from 24h. The results revealed that dopaminergic neurons decrease as astroglial response increases. Moreover, no significant differences were found in the expression phospho-ERK while the levels of phospho-p38 increased from 4h in the nigrostriatal pathway and, specifically, they became significant at 48h only in the striatum. The importance of these data lies in the description of the primary events triggered in old mice after the MPTP intoxication. In conclusion, these results open the door to deeper studies to evaluate the different metabolic pathways both upstream and downstream together with their comparison between different intoxication regimens (acute and chronic).

In this project, treatments are designed based on the use of anti-inflammatories and/or antioxidants to see its effect on dopaminergic neuronal death and on the activation of neuroinflammatory processes in parkinsonian mice (young and old). The importance of these studies lies in the possibility of elucidating the underlying mechanisms of neurodegenerative processes to improve the quality of life of the patient and provide knowledge for the search for solutions that slow down the development and progression of Parkinson's disease. Thus, this strong clinical relevance is translated into the development of tools for early diagnosis and design of more personalized therapeutic strategies. This dissertation provides different observations that highlight the importance of the involvement of neuroinflammatory processes, mediated by glial cells, in the development and exacerbation of neurodegenerative processes in Parkinson's disease. In addition, it launches the question to the questionable positive effect in some combinations of drugs, commonly used, may accelerate the onset of neurodegenerative diseases related to age, such as Parkinson's disease.

RESUMEN

Resumen

Diferentes estudios señalan que los procesos neuroinflamatorios tienen un papel fundamental en el inicio y en la progresión de la enfermedad de Párkinson. Estos mecanismos están regulados principalmente por las células de la glia, de las que destacan las células microgliales y los astrocitos. Estas células son los principales componentes que intervienen en los procesos neuroinflamatorios desencadenados tras una lesión en las neuronas dopaminérgicas. Es por esto que una de las consideraciones que merecen más atención, pues no están del todo claras, es el estudio de las interacciones que se establecen entre las células gliales y las neuronas dopaminérgicas, tanto en condiciones de homeostasis como patológicas. En esta línea, los modelos experimentales basados en la inducción de parkinsonismo por una neurotoxina, como 6-OHDA o MPTP, son muy útiles para dilucidar estos mecanismos con el fin de encontrar nuevas dianas moleculares. Asimismo, se investigan posibles estrategias terapéuticas basadas en anti-inflamatorios y/o anti-oxidantes para diseñar tratamientos más efectivos que reduzcan o eviten los efectos secundarios producidos por los fármacos actuales comúnmente utilizados en la enfermedad de Párkinson. Con esto en mente, los trabajos presentados en esta tesis examinan la implicación de los procesos neuroinflamatorios en la neurodegeneración dopaminérgica producida por la inducción de parkinsonismo por la administración de MPTP en ratones jóvenes y viejos.

En primer lugar, el Capítulo 1 familiariza al lector con la patología y la patogénesis de la enfermedad de Párkinson. A lo largo del capítulo se va focalizando la atención sobre el principal problema a abordar en este trabajo. Seguido de esta breve introducción, el Capítulo 2 presenta una amplia descripción de la importancia de la neuroinflamación en la enfermedad de Párkinson. En esta revisión se recogen las principales contribuciones desde los primeros trabajos que sugirieron la implicación de los procesos

neuroinflamatorios en la enfermedad de Párkinson hasta las principales cuestiones todavía por resolver. Se exponen que los principales desafíos que debe superar la investigación están centrados en las estrategias terapéuticas para tratar a los pacientes con EP. Como alternativa, se presentan las diferentes ventajas que ofrece el "reposicionamiento de fármacos" como evitar posibles efectos secundarios, ya que consisten en compuestos que han superado los estudios de seguridad en Fase I, y acelerar la identificación de nuevas dianas farmacológicas.

