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Neuroprotective and immunomodulatory properties of the new PPARγ agonist MDG548: an *in vitro* and *in vivo* study in PD models.

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ABSTRACT

Neuroinflammation is a key component of Parkinson's disease (PD) neuropathology. Skewed microglia activation with pro-inflammatory prevailing over anti-inflammatory phenotypes may contribute to neurotoxicity via the production of cytokines and neurotoxic species. Therefore, microglia polarization has been proposed as a target for neuroprotection. The peroxisome proliferator-activated receptor gamma (PPARγ) is expressed in microglia and peripheral immune cells, where it is involved in macrophages polarization and in the control of inflammatory responses, by modulating gene transcription. Several studies have shown that PPARγ agonists are neuroprotective in experimental PD models in rodents and primates. however safety concerns have been raised about PPARγ agonists thiazolidinediones (TZD) currently available, prompting for the development of non-TZD compounds.

Aim of this study was to characterize a novel PPARγ agonist non TZD, MDG548, for its potential neuroprotective effect in PD models and its immunomodulatory activity as the underlying mechanism of neuroprotection. The neuroprotective activity of MDG548 was assessed *in vivo* in the subacute MPTP model and in the chronic MPTP/probenecid (MPTPp) model of PD. MDG548 activity on microglia activation and phenotype was investigated in the substantia nigra pars compacta (SNc) via the evaluation of pro- (TNF-α and iNOS) and anti-inflammatory (CD206) molecules, with fluorescent immunohistochemistry. Moreover, cultured murine microglia MMGT12 were treated with MDG548 in association with the inflammagen LPS, pro- and anti-inflammatory molecules were measured in the medium by ELISA assay and phagocytosis was evaluated by fluorescent immunohistochemistry for CD68.

MDG548 arrested dopaminergic cells degeneration in the SNc in both the subacute MPTP and the chronic MPTPp models of PD, and reverted MPTPp-induced motor impairment. Moreover, MDG548 reduced microglia activation, iNOS and TNF-α production, while induced CD206 in

microglia. In cultured unstimulated microglia, LPS increased TNF-α production and CD68 expression, while decreased CD206 expression. MDG548 reverted LPS effect on TNF-α and CD206 restoring physiological levels, while strongly increased CD68 expression.

Results suggest that the PPARγ agonist MDG548 is neuroprotective in experimental models of PD. MDG548 targets microglia polarization by correcting the imbalance between pro- over anti-inflammatory molecules, offering a novel immunomodulatory approach to neuroprotection.

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ACRONYMS AND ABBREVIATIONS

6-OHDA 6-hydroxydopamine

AADC aromatic amino acid decarboxylase

AD Alzheimer's disease

ALS amyotrophic lateral sclerosis

AP-1 activator protein 1

ATP adenosine triphosphate

CD11b integrin alpha M

CD206 mannose receptor

CNS central nervous system

COMT catechol ortho-methyltransferase

CSF cerebrospinal fluid

DA dopamine

DAT membrane dopamine transporter

DOPAC 3, 4-dihydroxyphenylacetic acid

GPe external Globus pallidus

GPi internal Globus pallidus

HVA homovanillic acid

IL interleukin

iNOS inducible nitric oxide synthase

L-DOPA l-dihydroxyphenylanaline

LPS lipopolysaccharide

LRRK2 Leucine-rich repeat kinase-2

MAO monoamine oxidase

MHC major histocompatibility complex

MPDP+ 1-methyl-4-phenyl-3,4,-dihydropyridine

MPP+ 1-methyl-4-phenylpyridium ion

MPPP 1-methyl-4-phenyl-4-propionoxy- piperidine

MPTP 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine

MPTPp MPTP plus probenecid

MS multiple scerosis

NF-kB nuclear factor kappa-light-chain-enhancer of activated B cells

NMS Non-motor symptoms

NOS nitric oxide synthase

NSAID Nonsteroidal anti-inflammatory drugs

PD Parkinson's Disease

PPAR Peroxisome Proliferator-activated receptors

PPREs peroxisome proliferator response elements

RBD REM behavior disorders

RLS Restless Legs Syndrome

RNS Reactive Nitrogen Species

ROS Reactive Oxygen Species

RXR retinoic acid receptor

SN Substantia Nigra

SNc Substantia Nigra *pars compacta*

SNr Substantia Nigra *pars reticulata*

STAT3 Signal transducer and activator of transcription 3

STN Subthalamic nucleus

TGF transforming growth factor

TH tyrosine hydroxylase

TLR-4 toll- like receptor 4

TNF tumor necrosis factor

TZD thiazolidinediones

UCH-L1 Ubiquitin carboxy-terminal hydrolase L1

VMAT2 vesicular monoamine transporter 2

YM1 Chil-3 chitinase-like 3

INTRODUCTION

1. Parkinson's Disease

1.1 History and epidemiology

Parkinson's Disease (PD) was first described in 1817 by an English physician, Dr. James Parkinson, in his monograph “An essay on the Shaking Palsy”. First defined as “Paralysis agitans”, PD is the most common movement disorder (Lee et al., 2009) and the second most common neurodegenerative pathology after Alzheimer's Disease.

Since incidence of PD is age-related, its social and economic impact is continually increasing with population ageing (Willis, 2013); PD distribution ranges approximately between 0.5 and 1% of the population over 60 years (Nussbaum and Ellis, 2003), 1.5- 2% over 65 years (de Rijk et al., 2000), rising to over 4% in the oldest population (Tanner and Goldman, 1996; de Lau and Breteler, 2006). Related with estimated increase in number of western population aged over 60 within the next 50 years, an increase in PD spread is expected. Some studies reported a different prevalence between males and females, with a higher incidence in men than in woman, probably due to potential neuroprotective effect of oestrogens (Saunders-Pullman, 2003).

1.2 PD symptomatology: motor and non-motor deficits

PD is clinically characterized by a spectrum of motor and non-motor symptoms, distinctive of different development phases of pathology.

- *1.2.1 Motor symptoms*

The cardinal motor symptoms of PD include:

- tremor - the most noticeable early symptom. Tremor, or shaking, often begins in a localized region – a finger, a hand, an arm- and it usually occurs when the individual is sitting or standing still;
- akinesia - absence of ability to start voluntary movements. Often begins from legs and arms (typical is the loss of oscillatory movements of arms in walking). When it affects facial muscles is cause of hypomimia (“mask face” or “stone face”), a reduced degree in facial expression;
- bradykinesia - slowness in execution of movements. They become slower and over time muscles may randomly "freeze”;
- rigidity - involuntary amount in muscle tone, which inhibits ability in free movements;
- gait and postural abnormalities, as balance dysfunctions.

- *1.2.2 Non-motor symptoms*

Non-motor symptoms (NMS) can be present at any stage of pathological development of PD; usually, they rise during the early stages of disease and precede the onset of motor signs, reason why they are also defined as “pre-motor symptoms” (Chaudhuri et al. 2006; Chaudhuri and Naidu, 2008). NMS comprise a large variety of symptoms, consisting of cognitive, autonomic and neuropsychiatric disorders (Chaudhuri and Schapira, 2009; Boeve BF, 2013; Kaufmann and Goldstein, 2013).

The most frequent non-motor symptoms include:

- hyposmia – olfactory dysfunction characterized by a reduction of smell. It's a very early symptom of PD;
- sleep behavior disorders (RBD) – difficulties with falling asleep (sleep- onset insomnia) and staying in sleep (sleep- maintenance insomnia), probably associated with other symptoms such as nocturnal akinesia, nocturia or RLS (Maass and Reichmann, 2013);
- bladder and urinary dysfunctions – primarily irritative symptoms, as increase in urinary frequency and urgency, incontinence, but also difficulty in initiating urination and incomplete emptying of the bladder (Singer, 1998; Chadauri and Schapira, 2009). Most of patient also complain nocturia;
- gastrointestinal symptoms – constipation, due to decrease on bowel movements frequency and impairment in rectal sphincter muscles function;
- sexual dysfunction – erectile dysfunctions, loss of libido or hypersexuality;
- blood pressure – orthostatic and postprandial hypotension, nocturnal hypertension (Ziemssen and Reichmann, 2010);
- restless legs syndrome (RLS) – RLS is defined as the urge to move legs, accompanied or caused by unpleasant and uncomfortable sensations. It's more frequent during the night, or in general after periods of inactivity (Allen et al., 2013);
- dysphagia and dysarthria– difficulty on swallowing and on spoken communication, due to deficit in muscular function (Tjaden, 2008);
- depression – can affect up to 45% of PD patients (Chaudhuri et al., 2006). Depression in PD patient is typically characterized by a higher frequency of dysphoria, sadness, and suicidal ideation, and reduced frequency of guilt, self-blame and suicide (Brown et al., 1988);
- anxiety – common in PD patients, especially in people with depressive disorders: comorbidity has been reported between anxiety and depression in PD patients (Henderson et

al., 1992);

- cognitive impairment – a cognitive decline is observed in PD patients. Deficits on memory and learning functions, executive functions and slowness of thinking are frequent (Aarsland et al., 2004).

Non-motor symptoms are often under- recognized and, consequently, under- treated as compared with motor symptoms, causing a considerably worsening in quality of life of PD patients.

1.3 Histological and biochemical hallmarks of PD

Main pathological feature of PD is the progressive loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNc, part of Basal Ganglia), accompanied by the presence of typical neuronal intracellular proteinaceous inclusions containing α-synuclein, well-known as Lewy's bodies.
The neuronal death results in a progressive reduction in dopamine (DA) levels, with a consequent deficiency of dopaminergic transmission in nigrostriatal pathway.

The Basal Ganglia are a group of interconnected subcortical nuclei, comprising four structures:

- Nucleus Striatum, composed by caudate nucleus, putamen and ventral striatum;
- Globus pallidus, with an external and an internal section (GPe and GPi);
- Subthalamic nucleus (STN);
- Substantia Nigra, divided in pars reticulata (SNr) and pars compacta (SNc).

In physiological conditions, dopaminergic neurons of SNc project primarily to the Nucleus Striatum and, to a lesser extent, to GPe and GPi, STN and SNr (Forno*,* 1996*)*
Because of its strong interconnection with other brain structures as cortex and thalamus, basal

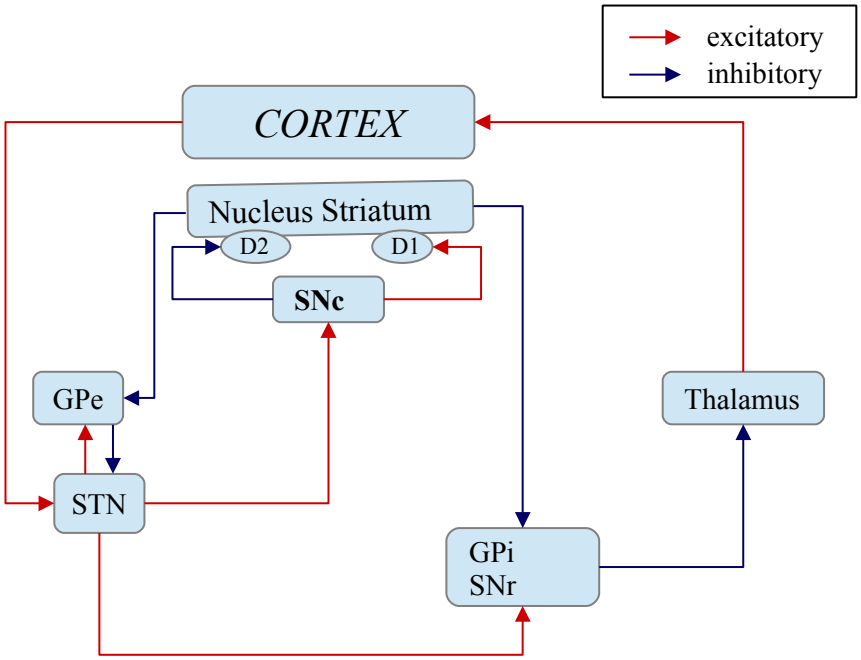
ganglia circuit is involved in modulating several functions, such us procedural learning, motivation, cognitive and emotional functions, eye movement and voluntary motor control. This last point is the main involved in progression of PD.

Two distinct pathways process the signal through the basal ganglia circuit:

- direct pathway, characterized by a prevalence of excitatory signals mediated by dopaminergic D1 receptor;
- indirect pathway, characterized by a prevalence of inhibitory signals mediated by dopaminergic D2 receptor.

Both pathways project to thalamic neurons which make excitatory connections onto cortical cells. In physiological conditions, the two pathways coexist in balance, with an excitation in cerebral cortex as final result. In PD, the dopaminergic neurons death in SNc results in an imbalance between the two pathways in favour of the inhibitory signal, with consequent deficit on cortical transmission and, finally, in movements control.

MOTOR CIRCUIT- NORMAL



MOTOR CIRCUIT- PD

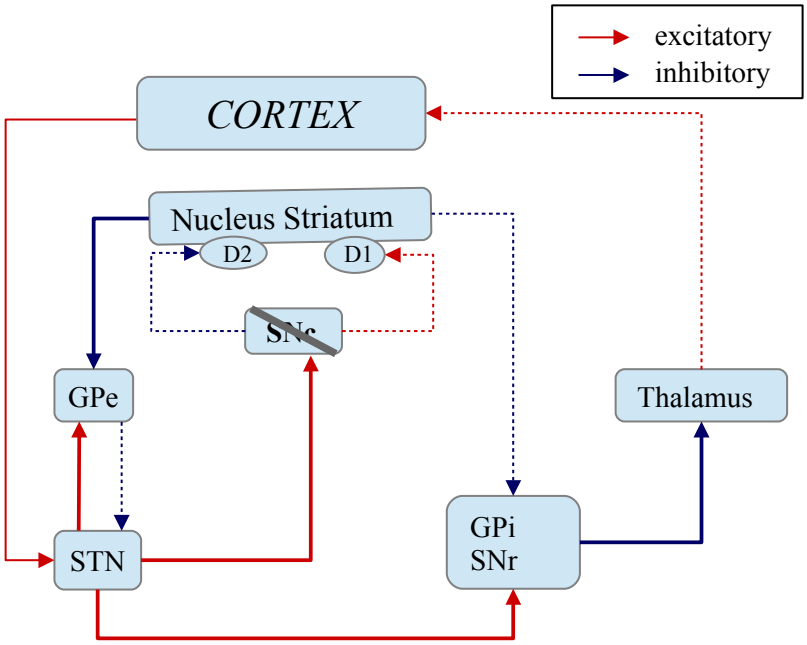


Fig.1 Basal ganglia circuit. *The picture represents the motor connections in physiological and pathological conditions, the latter characterized by the unbalance of excitatory (red) and inhibitory (blue) signalling.*

1.4 Etiology

The etiology of PD is not fully understood, but the main hypothesis considers both environmental and genetic factors as contributory causes on the disease development.

- *1.4.1 Environmental factors*

As regard to environmental factors, exposure to a large variety of compounds has been investigated as a contributing factor in the etiology of PD (Cannon and Greenamyer, 2013). The main event which led to the hypothesis about a potential involvement of environmental toxins in PD development, was the discover of 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine (MPTP) effect in rising of parkinsonian symptomatology. In 1983, several people showed the typical PD symptoms after intravenous injection of synthetic meperidine contaminated by the neurotoxic compound MPTP. This event suggested, for the first time, a correlation between environmental factors and PD pathology (Burns et al., 1984).

Moreover, a role of pesticides such as rotenone and paraquat was considered for their involvement in inhibition of mitochondrial complex I and in oxidative stress, showing a positive correlation between their exposure and PD (Tanner et al.,2011; McCormack et al., 2005). Because of their capacity to mimic the pathological hallmarks of PD, such as neuronal death in SNc and formation of alpha-synuclein inclusions, these compounds are used as experimental models of PD (Langston et al, 1984; Betarbet et al., 2000; McCormack et al., 2002).

Heavy metals such as iron, manganese, copper, lead, aluminium, or zinc have also been hypothesized to increase the risk of PD by accumulation in the Substantia Nigra and by increasing oxidative stress (Lai et al, 2002), even if epidemiological evidence of an association between metal exposure and risk of PD has not been totally demonstrated.

- *1.4.2 Genetic factors*

Regarding genetic factors, although most cases (about 90%) of PD are sporadic, several single gene mutations have been identified in familiar PD. A correlation between genetic factors and the disease seems to be especially important in cases of young-onset PD (Tanner et al., 1999). Most of identified mutations are presents in genes encoding for proteins involved in oxidative stress and mitochondrial function.

In genetic PD nomenclature, 18 chromosomal loci linked to the disease named as PARK have been identified and numbered in chronological order of their identification; nevertheless, up to date not all of them have been well-characterized .

The gene PARK1-PARK4/SNCA is involved in alpha-synuclein mutations (Polymeropoulos et al. 1997 ; Krüger et al., 1998). Mutations in this gene were detected in a large Italian-American family (Polymeropoulos et al., 1996), and subsequently in unrelated Greek and German families (Polymeropoulos et al., 1997; Krüger et al., 1998). Patients had a relatively early onset; they also showed typical clinical and pathological features of PD, including Lewy's bodies. These mutations result in an autosomal dominant pattern, characterized by increased levels of protofibrils, considered as the most toxic form of the protein which is involved in the formation of Lewy's bodies.

Gene PARK2 encodes for Parkin, a component of E3 ubiquitine ligase complex involved in proteins degradation by proteasome (Shimura et al., 2000). Mutations in this gene are associated with autosomal recessive juvenile PD (Kitada et al., 1998; Matsumine et al., 1998).

Gene PARK 5 encodes for Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), a protein with enzymatic activity involved in the development of ubiquitin monomers and in ubiquitin/proteosomal function. A missense mutation in UCH-L1 was detected in two members of a German family showing autosomal dominant inheritance (Leroy et al., 1998), suggesting a potential

role of ubiquitine/proteosomal system in disease pathogenesis. However, no other cases of PD induced by this kind of mutation have been described, so the real implication of UCH-L1 gene is far to be demonstrated.

Gene PARK 6 is implicated in dysfunction of PTEN-induced putative kinase 1 (PINK1), a serine/threonine-protein kinase localized in mitochondria. The main hypothesis is that PINK1 is involved in cell protection from mitochondrial dysfunction, triggering autophagy of damaged mitochondria (Narendra et al., 2010). Mutations in this gene give rise to an autosomal recessive early-onset PD (Valente et al., 2004).

Protein deglycase DJ-1 plays a protective role within neurons from oxidative stress, showing an antioxidant effect against ROS (Canet-Aviles et al., 2004). Mutations in the encoding gene for this protein (PARK 7) are associated with autosomal recessive early-onset PD (Bonifati et al., 2003).

Leucine-rich repeat kinase-2 (LRRK2) is an enzymatic protein with several functions. It is involved in several activities requiring protein-protein interactions, such as assemblage of cytoskeletal structures, vesicle trafficking, stimulation of stress-activated kinase (Ridley, 2001). Mutations in the LRRK2 (PARK 8) gene seem to be the most common among identified mutations, in inherited or sporadic PD: G2019S mutation alone has been reported in 2% to 6% of autosomal dominant PD families (Di Fonzo et al., 2005; Nichols et al., 2005) and in 2% to 8% of sporadic cases (Gilks et al., 2005; Deng et al., 2005).

1.5 Pathogenesis

Most of environmental toxins and genetic mutations seem to lead to neuronal degeneration by inducing oxidative stress (reactive oxygen or nitrogen species- ROS and RNS- production) as final

result of different mechanisms, such as mitochondrial dysfunctions, abnormal protein aggregation, modification on DA metabolism and neuroinflammation (Blesa et al., 2015; Bhat et al., 2015).

