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METFORMIN TREATMENT DECREASES MICROGLIAL ACTIVATION IN THE *IN VIVO* LPS MODEL OF PARKINSON'S DISEASE

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INFORMA:

Que D.ª Khadija Tayara ha realizada el trabajo experimental que ha llevado a la redacción de la presente memoria de Tesis Doctoral bajo la dirección del Dra. Rocio Martinez De Pablos y el Dra. Ana Maria Espinosa Oliva.

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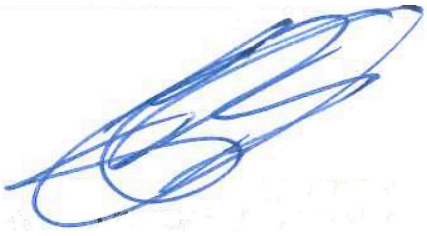

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La Dra Rocio Martinez De Pablos, profesora Contratada Doctora en la Universidad de Sevilla, y la Dra. Ana Maria Espinosa Oliva, profesora Contratada Doctora interina en la Universidad de Sevilla

INFORMAN:

Que D.ª Khadija Tayara ha realizado bajo nuestra dirección el trabajo experimental que ha llevado a la redacción de la presente memoria de Tesis Doctoral titulada METFORMIN TREATMENT DECREASES MICROGLIAL ACTIVATION IN THE *IN VIVO* LPS MODEL OF PARKINSON´S DISEASE"

Considerando que constituye el trabajo de tesis doctoral, autorizamos su presentación para optar al grado de Doctor.

Para que así conste y surta los efectos oportunos, firmamos el presente informe en Sevilla a 26 de Junio de 2018.

Fdo. Dra. Rocio Martinez de Pablos **Fdo. Dra. Ana Maria Espinosa Oliva**

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Contents

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
Ach	Acetylcholine
AGE	Glycosylation end product
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BBB	Blood brain barrier
Calmodulin	Calcium modulated protein
CB	Calabinin
CCK	Cholecystokinin
cDNA	Complementary DNA
CNS	Central nervous system
COX-2	cyclooxygenase 2
CR	Calretinin
CSF	Cerebrospinal fluid
cSNc	Caudal substantia nigra pars compacta
CTCF	Corrected total cell fluorescence
CXCR1	CX3C chemokine receptor 1
DA	Dopamine
DAergic	Dopaminergic
DAGs	Diacylglycerols
DBS	Deep brain stimulation
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's médium
dmSNc	Dorsomedial substantia nigra pars compacta

DMSO	Dimethyl sulfoxide
ECSIT	Evolutionarily conserved signaling intermediate in Toll pathways
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ERK1	Extracellular signal-related kinases 1
ERK2	Extracellular signal-related kinases 2
FBS	Fetal bovine serum
FoxO	Forkhead O family
FTMA	Mitochondrial transcription factor A
GABA	Gamma-aminobutyric acid
GDNF	Glia-derived neurotrophic factor
GFAP	Glial fibrilar acid protein
Glu	Glutamate
GP	Globus pallidus
GPe	Globus pallidus external
GPi	Globus pallidus internal
GPI	Glycosylphosphatidylinositol
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GT1b	Trisialoganglioside
H_2O_2	Hydrogen peroxide
HClO	Hypochlorous acid
hNSCs	human neuronal stem cells
HVA	Homovanilic acid
I.P	Intraperitoneal
IFN-γ	Interferon gamma

IgGs	Immunoglobulins G
IL-1	Interleukin-1
IL-10	Interleukin 10
IL-1β	Interleukin 1beta
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor
JNK	c-Jun N-terminal kinase
K.O	Knockout
KDO	2-keto-3-deoxyoctanoic acid
L-DOPA	Levodopa
LB	Lewy bodies
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MAO-B	Monoamine oxidase B
MAPKs	Mitogen-activated protein kinases
mCD14	Membrane-bound
MCP-1	Monocyte chemoattractant protein 1
MFB	Medial forebrain bundle
MGP	Medial globus pallidus
MHC II	Major histocompatibility complex class II
MLR	Mesencephalic locomotor command region
MPDP +	1-methyl-4 phenyl-2,3-dihydropyridine
MPO	Myeloperoxidase
MPP+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionpiperidine

MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MyD88	Myeloid differentiation primary response gene 88
NA	Noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NADPHox	NADPH oxidase
NF-κβ	Nuclear Factor kappa β
NLR	NOD-like receptor
NLRP3	Nucleotide-binding oligomerization domain-like receptor domain 3
NM	Neuromelanin
NMDA	N-methyl- D-aspartate
NO	Nitric oxide
NO2	Nitrite
NOS	Nitric oxide synthase
NOX	NADPH oxidases
NSAID	Non-steroidal anti-inflammatory drugs
O2-	Superoxide ion
OCT 1	Organic cation transporters 1
OH−	Hydroxyl radical
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PD	Parkinsons disease
PET	Positron emission tomography
PG	Prostaglandin
PGC-1α:	Peroxisome proliferator-activated receptorγ Coactivator 1 α
PI3K	Phosphatidylinositol 3-kinase
PK	Protein kinase
PKC	Protein kinase C

PLA2	Phospholipase A2
PLC	Phospholipase C
PPN	Pedunculopontine tegmental nucleus
PPT	Protein phosphatases
PV	Parvoalbumin
REM	Rapid eye movement
ROS	Reactive oxygen species
RRF	Retrorubural field
rSNc	Rostral substantia nigra pars compacta
S.C	Subcutaneous
sCD14	Soluble CD14
SM	Sphingomyelitis
SMOC	Supramolecular Organizing Center
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNl	Substantia nigra lateral part
SNr	Substantia nigra pars reticulate
SPECT	Single photo neuropathology studies tomography
STN-HFS	Subthalamic nucleus high-frequency stimulation
STN	Subthalamic nuclei
TAK1	Transforming growth factor-β-activated kinase 1
TBS	Tris-buffered saline
TF	Tissue factor
TGB	Transforming growth factor
TH	Tyrosine hydroxylase
TIR	Toll / IL-1 cytoplasmic receptor
TIRAP	TIR domain-containing adaptor protein

TK	Tyrosine kinases
TLR4	Toll like receptor
TNF-α	Tumor necrosis factor-alpha
TNFR 1	TNF receptors 1
TRAF6	TNF receptor-associated factor 6
UPS	Ubiquitin-proteosome system
VMAT	Vesicular monoamine transporter
VTA	Ventral tegmental área
α-sin	Alpha synuclein