El primer trabajo experimental de esta tesis, recogido en el Capítulo 3, comienza con una investigación sobre el efecto de la inflamación sistémica en la neurodegeneración y en la activación glial en ratones parkinsonianos. La hipótesis de este trabajo está inspirada en publicaciones que sugieren que tanto la inflamación cerebral como la periférica podrían desempeñar un papel clave en la progresión de este trastorno. Para llevar a cabo este trabajo, se utilizó un modelo de colitis ulcerosa inducido por la administración de DSS y se combinó cuidadosamente con un modelo experimental de parkinsonismo inducido por la intoxicación de MPTP. Por lo tanto, este capítulo tuvo como objetivo analizar el efecto de una inflamación sistémica, provocada por una lesión circunscrita en el colon, sobre la muerte neuronal dopaminérgica y la respuesta glial en ratones parkinsonianos. El estudio se realizó en 22 ratones machos (C57BL/6, 4 meses de edad) distribuidos en cuatro grupos: (i) Control, ratones que bebían agua del grifo; (ii) DSS, ratones que bebían 2-2.5% de DSS; (iii) MPTP, ratones que bebían agua del grifo y recibieron inyecciones de MPTP; y (iv) MPTP+DSS, ratones que bebían 2-2.5% de DSS y recibieron inyecciones de MPTP. El desarrollo de la colitis ulcerosa se monitorizó minuciosamente durante los 8 días de la intoxicación. Los resultados *in vivo* de los animales tratados con DSS presentaron claras características clínicas de colitis ulcerosa, que se confirmaron posteriormente mediante el análisis histopatológico de las secciones de colon. Por otro lado, el comportamiento motor se evaluó mediante la prueba de Rotarod. Los datos mostraron un rendimiento significativamente menor en la coordinación motora en animales que fueron intoxicados con MPTP en comparación con los grupos no parkinsonianos. Los estudios *post-mortem* de las secciones de cerebro mostraron una disminución muy significativa de las neuronas dopaminérgicas en la Substancia Nigra pars compacta (SNpc), así como un descenso de las terminaciones dopaminérgicas al nivel del estriado, especialmente significativo en los ratones tratados con MPTP+DSS en comparación con los animales control. Respecto a la respuesta glial, se observó una exacerbación en la activación de células de la microglia y astrocitos en el grupo MPTP+DSS.

En general, los datos indican que una lesión gastrointestinal específica, que induce una respuesta inflamatoria sistémica, puede exacerbar los mecanismos de muerte celular de las neuronas dopaminérgicas restantes desencadenando en una degeneración progresiva en la enfermedad. Estos resultados dejan abiertas nuevas líneas de investigación sobre el papel de la inflamación sistémica, desencadenada por una lesión gastrointestinal, en la progresión de la degeneración dopaminérgica nigrostriatal.

La segunda parte de esta tesis recoge estudios experimentales enfocados en el diseño de estrategias terapéuticas basados en el "reposicionamiento de fármacos" pues la re-utilización de agentes anti-inflamatorios y anti-oxidantes supone una gran apuesta para frenar la progresión de los trastornos neurodegenerativos. De esta manera, en el Capítulo 4, se evaluó el posible efecto neuroprotector de la combinación de dos fármacos comunes: (i) la N-acetilcisteína (NAC), un agente anti-oxidante, precursor del glutatión e inhibidor de la expresión de JNK, y (ii) HA-1077, un anti-inflamatorio, inhibidor de la ROCKinase y polarizador de microglia. Junto con esto, se tuvo en cuenta el hecho de que, a pesar de que la enfermedad de Párkinson es una patología relacionada con el envejecimiento, casi todos los estudios experimentales se llevan a cabo en animales jóvenes. Con esto en mente, se evaluó el efecto de la combinación de NAC y HA-1077 sobre la neurodegeneración y la respuesta glial en ratones añosos tratados con 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP). Se utilizaron 74 ratones machos (C57BL/6, 20 semanas de edad) y se dividieron al azar en dos grupos: veinticuatro de ellos eran ratones sin MPTP y los cuarenta restantes se intoxicados de forma aguda con MPTP. Al mismo tiempo, los animales se dividieron en cuatro subgrupos de acuerdo con los diferentes tratamientos: (i) Control (sin tratar); (ii) MPTP+NAC; (iii) MPTP+HA-1077; (iv) MPTP+NAC+HA-1077. Sorprendentemente, los estudios *post-mortem* mostraron que el tratamiento basado en NAC+HA-1077 producía un aumento significativo en la degeneración de las terminales dopaminérgicas al nivel del estriado. Junto a este hecho, se observó un aumento en la activación microglial y astroglial en la SNpc y en el estriado de los ratones parkinsonianos en comparación con sus grupos control. Además, se realizó un análisis más amplio enfocado en la respuesta astroglial mediante la inmunotinción de GFAP, S100b y p-JNK, y la cuantificación de la expresión de los niveles de JNK, como posible vía metabólica alterada. Así, se observó que la expresión de JNK y p-JNK se exacerbaba particularmente en todos los grupos intoxicados con MPTP, mientras que los niveles se mantenían bajos en el grupo MPTP+NAC. En general, los inesperados efectos tóxicos encontrados tras la administración combinada de NAC y HA-1077 en ratones viejos