- *1.5.1 Dopamine metabolism*

DA is synthesized from aminoacid tyrosine in two steps: (i) hydroxylation of tyrosine to l-dihydroxyphenylanaline (L-DOPA), by tyrosine hydroxylase (TH) and (ii) decarboxylation of l-dopa to dopamine, a reaction catalyzed by aromatic amino acid decarboxylase (AADC); then it is stored in synaptic vesicles through the vesicular monoamine transporter 2 (VMAT2). In physiological conditions, DA is easily metabolized via monoamine oxidase (MAO) and catechol ortho-methyltransferase (COMT) to form the main metabolites 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) as main molecules. Nevertheless, since DA is a relatively unstable molecule, it may also be susceptible to auto-oxidative mechanisms, with consequent production of DA quinones (o-quinone), which may alter in irreversible manner protein functionality via 5-cysteinyl-cathecols production (Miyazaki et Asanuma, 2008), and free radicals (O_2, H_2O_2).

- *1.5.2 Mitochondrial dysfunction*

The main evidence about a role of mitochondrial dysfunction in PD pathogenesis was based on the observation that several complex I inhibitors, in particular MPTP, paraquat and rotenone, are able to reproduce the dopaminergic cell loss in the SNc, in vitro as well as in vivo, in mice and primates (Manning-Bog et al.,2002; Forno et al., 1986). Moreover, a reduction in complex I activity in the SNc of PD patients has been well described (Schapira et al., 1990; Hattingen et al., 2009).

The inhibition of mitochondrial respiratory chain leads to ATP depletion, with consequent

impairment of all ATP-dependent cellular processes, and to the production of free radicals, with final promotion of oxidative stress. Inhibition of complex I increases the production of ROS superoxide, which may form hydroxyl radicals or react with RNS, to form peroxynitrite: these molecules, beyond worsening mitochondrial dysfunction by the action on the electron transport chain itself (Cohen, 2000), may induce cellular damage by acting on lipids, proteins and nucleic acids (Dauer and Przedborski, 2003).

- *1.5.3 Abnormal protein aggregation: role of α-synuclein*

The abnormal deposit of protein aggregate is a typical feature of several neurodegenerative diseases, including PD. In particular, evidences of an association between genetic mutations and presence of abnormal proteins (e.g. misfolding and aggregation of α-synuclein), or mutant forms of enzymes ubiquitin-related (parkin, UCH-L1), reveal how modifications in the activity of ubiquitin-proteasome system (UPS) may play a main role in pathogenesis of PD (McNaught et al., 2001).
Alpha-synuclein is a 140-amino acid protein first described in *Torpedo californica* which has a possible role in the regulation on dopamine transmission clustering synaptic vesicle by promoting presynaptic SNARE-complex assembly (Diao et al., 2013). It is abundantly expressed in nervous system, with a presynaptic localization in neuronal cells in brain (Tofaris and Spillantini, 2005). Structurally, α-synuclein does not assume a consistent conformation in aqueous solution, reason why it is defined as “natively unfolded protein”, but it can acquire an alpha-helic structure after interaction with lipid membranes (Davidson et al., 1998). In certain pathological conditions, such as PD, α-synuclein is characterized by conformational modifications, assuming an oligomeric configuration termed as protofibrillar. Those protein aggregates is resistant to ubiquination and contribute to Lewy 's body production in PD (Spillantini et al., 1997), promoting oxidative stress.

As a final result, these mechanisms cause a general increase of free radicals level, such as ROS and RNS, which promote oxidative stress, and of pro-apoptotic factors release, provoking an irreversible damage to cellular microenvironment and apoptotic cell death.

In the last years, a large number of studies focused their attention on neuroinflammation as a potential underlying mechanism promoting neurodegeneration. Inflammatory processes are not specific for PD, but contribute to development of several neurodegenerative disorders. Even though it is not clear whether neuroinflammation represents a first causative event or a consequence of cellular damage, a body of evidence suggest how neuroinflammation plays a pivotal role in the progression of neuronal degeneration by producing deleterious proinflammatory and toxic molecules. Since modulation of the neuroinflammatory response is the neuroprotective mechanism postulated for the class of drugs investigated in the present study, the following chapter will focus on general aspects of immune cells in the brain and on the role of these cells in PD.

2. Neuroinflammation

The term “neuroinflammation” includes all the inflammatory processes in central and peripheral nervous system. The inflammatory process may be caused by a large variety of stimuli, such as trauma, microbial infections, autoimmunity, toxic metabolites, or it may be part of a degenerative process (Gendelman, 2002).

Neuroinflammation has become a hot topic in modern neuroscience, with a particular focus on its potential involvement in development and progression of neurodegenerative disorders such as PD, Alzheimer's disease (AD), Huntington’s disease and amyotrophic lateral sclerosis (ALS) (McGeer and McGeer, 2004). Although the role of neuroinflammation in degenerative mechanisms is not totally understood, evidence of its involvement has opened to the possibility of new therapeutic strategies for neurodegenerative disorders (Joers et al, 2016, in press; Hirsch and Hunot, 2009; Akiyama et al., 2000).

The first evidence about an involvement of neuroinflammatory mechanisms in PD has been provided by post- mortem studies showing the presence of activated microglia in the SN of patients with PD and parkinsonism with dementia, (McGeer et al., 1988).

2.1 Microglia and neuroinflammation

- *2.1.1 Microglial cells: role in the healthy brain*

Microglia are the resident cells of the central nervous system (CNS) with an immune-competent role, characterized by an immediate activation in response to even minor pathological changes in the nervous tissue (Kreutzberg, 1996). This cell population was first defined as “the third element”

by Ramon y Cajal, to differentiate them from the “first element” (neuron) and “second element” (astrocyte) (Cajal, 1913). In 1921, Pio del Rio- Hortega, a Cajal's disciple, carried a study about the “third element”, describing microglia and oligodendrocytes as two separate cell populations (del Rio-Hortega, 1921).

Microglia represent approximately 10-12% of the whole brain cells and have a mesodermic origin (del Rio-Hortega, 1932). They derive from hematopoietic precursors, which enter in CNS through the blood, ventricles and meninges (Cuadro et Navascues, 1998). In the brain, these cells migrate and proliferate in the form of ameboid microglia, which differentiate into ramified microglia when they reach their final location, after a period of migration (Dalmau et al., 2003).

Microglial cells play two main functions in the CNS: tissue maintenance, by keeping homeostatic balance, and immune surveillance and defense, by responding to environmental and immunological stimuli through several effector mechanisms (Ransohoff and Perry, 2009; Nakamura, 2002).

In the healthy brain, microglia are in a “resting” state showing the typical physiological morphology with a small rod-shaped soma, ramified and fine cellular processes, even if different variations of morphology have been observed also in the quiescent state (Kreutzberg, 1996). Actually, the definition of “surveillance” cells seems to fit better than “resting”: hence microglia are not in a static condition, being characterized by a constant supervising activity of surrounding area for changes in microenvironment. In a study of 2005, Nimmerjahn showed by using in vivo two-photon imaging how microglial cells look highly active in their surveillance state: in particular, although their soma generally remained fixed with few signs of migration, processes are impressively movable, with rapid extensions and retractions in response to environmental stimuli (Nimmerjahn et al., 2005). Functionally, one way that microglia remain in a quiescent state is via the interaction between the glycoprotein CD200 located on surrounding neurons and the receptor CD200R located on microglia.

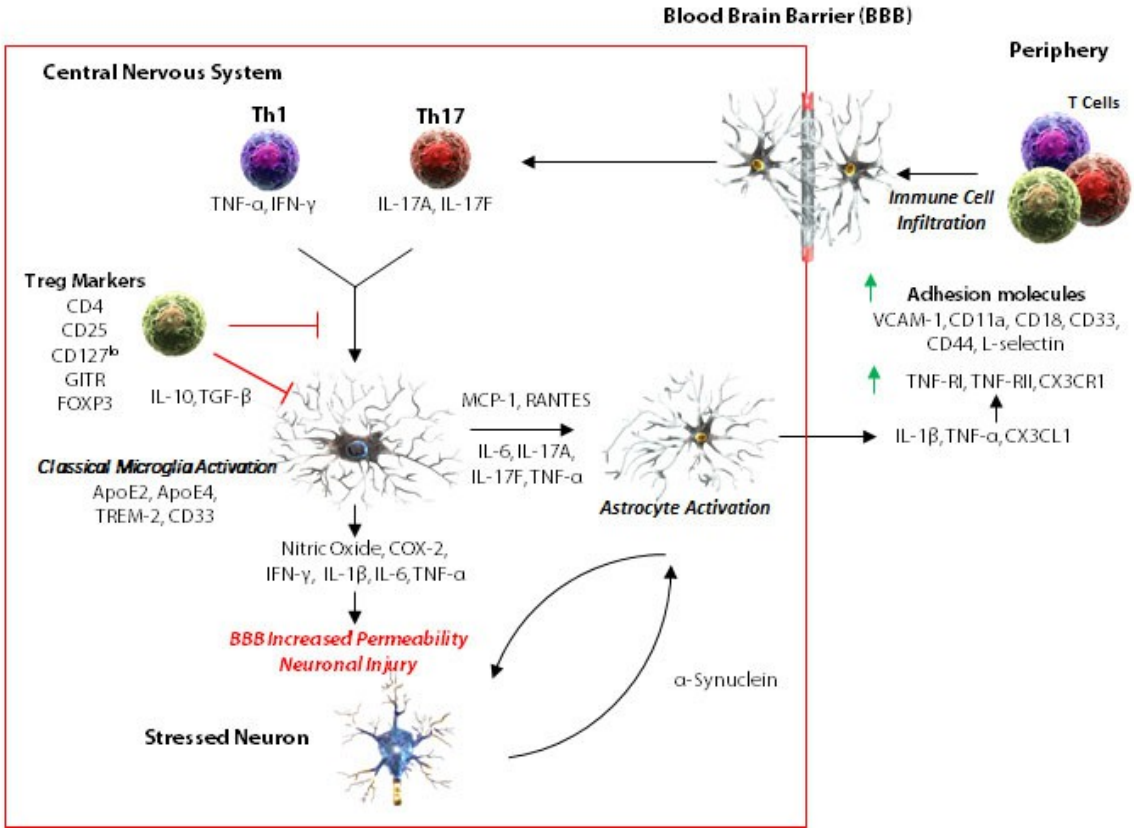


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Fig.2. Schematic representation of neuroinflammation pathways. *After compromise of the BBB, T cells can infiltrate the CNS, releasing cytokines that contribute to neuronal inflammation by activating microglia, astrocytes, T cells and other immune cells. Chronic neuronal inflammation can lead to release of toxic factors and neuronal death.*

- *2.1.2 Activated microglia display morphological and phenotypical changes*

A large variety of stimuli such as infections, trauma, toxins, changes in neuronal activity or dysfunction in physiological brain homeostasis that suggest a potential danger for cerebral tissue, may bring strong modifications in microglia morphology and function, a process known as "microglial activation". One of the most common methods to study this process *in vivo* and *in vitro* is the employment of the endotoxine lipopolysaccharide (LPS), which binds the toll- like receptor 4 (TLR 4) expressed on the microglial surface, a member of the TLR family involved in the detection of microbial intruders (Jack et al., 2005).

The earliest and most evident feature of activated microglia are the morphological changes, which may assume different features as activation progresses. The classical classification defines as stage 1, early activated microglia, characterized by an enlarged soma and partially retracted and ticker processes; stage 2, "bushy" or amoeboid microglia, presents short ticker processes and a round-shaped soma; in stage 3, phagocytic microglia, cells look like large and round- shaped, indistinguishable from blood macrophages (Kreutzberg, 1996; Ladeby et al., 2005a). However, a more recent view argues whether different morphologies should be considered as stages of the activation process or rather differential phenotypes depending on the injury or disease type (Perry et al., 2010).

Over the last decade the important concept has emerged that microglia, similar to other tissue macrophages, assume different phenotypes and perform specific effector functions depending on the precise nature of the stimulus, its intensity and duration (Perry et al., 2010).

Based on the similarities between microglia and macrophages, microglia activation responses were initially classified using M1/M2 polarization terminology. Based on this classification, microglia may assume two main activated phenotypes. Classical activation M1, promotes neurotoxicity and pro-inflammatory responses through the release of neurotoxic molecules such as pro-inflammatory

cytokines, nitric oxide synthase (NOS), reactive oxygene species (ROS), and the upregulation of cell surface markers such as major histocompatibility complex-II (MHC-II) and the marker for phagocytosis CD86. Alternative activation M2, associated with repair and healing or an anti-inflammatory phenotype, via the production of anti- inflammatory cytokines and neurotrophic factors (Varnum and Ikezu, 2012). More recently it has become clear that describing the states of microglia activation, as well as peripheral macrophages, by the M1 and M2 phenotypes is not accurate, as evidence suggests there are many degrees of activation, regarded more as a continuum, especially when considering the complexity of an *in vivo* environment. For instance, M1 macrophages have been defined as M (LPS) or M (INFγ), based on the associated activating factor. While both display a pro-inflammatory phenotype, different pathways of activation lead to expression of different markers. Similarly, activated microglia may be characterized by the presence of various markers depending on the stimulating factor (Murray et al., 2014).

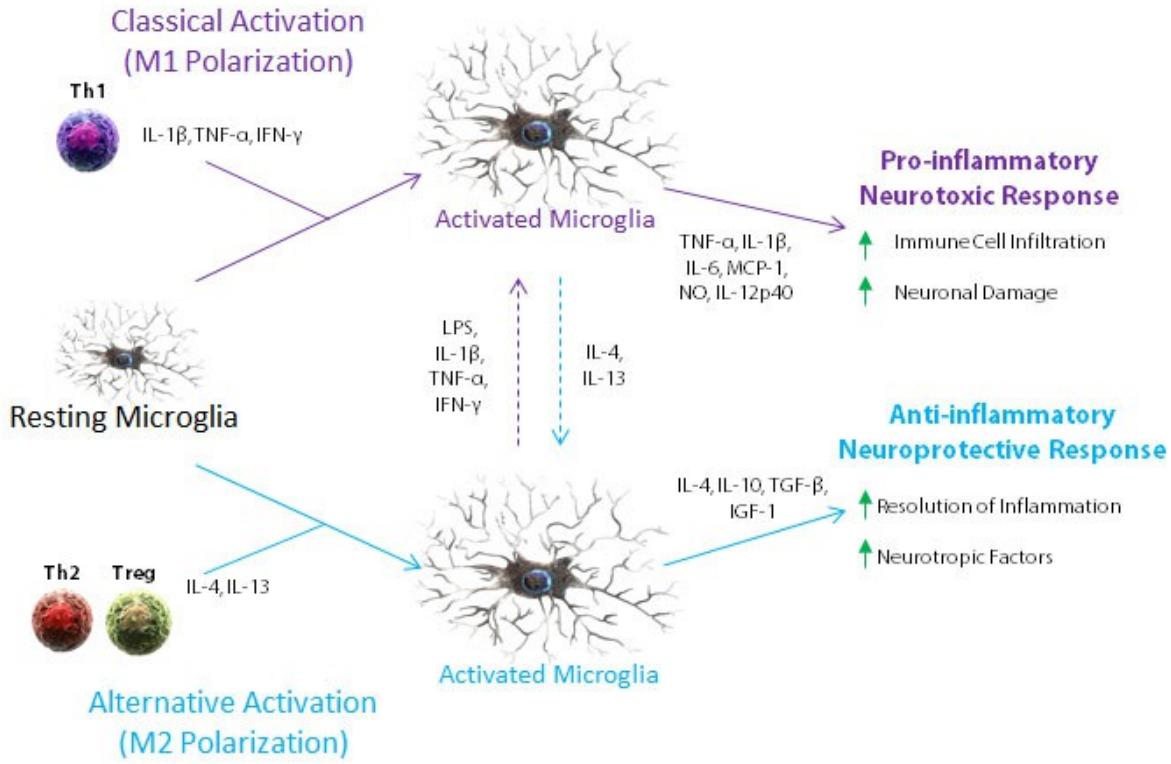

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Fig.2. Schematic representation of microglial activation processes. *The microglia is the resident macrophage of the CNS which can have neuroprotective or neurotoxic properties. Microglia, under*

classically activating conditions such as stimulation by TNF-α and IFN-γ, adopt an inflammatory phenotype while stimulation by IL-4 and IL-13 leads to an alternative activated state that reduces inflammation.

- *2.1.3 Microgliosis and microglia phenotypes in PD*

Multiple studies have described reactive microglial in post-mortem brain samples of PD patients. Specifically, major histocompatibility complex class II (MHC-II) immunoreactive microglia were identified in areas of the SN and striatum in PD patients and in patients with induced parkinsonism from self-administered MPTP (Langston et al., 1999; McGeer et al., 1988). Located in the vicinity of the few remaining nigral DA neurons, these microglia displayed morphologies characteristic of activated and phagocytic cells. More recently, in vivo microglia activation in PD has been described by increased binding of the marker for activated microglia ^{11}C-PK11195, in the pons, basal ganglia and frontal and temporal cortex of PD patients compared to age-matched normal controls (Gerhard et al., 2006). Is is interesting to note that in this study the longitudinal evaluation of ^{11}C-PK11195 binding suggested that there was not a correlation with disease progression. While microglia proliferation and activation have been described in PD, functional polarization is still little investigated due to the unavailability of proper *in vivo* markers for multiple microglia phenotypes, and to the fact that most studies are performed in the late disease stage. Therefore, it remains unclear whether microglia activation may play a protective or cytotoxic role during the earliest phases of the pathology. These studies have shown that both pro- and anti- inflammatory cytokines are elevated in the ventricular cerebrospinal fluid (CSF) of parkinsonian patients, suggesting that pro-and anti-inflammatory microglia may coexist in parkinsonian brain (Rojo et al., 2010). Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-4, and IL-6 have been found in serum and CSF as well as in post-mortem brain of PD patients (Boka et al., 1994; Mogi et al., 1994a,b; Brodacki et al., 2008). In particular, TNF-α and IL-1β seem to be

mainly involved in the pathogenesis of neurodegenerative disorders, since it has been shown that high levels of soluble TNF (solTNF) are a marker of acute and chronic inflammation in PD as well as in several neurodegenerative conditions such as AD, ALS and multiple sclerosis (MS). Higher concentrations of IL-1β have been described in dopaminergic striatal regions of PD patients brain as compared with controls (Mogi et al., 1994a). Not only pro-inflammatory cytokines, but also anti-inflammatory species including transforming growth factor (TGF)-β and IL-10 occur in CNS or serum of PD patients, in agreement with the concurrent presence of pro- and anti-inflammatory microglia (Mogi et al., 1994a; Brodacki et al., 2008). Supporting the hypotheses that an unbalanced occurrence of pro- and anti-inflammatory microglia may sustain PD neuropathology, higher levels of pro-inflammatory over anti-inflammatory species has been observed in other neurodegenerative disorders, such as Alzheimer's disease (Hoozemans et al., 2006; Lund et al., 2006; Sanchez-Guajardo et al., 2013).