ABSTRACT

Metformin is a widely used oral antidiabetic drug with known anti-inflammatory properties due to its action on AMP kinase. This drug has shown a protective effect on various brain tissues, including cortical neurons. However, the effect of metformin on the substantia nigra (SN), the main structure affected in Parkinson's disease (PD), has not yet been studied. Accumulating evidence suggest that inflammation may play a central role in the cell loss that occurs in PD. The aim of this work was to determine the effect of metformin on the microglial activation of the SN of rats using the animal model of PD based on the injection of the pro-inflammogen lipopolysaccharide (LPS). *In vivo* and *in vitro* experiments were used to study the activation of microglia at both the cellular and molecular levels. Our results indicate that metformin overall inhibits microglia activation measured by OX-6 (MHCII marker), IKKβ (pro-inflammatory marker) and arginase (anti-inflammatory marker) immunoreactivity. In addition, qPCR experiments reveal that metformin treatment minimizes the expression levels of several pro- and anti-inflammatory cytokines. Furthermore, the drug decreases the phosphorylated forms of MAPKs as well as ROS generation through the inhibition of the NADPH oxidase enzyme. However, metformin treatment fails to protect the dopaminergic neurons of SN. These findings suggest that metformin could have both beneficial and harmful pharmacological effects and arise the question about the potential use of metformin for the prevention and treatment of PD.

RESUMEN

La metformina es un fármaco antidiabético oral ampliamente utilizado con propiedades antiinflamatorias conocidas debido a su acción sobre la AMP cinasa. Esta droga ha mostrado un efecto protector en varios tejidos del cerebro, incluidas las neuronas corticales. Sin embargo, el efecto de la metformina sobre la sustancia negra (SN), la principal estructura afectada en la enfermedad de Parkinson (EP), aún no se ha estudiado. Estudios recientes sugieren que la inflamación puede desempeñar un papel central en la pérdida celular que ocurre en la EP. El objetivo de este trabajo fue determinar el efecto de la metformina sobre la activación microglial de la SN de ratas utilizando el modelo animal de EP basado en la inyección del pro-inflamamógeno lipopolisacárido (LPS). Se usaron experimentos *in vivo* e *in vitro* para estudiar la activación de la microglía tanto a nivel celular como molecular. Nuestros resultados indican que la metformina inhibe de manera general la activación de la microglía medida a través de inmunocitoquímica contra OX-6 (un marcador del complejo mayor de histocompatibilidad tipo II), IKKβ (un marcador proinflamatorio) y la arginase (un marcador antiinflamatorio). Además, los experimentos de qPCR revelan que el tratamiento con metformina minimiza los niveles de expresión de varias citocinas pro y antiinflamatorias. Además, este fármaco disminuye las formas fosforiladas de MAPKs así como la generación de ROS a través de la inhibición de la enzima NADPH oxidasa. Sin embargo, el tratamiento con metformina no protege las neuronas dopaminérgicas de la SN. Estos hallazgos sugieren que la metformina podría tener efectos farmacológicos beneficiosos y perjudiciales y plantea la pregunta sobre el posible uso de la metformina para la prevención y el tratamiento de la EP.

I. INTRODUCTION

1. BASAL GANGLIA

1.1. History of basal ganglia discovery

In 1664, the English anatomist Thomas Willis was the first to provide a clear identification of the different subcortical structures. What is now known functionally as basal ganglia was then called the corpus striatum. It occupied a central position, connected with a wide variety of cortical fibers and to the brainstem, which at that time was believed to be the "sensorium commune" defined by Aristotle. It was thought that this structure received all the sensorial modalities and initiated all the motor acts. This idea appeared to be anatomically reinforced by its ventral position and by clearly visible ascending and descending fiber systems (Parent and Hazrati 1995).

One of the first descriptions of the basal ganglia by Willis in the 17th century intimated a role of the basal ganglia in the control of movement and in neurological disorders: *''To the corpus callosum are attached the corpora striata connecting the cerebrum to the legs of the medulla oblongata. In these corpora there are some striae passing upwards and others downwards and through them the spirits and images of sensible things pass from the medulla oblongata into the cerebrum, while spirits initiating movement descend into the medulla oblongata. In those who suffer or have died from paralysis, I have often observed that these corpora are affected: they became flaccid and their striae are almost obliterated''* (Smith, et al. 1998)

Two facts contributed to relegate the corpus striatum to a dark and less defined position: the attractive histological organization of the cortex, and the possibility of locating higher mental functions in the cortex, which led many neurologists of the 18th and 20th centuries to study the issue. Those who continued to study the corpus striatum suddenly discovered that the neighboring corticospinal pathways, in fact owned many of the functions originally assigned to this structure. As Wilson described in 1914, *"the corpus striatum has been depreciated in its physiological significance."* At the beginning of the 20th century there were serious attempts to provide a comparative and detailed description of the corpus striatum (Ramón y Cajal, 1911, Wilson, 1914).

It began to gain importance again with the discovery that lesions of these areas in humans often resulted in disorders of motor functions. The corpus striatum became the main component of the extrapyramidal motor system. This term, in a somewhat indefinite form, groups the corpus striatum with a series of nuclei of the brainstem, and reflects the presumption that this grouping constituted a complete and independent motor unit. Nowadays, the basal ganglia are defined as a group of subcortical telencephalic nuclei spanning the telencephalon, diencephalon, and midbrain at the base of the forebrain that are highly connected with the cortex.

1.2. Anatomy of the basal ganglia

The four principal nuclei of the basal ganglia are (Fig. 1):

(1) The striatum. The striatum consists of three important subdivisions: the caudate nucleus, the putamen, and the ventral striatum. Except at its most anterior pole, the striatum, where it is known as the neostriatum, is divided into the caudate nucleus and putamen by the internal capsule, a major collection of fibers found between the neocortex and the thalamus in both directions (Gerfen 1992).

(2) The globus pallidus (GP). The GP is divided into two segments, the external (GPe) and the internal (GPi); in the rat the latter is called the entopeduncular nucleus.

(3) The subthalamic nucleus (STN). The STN of Luys lies inferiorly to the thalamus at the junction of the diencephalon and the mesencephalon (midbrain).

(4) The substantia nigra (SN). Although the SN is presenting a more caudal situation, it is included within the structures of the basal ganglia because of its intimate anatomical and functional relationship. It is a mesencephalic nucleus, which appears as a flattened oval structure on the dorsal aspect of the base of the cerebral peduncle. SN lies dorsal to the crus cerebri, a nervous tract that lies between the brain and the medulla oblongata, and extends to the midbrain; it plays a central role in movement control, and acts as the primary output and entrance center of the basal ganglia (Fink

and Smith 1980; Parent, et al. 1983). SN has been divided cytoarchitectonically into three different parts:

1) SN pars compacta (SNc), formed by a horizontal layer packed with long and medium cells. This part of SN uses dopamine (DA) as neurotransmitter. SN derives its name from the presence of the black pigment neuromelanin, contained in the neurons of the SNc.