parkinsonianos, resaltan la importancia de tener en cuenta la cuestionable acción terapéutica al combinar ciertos fármacos en pacientes ancianos con enfermedad de Párkinson. Esto es, diferentes alteraciones relacionadas con la edad pueden tener efectos secundarios que pueden resultar en la exacerbación del proceso neurodegenerativo. Como alternativa para evitar estas situaciones, es importante apoyar la investigación centrada en el desarrollo de estrategias terapéuticas más personalizadas.

El Capítulo 5 cubre una línea de investigación similar a la del capítulo anterior. En base a los efectos neuroprotectores obtenidos por la administración de NAC, este estudio tuvo como objetivo analizar si se produce un efecto sinérgico positivo del tratamiento con NAC al combinarlo con actividad física voluntaria (PA) sobre la neurodegeneración dopaminérgica y la respuesta glial en un modelo de parkinsonismo inducido por 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP). Entre otras estrategias, la actividad física se ha descrito como un tratamiento no farmacológico que participa en diferentes funciones cerebrales, especialmente, mediante la modificación de la respuesta inflamatoria. Para llevar a cabo este estudio, se utilizaron 48 ratones machos (cepa C57BL/6, 8 semanas de edad). 24 de ellos se colocaron individualmente en jaulas donde la actividad física voluntaria se controló automáticamente durante 30 días. A su vez, se dividieron en cuatro subgrupos en función de los tratamientos: (i) Control; (ii) NAC; (iii) MPTP, y (iv) MPTP+NAC. Los otros 24 ratones restantes se dividieron en los mismos cuatro subgrupos pero sin actividad física. Los datos recopilados durante el período de actividad física mostraron que hubo un aumento general en la distancia total recorrida en todos los grupos con actividad física, incluidos los animales parkinsonianos. Sin embargo, los datos de la monitorización diaria mostraron que la rutina de actividad de los grupos MPTP y MPTP+NAC se veía alterada por cambios en el ritmo circadiano debido a la intoxicación por MPTP. Los resultados de los estudios *post-mortem* en la SNpc y en el estriado mostraron una disminución significativa en el número de células TH+ en todos los grupos intoxicados por MPTP. Por lo tanto, el tratamiento combinado no protege a las neuronas dopaminérgicas contra una intoxicación subcrónica de MPTP. Con respecto a la respuesta glial, los resultados obtenidos del análisis microglial no muestran un aumento significativo en el número de células Iba-1+ en los grupos MPTP+NAC y MPTP+PA+NAC. En el estriado, se observa una disminución significativa solo en el grupo MPTP+NAC en comparación con el grupo MPTP. Los resultados microgliales se ven reforzados por los obtenidos del análisis de la activación astroglial, en la que se observa una disminución en la expresión de células GFAP+ en