More insights on the multiplicity of microglia phenotypes in PD have been provided by studies in animal models. An increase in surface markers such as major histocompatibility complex class I and II (MHC-I and MHC-II) and CD68 has been described in different PD models in both rodents and primates. In addition to cell surface molecules, a number of studies have investigated soluble factors such as cytokines and chemokines, following administration of different neurodegeneration-inducing neurotoxins and inflammagens, highlighting their role in DA degeneration and revealing dynamic temporal changes in the production of these molecules coincident with the neurodegenerative process. Deficiency of TNF receptors has a neuroprotective role in PD animal models (McCoy and Tansey, 2008; Sriram et al., 2006). Moreover an overproduction of IL-1β has been observed in the 6-OHDA model of PD (Pott Godoy et al., 2008). Infusion of TGF-β displays a dopaminotrophic effect after intrastriatal infusion of 6-OHDA (Gonzalez- Aparicio et al., 2010), while in the MPTP model of PD TGF-β mediates GDNF-induced neuroprotection (Schober et al., 2007). IL-10 is neuroprotective both in the 6-OHDA rat model of PD (Johnston et al., 2008) and

against the LPS-induced degeneration of the SN (Arimoto et al., 2007). In summary, studies in animal models of PD suggest that pro-inflammatory microglia may progressively prevail over the anti-inflammatory phenotype along the disease (Pisanu et al., 2014). Overproduction of inflammatory cytokines is an early and persistent event that is associated with microglia activation from the early stages of injury, preceding and likely driving microglia polarization towards a phagocytic, CD68-expressing phenotype present in later stages of neurodegeneration. Increased phagocytic function clearly is not a detrimental feature per se, however the upregulation of phagocytosis in microglial cells which have mostly assumed a pro-inflammatory phenotype in earlier stages of the disease is likely to contribute to progressive neuronal loss (Joers et al, 2016, in press).

3. Animal models of Parkinson's Disease

Several types of PD experimental models may be employed in order to reproduce a pathological condition which models the main features of the disease, with the aim to analyse biochemical and behavioral changes typical of the disorder.

The main requirement for a model of PD is the presence of a relatively specific and progressive dopaminergic neuron degeneration in SNc, with an estimable motor impairment as behavioral correlation of nigrostriatal pathway deficit (Dauer et Przedborski, 2003).

Typically, they can be divided into genetic models, which reproduce PD-related mutations, or pharmacological models, which use environmental or synthetic neurotoxins.

Genetic models are based on the involvement of different mutations (α-synuclein, Parkin, LRRK2, PINK1, DJ1) in familiar PD cases (Dawson et al., 2010). Although inheritance in the disease is quite rare and only 10% of cases may be considered as "genetic" PD, the use of these models allows to analyse the common mechanisms shared by genetic and sporadic PD, in order to identify biochemical and molecular alterations involved in disease pathogenesis.

The most largely used neurotoxin- based models are obtained by the administration of 6-hydroxydopamine (6-OHDA) or 1- methyl -1,2,3,6- tetrahydropyridine (MPTP) The herbicide paraquat and more recently the natural pesticide rotenone are also used to induce a parkinsonian syndrome in rodents (Dauer and Przedborski, 2003).

3.1 MPTP model

- *3.1.1 MPTP: finding and history*

1 -Methyl-4-phenyl- 1,2,5,6-tetrahydropyridine (MPTP) is a highly lipophilic neurotoxic compound. It was first synthesized and tested as analgesic in 1947, in order to find a pain-killer less addictive than morphine, (Lee et al., 1947), but experimental procedures were interrupted when Parkinson-like symptoms were observed in primates object of experiments. 30 years later, the 23-years-old chemistry graduate student Berry Kidston synthesized and self-injected MPPP; within few days after the first injection, he began to show parkinsonian symptoms. After this event, the National Institute of Mental Health tested the compound on rats, but the study failed because of rat tolerance to this neurotoxin.

MPTP neurotoxicity became again object of studies since 1982, when four people in Santa Clara County, California, US, developed a juvenile severe parkinsonism after accidental intravenous injections of MPTP derived from the synthesis of 1-methyl-4-phenyl-4-propionoxy- piperidine (MPPP), a meperidin analogue sold as “synthetic heroin” (Langston et al., 1983).

Patients showed first symptoms within a week from the first injection, developing a generalized motor impairment after 4-14 days; symptoms continued to evolve even after the last injection.

MPTP administration reproduces the main pathological hallmarks of PD causing a strong loss of dopaminergic neurons in SNc and compromising the nigrostriatal dopaminergic pathway, with the development of typical symptoms including tremor, akinesia and rigidity. The presence of inclusion bodies has been observed in a progressive model of PD (Meredith et al., 2002), even if there are conflicting opinions regarding this evidence (Shimoji et al., 2005). In any case, although no Lewy's bodies have so clearly been detected in these models, immunoreactivity for α-synuclein in brain regions of MPTP-treated primates has been observed (Vital et al., 2010).

- *3.1.2 Mechanism of neurotoxicity*

MPTP is a protoxin: after easily crossing the blood- brain barrier because of its high lipophilicity, the compound is converted to its quaternary derivative 1-methyl-4-phenylpyridium ion (MPP+), the active metabolite (Langston et al., 1984), by monoamine oxidase type B (MAO B) (Chiba et al., 1984) in astrocytes and glial cells, with 1-methyl-4-phenyl-3,4,-dihydropyridine (MPDP+) as an intermediate. Thereafter, MPP+ is released in the extracellular space by a still unknown mechanism; here, because of its high affinity for membrane dopamine transporter (DAT), MPP+ is uptaken by dopaminergic neurons, where it exerts the neurotoxic activity. The main cellular target of neurotoxic damage is the mitochondria: MPP+ compromises mitochondrial respiration processes by inhibiting complex I associated to electron transport chain and disrupting the electron flow along the chain (Nicklas et al., 1985; Mizuno et al., 1987), with a resulting impairment in oxidative phosphorylation and ATP synthesis. Deficit in ATP-mediated processes, with an increased production of reactive oxygen species (ROS) as a consequence of complex I inhibition (Parihar et al., 2009) are the two main well-known mechanisms involved in MPTP-related toxicity, even if recent studies suggest how damages in mitochondrial function can be related to activation of pro-apoptotic mechanisms. In particular, it has been shown that MPP+ may promote the release of cytocrome C to the cytosol, where it can activate caspase activation and consequent apoptotic process (Przedborski and Vila, 2001).

These evidences suggest that the MPTP neurotoxic effect is mediated by several mechanisms; this is a common point with the pathogenesis of PD which make it an excellent experimental model of PD. Vulnerability to MPTP toxicity is not the same in all animal species: most of rodents seem almost immune to MPTP damage, for different metabolic mechanisms (Heikkila et al., 1984; Kopin, 1987); in particular, rats are strongly resistant to MPTP treatment likely because of their low concentration of MAO-B. The most used species for MPTP models are mice and monkeys, in which PD

symptoms and mechanisms are better reproduced. The non-human primate model is the one that better reproduces all the features of human PD, however the development of a comparable model in small animals such as rodents is fundamental for ethical and economical reasons.

- *3.1.3 MPTP acute and subacute protocols*

Acute and subacute MPTP mouse model have been the first protocols of administration tested in mice. Although different protocols have been used in both models, generally the acute MPTP model follows the protocol originally developed by Sonsalla and Heikkila (1986), consisting in 4 injections of MPTP 20 mg/kg at 1h-2h intervals within a day, characterized by a high mortality; in the subacute model, mice are typically injected once a day at a dose range from 30 to 20 mg/kg for 4-10 days. Even though acute and subacute treatments may result in a neuronal death in SNc and a decrease in striatal dopamine levels, it has been shown that these protocols produce a transient damage, which reverse spontaneously without long term consequences (Petroske et al., 2001).

- *3.1.4 MPTP chronic protocols*

The chronic MPTP model is the most clinically relevant neurotoxin model, since it reproduces several typical features of human PD, in particular the slow, progressive and non-reversible neurodegeneration of SN neurons. In this sense, improved results has been obtained by administration of MPTP simultaneously with probenecid (MPTPp), an adjuvant which inhibits the rapid renal clearance of the toxin and its metabolites (Betarbet et al., 2002; Petroske et al., 2001; Meredith et al., 2008; Schintu et al, 2009b). The MPTPp model is carried out for a period of 5 weeks, with twice injections a week, using MPTP at the dose of 25 mg/kg. This model induces a progressive and irreversible degeneration of SNc dopaminergic cells of 40-50% and a depletion of

striatal DA of 70-80%, which is reflected by progressive appearance of motor impairment (Meredith et al., 2008; Schintu et al., 2009b); moreover, a progressive increase of neuroinflammation has been observed, in terms of microglia activation, unbalance in microglia polarization toward the M1 over the M2 phenotype, as shown by an increased production of pro-inflammatory cytokines (TNF-α, IL-1β) and, on the other hand, a decrease of anti-inflammatory cytokines levels (TGF-β, IL-10) (Pisanu et al., 2014).

Pro-inflammatory cytokines are soluble factors which promote neurodegeneration mostly via a direct receptor-dependent mechanism, which trigger initiation of pro-apoptotic pathways and further activation of NF-kB. In addition, in 1999 Liberatore et al showed how in MPTP-treated cytokines stimulate the expression of inducible nitric oxide synthase (iNOS), and the consequent increase of nitric oxide (NO). NO may then diffuse to neuronal cells where could increase the levels of nitro-tyrosine, worsen oxidative stress and alter iron metabolism, which is in turn involved in oxidative stress (Liberatore et al., 1999; Hirsch and Hunot, 2000).

4. Peroxisome proliferator-activated receptor (PPAR)

Peroxisome proliferator-activated receptors (PPAR) belong to the type II nuclear hormone receptor superfamily; they include alpha, gamma and delta/beta subtypes, encoded by different genes. PPARs act as ligand-dependent nuclear transcriptional factors, modulating gene transcription by binding to conserved DNA sequences termed peroxisome proliferator response elements (PPREs), as an heterodimer with the retinoic acid receptor (RXR) (Kliewer et al., 1992; Berger and Moller, 2002; Michalik et al., 2006).

4.1 *PPARγ*

PPARγ exists in two isoforms, detected in humans and mice: PPARγ 1, ubiquitously expressed in all tissues, including the brain (localized in several cell types, such as microglia and neurons), and PPARγ 2, primarily expressed in adipose tissue (Fajas et al., 1997; Cristiano et al., 2001).

PPARγ were historically studied for their action in regulation of glucidic and lipidic metabolism, mediated by endogenous ligands such as arachidonic acid metabolites 15-deoxy-delta 12, 14 prostaglandin J2 (Forman et al., 1995); experimental data have shown that PPARγ knockout mice fed with a high-fat diet were protected against obesity and insulin resistance as compared with controls (Jones et al., 2005).

Besides endogenous ligands, PPARγ is also activated by the synthetic class of agonists thiazolidinediones (TZDs) and few nonsteroidal anti-inflammatory drugs, such as ibuprofen, fenoprofen and indomethacin (Bernardo and Minghetti, 2008).

In the last decade, beside the main effects as modulators of glucidic and lipidic metabolism, particular attention has been given to PPARγ agonists for their neuroprotective properties in neurodegenerative disease models; evidences about this effect has been shown in several

neurodegenerative conditions, such as PD, AD, ALS and cerebral ischemia (Breidert et al., 2002; Heneka et al., 2007; Schintu et al., 2009a).

As regard to PD models, the neuroprotective action has been observed in various kind of MPTP protocols based on acute and subacute regimen, as well the chronic regimen. In acute MPTP protocols, the PPARγ agonists pioglitazone prevented dopaminergic loss in SNc and striatal DA decrease, and attenuated the MPTP-mediated glial activation (Breidert et al., 2002; Dehmer et al.,2004). In the chronic MPTPp protocol, TZD rosiglitazone has shown neuroprotective action by completely preventing or arresting TH-positive cell death in SNc and partially counteracting loss of striatal DA. Biochemical results matched with behavioral data: chronic administration of rosiglitazone totally prevented olfactory and motor impairment (Schintu et al., 2009b).

As an underlying mechanism of neuroprotection, PPARγ modulation of inflammatory processes has been proposed.

Modulation of target genes involved in the inflammatory response includes suppression of pro-inflammatory cytokines and induction of anti-inflammatory molecules, upregulation of antioxidant enzymes, upregulation of scavenger receptors such as CD36. A well-established mechanism through which PPARγ regulates inflammatory responses consider the negative modulation of pro-inflammatory pathways, such as NF-kB, STAT-3, AP1, and consequent decrease on the production of pro-inflammatory molecules (Chinetti et al., 2003b). First evidences reported an anti-inflammatory activity of PPARγ agonists *in vitro,* by showing that PPARγ agonists inhibit inflammatory processes in various cell types, including monocytes/ macrophages (Ricote et al., 1998) and microglial cells (Combs et al., 2000); moreover, PPARγ activation in microglia prevented LPS-induced release of nitric oxide, pro-inflammatory cytokines and MHC-II expression (Bernardo et al., 2000; Luna-Medina et al., 2005).

Moreover, studies on peripheral immune cells have demonstrated that PPARγ activation stimulates the polarization of circulating monocytes to macrophages with an M2 anti-inflammatory phenotype,

which in turn promote the switch of polarization from a pro- to an anti-inflammatory profile (Bouhlel et al., 2007). Based on these evidence, the intriguing hypothesis has emerged that the potent neuroprotective activity of PPARγ agonists may rely on the ability of polarize immune cells toward a anti-inflammatory phenotype, while suppressing pro-inflammatory cells. This properties highly differentiate these drugs from anti-inflammatory drugs, such as NSAID, whose activity consists on the mere inhibition of inflammatory responses, and would give a likely explanation for the failure of NSAID to prevent or counteract neurodegeneration. A large number of *in vivo* studies support the modulation of immune responses as a mean of neuroprotection in several neurodegenerative pathologies. In experimental models of PD, autoimmune encephalomyelitis, amyotrophic lateral sclerosis and spinal cord injury, the neuroprotective effect of PPARγ agonists has been associated with a decrease of pro-inflammatory and increase of anti-inflammatory cytokines production (Diab et al., 2004 ; Kiaei et al., 2005; Park et al., 2007; Pisanu et al., 2014).

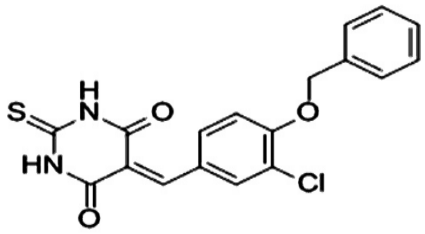
In the mice MPTPp model of PD, the chronic administration of rosiglitazone induced an increase of CD206 levels, a M2 microglia/ macrophage marker, and anti-inflammatory molecules such as TGF-β and IL 10, together with a decrease of pro-inflammatory cytokines such as TNF-α and IL-1β (Pisanu et al., 2014). Therefore, in this study, the PPARγ agonist completely reverted the MPTP-induced increase of pro-inflammatory microglia in favor of anti-inflammatory phenotype. Boosting of M2 phenotype, suggesting microglial polarization as a key mechanism for the anti-inflammatory effect of PPARγ agonists.

5. MDG548

TZDs such as rosiglitazone, pioglitazone and troglitazone were originally introduced in the market in 1990s as insulin-sensitizing agents for the treatment of type 2 diabetes (Barnett, 2009). After the discovery of their neuroprotective potential, this class of compound started to be employed in preclinical models of a large number of acute and chronic neurodegenerative conditions (Breidert et al., 2002; Heneka et al., 2010; Park et al., 2007; Schintu et al., 2009b): rosiglitazone effects have been clinically evaluated in patient with ischemia and AD (Irizarry et al., 2008).

Over time, safety concerns have been raised about TZDs safety in diabetic patients: troglitazone was removed from the market because of its hepatotoxicity (Smith, 2003), and rosiglitazone and pioglitazone were objects of multicentre studies with the purpose to analyse a potential involvement in cardiovascular disorders in patients with diabetes (Home et al., 2009; Dormandy et al., 2005). Both rosiglitazone and pioglitazone have been related to an increase of LDL cholesterol levels, fluid retention and increased body weight (Nesto et al., 2003) and, moreover, TZDs have been associated with an increase of cardiovascular risk by promoting high levels of blood pressure and coagulation factors (Komajda et al., 2008).

This limitation has prompted for the search of novel non-TZD compounds void of such side-effects. MDG548 is a novel thiobarbituric-like compound recently identified by computational integrated virtual screening, displaying specific PPARγ binding with an affinity approximately double that of rosiglitazone (Nevin et al., 2012). The neuroprotective potential of MDG548 was evaluated by *in vitro* and *in vivo* tests. The high potency of the compound, which permit to employ lower doses than TZDs, associated with the non-TZD structure, might reduce the risk of unwanted side effects.



MDG 548

Part of the data discussed in this thesis has been already published in the paper "Neuroprotective and anti-inflammatory properties of a novel non-thiazolidinedione PPARγ agonist *in vitro* and in MPTP-treated mice". That study has been conducted in collaboration with the Biochemistry Group of the Trinity College of Dublin, where TCD performed all in vitro experiments aimed at assess effective doses and the neuroprotective potential of MDG548, while our group investigated the neuroprotective and anti-inflammatory properties of the compound *in vivo* in a subacute MPTP mouse model of PD. For clearness, results of *in vitro* experiments conducted by TCD have been inserted in the present thesis, representing the basis for a rational *in vivo* testing. The remaining data presented in the thesis have been submitted for publication in Experimental Neurology Journal.

AIM OF THE STUDY

Therapeutic management of neurodegenerative disorders is a crucial social aim, because of improvement of life expectation, and consequent increase of age-related pathologies.

Currently, available therapies for PD are strictly symptomatic. Since the first signs of the disease appear when the dopaminergic degeneration in SNc is already in a late stage (about 70%) (Dauer and Przdeborski, 2003), current therapeutic approaches focus on the symptomatic control of motor symptoms. Nowadays, the gold standard therapy for PD patients is the DA precursor L-DOPA. Nevertheless, a long-term use is usually associated with the onset of motor complications, including dyskinesia (Olanow et al., 2001).

Therefore, research is highly focused on the understanding of neuropathological mechanisms of PD which may serve as targets for disease-modifying therapies aimed at arrest disease progression. PPARγ agonists hold a promising disease-modifying potential, since they are neuroprotective in several preclinical models of PD.

Aim of the present study was to characterize the neuroprotective properties of the PPARγ agonist MDG548, a new no TZD compound displaying high selectivity and binding affinity for PPARγ, in a preclinical model of PD. Immunomodulatory properties of MDG548 were investigated as a supposed underlying mechanism of neuroprotection. Microglia phenotypes were investigated in *in vivo* PD models. Moreover, since the quite ubiquitous localization of PPARγ in CNS cells does not allow to investigate direct effects selectively on microglia, *in vitro* studies were carried to deeply investigate PPARγ effect on these cells.

The whole study was made in collaboration with local and international research groups. All the experiments are presented in this thesis for completeness and better understanding of the data. Roles of our group as well as collaborators are specified below.

The group from the School of Biochemistry & Immunology of TCD performed *in vitro* experiments

aimed at assessing effective doses and the neuroprotective potential of MDG548 in cultured neurons.

Our research group performed all *in vivo* treatments and fluorescent immunohistochemistry experiments *ex vivo* as well as *in vitro* in cultured microglia.

Dr. Andrea Diana group, from our Department of Biomedical Sciences, prepared MMGT12 cultures and made in vitro pharmacological treatments.

Dr. Barbara Batetta group, from our Department of Biomedical Sciences, performed all ELISA assays in microglia collected medium.

MATERIALS AND METHODS

1. Drugs

For *in vitro* studies, MDG548 (Specs ID number: AN-698/ 15136006; 5-[4-(benzyloxy)-3-chlorobenzylidene]-2- thioxodihydropyrimidine-4,6(1H,5H)-dione; Mol. Weight: 372.83) and rosiglitazone (Santa Cruz) were dissolved in 0.1% dimethyl sulfoxide (DMSO).