2) SN pars reticulate (SNr), a more diffuse and poor cell division containing large neurons that project outside of the basal ganglia (Gulley and Wood 1971; Mink 1996).

3) SN lateralis (SNl), formed by a small group of medial cells extending face-caudally along the lateral border of SNc and SNr (Danner and Pfister 1982; Fallon and Seroogy 1985; McRitchie, et al. 1998; McRitchie, et al. 1995; Yelnik, et al. 1987).

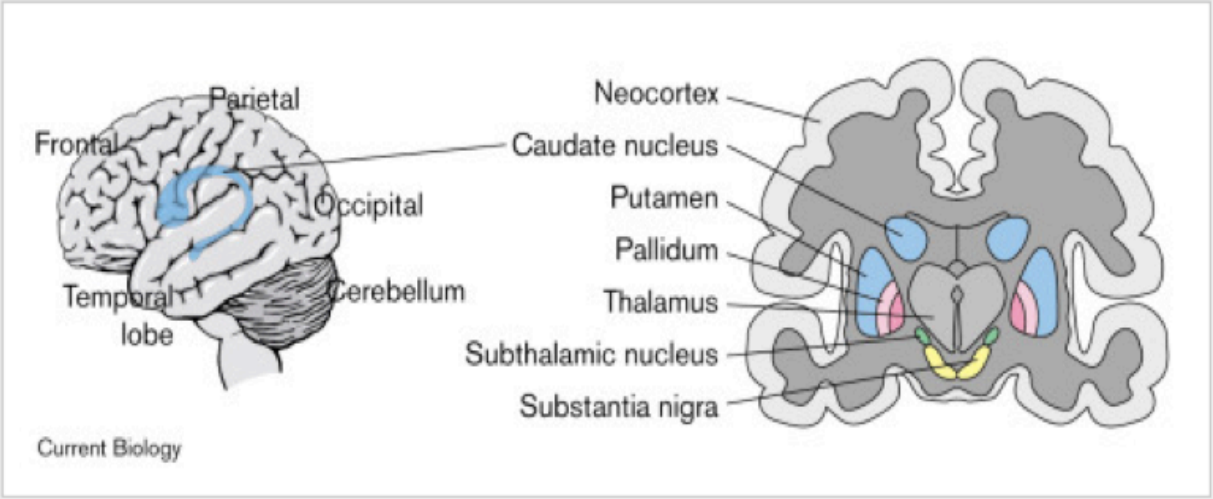

Figure 1. Basal ganglia main regions within the brain. The blue part is the striatum, which consists of the caudate nucleus and the putamen. The pink part is the globus pallidus. The green part is the subthalamic nucleus and the yellow part is the substantia nigra (Graybiel 2000)**.**

1.3. Basal ganglia neurotransmisors and neuromodulators

The main neurotransmitter of the basal ganglia is GABA, which has inhibitory action. In the striatum, GABA is found in the projection neurons, the middle type spiny neurons. These neurons, which have abundant dendritic spines, project their axons to the two GP segments (GPe and GPi) and SNr. In addition to GABA, the middle spiny neurons contain enkephalins or substance P and dynorphins. The projection neurons of the internal and external segments of GP and SNr also contain GABA. Most of the afferents to the SN are GABAergic and at least 70% of the synapses formed on nigral DAergic neurons are GABAergic (Grofova and Rinvik 1970; Gulley and Smithberg 1971). As a result, the basal ganglia exit nucleus is inhibitory. In addition, there are many neuromodulatory substances with slow synaptic actions, such as DA, acetylcholine (ACh), along with Glu (when acting on NMDA receptors) (Mugnaini 1985).

Glutamate, considered an excitatory neurotransmitter, is found in the pyramidal neurons, being the main afferent to the striatum (the main input nucleus to the basal ganglia), in the terminals of the thalamic neurons in the striatum and in the projection neurons in the STN (Rajakumar, et al. 1994).

ACh is also a normal neurotransmitter in the basal ganglia and is present in the striatal interneurons, where it plays an important role in the neural circuits function (Haber 1986). Its clinical effects are generally antagonistic to those of DA in various movement disorders, especially in Parkinson´s disease (PD) (Alexander and Crutcher 1990).

DA is the main neurotransmitter in SN, the structure focus of this work. The number of DAergic neurons in the SN ranges from about 10.000 (bilaterally) in adult mice to about 400.000 in adult humans (Brichta and Greengard 2014; Damier, et al. 1999), and they are predominantly found within the SNc.

Within the midbrain DA-releasing catecholaminergic neurons are part of the basal ganglia network, and are crucial for a variety of fundamental brain functions, such as voluntary movement, goal-

directed behavior and habit formation, motivation, emotion, cognition, reward, memory, associative learning, and decision making (Bjorklund and Dunnett 2007; D'Ardenne, et al. 2012; Gerfen and Surmeier 2011; Pignatelli and Bonci 2015).

Dahlström and Fuxe published the first detailed account of the distribution of catecholamines (DA and noradrenaline) and serotonin-containing neurons in the rat brain. Twelve groups of catecholaminergic cells (designated A1–A12) distributed from the medulla oblongata to the hypothalamus were identified (Dahlström and Fuxe 1964). The study provided an evidence for the existence of monoamine-containing neurons in the central nervous system (CNS) (Dahlstroem and Fuxe 1964). Since then this nomenclature has been retained and has proved advantageous because the catecholaminergic cell groups are in most cases not confined to single, defined anatomical structures. In addition, the distribution of cell bodies within each cell group varies markedly between different mammalian species (Fig. 2) (Bjorklund and Dunnett 2007).

There are two major DAergic neuron subtypes in the mesencephalon: the nigral A9 neurons and the A10 neurons of the VTA projecting to limbic and cortical areas along mesolimbic and mesocortical pathways. The DA of the SN DAergic neurons is contained in the axons and processes, classically identified as dendrites.

The SN DA-containing cell bodies are oval or multipolar, medium-sized that are found rather densely aggregated throughout the SNc and scattered in the SNr (Bjorklund and Lindvall 1975). DA-storing nerve terminals are found in the neostriatum (the caudate nucleus and the putamen), while the DA-containing nerve cells are in the SN (Andén, et al. 1964).