MPTP+NAC y MPTP+PA+NAC en comparación con los grupos MPTP tanto en la SNpc como en el estriado. Finalmente, a partir del estudio de la respuesta astroglial por la co-localización de GFAP/S100b, se describieron diferentes perfiles de expresión con respecto a la ubicación celular de estas proteínas según el grado de severidad producido por la intoxicación con MPTP. En definitiva, este estudio indica que la combinación de actividad física con un agente antioxidante no tiene un efecto neuroprotector sinérgico en la vía nigrostriatal. Los resultados muestran un efecto positivo potencial, solo debido al tratamiento con NAC, en la respuesta neuroinflamatoria después de una intoxicación por MPTP. Por lo tanto, la actividad física no es esencial, bajo estas condiciones. Sin embargo, creemos que la actividad física, utilizada con fines terapéuticos, tiene un efecto beneficioso a largo plazo. En esta línea, estos resultados sugieren que estudios más largos podrían demostrar su efecto como estrategia neuroprotectora.

La parte experimental final de esta tesis se presenta en el Capítulo 6, que profundiza en los cambios, a lo largo del tiempo, de los eventos relacionados con la degeneración dopaminérgica, la respuesta astroglial y la expresión de las MAPKinasas. Como se ha destacado a lo largo de esta disertación, es importante establecer nuevas estrategias terapéuticas que retrasen la muerte neuronal dopaminérgica y, especialmente, que tengan en cuenta los procesos deletéreos asociados con el envejecimiento. En esta línea, las proteínas quinasas activadas por mitógenos (MAPK) ha sido destacadas como una de las principales vías metabólicas involucradas en la regulación de la neuroinflamación y, como consecuencia, se han relacionado con diferentes enfermedades neurodegenerativas. Para definir la relación entre la expresión de MAPKs con la respuesta glial y la neurodegeneración dopaminérgica, 40 ratones machos (cepa C57BL/6, 20 semanas de edad) se distribuyeron al azar en dos grupos: grupo control (ratones no tratados) y grupo MPTP. Los animales se sacrificaron en diferentes puntos temporales después de la última inyección de MPTP: 4 h, 8 h, 24 h y 48 h. Los resultados mostraron que los procesos relacionados con la neurodegeneración y la respuesta astroglial comenzaron a detectarse significativamente, en comparación con los animales no tratados, a partir de las 24 h. Los resultados revelaron que las neuronas dopaminérgicas disminuyen a medida que aumenta la respuesta astroglial. Por otro lado, no se encontraron diferencias significativas en la expresión p-ERK mientras que los niveles de p-p38 se identificaron de manera aumentada desde las 4 h en la vía nigrostriatal siendo significativos a las 48 h en el cuerpo estriado. La importancia de estos datos radica en la identificación de los eventos primarios desencadenados en ratones viejos después de la intoxicación

con MPTP. En conclusión, estos resultados abren la puerta a estudios más profundos para evaluar las diferentes vías metabólicas tanto en aguas-arriba como aguas-abajo, junto con su comparación entre diferentes regímenes de intoxicación (aguda y crónica).

En este proyecto, los tratamientos están diseñados en función del uso de anti-inflamatorios y/o anti-oxidantes para ver su efecto en la muerte neuronal dopaminérgica y en la activación de procesos neuroinflamatorios en ratones parkinsonianos (jóvenes y viejos). La importancia de estos estudios radica en la posibilidad de dilucidar los mecanismos subyacentes de los procesos neurodegenerativos para mejorar la calidad de vida del paciente y proporcionar conocimientos para la búsqueda de soluciones que retrasen el desarrollo y la progresión de la enfermedad de Párkinson. Por lo tanto, esta fuerte relevancia clínica se traduce en el desarrollo de herramientas para el diagnóstico temprano y el diseño de estrategias terapéuticas más personalizadas. Esta tesis proporciona diferentes observaciones que resaltan la importancia de la participación de los procesos neuroinflamatorios, mediados por las células de la glía, en el desarrollo y la exacerbación de los procesos neurodegenerativos en la enfermedad de Párkinson. Además, expone, mediante resultados, el cuestionable efecto positivo de algunas combinaciones de medicamentos, de uso común, que pueden acelerar la aparición y progresión de enfermedades neurodegenerativas relacionadas con la edad, como la enfermedad de Párkinson.