For *in vivo* studies, MPTP-HCl (Sigma, Italy) was dissolved in saline. MDG548 was suspended in 30% DMSO, 10% polyethylene glycol (PEG) and water.

For chronic treatment, probenecid (Sigma Aldrich, Italy) was suspended in 5% NaHCO3; PPARγ antagonist GW9662 (Sigma Aldrich, Italy) was suspended in 5% DMSO and water.

2. In vitro tests

2.1 Primary culture of cerebral cortical neurons

Primary cerebral cortical neurones were obtained from postnatal 1-day-old Wistar rats. Cortical tissue was incubated with 2 ml sterile phosphate-buffered saline (PBS) containing trypsin (0.3%; Sigma–Aldrich, Dorset, UK) in a humidified chamber for 25 min at 37° C, followed by PBS containing soyabean trypsin inhibitor (0.1%), DNAse (0.2 mg/ml) and $MgSO_4$ (0.1 M) (all from Sigma–Aldrich, Dorset, UK). The cell suspension was gently filtered and centrifuged at 2000g for 3 min at 20° C. The pellet was resuspended in neurobasal medium (NBM, Invitrogen, Paisley, UK), supplemented with heat-inactivated horse serum (10%) penicillin (100 U/ml), streptomycin (100 U/ml) and glutamine (2 mM) (all from Gibco BRL, Maryland, USA). Resuspended neurons were

placed on coverslips at a density of $0.25x10^6$ and incubated with NBM containing 5 ng/ml cytosine-arabino-furanoside (ARA-C; Sigma–Aldrich, Dorset, UK) for 24 h to prevent proliferation of non-neuronal cells. Cells were grown in NBM (400 µl/well) media for up to 5 days post ARA-C treatment.

2.2 CalceinAM cell viability assay

Cells were treated with MDG548, rosiglitazone or vehicle (0.1% DMSO) and allowed to incubate for 24 or 48 h prior to the addition of calceinAM (2 µM) at the experimental endpoint. After incubation (30 min at 37° C), fluorescence intensity (Ex: 495 nm, Em: 515 nm) derived from cleaved calcein retained within viable cells was read on a Spectramax Gemini fluorometric plate reader using SOFTmax Pro (V4.0, Molecular Devices) software package. Data were presented in relative fluorescence units (RFU).

2.3 PrestoBlue cell viability assay

For the PrestoBlue assay, cortical neurons were analyzed in two experimental conditions: (A) pre-treatment with MDG548 or rosiglitazone for 24 h prior to exposure to H_2O_2 (50 µM), and (B) co-treatment with MDG548 or rosiglitazone and H_2O_2 (50 µM) at the same time point. In both instances, cortical neurons were incubated for a further 24 h prior to measurement of cellular viability. MDG548 or rosiglitazone were added at increasing concentrations (100 nM to 10 µM). Based on results of MDG548-mediated neuroprotection, in a following experiment PPARγ antagonist GW9662 was added 1 h prior to MDG548 and the experimental condition A was followed. PrestoBlue reagent is reduced to a highly fluorescent compound by the reducing the environment of the viable cell. Fluorescence intensity was assessed as a directly proportional

measure of the amount of viable cells.

2.4 Neutral red uptake assay for cell viability/cytotoxicity

PC12 cells, the rat adrenal pheochromocytoma cell line, were purchased from American Type Culture Collection. Cells were grown on poly-L-lysin-coated flasks (100 μg/ ml) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin (all from Sigma–Aldrich). All cells were cultured at 37° C with 5% CO_2. In order to ensure the expression of dopaminergic features, 104 cells per well were seeded in 24 well plates and immediately treated with NGF (50 ng/ml) every second day so that neuronal differentiation was achieved after 10 days. At that time, the complete medium was replaced by the same medium but with lower (1%) concentration of FBS to avoid the risk of any possible protein interference on the assay sensitivity. GW9662 (1 μM) or vehicle was added 1 h prior MDG548 (500 nM and 5 μM) plus MPP+ (500 μg/ml). After the scheduled treatment, cells were briefly washed with PBS and incubated with neutral red solution (40 μg/ml) for 3 h at 37° C in the dark. This assay is based on the ability of viable compartments (Repetto et al., 2008). After 3 h, neutral red solution was removed and after gentle immersion in PBS, neutral red destain solution (50% ethanol, 95%, 49% deionized water, 1% glacial acetic acid) was applied for at least 10 min in agitation to allow the complete extraction of the red chemical from cells and the formation of a homogeneous solution. Neutral red extract was measured at 540 nm in a microtiter plate reader spectrophotometer using blanks with no cells for measurement normalization. Finally, cell viability was expressed as a percentage of control cells (100%).

2.5 HEK-Blue NF-kB reporter assay

HEK-Blue reporter assay was performed using HEKBlue -hTLR4 cells (Invivogen, Carlsbad, CA, USA). Stimulation of TLR4 with LPS induces rapid activation of NF-kB and a correlated expression of Secreted Embryonic Alkaline Phosphatase (SEAP). HEK-Blue cells were seeded at a density of $0.25x10^5$ cells/well and pre-treated for 24 h with vehicle (0.1% DMSO), or increasing concentrations of MDG548 or rosiglitazone in association with vehicle or the PPARγ antagonist GW9662. Post-incubation, cells were treated with LPS (100 ng/ml) and further incubated at 37° C for 6 h. The proteasome inhibitor MG132 was included as a control. Quanti-Blue analysis was performed by incubating 20 µl of HEK-Blue supernatant with 180 µl of Quanti- Blue media in the dark at 37° C for 2–4 h. SEAP-induced changes from a pink to blue color were monitored by reading absorbance (650 nm) on a Versamax absorbance plate reader.

2.6 Culture of murine microglial cell and in vitro immunohistochemistry

The murine microglial cell line MMGT12 (Briers et al., 1994; Michelucci et al., 2009; Heurtaux et al., 2010), a generous gift from Dr. Michelucci was cultured in DMEM/F12, supplemented with 10% fetal bovine serum (FBS). No antibiotics were used. The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C and passaged twice a week. For experiments (both ELISA and immunocytochemistry), MMGT12 cells were seeded into 12 multiwell plates previously filled with coverslips 18 mm diameter size. Cellular density was set to $15x10^3$/well in order to ensure to reach 70% confluence by adhesion to the coverslips at 3 days in vitro (DIV) when most of cells presented a common morphological appearance. Cells were subjected to incubation in a medium containing 5% FBS according to table 1. After 24 h treatment, exposure media were saved for ELISA experiments and cells were washed in PBS. Then, all specimens were fixed with 4%

paraformaldehyde/PBS for 15 min at room temperature (RT) with gentle agitation. Fixative solution was replaced with PBS and immediately samples were processed for immunocytochemical technique. After three rinses in the same buffering solution, cells were transferred for 5 min in 0.2% TritonX-100 containing PBS and then preincubated with 10% normal non immune serum (Sigma) for 1 h. Primary antibodies were rabbit anti-CD68 (Abbiotec) diluted 1.200 in PBS/0.2% TritonX-100 overnight at 4°C. Subsequently, after 3x5 min. rinsing steps in the same PBS/triton solution, detection with secondary antibodies (Molecular Probes, USA) was carried out using Alexa Fluor 546 F(ab')2 fragment of goat anti-rabbit IgG (1:200) for 1 h incubation time at RT. Nuclear counterstaining was performed with DAPI, dihydrochloride solution (300nM in PBS, Molecular Probes, USA) and finally, samples were washed and mounted in 70% glycerol in PBS containing 1% DABCO (Sigma-Aldrich, U.S.A.) as anti-fading agent.

Observations and images were captured using ZOE fluorescent cell imager (Bio-Rad) as TIFF files with a resolution of 647 × 486 pixels.

Treatments	MDG548 concentration	MDG548 time administration
Controls (+0.1% DMSO as vehicle)		
LPS 1 µg/ml (1mg/ml DMSO)		
MDG548	*10 µM*	
MDG548	*50 µM*	
LPS 2h +MDG548s	*10 µM*	*2h after LPS*
LPS 2h +MDG548s	*50 µM*	*2h after LPS*
LPS 2h +MDG548c	*10 µM*	*contemporaneous with LPS*
LPS 2h +MDG548c	*50 µM*	*contemporaneous with LPS*

Table 1: MMGT12 cell treatment for immunocytochemistry and ELISA. *LPS and MDG548 were solved in DMSO (0.1% final concentration).*

2.7 Cytokines quantification by ELISA assay

Cells were removed by 400 g centrifugation for 10 min and supernatants frozen at 80°C for subsequent determination of mouse TNF-α, Ym1 (EIAab Science, Wuhan, China), CD206 (Cloud-Clone Corp., TX, USA). Cytokines were assessed with a sandwich ELISA test as described by manufactures guidelines. Absorbance at 450 nm for all cytokines was measured with a microplate reader, model 680 (Bio-Rad, Hercules, CA). A standard curve was prepared by plotting absorbance value of the standard cytokine versus the corresponding concentration (pg/ml or ng/ml). The range of assay for cytokines: 15.6–1,000 pg/ml for TNF-α, 78.0-2500 pg/ml for Ym1 and 15.6-1000 pg/ml for CD206.

3. In vivo methods

3.1 Subacute treatment

Three-month-old male C57BL/6J mice (Charles River, Italy) were divided into six groups (n=4/5 for each group) and housed with a 12:12-h light/dark cycle with food and water ad libitum. Mice received multiple injections treatment with saline as a vehicle, MPTP-HCl (20 mg/kg i.p.) once a day for 4 days, or MDG548 (2, 5 or 10 mg/kg i.p.) plus MPTP (20 mg/kg i.p.) once a day for 4 days. MDG548 was injected 15 min before MPTP administration. Three days after discontinuation of treatment, mice were anaesthetized with chloral hydrate (400 mg/kg i.p.) and transcardially perfused with 4% paraformaldehyde (PFA)/0.1M in phosphate buffer (PBS).

3.2 Chronic treatment

Three-month-old male C57BL/6J mice (Charles River, Italy) were divided into six groups (n=5-10 for each group) and housed with a 12:12-h light/dark cycle with food and water *ad libitum*. The control group received saline as a vehicle, groups MPTP3, MPTP7 and MPTP10 received 3, 7 and 10 doses of MPTP (25 mg/kg i.p.) respectively, plus probenecid (100 mg/kg i.p.) (MPTPp). MPTPp was injected twice a week up to 5 weeks. Group MPTP+MDG548 (MM) received 10 doses of MPTPp over 5 weeks and the PPARγ agonist MDG548 (2 mg/kg i.p., 15 minutes before MPTPp injection) starting on the 2nd day after the seventh MPTPp administration. Group MPTP+MDG548+GW9662 (MMG) received MPTPp+MDG548 at the same conditions of MPTP+MDG548 group, with administration of PPARγ antagonist GW9662 (5 mg/kg i.p.) 15 minutes before MDG548 injection. For both MM and MMG groups, MDG548 was administered daily, 15 minutes before MPTPp and until sacrifice. Three days after discontinuation of MPTPp treatment, part of mice were anaesthetized with cloral hydrate (400 mg/kg i.p.) and transcardially perfused with 4% PFA /0.1 M PBS. Brain were removed, postfixed in PFA for 2 hours and stored in 0.1% sodium azide PBS at 4°C until immunohistochemical processing. All animal experimentation has been conducted in accordance with the guidelines for care and use of experimental animals of the European Communities Council Directive of 24 November 1986 86/609/EEC and the National Institutes of Health.

3.3 Beam traverse test

The beam was constructed as described (Fleming et al., 2004). Mice were trained for two consecutive days to traverse the beam. On the test day a grid (1 cm^2) of corresponding width was

placed 1 cm above the beam, mice were videotaped while traversing it for a total of five trials, and errors were calculated (when a limb slipped through the grid). By scoring each limb slip individually, the severity of the error could be measured (Fleming et al., 2004).

3.4 Immunohistochemistry/Immunofluorescence

Coronal sections from the SNc (40 μm thick) were cut on a vibratome and immunoreacted against tyrosine hydroxylase (TH), CD11b, iNOS, TNF-α and CD206.

TH-positive cells were visualized using a TH antibody (anti- TH monoclonal antibody produced in rabbit 1:1000, Sigma-Aldrich, Italy). After washing in PBS, sections were incubated for 1 hour with the secondary antibody (IgG (H+L) Biotin-Goat anti-rabbit 1:500, Invitrogen, Italy) and the classic avidin-peroxidase complex (ABC, Vector, UK) protocol was applied, using 3,30- diaminobenzidine (Sigma) as a chromogen.

For CD11b and iNOS immunofluorescence, sections were incubated overnight with a CD11b antibody (anti- CD11b monoclonal antibody produced in rat 1:1000, Serotec, Oxford) or iNOS (anti-iNOS monoclonal antibody produced in rabbit 1:400, Santa Cruz, Germany) plus TH antibody (anti-TH monoclonal antibody produced in mouse 1:1000, Sigma Aldrich, Italy). After PBS washing, sections were incubated with a goat anti-rat secondary antibody AlexaFluor 594-conjugated immunoglobulin G (IgG, 1:200, JacksonImmunoResearch Europe, UK) for 4 hours for CD11b.

For iNOS/TH double labeling, AlexaFluor 594-conjugated immunoglobulin G (IgG (H+L) donkey anti-mouse 1:500, Invitrogen, Italy) was used to detect TH, while a three-step detection was used to increase the signal of iNOS by biotin-conjugated IgG (biotin-conjugated donkey anti-rabbit 1:200, Jackson ImmunoResearch Europe, UK) and treptavidin–fluorescein (1:200, Vector, UK).

For CD11b/TNF-α double labelling, sections were incubated 48 hour with CD11b antibody and a

TNF-α antibody (TNF-α polyclonal rabbit anti-TNF-α 1:800, Abbiotec, USA); after PBS washing, sections were incubated with secondary antibody for CD11b while three-step detection was used to increase the signal of TNF-α by biotin-conjugated IgG (IgG (H+L) Biotin-Goat anti rabbit 1:500, Invitrogen, Italy) and streptavidin–fluorescein (1:200, Vector, UK).

For CD11b/CD206 double labelling, sections were incubated 48 hour with CD11b antibody and CD206 antibody (CD206 polyclonal goat anti CD206 1:500, Santa Cruz Biotecnology, CA); after washing in PBS / 0.2% Triton X-100 sections were incubated with secondary antibody for CD11b, while three-step detection was used to increase the signal of CD206 by combining biotin-conjugated IgG (IgG (H+L) Biotin-rabbit anti goat 1:500, Invitrogen, Italy) and streptavidin–fluorescein (1:500, Vector, UK).

To allow visualization of cell nuclei (not shown in figures), sections were incubated for 5 minutes in 5 μM Hoechst 33258 (H 33258) solution (Sigma, Italy).

3.5 Stereological counting of TH-immunoreactive and Nissl-stained neurons

TH-immunoreactive and Nissl-stained cells were counted on both hemispheres. Each mounted section was numbered following rostro-caudal levels corresponding to the mouse brain atlas (Paxinos and Franklin, 2001) and every sixth section was used for cell number estimation. We used a dedicated software (Stereologer, System Planning and Analysis, Inc., Alexandria, VA, USA) linked to a motorized stage on the BX-60 Olympus light microscope. The total number of TH-immunostained cells was estimated by means of the Optical Fractionator method, which combines the optical dissector with the fractionator sampling scheme, giving a direct estimation of the number of 3-D objects unbiased by their shape, size and orientation (Mouton, 2002). After delimitation of the area of interest based on clearly visible cues using a 2 objective, a systematic random sampling of cells was achieved by positioning a sampling grid over the area on each section, automatically

operated by ‘‘Stereologer’’ program. The grid was divided into equidistant counting frames (frame area=50 μm^2). The sampling fraction was delimited at low power and cells were sampled with a 40 oil immersion objective through a defined depth with a guard zone of 2 μm. The coefficient of error (CE) for each estimation and animal ranged from 0.05 to 0.1.

3.6 CD11b analysis

CD11b-positive microglia were identified at ×100/1.25 oil magnification. For each animal six fields of SNc were captured from both the left and right SNc and analyzed; for each microglial cell, the body and primary processes were outlined, and the area occupied by CD11b immunoreactivity (IR) was measured using NIH software ImageJ 1.47v.

3.7 iNOS analysis

Images of iNOS-positive cells were captured at ×40 magnification. The SNc was surrounded and the same boundary applied to each section; for each animal, three fields were sampled in both left and right SNc. Cells were automatically counted using Media Cybernetics software Optimas 6.51.

3.8 CD11b/TNF-α and CD11b/CD206 colocalization analysis

Qualitative and quantitative analyses for CD11b and CD206 were performed using a Leica 4D confocal laser scanning microscope, equipped with an argon–krypton laser. Images were digitized 24 h after the immunofluorescence procedure. Surface rendering, maximum intensity, colocalization, and simulated fluorescence process algorithms were used (ImageJ 1.48q and Imaris

7.0). Volume of colocalized elements was determined as follows: for each dataset (40–60 images), a colocalization channel was automatically composed by Imaris 7.3. In the resulting stacks, four regions of interest (x= 40 μm; y= 40 μm; z= 10 μm) were randomly chosen and volume of the elements of interest was calculated, summed and expressed as volume/μm^3 (n= 200).

4. Statistics

For *in vitro* assays, MDG548-treated cells were compared to the relative control cells by using a two-way analysis of variance (ANOVA) with Bonferroni post hoc test. Stereological measures of TH and Nissl-positive cells in the SNc, as well as CD11b and iNOS data were statistically compared with a one-way ANOVA followed by Tukey's post hoc test.

For behavioral test, number of errors was statistically compared with a one-way ANOVA followed by Tukey's post-hoc test.

RESULTS

1. Neuroprotective potential and immunomodulatory properties of MDG548 in cultured cells

Determination of MDG548 cytotoxicity against rat cortical neurons

In order to determine a concentration range void of cytotoxicity in which to assay the neuroprotective effects of MDG548, the cell viability of cortical neurons was assessed at 24 and 48 h after treatment with increasing MDG548 concentrations. The CalceinAM cell viability assay showed a dose-dependent cytotoxicity for MDG548 (Fig. 3). When compared to the control (0.1% DMSO) MDG548 induced a significant reduction in viability at 50 μM 24h ($p<0.05$) and 48 h ($p<0.001$) post-treatment, while lower concentrations were void of any cytotoxicity (Fig. 3A, B). Rosiglitazone, a marketed drug that acts as a high affinity PPARγ agonist displayed an effect on cell viability similar to MDG548 (Fig. 3A, B).