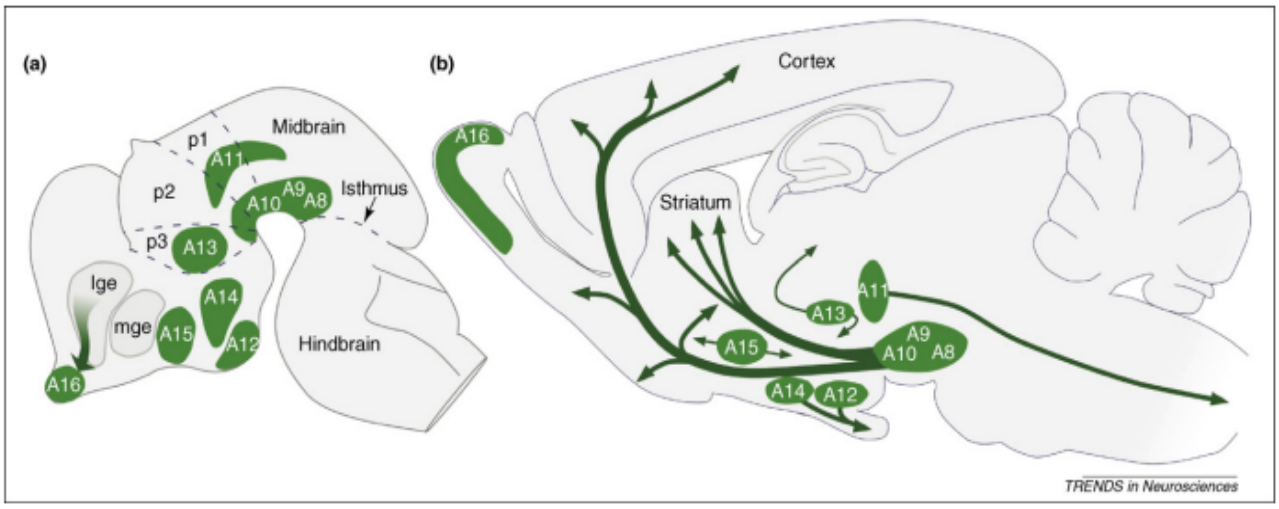

Figure 2. Schematic representation of DAergic neuron cell groups distribution in the developing (a) and adult (b) rodent brain. The DAergic neurons in the mammalian brain are localized in nine distinctive cell groups, distributed from the mesencephalon to the olfactory bulb, as illustrated in a sagittal view, in (a) the developing and (b) the adult rat brain. The numbering of the cell groups, from A8 to A16, was introduced in the classic study of Dahlstrom and Fuxe in 1964 (Dahlström and Fuxe 1964). (b) The DAergic cell groups principal projections are illustrated by arrows. (Bjorklund and Dunnett 2007).

1.4. Main connections of basal ganglia

The basal ganglia may be viewed as two primary input structures, two primary output structures, and two intrinsic nuclei (Fig. 3). The input structures of the basal ganglia are the striatum (caudate and putamen) and the STN. The striatum receives excitatory inputs from virtually all areas of cerebral cortex apart from the primary visual, auditory, and olfactory cortices (Fig. 4). The caudate and putamen are reciprocally interconnected to the SN through the nigrostriatal tract (Grahn, et al. 2008).

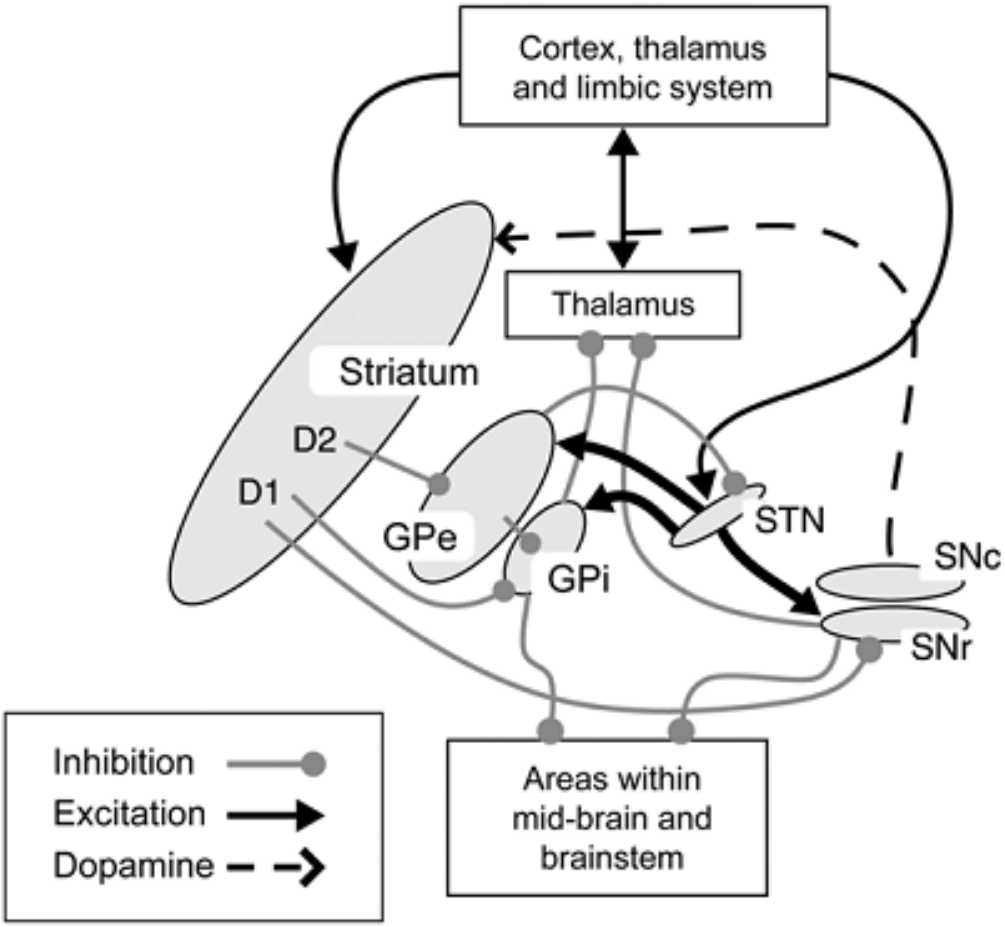

Figure 3. Different types of connections between the basal ganglia. D1, D2, striatal neurons preferentially expressing dopamine receptors subtypes D1 and D2. (Chersi, et al. 2013).

There are different connections that travels from the cortex to the basal ganglia, and back to the cortex after processing the information. The most important of these connections regarding the subject of this work is the nigrostriatal pathway consisted of DAergic neurons of SN that project to the striatum. This nigrostriatal DAergic pathway, in a restricted sense, is derived from neurons located in both the dorsal and ventral tiers of the SNc, and is the predominant source of DA innervation of the sensorimotor striatum (Bjorklund and Dunnett 2007).

This nigrostriated projection is mainly ipsilateral, where only 5% of its fibers cross-contralateral neoestriate (Parent, et al. 1983). These DAergic projections establish connections with various neuronal systems, including cholinergic interneurons (McGeer and McGeer 1980). Thus, from 30 to 50% of the caudate nucleus cells project to the SN (Bunney and Aghajanian 1976). In addition, there are also GP neurons that project to the SN (Grofová 1975).