CURRICULUM VITAE

Curriculum Vitae

Ana Luisa Gil Martínez was born on January 19th 1992 in Murcia, Spain. She became interested in science when she was almost 9 years old. Together with her father, she was a great enthusiastic about physics, specifically astronomy. As a result of her "guilty pleasure", she went to various courses and conferences related to that field. Once in high school, she opened the spectrum of knowledge and concern to other subjects such as Biology and Chemistry. In the last courses, she was selected to carry out a special program entitled "Research Baccalaureate" in the Category of Health Science where she got her first chance to perform a research project entitled "*Laces, zippers and Nobel Prize in Medicine*".

Thanks to this work, she did not hesitate to study in depth the molecular foundations of biological systems and, she enrolled in the Bachelor's Degree of Biochemistry (School of Chemistry, University of Murcia). During the four years of the bachelor's degree, she found different subjects of interest such as Molecular Biology, Molecular Genetics, Chemistry or Bioinformatics. Thus, she joined the Department of Inorganic Chemistry where she completed her final Bachelor Thesis project entitled "*Silk fibroin nanoparticles loaded with a prodrug of platinum (IV) as an alternative in antitumor therapy*". This work was of great interest which resulted in a publication in Dalton Transactions (2015). The project consisted of synthesizing an inorganic compound with antitumor properties and encapsulating it in silk fibroin nanoparticles. Due to the great impact of the previous study, she decided to continue investigating what was the specific response of the tumour and healthy cells after the administration of the loaded nanoparticles. From there, her Master project in Molecular Biology and Biotechnology emerged. This period gave her the skills related to the management of cell cultures, flow cytometry and confocal microscopy. However, due to the lack of funding, she had to leave the laboratory when she finished her Master's Degree Thesis. It was then, when neuroscience and her, met for the first time. In October 2015,

Dr Maria Trinidad Herrero offered a contract for a biochemist to do the PhD. After the job interview, she was selected and in December 2015, she started her PhD focused on the effect of neuroinflammation in Parkinson's Disease.

From there, she was trained in different essential techniques to carry out her research, such as stereology, image analysis or statistics. During the first year, she was selected to take a course in the CAJAL Advanced Neuroscience Training Program entitled "*Glial cells in health and disease*" (Bordeaux, France). In 2016, she presented a poster in the 10th FENS-Forum held in Copenhagen (Denmark) where it was agreed to do a joint doctorate with Dr Harry Steinbusch from Maastricht University. Her work has been presented as posters and oral presentations in different national and international conferences, including the European Meeting on Glial Cells in Health and Disease (Edinburg, Scotland) or The Annual MHeNs Research Day (Maastricht, The Netherlands). Her delight to give oral communications have made her selected as a Stellar Communication in the LXVIII Annual Meeting of the Spanish Society of Neurobiology (Valencia, Spain) as well as being awarded with the prize for Best Oral Communication in the Pre-meeting School: Neuroglia in Health & Disease (Florianópolis, Brasil). Thus, she has participated in different scientific dissemination events such as the international event Pint of Science, Researchers' Night or Day of Women and Girls in Science.

Ana Luisa has also been practical supervisor in Neuroanatomy and Human Anatomy in Medicine and Pharmacy, respectively at the University of Murcia, and she has co-tutored many Bachelor and Master students during her thesis. Far from teaching others, Ana Luisa also continued expanding her own skills, taking courses and workshops in bioinformatics and statistics. In this line, her future perspectives are focused on learning and study in-depth the role of epigenetics in the onset and progression of Parkinson's disease based on bioinformatics approach techniques.

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ACKNOWLEDGMENTS

Acknowledgments

Este proyecto no hubiera sido posible sin todas las personas que me han rodeado y que, de una manera u otra, me han hecho más fuerte para llevarlo a cabo. Por eso, a todos ellos, me gustaría darles las gracias.