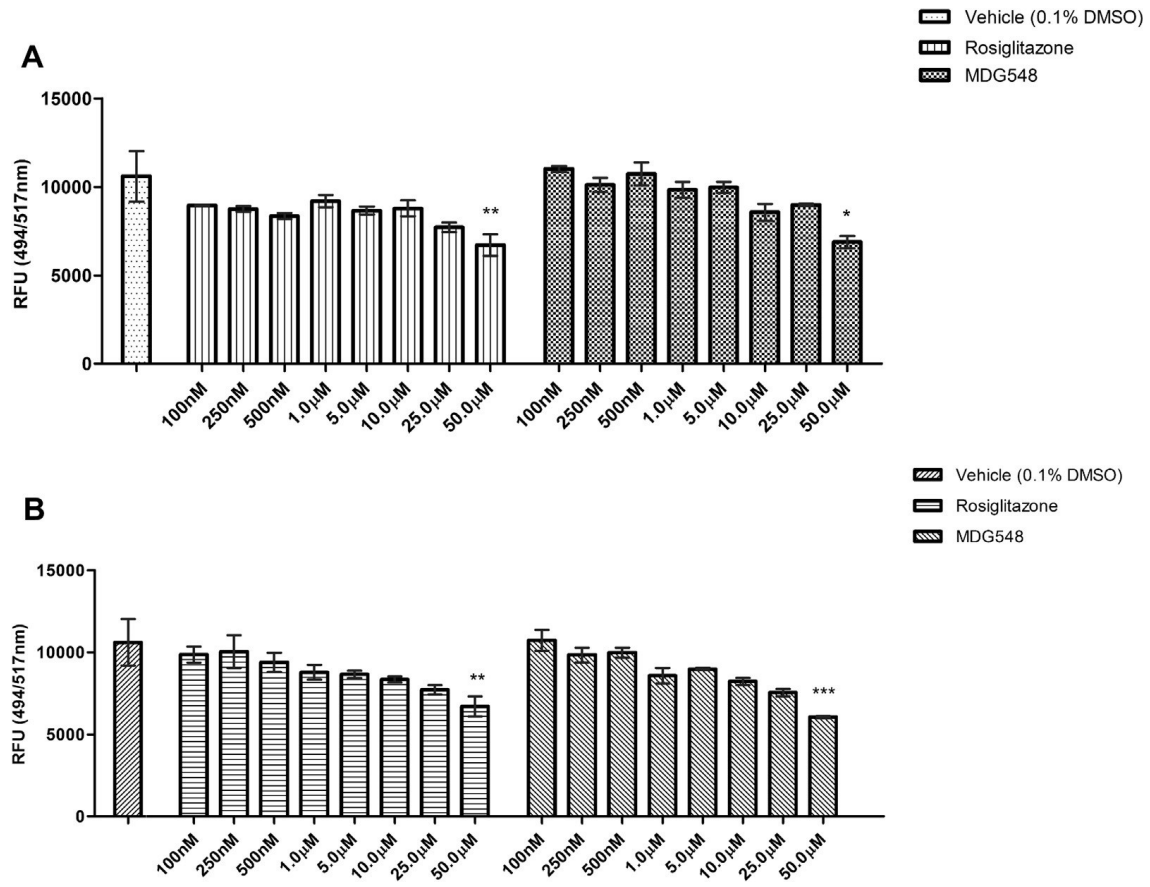


Fig. 3. Determination of MDG548 cytotoxicity against rat cortical neurons*. Cells were treated with vehicle (DMSO 0.1%) or increasing doses of MDG548 or rosiglitazone, and allowed to incubate for 24 (A) or 48 h (B) prior to addition of CalceinAM (2 μM) at the experimental endpoint. Data are presented in relative fluorescence units (RFU). Test wells were compared to vehicle treated (0.1% DMSO) using a two-way ANOVA with Bonferroni post hoc test. *p<0.05, **p<0.01, ***p<0.001. Error bars represent the Standard Error of the Mean (SEM). Experiments were carried out in triplicate in at least three independent experiments.*

MDG548 exposure protected rat cortical neurons from H_2O_2-mediated injury

The protective effect of MDG548 was assessed in cultured rat cortical neurons exposed to H_2O_2. Two experimental conditions were studied, where neurons were exposed to MDG548 or

rosiglitazone either 24 h prior to H_2O_2 exposure (Fig. 4A) or at the same time point (Fig. 4B), and incubated for a further 24 h prior to measurement of cellular viability via PrestoBlue assay. The non-cytotoxic concentration window previously determined was used for MDG548 and rosiglitazone (100 nM–10 μM). In both experimental conditions, neuronal viability was significantly reduced after exposure to H_2O_2 when compared to the control vehicle (0.1% DMSO) ($p<0.001$). Pre-treatment (24 h) of cortical neurons with increasing concentrations of MDG548 resulted in a dose-dependent raise in cell viability as compared to H_2O_2-treated cells, displaying significant values at 500 nM concentration ($p<0.05$) and 1.0 to 10 μM concentration ($p<0.001$) (Fig. 4A). Pre-treatment (24 h) with rosiglitazone also displayed significant neuroprotective effects against H_2O_2 at 1.0 μM ($p<0.05$) and 5.0 to 10 μM ($p<0.001$) doses (Fig. 4A). MDG548/H_2O_2 co-treatment induced a limited increase in cortical neurons viability at 1 μM ($p<0.01$) and 5 to 10 μM concentrations ($p<0.001$) when compared to H_2O_2 (Fig. 4B). In this experimental condition rosiglitazone increased cell viability at 10 μM concentration only and to a very small extent ($p<0.05$). Exposure to the PPARγ antagonist GW9662 (1 μM) for 1 h prior to incubation with MDG548 in experimental condition A abrogated the neuroprotective effect (Fig. 5). The PPARγ antagonist was not tested in experimental condition B since MDG548 yielded a very small effect in this test while rosiglitazone mostly displayed no effect. Neuronal viability was not significantly different following exposure to the antagonist alone (Fig. 5).

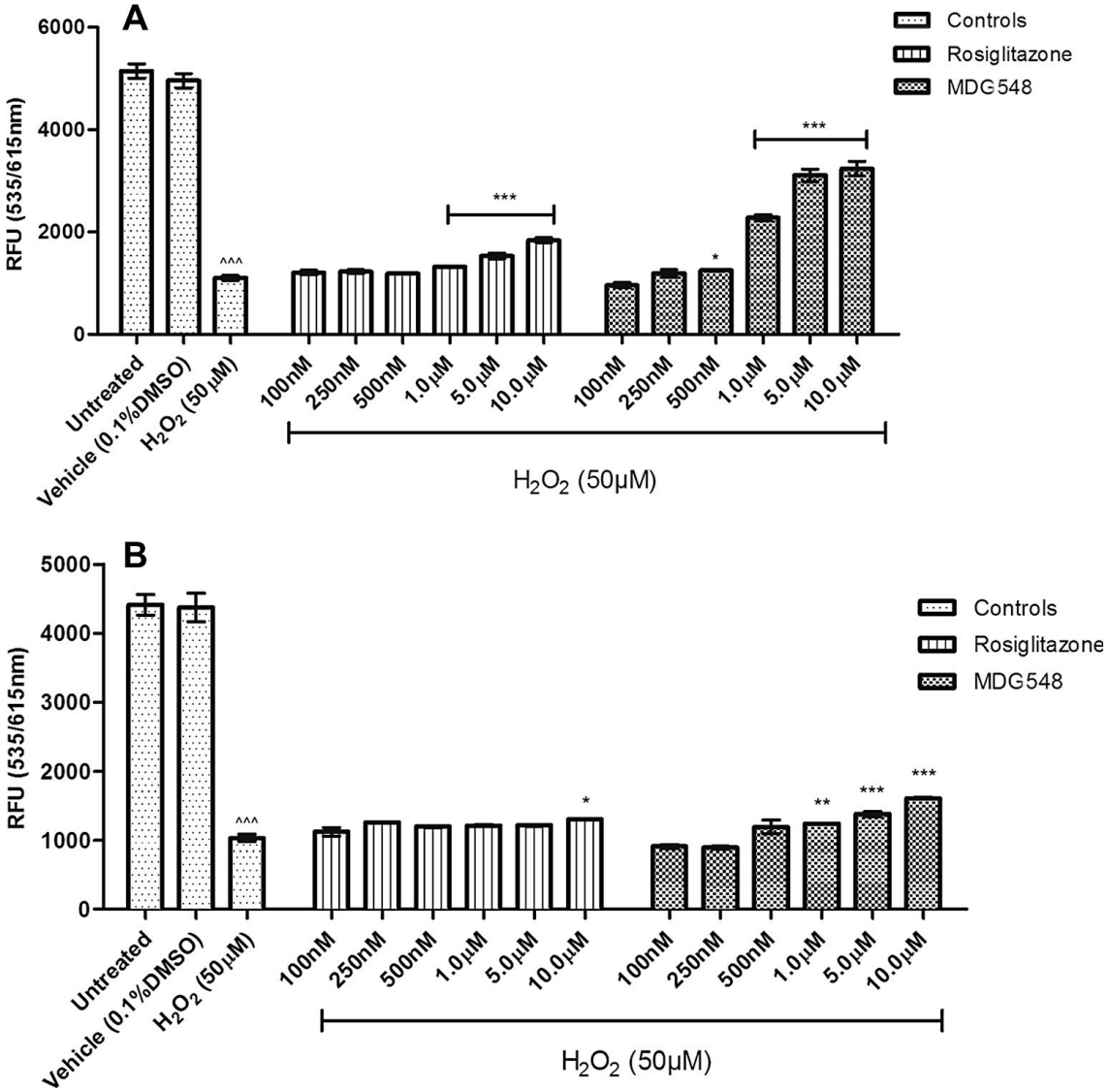


Fig. 4. MDG548 exposure protects rat cortical neurones from H_2O_2-mediated injury*. Cells were treated with vehicle (0.1% DMSO), MDG548 or rosiglitazone 24 h prior to addition of H_2O_2 (50 μM) (A) or at the same time-point than H_2O_2 (B). Data are presented in relative fluorescence units (RFU). Test wells were compared to H_2O_2 treated (0.1% DMSO) using a two-way ANOVA with Bonferroni post hoc test. *p<0.05, **p<0.01, ***p<0.001, ^^^p<0.001 versus vehicle (0.1% DMSO). Error bars represent the Standard Error of the Mean (SEM). Experiments were carried out in triplicate in at least three independent experiments.*

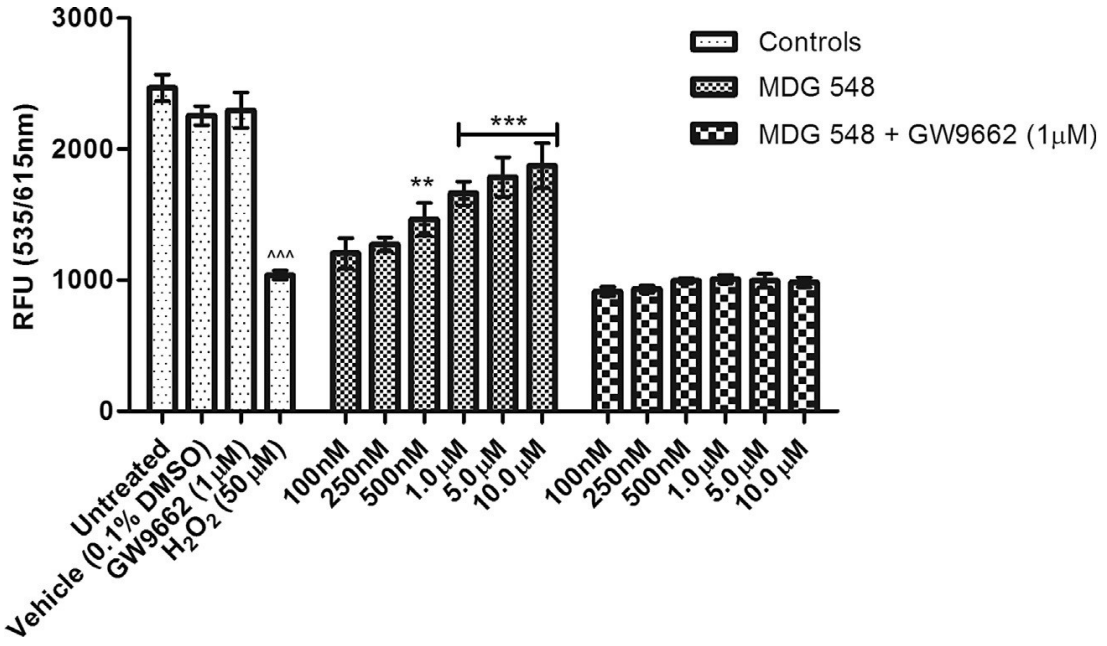


Fig. 5. PPARγ antagonist GW9662 inhibits the neuroprotective effects of MDG548*. Cells were treated with GW9662 (1 μM) or vehicle (0.1% DMSO) for 1 h prior to treatment with MDG548 or vehicle, and allowed to incubate for 24 h prior to addition of H_2O_2 (50 μM). Data are presented in relative fluorescence units (RFU). Test wells were compared to H_2O_2-treated cells (0.1% DMSO) using a two-way ANOVA with Bonferroni post hoc test. **p<0.01, ***p<0.001. ^^^p<0.001 versus vehicle (0.1% DMSO). Error bars represent the Standard Error of the Mean (SEM). Experiments were carried out in triplicate.*

MDG548 protected PC12 dopaminergic-like cells against MPP+-induced toxicity

Neuroprotection by MDG548 was assessed in NGF differentiated PC12 cells exposed to MPP+ (Fig. 6). NGF was added in order to confer dopaminergic-like features to cells. Neuronal viability was significantly reduced after exposure to MPP+ when compared to the control vehicle (0.1% DMSO) ($p<0.001$). Exposure to GW9662 alone also reduced cell viability, although to a lesser extent than MPP+ ($p<0.01$). Treatment of PC12 cells with increasing doses of MDG548 induced an increase of cell viability ($p<0.001$), which was attenuated by exposure to GW9662 (1 μM)

(p<0.001) (Fig. 6).

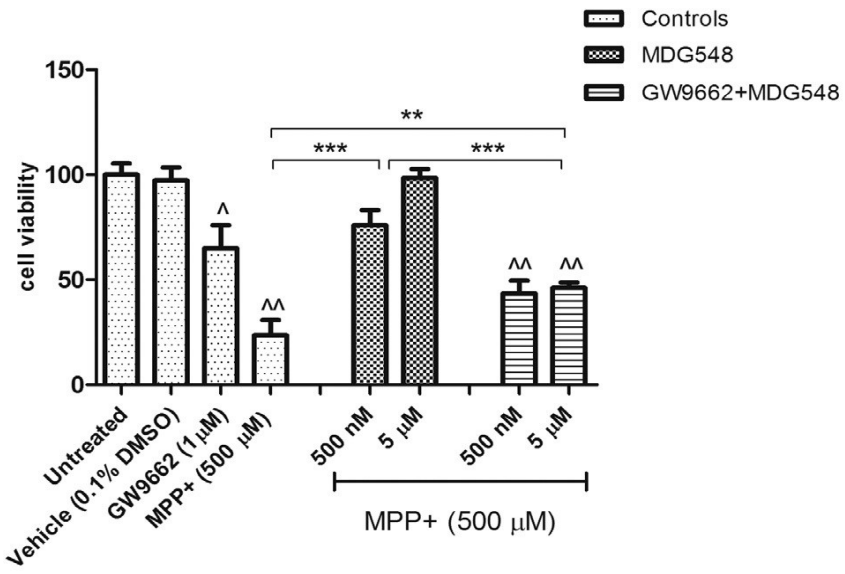
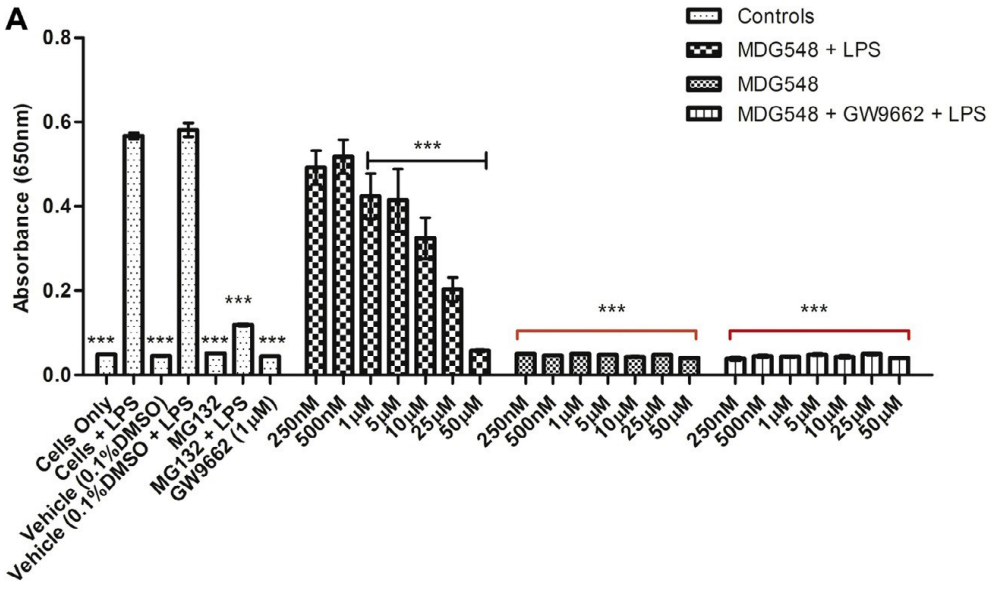


Fig. 6. MDG548 exposure protected PC12 dopamine-like cells from MPP+ toxicity. *Cells were treated with GW9662 (1 μM) or vehicle (0.1% DMSO) for 1 h prior to treatment with MDG548 or vehicle plus MPP+. Data are presented in % of viable cells, and a two-way ANOVA with Bonferroni post hoc test was applied for statistical analysis. ^; ^^p<0.01 or 0.001 versus vehicle (0.1% DMSO), **p<0.01, ***p<0.001. Error bars represent the Standard Error of the Mean (SEM). Experiments were carried out in triplicate in at least three independent experiments.*

MDG548 inhibited LPS-induced activation of NF-kB

HEK Blue cells were treated with increasing concentrations of MDG548 or rosiglitazone for 24 h prior to stimulation with LPS (100 ng/ml) for a further 6 h. MG132, a known inhibitor of NF-kB activation was also included alongside LPS-stimulated/unstimulated controls. Stimulation with LPS but not LPS and MG132 resulted in activation of NF-kB compared to controls (p>0.01), as reported by the increase in absorbance. Pre-treatment with MDG548 induced a dose-dependent decrease in LPS-stimulated NF-kB activation as compared to LPS alone, which was significant at

concentrations 1.0 to 50 μM ($p<0.01$) (Fig. 7A). MDG548 itself did not induce NF-kB activation in non-LPS-stimulated treatments, at any tested concentration ($p >0.01$). As expected, rosiglitazone also showed a reduction in NF-kB activation, but only at 25 and 50μM ($p<0.01$) (Fig. 7B). Inclusion of the PPARγ antagonist GW9662 did not abolish MDG548 and rosiglitazone effect on NF-kB activation ($p<0.01$) (Fig. 7A, B).