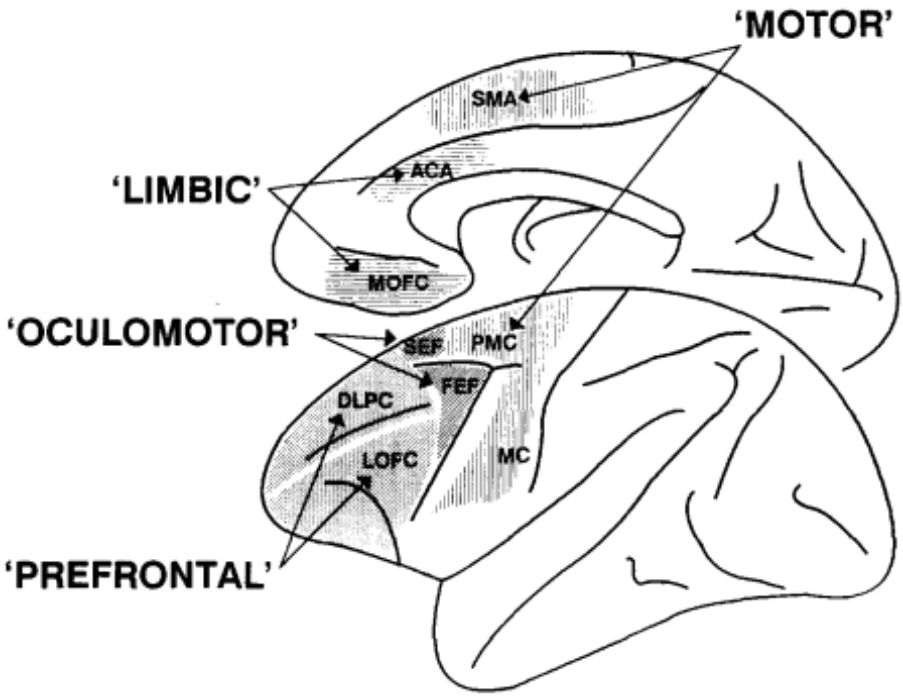

Figure 4. Schematic representation of the different cortical areas which receive output from separate basal ganglia thalamocortical circuits. The "motor" circuit is focused on the precentral motor fields, the "oculomotor" circuit on the frontal and supplementary eye fields, the two "prefrontal" circuits on the dorsolateral prefrontal and lateral orbitofrontal cortex, respectively, and the "limbic" circuit on the anterior cingulate and medial orbitofrontal cortex. Taken from (Alexander and Crutcher 1990).

The circuit that begins in the cortex, innervates the striatum and reaches the SN, is closed by the GABAergic (and therefore inhibitory) neurons of the SNr and the internodal segment of the GP which constitute the greatest aference of the basal ganglia, innervating the mediodorsal and ventromedial thalamic nuclei (which projects to the frontal cortex) and intralaminar (which projects to the striatum), the superior colliculus and the peduncle-pontine nucleus. These neurons are tonically active and will therefore produce the inhibition of the thalamic cells that return the projection to the areas of the cortex involved in motor control. The movement, at the level of the basal ganglia, originates when the thalamic neurons are released from this tonic inhibition with the consequent motor activity of the cortex. This inhibition of thalamic neurons can occur through two pathways that connect the striatum and the SN, the nigrostriatal (direct) and the palidostriatal (indirect).

The striatal projection neurons belonging to the direct pathway are connected with the inner pale neurons, which in turn, project to the lateral ventral and anterior ventral nuclei of the thalamus (Fig. 5). This circuit contains two inhibitory neurons, in the striatum and the GP. Thus, the cortical excitation of the striatum is first transformed into an inhibitory message that reaches the GPi. However, the output of the GPi is also inhibitory. Consequently, the inhibition of the striatum reduces the inhibition of the thalamus from the GPi. The inhibition of an inhibitory signal is called disinhibition; functionally this "double negative" is equivalent to excitation.

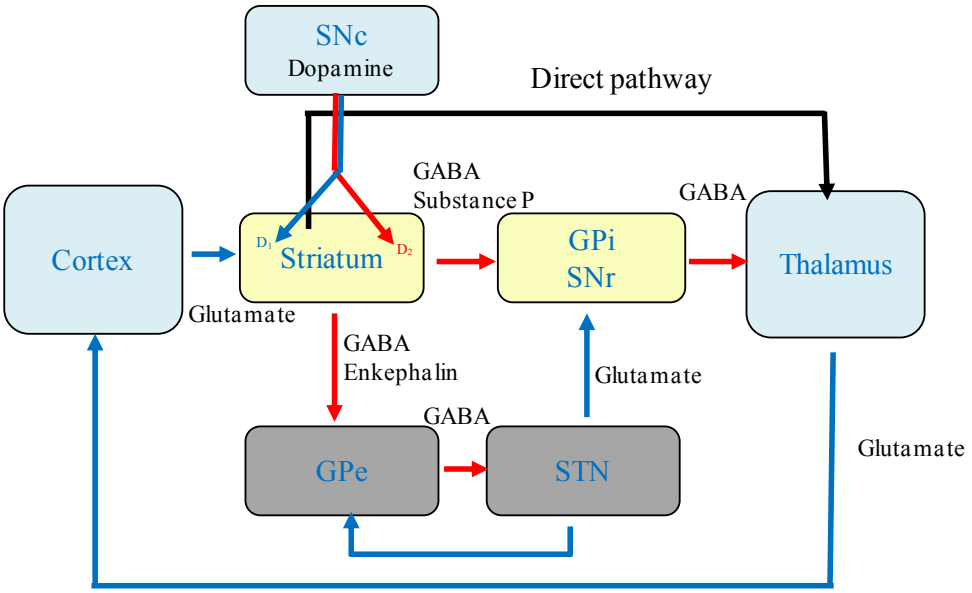

Figure 5. Schematic diagram of the direct connection of the basal ganglia. The blue arrows indicate exciter type connection, and the red arrows indicate inhibitor type connection. GABA, γ-aminobutyric acid; GPi, internal segment of the globus palidus; GPe, outer segment of the globus palidus; SNr, substantia nigra pars reticulata; SNc, substantia nigra pars compacta; NST, subthalamic nuclei.

The indirect pathway has an opposite effect on the thalamus and cortex than the direct pathway (Fig. 6). The key to understanding the indirect pathway is that the STN is excitatory. The striatal neurons of the indirect pathway, which are inhibitory, project to the GPe, whose output is equally inhibitory. Since the GPe projects to the STN, the putamen disinhibits the STN. This disinhibition excites the output of the STN, the GPi and the SNr, thus increasing the strength of the inhibitory signal of output that goes to the thalamus.