A mi directora, la Dra. María Trinidad Herrero, por confiar en mí para llevar a cabo este proyecto y darme la oportunidad de desarrollarme como investigadora y como persona. No ha sido un camino fácil, pero me has demostrado que con esfuerzo y perseverancia se pueden superar todos los obstáculos y conseguir cualquier cosa.

I want to thank to my supervisor, Dr. Harry Steinbusch, for his dedication in this project and for all the nice talks about science and life during my period in Maastricht. I will never forget the great hospitality that Gonny and you had with me.

A Lorena Cuenca y a Consuelo Sánchez. GRACIAS. Vuestro apoyo diario e incuestionable me ha obligado a dar lo mejor de mí misma constantemente. Vuestras risas han convertido los peores momentos en una anécdota más. Espero haberos dado la mitad de lo que me habéis aportado. En este trabajo queda reflejado un antes y un después de nosotras. Lorena, ahora te toca a ti darlo todo, permítete disfrutar de tu fortaleza interna y no dejes de luchar por aquello que te hace feliz. Consuelo, mi pequeño gran saltamontes, sigue con tu entusiasmo y tu pasión porque es lo que te hace poder llegar hasta donde tú quieras.

Thanks to Cate and Martyna for making Maastricht an unforgettable experience (we still have a road trip to do). To Víctor and Yiyong, because together we built a small home in Maastricht.

A mis estudiantes, Juanan, Manu, Iris, Rocio, Clivia, Armando, Angela, Marina y Lorena. A todos vosotros, porque me hicisteis conscientes de las dificultades que conlleva enseñar. Sin embargo, juntos aprendimos que

el secreto para superarlas estaba en la constancia y en la organización. Que no hacía falta sacrificar nada, porque en el momento que lo hacíamos, dejábamos de disfrutar. Me permitisteis salir de mi zona de confort. Entendimos el fundamento de muchas cosas y compartimos todo lo que aprendíamos. Me pedisteis ser detallista con vuestros proyectos y, al final, todos acabamos siendo perfeccionistas. Especialmente, a Lorena Cano, no pares de luchar por lo que crees y no pierdas la confianza en ti misma, tampoco permitas que nadie te la quite.

A María Vera, Ana Zamora, Paula Gómez, Gloria Vigueras y Arturo Rocamora. Mi "Familia Hermsworth", no sabéis la culpa que tenéis en todo esto. Con vosotros aprendí que los compañeros de laboratorio pueden formar una gran familia. Gracias por todas las locuras, las risas y las infinitas conversaciones entre experimentos y cafés.

A Raquel y Marina. Mis incansables compañeras, hemos compartido dificultades que hemos convertido en retos. Retos que no han hecho nada más que unirnos, y es que, "la unión hace la fuerza" y juntas, siempre lo hemos conseguido. Gracias por entenderme en cada situación y ayudarme con tanto cariño.

A Josedu. Gracias por todas esas horas de conversaciones y consejos. Gracias por escucharme y permitir que me escuchara porque hacía que los buenos pensamientos se hicieran más fuertes y los malos se debilitaran.

A mis amigos. Jorge Tárraga, Ana García, María González, Pablo Piqueras, Arturo García, Gema Pardo, Beatriz Lorente, Paula Ruiz, Carmona y Pablo Mínguez. A vosotros, porque nos hemos visto crecer. Gracias por todos esos años con sus muy buenos y no tan buenos momentos porque han sido cruciales en mi forma de pensar y de sentir.

A mis tíos y a mis primos, por haber sido tan pacientes cuando un "hoy no puedo, tengo que estudiar" hacía que hubiera una silla vacía en la mesa. Prometo recuperar todas las comidas/cenas que me he perdido.

Finalmente, quiero terminar con los pilares de mi vida, las personas que viven como si les tocara a ellas mismas mis alegrías y mis tristezas.