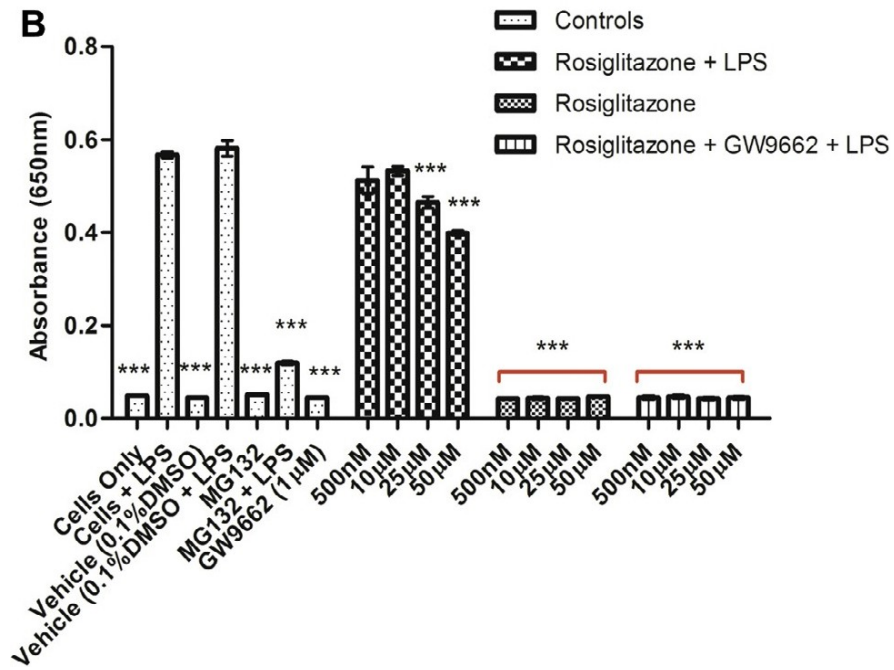


Fig. 7. MDG548 inhibits NF-kB activation in a dose-dependent manner. *Cells were treated with control solutions or increasing concentrations of MDG548 or MDG548+GW9662 (1 μM) (A), or increasing concentrations of rosiglitazone or rosiglitazone+GW9662 (1 μM) (B), and allowed to incubate for 24 h prior to addition of LPS (100 ng/ml). Data are presented in arbitrary absorbance units. Test wells were compared to 0.1% DMSO+LPS-treated cells using a two-way ANOVA with Bonferroni post hoc test. ***p<0.01. Error bars represent the Standard Error of the Mean (SEM). Experiments were carried out in triplicate in at least three independent experiments. C depicts an XY plot of absorbance versus log concentration (M) of MDG548 and is representative of a decrease in NF-kB activation under MDG548+LPS and MDG548-only treatments.*

MDG548 altered microglia phenotype in LPS-stimulated MMGT12

In murine microglial cells MMGT12, MDG548 showed a dose-dependent effect on the levels of pro- and anti-inflammatory molecules measured by ELISA. 10 μm but not 50 μm of MDG548 significantly decreased TNF-α levels and increased CD206 levels in untreated cells, as compared with vehicle ($p< 0.05$ for TNF-α; $p< 0.001$ for CD206) (Fig.8A). IL-1β levels were in the low physiological range and undetectable (data not shown), Ym1 was unaffected by MDG548.

Moreover, LPS increased TNF-α and IL-1β, and decreased CD206 secretion, leaving unaltered Ym1 levels. MDG548 reverted LPS effect on TNF-α and CD206 restoring physiological levels when added 2 hours after LPS ($p< 0.001$ and $p<0.005$ VS LPS), while did not affect IL-1β and Ym1 levels (Fig.8B). When MDG548 was incubated at the same time point than LPS, only the effect on CD206 was maintained.

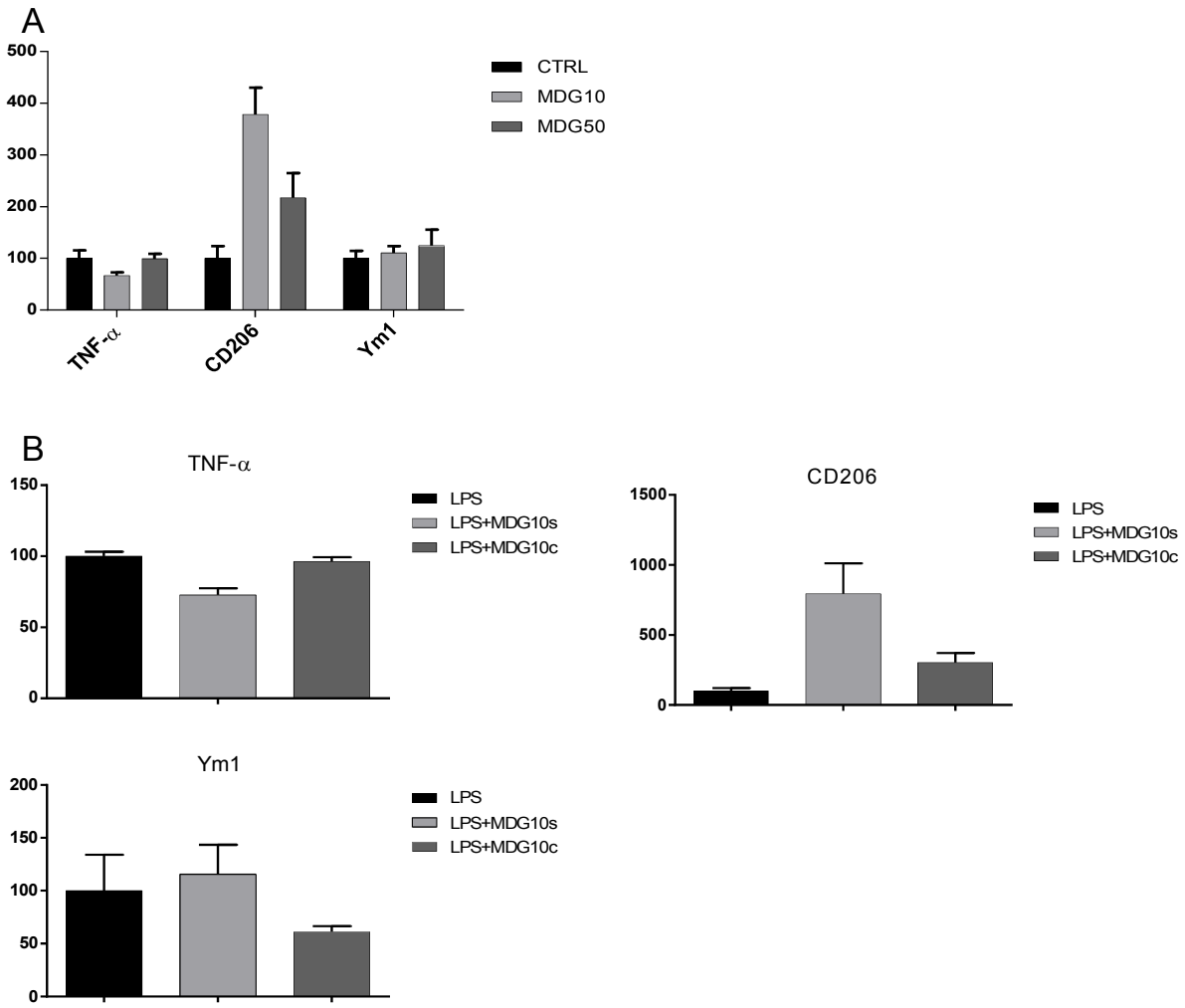
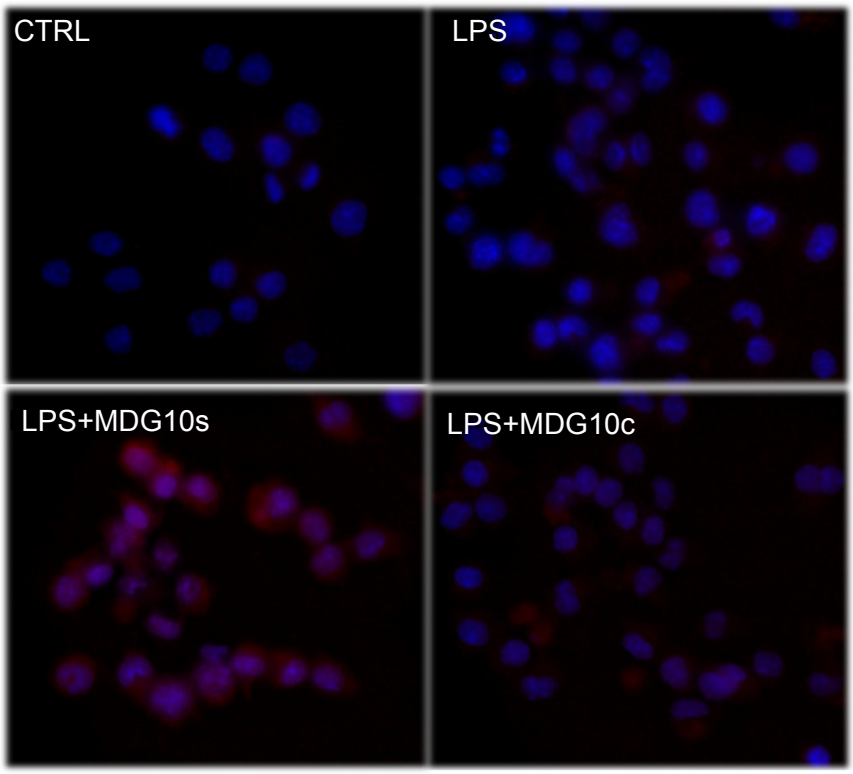


Fig.8. MDG548 modified the production of TNF-α and CD206 in MMGT12 cells. *Cells were treated with MDG548 at the doses of 10 μm and 50 μm (MDG10 and MDG50, respectively). TNF-α, CD206 and YM-1 were measured by ELISA increased in the group MDG548 at the dose of 10 μm (Fig. 8A). Cells were treated with LPS in association with MDG548 10 μm 2 hrs later (s) or at the same time point (c). (*p< 0.05 for TNF-α; p<0.001 for CD206) (Fig.8B).*

MDG548 affected phagocytosis in MMGT12 cells.

Qualitative evaluation of IR in MMGT12 cells showed that LPS slightly increased CD68 IR, while MDG548 at the dose of 10 μm dramatically enhanced CD68 IR, particularly when administered 2 hrs after LPS. 50 μm of MDG548 displayed a similar but attenuated effect as compared to the low dose (Fig.9).



2. Neuroprotective properties of MDG548 in MPTP models in mice

MDG548 reverted MPTPp-induced motor impairment.

MDG548 prevented motor impairment induced by MPTPp treatment. MPTPp chronic treatment induced a significant increase on the number of errors per step as compared with vehicle (by Tukey's post-hoc test), in agreement with previous characterization of this protocol (Schintu et al. 2009a). Combined treatment with MDG548 in the last part of the MPTPp treatment totally prevented the increase of errors induced by MPTPp treatment ($p<0.05$ by Tukey's post-hoc test).

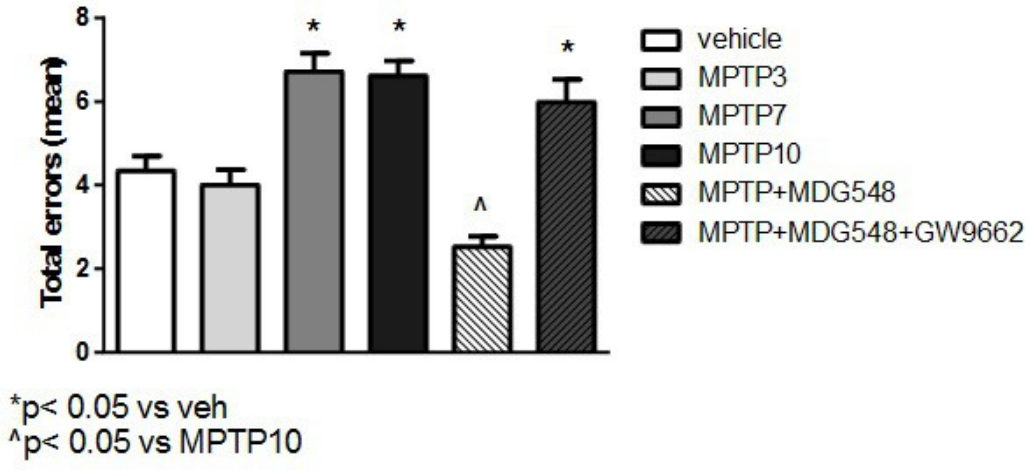


Fig. 10. MDG548 prevented MPTPp-induced motor deficits. *Motor impairment was expressed as total step errors measured by the beam walking test. Combined treatment with MDG548 plus MPTPp prevented the increase of total errors. *p<0.05 versus vehicle-treated mice, by Tukey post-hoc test; ^p<0.05 versus MPTP10, by Tuckey post-hoc test (n= 5-10 per group).*

MDG548 prevented MPTP-induced degeneration of the SNc in the subacute MPTP protocol.

In the subacute MPTP treatment, stereological evaluation of TH-IR and Nissl staining showed a reduction in the number of neurons in the SNc of MPTP-treated mice by about 50% as compared to vehicle ($p<0.001$ by Tukey's post hoc test) (Fig. 11A, B). The lowest MDG548 dose was chosen based on a pilot study where we found that MDG548 at the dose of 0.5 mg/kg was ineffective on MPTP-induced nigral neurodegeneration. Moreover, in vitro assays showed that MDG548 was more potent that the control compound rosiglitazone, which we have found to be neuroprotective at 10 mg/kg in vivo in several previous studies (Schintu et al., 2009b; Carta et al., 2011; Pisanu et al., 2014). We therefore further narrowed dose selection for MDG548 by using rosiglitazone effective dose as the highest tested dose. The administration of MDG548 to MPTP-treated mice inhibited the dopaminergic degeneration to a similar extent at all tested doses of 2, 5 and 10 mg/kg, with no significant differences from the vehicle group (Fig. 11A, B).

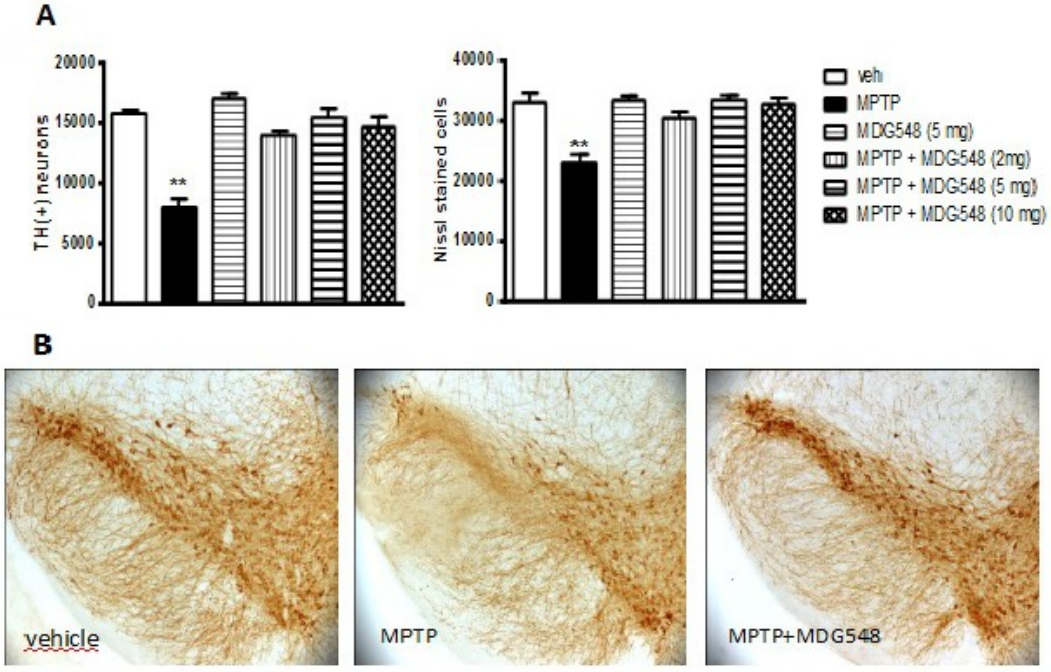


Fig. 11. MDG548 prevented dopaminergic degeneration of the SNc in the subacute MPTP protocol. *Representative images of the SNc of mice treated with vehicle, MPTP or MPTP+MDG548 (2 mg/kg). Magnification 20X (A). Total number of TH+ and Nissl-stained cells in the SNc, as measured by unbiased stereological analysis (B). **$p<0.05$ versus vehicle-treated mice, by Tukey's post hoc test (n=4–5 per*

group).

MDG548 arrested MPTP-induced degeneration of the SNc in the chronic MPTPp protocol.

When tested in the chronic MPTPp protocol, MDG548 arrested dopaminergic neurodegeneration in the SNc. Stereological evaluation showed a reduction in TH-positive cells in the SNc of MPTPp chronically-treated mice as compared to vehicle ($p < 0.01$). Neurodegeneration was progressively increased during the chronic MPTPp treatment, with a slight reduction in TH-positive cells after three MPTP injections (MPTP3), and a larger neuronal loss after subsequent injections (MPTP7, MPTP10), in agreement with the previous characterization of this MPTPp protocol (Schintu et al., 2009a). The repeated administration of MDG548 starting after the seventh MPTPp injection (MPTP+MDG548) arrested the dopaminergic cell death, with no significant differences in number of TH+ cells as compared to vehicle. Simultaneous administration of PPARγ antagonist GW9662 (MPTP + MDG548 + GW9662), counteracted the neuroprotective effect of the drug, with levels of neuronal loss similar to MPTP10-treated rats ($p< 0.05$).

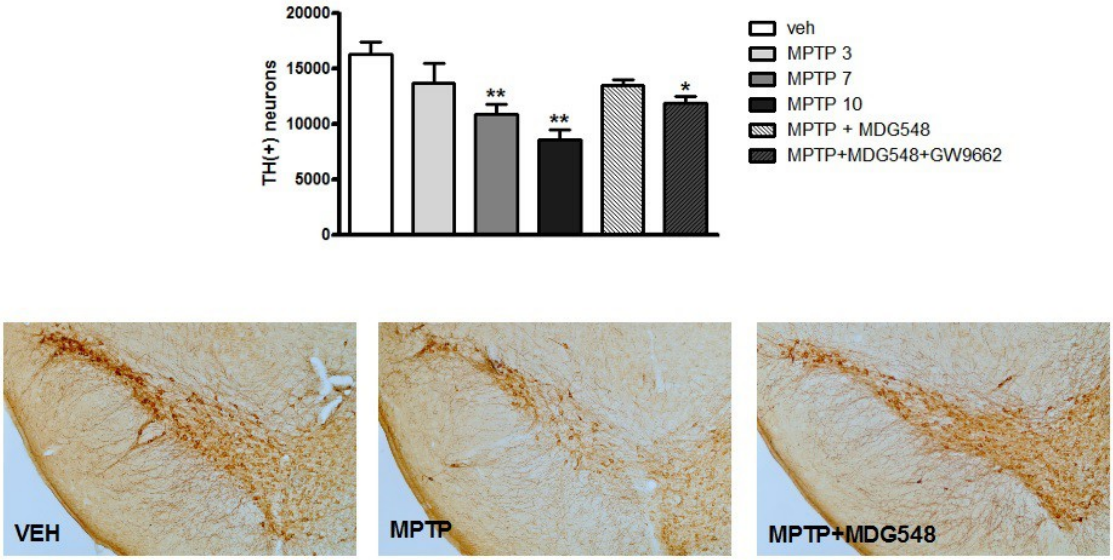


Fig. 12. MDG548 arrested dopaminergic degeneration of the SNc in the chronic MPTPp protocol. *Total number of TH+ cells measured by stereological counting. Representative images of SNc in mice chronically treated with vehicle, MPTP or MPTP+MDG548 (magnification 20X). *p<0.05 versus vehicle-treated mice, by Tukey post-hoc test; **p<0.01 versus vehicle-treated mice, by Tukey post-hoc test (n=5-10 per group).*

3. Modulation of the neuroinflammatory response by MDG548 in MPTP models

Changes in microglia activation and iNOS induction in the subacute MPTP protocol.