At present, this model is allowing us to understand the mechanisms of some hypokinetic and hyperkinetic signs observed in the pathology of the basal ganglia. Thus, in PD, there is a deficiency of DA, which produces hypokinetic signs. It is thought that this neurotransmitter excites the neurons of the direct pathway and inhibits the striated neurons of the indirect pathway.

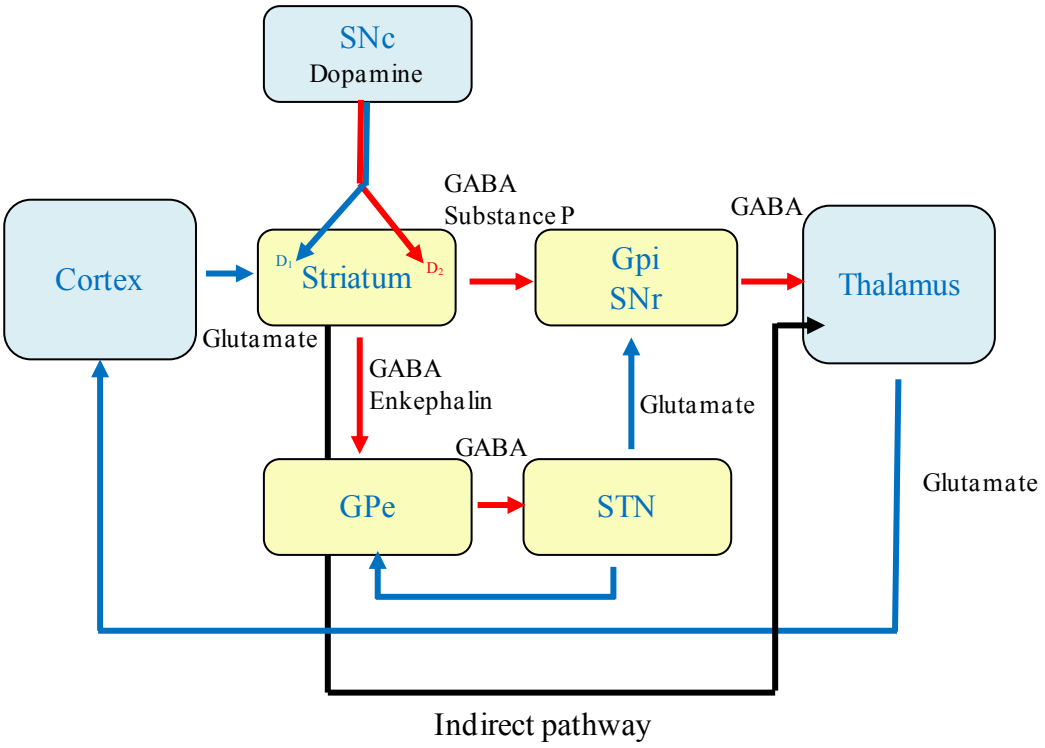

Figure 6. Schematic diagram of the indirect connection of the basal ganglia. The blue arrows indicate exciter type connection, and the red arrows indicate inhibitor type connection. GABA, γ-aminobutyric acid; GPi, internal segment of the globus palidus; GPe, outer segment of the globus palidus; SNr, substantia nigra pars reticulata; SNc, substantia nigra pars compacta; NST, subthalamic nuclei.

1.5. Functions of basal ganglia

Basal ganglia are the largest subcortical group of structures in the human forebrain, which gives a clue to their motor and cognitive functions. The basal ganglia receive inputs from the neocortex, and sends its output to the thalamic nuclei, which project to the frontal cortex. As a result of this anatomy, the basal ganglia is in a prime position to influence the executive functions of the forebrain, such as movement planning and cognitive behavior (Graybiel 2000). Thus the basal ganglia appear to be capable of concurrent participation in a number of separate functions (including skeletomotor, oculomotor, cognitive and “limbic” processes), due to the parallel structure of the individual basal ganglia thalamocortical circuitry

It is generally assumed that the basal ganglia express their influence on different neuronal systems of the brain by disinhibiting their target cells in the thalamus and the brainstem. Therefore, one of the general effects of diffuse excitation of the basal ganglia is to inhibit muscle tone throughout the body. This effect is the result of inhibitory signals transmitted from the basal ganglia to both the motor cortex and the lower part of the brainstem. Muscle tone can be regulated by a counterbalance between the inhibitory and the facilitatory systems (Takakusaki, et al. 2003). Under resting conditions, the motor brainstem centers are tonically inhibited by the basal ganglia outputs, which in turn will be disinhibited when called into action (DeLong 1990; Grillner and Robertson 2015). Consequently, when extensive destruction of the basal ganglia occurs, muscle rigidity is caused throughout the body. However, stimulation of certain specific areas within these ganglia can trigger muscle contractions and sometimes complex movement patterns (Parent and Hazrati 1995).

The basal ganglia also contribute to the planning and execution of voluntary movements via a series of parallel basal ganglia thalamocortical loops (DeLong 1990; Middleton and Strick 2000).

In addition, there is a clear link between the caudate and frontal lobe regions known to be responsible for “executive” functions, which generally require the generation and monitoring of appropriate strategies, and evaluation of potential outcomes for successful performance.

The GP plays an important role in movement regulation; this is done through its inhibitory action that balances the excitatory action of the cerebellum. The harmony between these two systems allows smooth and controlled movements. Injuries in the GP seriously interfere the movements associated with precise activities, so it would be difficult or impossible to do defined activities. The electrical stimulation of the GP while an abrupt body movement is taking place will often stop the movement in a static position, holding that position for a few seconds while the stimulation continues. Therefore, the GP is involved in some type of feedback control motor system that is able to block the different parts of the body in specific positions (Parent, et al. 1983).

Finally, SNc target the striatum and are involved in control of movement. Although being one of the major DA-producing parts of the brain, SN functions extend beyond motor control. Neurons of the SNr project to different parts of the brain stem and the thalamus, thus controlling different functions. SNr neurons project to the mesencephalic locomotor command region (MLR) for postural control, to the optic tectum for eye and orienting movements, to the periaqueductal grey for vasodialation and to the thalamus for hand and finger movement (Grillner and Robertson 2015).

2. CLINICAL SYNDROMES RESULTING FROM DAMAGE TO BASAL GANGLIA

Taking into account the afore mentioned connections and functions of the basal ganglia is not a surprise that damage in this brain region produces alterations in several motor, cognitive and behavioral functions (Middleton and Strick 2000). Clinical syndromes resulting from damage to basal ganglia include Parkinson´s disease (PD), Huntington's disease, athetosis and hemibalism. In the coming section we will talk about PD, the most prevalent disease related with the basal ganglia.