A mis abuelos. A Lala, a ti, tu voz me guía desde el cielo. A Lolo, tu esfuerzo y tu bondad me enseñan que siempre puedo ser mejor persona. A Lady, por tu infinita fortaleza y por tu inquietud interna tan reveladora, solo personas tan pasionales como tú aman de manera tan incondicional. Y así, a tu amor, a Pepe, también se lo agradezco.

A Elena, a mi hermana, a mi mitad. Tu existencia, nuestra conexión, me dan la fortaleza para ir junto a ti y poder superar cualquier cosa.

A mi madre y a mi padre. Sois la génesis de todos mis proyectos y mis sueños, mi inspiración. Gracias por darme, demostrarme y enseñarme todo. No existen palabras que expresen la gratitud que siento, creo que sólo los hechos os podrán demostrar lo que habéis conseguido. Este trabajo es sólo una mínima parte. Este trabajo es vuestro.

A Miguel Ángel. Las palabras de agradecimiento se quedan cortas en comparación a su significado. En comparación a todas las horas, las ganas y, sobretodo, el amor que me has dado durante todo este tiempo. Has sido y eres una parte fundamental de mí y, por tanto, de este trabajo. Gracias, porque has sabido animarme cuando más hundida me encontraba, has hecho que disfrutase de mi risa cuando más feliz me sentía, y lo más importante, has hecho que me encontrara cuando más perdida estaba. Gracias por haber sabido estar en cada momento, lejos o cerca de mí, siempre has estado a mi lado.

ABBREVIATIONS

6-OHDA: 6-hydroxydopamine

[18F]DPA-714: N,N-diethyl-2-[4-(2-fluoroethoxy)phenyl]-5,7-dimethylpyrazolo[1,5-a]pyrimidine-3-acetamide

α-syn: alpha-synuclein

ANOVA: analysis of variance

ATP: adenosine triphosphate

BBB: blood-brain barrier

BP: biological process

C57BL/6 mice: C57 black 6 inbred mouse strain

CC: cellular component

CNS: central nervous system

COMT: catechol-omicron-methyltransferase

DA: dopaminergic

DAB: 3,3'-diaminobenzidine

DAI: disease activity index

DAT: dopamine active transporter

DEGs: differentially-expressed genes

DSS: dextran sodium sulfate

EMA: European Medicines Agency

ERK: extracellular signal-regulated kinase

FDA: Food and Drug Administration

FDR: false discovery rate

GFAP: glial fibrillary acid protein

GO: Gene Ontology

GWAS: genome-wide association study

HA-1077: fasudil

HLA: human leukocyte antigens

IBA-1: ionized calcium-binding adapter molecule 1

IL: interleukins

IR: immunoreactive

JNK: c-Jun NH2-terminal kinase

LRRK2: leucine-rich repeat kinase 2

LPS: lipopolysaccharide

MAPKs: mitogen-activated protein kinases

MPDP+: 1-methyl-4-phenyl-2,3-dihydropyridinium

MPP+: 1-methyl-4-phenylpyridinium

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

MF: molecular function

MFB: medial forebrain bundle

MAO-B: monoamine oxidase B

NAC: *N*-acetyl-L-cysteine

NSAIDs: non-steroideal anti-inflammatory drugs

NE: norepinephrine

NET: norepinephrine transporter

NO: nitric oxide

PA: physical activity

PCA: principal component analysis

Abbreviations

PD: Parkinson's disease

PET: positron emission tomography

PERC: perchloroethylene

PINK1: PTEN induced putative kinase-1

ROS: reactive oxygen species

SN: substantia nigra

SNpc: substantia nigra pars compacta

SOD: superoxide

TCE: trichloroethylene

TGF-β: transforming growth factor beta

TH: tyrosine hydroxylase

TNF-α: tumour necrosis factor alpha

VTA: ventral tegmental area

SUPPORTING INFORMATION

Supporting Information Chapter 4

The Supplementary Material corresponding for this Chapter can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2018.00451/full#supplementary-material

Supporting Information Chapter 5

The Supplementary Material corresponding for this Chapter can be found online at: https://jneuroinflammation.biomedcentral.com/articles/10.1186/s12974-018-1357-4