Following subacute MPTP treatment, CD11b-IR was increased as compared to vehicle, in terms of total IR area in the analysed region ($p<0.05$ by Tukey's post hoc test), as well as mean IR area/cell, suggesting microglia proliferation and morphological changes respectively ($p<0.05$ by Tukey's post hoc test). Hence, in the vehicle-treated SNc the majority of microglia displayed a resting morphology with a small body, long and ramified processes, while after MPTP treatment microglia was increased in number and displayed an activated morphology, characterized by a larger body with short and thicker processes. In contrast, after MDG548/MPTP co-treatment, microglia proliferation as well as morphological changes were inhibited and cells displayed a resting-like morphology (Fig. 13). iNOS-positive cells were significantly increased by the MPTP treatment in the SNc, while co-treatment with MDG548 attenuated the MPTP effect ($p<0.05$ by Tukey's post hoc test) (Fig. 14). As expected, double IR confirmed that iNOS immunostaining avoided TH-positive neurons, suggesting that changes occurred in glial cells (Fig. 14).

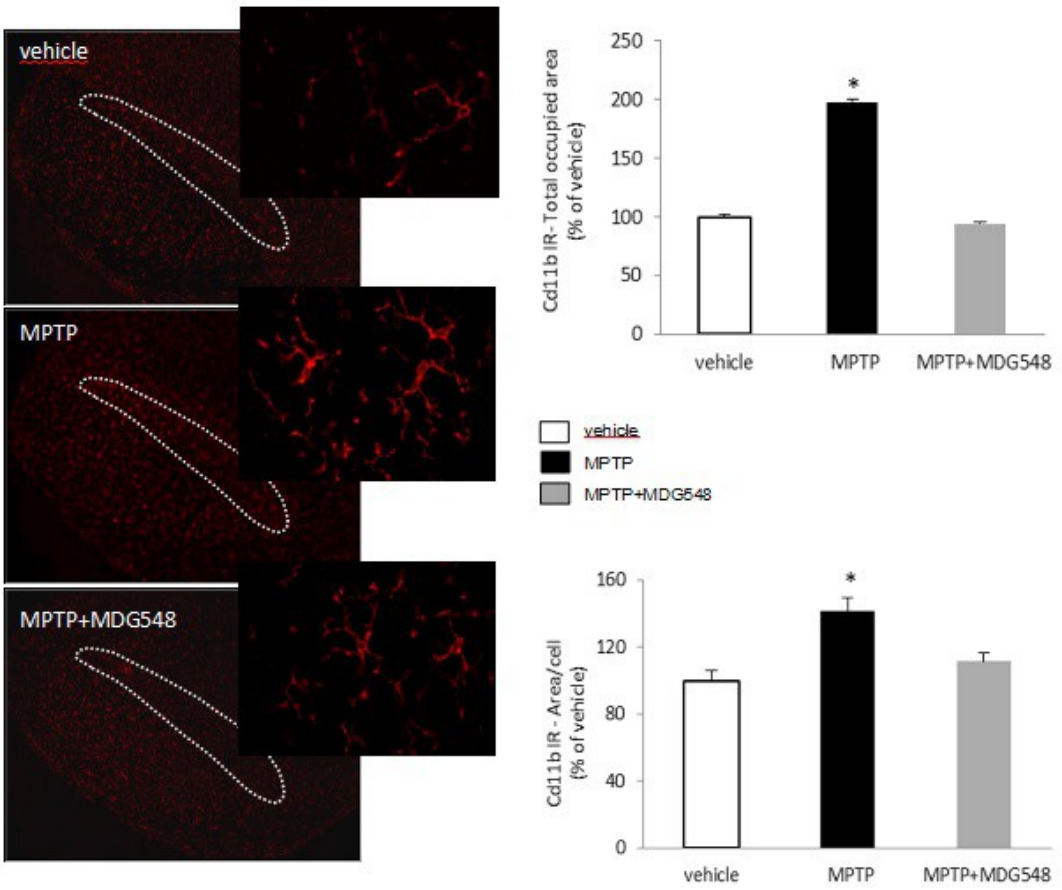


Fig. 13. MDG548 inhibited microglia reactivity in the SNc. *Representative images of CD11b+ cells in the SNc of vehicle, MPTP and MPTP+MDG548 (5 mg/kg)-treated mice. Magnification 100X (A). CD11b in the SNc was measured as the total area occupied by CD11b+ cells in the region of SNc analyzed (see text for details) (B), and as the mean CD11b+ area/cell (C). *p<0.05 versus vehicle-treated mice by Tukey's post hoc test (n=4–5 per group).*

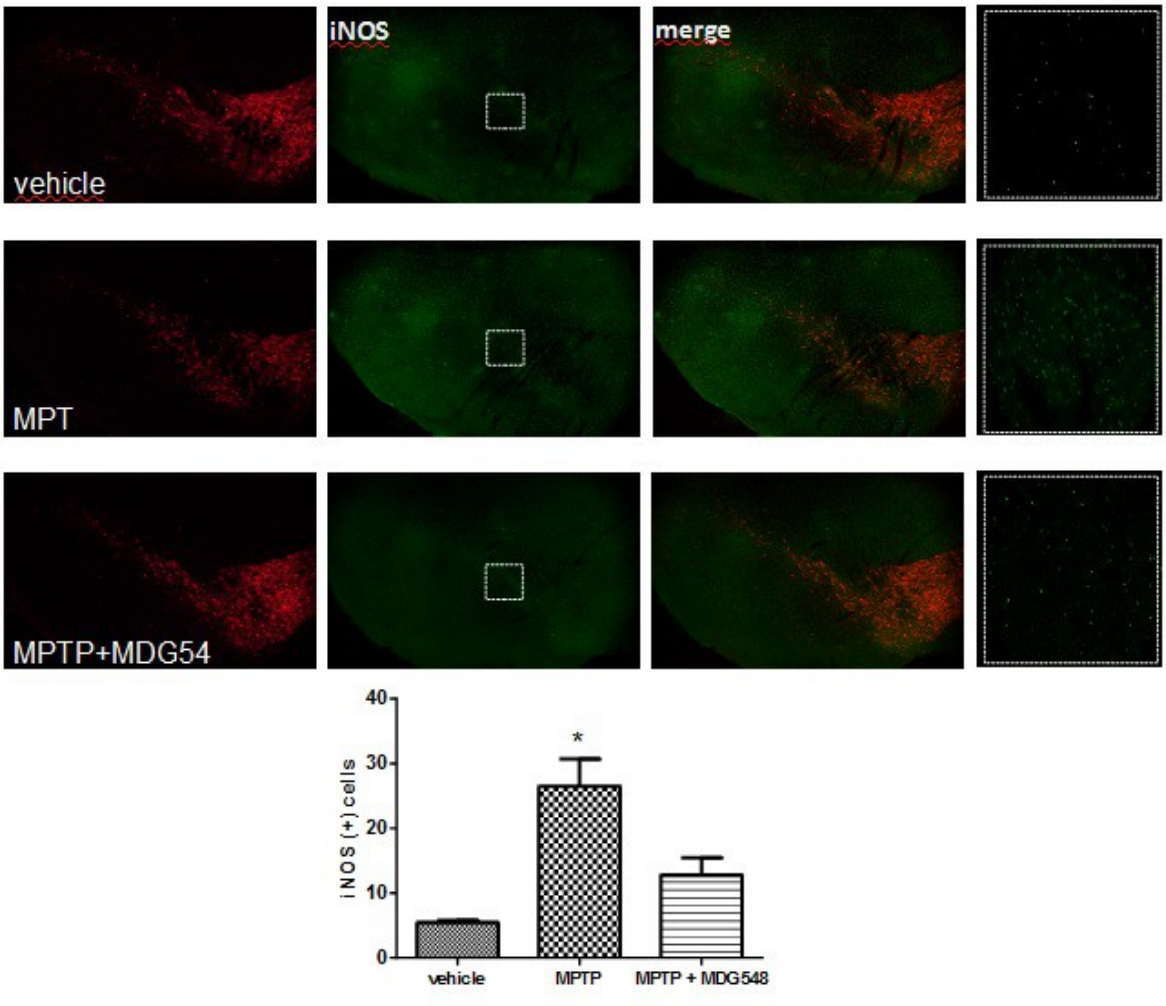


Fig. 14. MDG548 attenuated iNOS induction in the SNc*. Representative images of iNOS+ cells in the SNc of vehicle, MPTP and MPTP+MDG548 (5 mg/kg)-treated mice. Magnification 40X (A). iNOS+ cells were automatically counted by Optimas image analyzer within a fixed area (see methods for details) *p<0.05 versus vehicle-treated mice by Tukey's post hoc test (n=4–5 per group).*

Changes in microglia activation and cytokines production in the chronic MPTPp protocol.

MDG548 attenuated microglia activation in the SNc. The MPTPp treatment produced a progressive increase of reactive microglia. CD11b-IR was increased in MPTP7 and MPTP10-treated mice as compared to vehicle, in terms of IR area/cell ($p< 0.05$ by Tukey's post-hoc test), as well as mean total IR area in the analyzed region of SNc, suggesting microglial proliferation and morphological changes, respectively ($p< 0.05$ by Tukey's post-hoc test). After MPTPp+MDG548 co-treatment, microglia activation was attenuated.

IR for the pro-inflammatory cytokine TNF-α increased in microglia along with the chronic MPTPp treatment ($p< 0.05$ by Tukey's post-hoc test). Co-administration of MDG548 in the last part of the neurotoxin treatment reduced the levels of CD11b/TNF-α IR colocalization, reverting it to vehicle values ($p< 0.05$ vs MPTP10 by Tukey's post-hoc test). Administration of the PPARγ antagonist GW9662 reverted MDG548 effect ($p< 0.05$ vs veh by Tukey's post-hoc test).

IR for the anti-inflammatory cytokine CD206 in microglia decreased along with the chronic MPTPp treatment, reaching 50% of colocalization in CD11b(+) cells after ten MPTPp injections,as compared with vehicle ($p< 0.05$ vs veh by Tukey's post-hoc test). MDG548 reverted this trend, by reverting colocalization levels to vehicle values ($p< 0.05$ VS MPTP10 by Tukey's post-hoc test).

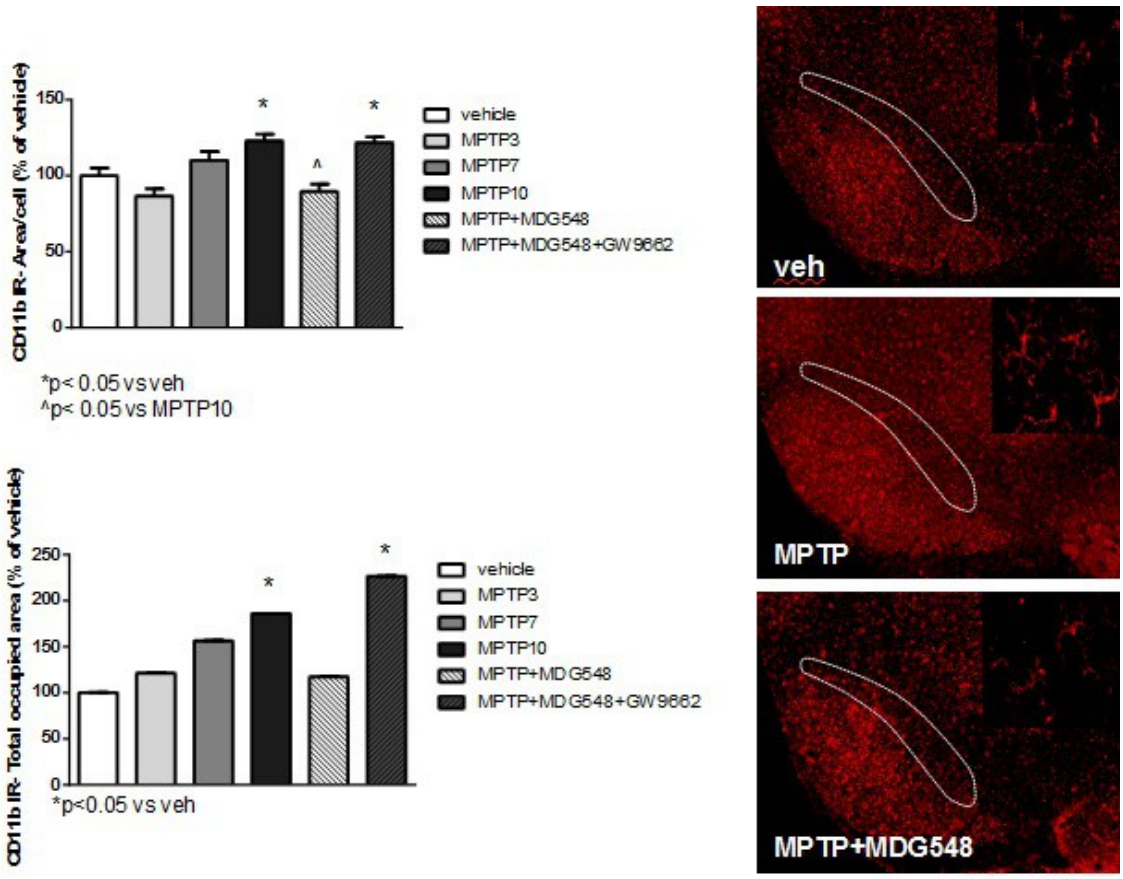


Fig. 14. MDG548 decreased MPTPp-induced microglia activation. *CD11b IR was measured in the analyzed region of SNc as Cd11b+ mean area/cell (*p<0.05) and as total area occupied by Cd11b+ cells (*p<0.05). Representative images of Cd11b+ cells in SNc of mice chronically treated with vehicle, MPTP and MPTP+MDG548 (magnification 100X).*

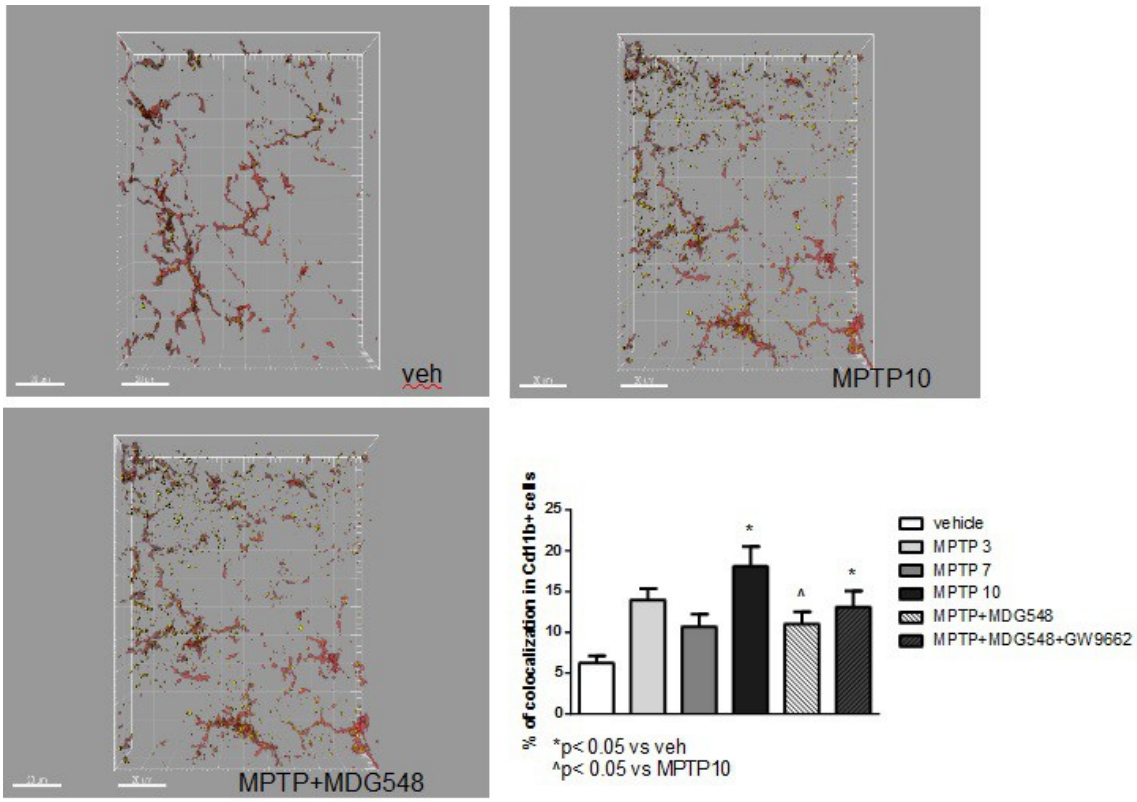


Fig. 15. MDG548 inhibited the MPTPp-induced production of TNF-α in microglia. *Representative confocal images showing colocalized elements (yellow) in CD11b (red) positive cells in the SNc (A). Percentage of colocalization of TNF-α IR in CD11b positive cells. *p<0.05 versus vehicle-treated mice, by Tukey post-hoc test; ^p<0.05 versus MPTP10, by Tuckey post-hoc test (n= 5-10 per group) (B).*

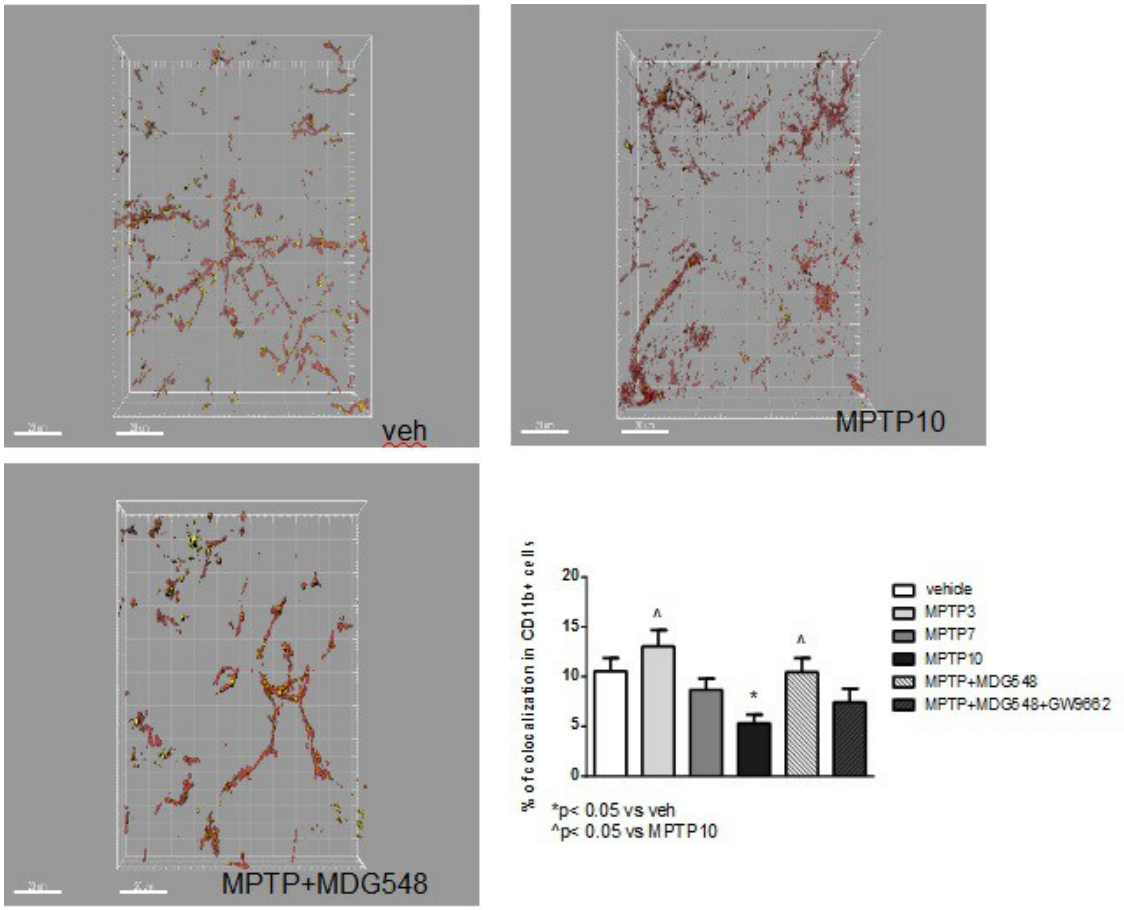


Fig. 16. MDG548 increased CD206 IR in microglia. *Representative confocal images showing colocalized elements (yellow) in CD11b (red) positive cells in the SNc (A). Measure is expressed as percentage of colocalization of CD206 IR in CD11b(+) cells (B). *p<0.05 VS vehicle-treated mice, by Tukey post-hoc test; ^p<0.05 vs MPTP 10, by Tuckey post-hoc test (n= 5-10 per group).*

DISCUSSION

The present study investigated the neuroprotective properties of the novel non-TZD compound MDG548, in relation with its ability to modulate the neuroinflammatory response in dopaminergic areas. The whole study has been subdivided in three phases, where a dose-response study and the potential neuroprotective efficacy of MDG548 were tested *in vitro* and *in vivo* in the first phase, while the neuroprotective/disease modifying properties and immune-modulatory activity were investigated in a clinically relevant *in vivo* model of PD in a second set of experiments. Finally, mechanisms of immunomodulation were investigated in more depth in cultured microglia cells.
Part of the data presented here, concerning results of the *in vitro* dose-response study and *in vivo* testing of MDG548 in the subacute MPTP treatment, have been recently published in Neuroscience (Lecca et al, 2015).