2.1. Parkinson's disease

PD is a terminal progressive and chronic neurodegenerative disease that affects the SNc. PD affects people of all races. Around ten million people worldwide have the disease (EPDA 2018), making it the most common neurodegenerative disease after Alzheimer´s disease. Historical references of motor symptoms similar to those of PD have been found in traditional Indian and Chinese texts from approximately 1000 BC (Manyam 1990; Zhang, et al. 2006). Later on, James Parkinson was the first to describe PD as a neurological syndrome in 1817. Parkinson described the clinical symptoms in pithy English:

"Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured".

In 1872 Jean-Martin Charcot distinguished bradykinesia as a feature of PD. He was the first to suggest the term "Parkinson's disease" rejecting the earlier nomenclature of paralysis or shaking palsy, because he noticed that PD patients are not weak and do not necessarily have tremor (Charcot 1872).

The mean age is around 60 years (de Rijk, et al. 1997). Most cases of PD are sporadic or idiopathic, whereas in rare cases it is inherited, known as familial PD. The underlying cause of sporadic PD remains to be explored. Age is considered the most important documented risk factor, in addition to other risk factors such as gender, pesticide exposure, and brain injury (Kieburtz and Wunderle 2013), while smoking, caffeine, and non-steroidal anti-inflammatory drugs (NSAID) were found to lower PD's risk. Slightly men are more affected than women.

Prior to the onset of the motor phase symptoms there is a prodromal phase of several years duration (Hawkes, et al. 2010). These prodromal symptoms are mainly non-motor, manifested as rapid eye movement (REM) disorders, hyposmia, constipation, apathy pain, and depression (Chaudhuri and Schapira 2009; Leentjens, et al. 2003). The clinical onset of PD starts once the motor symptoms appeared (Fig. 7).

From the clinical point of view, PD is characterized by:

1) Muscle rigidity in large areas of the body or in isolated areas;

2) Resting tremor of the involved areas, in most but not all cases;

3) Serious inability to initiate movement, called akinesia.

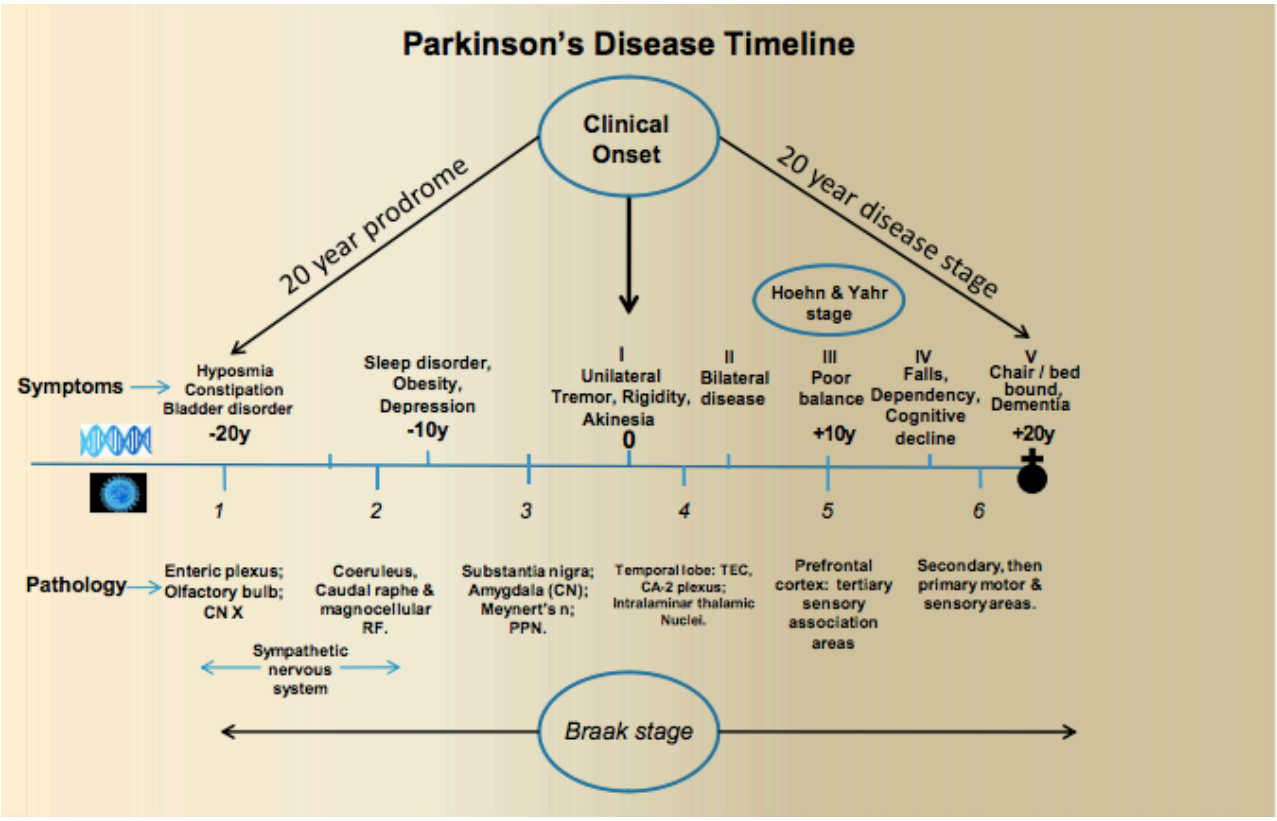

Figure 7. Proposed timeline from onset to death in classical PD. A 40-year course is assumed although it is inevitable that there will be considerable individual variability. The various steps are presented in linear fashion, but the disease is likely to show an accelerated course in the latter years. Braak pathological staging is indicated in Arabic numerals below the horizontal line. Above this line are the proposed symptoms in the prodromal phase and to the right of this, the major features according to Hoehn & Yahr staging (Roman numerals). The two symbols at the extreme left of the timeline represent two possible causative agents: genetic mutations or viral infections. It is uncertain whether the sympathetic nervous system is involved in Braak stage 1 or 2; hence arrows straddling both of these phases indicate the pathology. Abbreviations: CA2 ¼ second section of the Ammon's horn; CN X ¼ motor component of cranial nerve X; RF ¼ reticular formation; CN ¼ central subnucleus of the amygdala; Meynert's n ¼ basal nucleus of Meynert; PPN ¼ pedunculopontine tegmental nucleus; TEC ¼ transentorhinal cortex. (Hawkes, et al. 2010).