1. MDG548 is neuroprotective in PD models

Neuroprotective activity of MDG548 was first observed *in vitro* against H_2O_2 or MPP+ toxicity on cultured neurons. Thereafter, neuroprotection was found *in vivo* in both the subacute and the chronic MPTP mouse model of PD.

1.1 In vitro studies

Cell viability assays showed a cytotoxic effect of MDG548 only at the higher concentration tested, above 25 μM. Based on these data, a dose-range up to 10 μM was used in neuroprotection studies, in order to avoid any possible confounding cytotoxic effects of MDG548 on neuronal cells. Importantly, MDG548 was toxic at a dose range equivalent to that of the marketed drug

rosiglitazone, a PPARγ agonist extensively investigated for its neuroprotective properties in PD models. MDG548 effect against H_2O_2 insult in cultured neurons was assayed in order to evaluate possible neuroprotective activity of the compound prior to *in vivo* testing. Results showed a dose-dependent protection by MDG548, which was effective in preventing and, to a lesser extent, counteracting the H_2O_2-induced cell death, when administered prior or together with the neurotoxicant, respectively. As compared to rosiglitazone, MDG548 displayed a protective activity against H_2O_2 insult at lower doses, in agreement with the higher PPARγ affinity of MDG548 reported in a previous study (Nevin et al., 2012). Relevant to subsequent *in vivo* experiments, MDG548 was neuroprotective when co-administered with MPP+ in PC12 cells. Moreover, MDG548 protective effect was abolished by the specific PPARγ antagonist GW9662. Interestingly, the protective effect against H_2O_2 required a 24-h pre-exposure to MDG548. Multiple PPARγ-mediated mechanisms, either direct and indirect, have been indicated to contribute to the neuroprotective efficacy (Zhao et al., 2015). These include not only direct post-translational mechanisms such as PPARγ inhibitory binding to transcription factor NF-kB, but also modulation of the expression of genes involved in oxidative stress pathways such as antioxidant enzyme catalase, a mechanism which may require drug pre-exposure. This last mechanism, by increasing the H_2O_2 buffering capacity of neurons, may be the prevalent mechanism for neuroprotection against acute H_2O_2 insult in the present study.

1.2 In vivo treatments

In vivo, neuroprotection was tested in two classical mouse models of dopaminergic degeneration induced by MPTP administration (Carta et al., 2009).

Firstly, a subacute MPTP model consisting in 4 injections once a day for 4 consecutive days, at the dose of 20 mg/kg, was used. This protocol is suitable for a preliminary and rapid screening of novel

potentially neuroprotective compounds (Vila et al., 2000; Carta et al., 2009; Blesa et al., 2012).

In a second set of experiments, the neuroprotective effect of MDG548 was investigated in the chronic MPTPp model. The MPTPp model reproduces several features of PD, particularly the progressive nature of dopaminergic degeneration, associated with a slow development of motor impairment, which makes testing of drugs in this model clinically relevant (Petroske et al., 2001; Carta et al., 2011). The progressive nature of this model allowed us to test MDG548 in presence of a partial and ongoing degeneration of nigrostriatal neurons, reproducing more closely the disease stage of parkinsonian patients at the moment of diagnosis. Moreover, in the MPTPp chronic model, neuroinflammatory responses display a dynamic development as the neurodegeneration progresses, offering the possibility to investigate changes in neuroinflammation related with the different disease stages (Pisanu et al, 2014).

In the subacute and chronic MPTP models, MDG548 administration was effective in preventing the neurotoxin-induced neurodegeneration, as shown by the stereological counting of both TH-positive and Nissl-positive cells in the SNc. Moreover, the neuroprotective effect was observed 4 days after the last administration, indicating that MPTP-induced degeneration was actually inhibited by MDG548 rather than simply delayed.

Using this subacute protocol, MDG548 did not display a concentration-dependent response since all tested doses 2, 5 and 10 mg/kg induced a comparable neuroprotective effect, suggesting that the lowest dose was already fully effective. On the other hand, in a pilot experiment we found 0.5 mg/kg of MDG548 ineffective (data not shown). Since the *in vitro* assay allowed to test a broader range of doses in order to obtain a concentration-dependent response, we can assume that a fine tuning down of the dose-range *in vivo* would provide a similar response pattern.

In the chronic MPTPp protocol, we used the lowest effective dose of MDG548, 2 mg/kg, tested in the previous experiment. As previously shown, MPTPp-mediated nigral neurodegeneration gradually increased at progressive time-points along the neurotoxin treatment. We found that

MDG548 arrested the neurodegenerative process when tested in the last part of the MPTPp treatment, in presence of a partial and ongoing degeneration, suggesting a disease-modifying property of this drug.

Confirming previous studies, the chronic MPTPp intoxication induced motor deficits which increased along with nigral degeneration (Meredith et al., 2008; Petroske et al., 2001; Schintu et al., 2009a). By using the beam-walking test, a highly sensitive test for motor deficits in rodents, we found that MDG548 completely prevented the development of MPTPp-induced motor impairment, suggesting that neuroprotection was reflected by the recovery of motor function (Fleming et al., 2004).

The selective PPARγ antagonist GW9662 counteracted MDG548-mediated neuroprotection. Recent studies reporting *in vivo* neuroprotection by non-selective PPAR agonists have suggested that different PPAR subtypes may be involved in this effect (Sadeghian et al., 2012; Swanson et al., 2013). MDG548 has potent and highly selective PPARγ agonist activity, therefore the present results strongly support the primary role of a PPARγ-dependent mechanism as a mediator of neuroprotection. (Nevin et al., 2012). Studies with TZDs compounds have suggested that receptor-independent mechanisms might be involved in neuroprotection, such as binding to mitochondrial protein mitoNEET, generating some doubt on the actual involvement of PPARγ in neuroprotection (Feinstein et al., 2005; Hunter et al., 2008). Although additional mechanisms cannot be excluded at this stage, our results provide evidence that specifically targeting PPARγ is sufficient to elicit neuroprotection in the PD models used here. The recent finding that dopaminergic neurons not expressing PPARγ appears more susceptible to MPTP neurotoxicity supports our results (Swanson and Emborg, 2014).

In previous studies we reported the neuroprotective efficacy of the TZD rosiglitazone in the same chronic MPTPp mouse model of PD used here (Schintu et al., 2009; Carta et al., 2011). Rosiglitazone showed neuroprotective properties at the lowest effective dose of 10 mg/kg i.p., while

lower doses such as 5 mg/kg were without an effect in that model (unpublished data). Therefore, in line with the *in vitro* results and along with the higher provisional blood brain barrier permeability displayed by MDG548, the present data suggest an increased *in vivo* potency of MDG548 against MPTP neurotoxicity as compared to rosiglitazone (Nevin et al., 2012).

Recently, concerns have been raised regarding TZDs rosiglitazone and pioglitazone safety in diabetic patients, including increased bladder cancer risk and cardiovascular complications (Home et al., 2009; Barbalat et al., 2012; Carta and Simuni, 2015). However, specifically designed studies have suggested that unwanted side effects of TZDs may be largely due to off-target binding and receptor-independent mechanisms (Cohen, 2005; Tseng and Tseng, 2012). These concerns, while lessening the enthusiasm for supporting research on neuroprotection by TZDs in CNS pathologies, has prompted for search of alternative non-TZDs PPARγ agonists specifically designed for neurodegenerative pathologies. MDG548 was specifically selected for this purpose from a broad spectrum of potentially active non-TZDs compounds. The potent and selective PPARγ activity of MDG548, by allowing the use of low doses to reach neuroprotection, may attenuate the risk of untoward side effects.

2. MDG548 displays immunomodulatory effects in microglia and in PD models *in vivo*.

2.1 Immunomodulatory effects in cultured microglia

Neuroinflammation is a key pathological event in the pathogenesis of PD. A chronic and dysregulated glial response is believed to contribute to the progression and spreading of nigral degeneration mainly by an excessive release of inflammatory cytokines and neurotoxic species such as reactive oxygen/nitrogen species, free radicals, iNOS induction, which confer to microglia a

toxic phenotype responsible for secondary damage to neighbouring neurons (Ramsey and Tansey, 2014). Therefore, targeting microglial response is considered a valuable strategy to modify disease progression (Nolan et al., 2013). PPARγ, being highly expressed in immune cells throughout the body including microglia, holds a pivotal role in the regulation of inflammatory responses mainly by the regulation of the activity of NF-kB transcription factor (Moraes et al., 2006; Straus and Glass, 2007), and several evidence have suggested that the regulation of neuroinflammatory responses may represent a main mechanism of neuroprotection by PPARγ agonists (Dehmer et al., 2004; Sundararajan et al., 2006; Pisanu et al., 2014).

Many of the proven immunomodulatory effects of PPARγ are mediated by innate immune cells, such as macrophages and monocytes (Reddy, 2008). In turn, these cells produce a number of PPARγ ligands, which can potentiate the anti-inflammatory and proresolving properties of this receptor on other immune cells. Importantly, targeting PPARγ is known to modulate the phenotype of peripheral macrophages. Hence, a number of studies have shown that PPARγ modulates the production of both pro- and anti-inflammatory cytokines by these cells, boosting the anti-inflammatory benefit while suppressing the harmful macrophage phenotype (Odegaard et al., 2007). We therefore hypothesized that the same role was carried by PPARγ expressed in microglia, were they may boost the "alternatively" activated M2 phenotype, that is associated with anti-inflammatory characteristics and is involved in the resolution phase of inflammation and in tissue repair (Croasdell et al., 2015).

We first investigated the effect of MDG548 in a classical inflammatory model such as LPS stimulation of cultured HEK-Blue-hTLR4 cells and found a dose-dependent inhibition of LPS-induced NF-kB activation. These results provided first evidence for an anti-inflammatory activity of MDG548. PPARγ may inactivate NF-kB by direct binding to p50 and p65 subunits (Rossi et al., 2000; Straus et al., 2000). In addition, it can induce an indirect NF-kB inhibition through several mechanisms including upregulation of inhibitor kappa B (IkB) protein or activation of transcription

factor Nrf2, which reduces NF-kB activation by dampening the oxidative load in the cell (Nolte et al., 1998; Delerive et al., 2000; Shih et al., 2005). Interestingly, unlike the neuroprotective activity in neurons, PPARγ antagonist was unable to counteract NF-kB inhibition by MDG548 in HEK-BluehTLR4 cells. Although additional studies will be needed to elucidate this result, which may be related to the experimental setting and the cell line used in the assay, it should be noticed that NF-kB was significantly inhibited at concentrations higher than those required for neuroprotection, suggesting that additional uninvestigated mechanisms may be recruited for a full NF-kB inhibition in TLR4 cells.

To investigate the immunomodulatory function of MDG548, we designed a series of *in vitro* experiments were the effect of MDG548 was investigated in pure cultured microglial cells. As compared to the complex *in vivo* system, where the diffuse PPARγ expression in different cell types does not allow to discriminate their function in selected cell population, the *in vitro* experiments allowed us to define the direct effect of PPARγ stimulation on microglia. MMGT12 cells were exposed to MDG548 in presence of the typical pro-inflammatory stimulus LPS and levels of pro-inflammatory and anti-inflammatory markers in the exposure media from these cells were measured by ELISA (Briers et al., 1994; Michelucci et al., 2009; Heurtaux et al., 2010). In particular, we assessed, after exposure to MDG548, the concentration of the pro-inflammatory cytokine TNF-α, and of the surface markers CD206 (mannose receptor) and Ym1, which are typically expressed by alternatively activated microglia. Moreover, we assessed the expression of the phagocytosis marker CD68 by immunocytochemistry. At the lowest dose tested, MDG548 decreased TNF-α and dramatically increased CD206 levels in untreated cells. A not significant or attenuated effect was observed with the highest dose tested, in line with the cytotoxic effect observed with this dose in cultured neurons (see above). Ym1 was not modulated by MDG548 at any tested dose. Furthermore, in LPS activated microglia MDG548 restored TNF-α and CD206 to vehicle values reverting the LPS effect. All these effects were more marked when MDG548 was added 2 hrs after

LPS stimulation, suggesting that the drug was most effective in activated microglia.

It is worth to notice the strong effect of MDG548 on CD206 levels, suggesting a main role of this protein in PPARγ-mediated effects. CD206, or the mannose receptor, is a pattern-recognition receptor involved in innate and adaptive immunity. In the brain the mannose receptor expressed by microglia mediates phagocytosis by these cells, and is regulated by various cytokines, being downregulated by pro-inflammatory cytokines and upregulated by anti-inflammatory cytokines (Régnier-Vigouroux, 2003; Zimmer et al, 2003). Accordingly, we found that CD68 immunoreactivity was increased by LPS, and further highly enhanced by the PPARγ agonist.

In contrast, Ym1 levels did not display any significant modification after MDG548 addition. Although Ym1 is a well-known marker of M2 macrophage a possible functional relation between PPARγ and Ym1 is still elusive and poorly investigated (Rőszer, 2015). Recent studies have clarified that microglia activation is a complex and dynamic process, depending on the type and intensity of the stimulus, which may lead to variable activation profiles. Based on this recent view, microglia activation cannot be simplified in M1 or M2 states, but there are many degrees of activation, especially when considering the complexity of an *in vivo* environment. The results of the present study show that stimulation of microglial PPARγ enhances the anti-inflammatory profile of these cells, together with the phagocytic activity, while suppressing the pro-inflammatory profile of activation. A number of studies have suggested that prevalence of anti-inflammatory microglia and enhanced phagocytic activity of these cells may sustain the deleterious neuroinflammatory response in PD, which contribute to the progression of neurodegeneration (Sanchez-Guajardo et al., 2010). Here, we propose that the phagocytic activity of microglia, sustained by increased CD206 expression, may hold a positive function when associated with an enhanced anti-inflammatory profile, and contribute to PPARγ-mediated neuroprotection.

2.2 Immunomodulatory effects in in vivo treatments

In vivo studies confirmed the observations made in cultured microglia. In both subacute and chronic MPTP protocols, MDG548 reduced MPTP-induced microglia reactivity, as shown by CD11b IR, both in terms of proliferation and changes in cell morphology. Based on Kreutzberg classification, vehicle- treated mice showed a stage of resting microglia, whereas after MPTP treatment a progressive increase of microglia activation has been clearly observed. When MDG548 was administrated (in co-administration with MPTP in subacute protocol, and after the seventh MPTPp injection in chronic protocol), morphological changes typical of activation processes were attenuated.

Moreover, in the subacute MPTP protocol MDG548 significantly attenuated MPTP-induced iNOS expression. In chronic MPTPp treatment, MDG548 decreased TNF-α IR while increasing CD206 levels, in accordance with the *in vitro* studies on cultured microglia.

In line with this result, previous studies showed the inhibitory activity of PPARγ agonists TZDs on NF-kB activity both *in vitro* and in *in vivo* models of PD (Dehmer et al., 2004; Moraes et al., 2006). Pioglitazone administration in a rodent PD model induced the expression of the inhibitory protein (I)-kB and the consequent inhibition of NF-kB activity, by blocking subunit p65 translocation into the nucleus (Dehmer et al., 2004). Moreover, TZDs have been shown to either reduce microglia activation and/or modify the microglial response in terms of cytokine production, both in rodent and primate models of PD (Cunard et al., 2002; Luna-Medina et al., 2005; Schintu et al., 2009; Ji et al., 2010; Swanson et al., 2011; Pisanu et al., 2014). Furthermore, previous studies have shown that PPARγ agonists inhibited LPS-induced iNOS expression both *in vitro* and *in vivo* models of PD (Kitamura et al., 1999; Hunter et al., 2007).

Among pro-inflammatory cytokines, TNF-α plays a pivotal role in neuroinflammatory response, and it has been functionally related to neurodegeneration in several disorders including PD, in many

experimental models. Moreover, an increase in its production has been observed in several studies of MPTP-induced neurotoxicity (Lofrumento et al., 2011; Luchtman et al., 2009); as confirm of these data, a deficiency in TNF receptors has been showed to inhibit microglial activation and to protect dopaminergic neurons against MPTP neurotoxicity (Sriram et al., 2006), while TNF administration elicit nigral degeneration (De Lella Ezcurra et al., 2010). TNF-α may induce NF-kB nuclear translocation via stimulating proteolytic degradation of inhibitory protein (I)-kB and activating apoptotic processes, in dopaminergic neurons (Wajant et al., 2003).

Targeting microglia activation in order to stimulate an alternative anti-inflammatory phenotype has been proposed as a possible strategy for neuroprotective approaches in AD (Hoozemans et al., 2006; Jankowsky et al., 2003). Moreover, an aberrant microglia polarization has been observed in LPS-treated mice and in the aged brain, suggesting that it may represent a damaging mechanism in neurodegenerative diseases and in ageing processes (Lund et al., 2006; Jimenez et al., 2008; Nolan et al., 2005). We propose that targeting microglia polarization by PPARγ agonists may provide neuroprotection by switching the aberrant microglia polarization observed in PD in an anti-inflammatory profile. Phagocytosis has been regarded as a deleterious mechanism in the PD brain, which may contribute to neurons destruction. We believe that enhancing the phagocytic activity of microglia while boosting an anti-inflammatory profile may help the restorative process by improving the scavenging process of debris or perhaps toxic forms of proteins.

CONCLUSIONS

MDG548 is a potent and selective PPARγ agonist, displaying higher receptor affinity for this receptor, compared with currently employed TZDs. MDG548 showed neuroprotective and immunomodulatory properties in *in vitro* as well as *in vivo* models of PD, at lower doses than TZDs. Such increased potency, together with the non-TZD structure of the compound, may reduce the risk of untoward side-effects related to use of excessive doses and/or to receptor-independent mechanisms. Results suggest that MDG548 exerts an immunomodulatory action via a modulation of microglia polarization correcting the imbalance between pro- over anti-inflammatory molecules, offering a novel immunomodulatory approach to neuroprotection in PD.

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