As the disease progresses dyskinesia, ataxia, pain and sensory complaints such as orthostatic intolerance and urinary incontinence, and neuropsychiatric manifestations (hallucinations, depression, and dementia) become outstanding. This happens due to the spread of the disease pathology to other parts of the brain (Chaudhuri and Schapira 2009).

The diagnosis of PD is generally made based on the clinical data exposed. However, new and modern imaging techniques, such as neurochemical positron emission tomography (PET) and single photo neuropathology studies tomography (SPECT) imaging studies, may play an important role in the differentiation of the various parkinsonian syndromes or in the preclinical diagnosis of this disease. This technique has complemented neuropathology studies by allowing *in vivo* assessment of the regional distribution and quantitative measurement of cholinergic terminal markers or receptors in the brain of PD patients or related syndromes. These imaging technologies give the opportunity to study cholinergic innervation *in vivo* at early stages of PD and other neurodegenerative disorders (Bohnen and Albin 2011; Forno 1982).

The medical treatment of PD is usually effective and must be continued for the rest of the patient's life. The degree of improvement achieved with the range of drugs used is variable in each case, indicating that these treatments are not curative at present, but palliative. In general, it is based on the following measures:

a) Anticholinergics

b) Amantadine

c) L-DOPA (or levodopa) and other DAergic drugs,

d) Other general measures.

However, although for most patients these measures maintain a good quality of life, when the disease progresses or when these drugs do not improve mobility or produce significant adverse

effects, surgical treatment based mainly on pallidotomy and thalamotomy, and high-frequency deep brain stimulation (DBS) of the subthalamic nucleus (STN-HFS) would be the preferred treatment for advanced PD (Benabid, et al. 2009).

2.2. Pathology of Parkinson's disease

In PD there is a severe pathology of the basal ganglia, which starts as an extranigral involvement in the disease. It starts in the lower brainstem (medulla) and the anterior olfactory nucleus regions. These regions include the periamigdaloid nucleus, the locus ceruleus, the dorsal motor nucleus of the vagal nerve, the innominate substance, the raphe nuclei, and the hypothalamus, which accounts for the non-motor manifestations of the disease. However, other authors (Braak, et al. 1996) have suggested that neurodegeneration begins in the entorhinal cortex, so that the cell death cascade begins in this area and continues with the degeneration of the SN. Therefore, the most important neuropathologic hallmark of PD is the loss of DAergic neurons of SNc. Actually, this loss of DA in the basal ganglia appears to produce the most debilitating neurological signs. Therefore, the fundamental neuropathological characteristic in PD is the bleaching of the SN band, usually gray or black by the presence of neuromelanin, due to the loss of myelinated neurons (Fearnley and Lees 1991; Jellinger 1991; Jellinger 2001). It requires the death of at least 50-60% of the SN DAergic neurons for the manifestation of clinical symptoms, which demonstrates the high plasticity of the nigrostriatal DAergic system.

Since the disease shows a special predilection for the ventrolateral zone of the SN, it is in this type of cells where the lesion begins, to progress later to the dorsal area of this structure. Cell loss in the SN causes depletion of striatal DA, which results in hyperactivity of striatal projection neurons belonging to the indirect pathway because the corticostriatal glutamatergic excitatory input is no longer inhibited. This might be one cause of hypokinesia.

At final stages the process enters the neocortex, at which all the clinical minifestations of the disease appears (Braak, et al. 2004).

Another evidence of PD is the presence of Lewy bodies in the CNS of individuals who are not yet diagnosed with PD. Although Fredrich Lewy was the first to describe them more than a century ago, their importance in the pathogenesis of PD is still unclear (Bohnen and Albin 2011; Goedert, et al. 2013). These intra cytoplasmic inclusions mainly contain filamentous forms with a dense center consisting of radially oriented filaments. It appears that their formation is the result of an abnormal accumulation of cytoskeletal proteins within the neuron, as well as α-synuclein (α-syn) and ubiquitinated proteins (Lees, et al. 2009). There is a debate about whether Lewy bodies directly cause neuronal death or perhaps isolate small aggregates of cellular toxins to preserve neuronal viability (Goldberg and Lansbury 2000). Some theories indicate that inclusions found in brain tissues with neurodegenerative diseases, including PD, are a mechanism to compartmentalize accumulated defective proteins and prevent them from obstructing the proper functioning of the cell (Goldberg and Lansbury 2000).

Another important feature that characterize PD is the presence of neuroinflammation

2.3. Neuroinflammation on Parkinson's disease

Neuroinflammation is a complex process involving cells of the immune system and the CNS, which have the purpose of repairing the damage produced. This inflammation could be both beneficial and detrimental to the brain. On the one hand it is part of a physiological repair process; however, when this process is not controlled, the inflammatory process is extended, thus the inflammation loses its repairing function and can be the cause of the damage. The main players in the inflammatory processes of the brain are the microglia and astrocytes. Microglia are the innate immune cells resident in the CNS. When they are at resting state they have a ramified morphology, but when they become activated upon stimulation, they transform into an amoeboid morphology (Fig. 8).

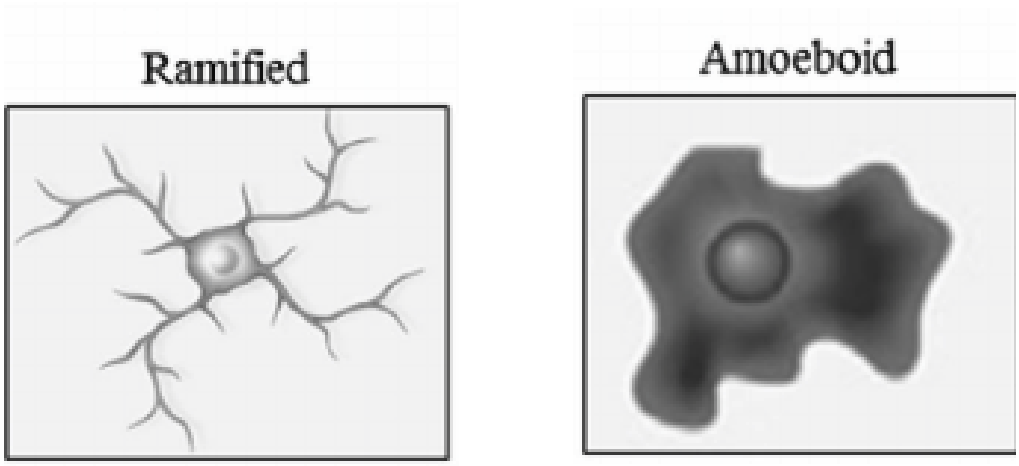

Figure 8. Morphological differences between resting and activated microglia

Besides, macrophages/microglia have diverse functions that range from fighting bacterial infection to tissue regeneration and wound healing. The diverse functions of microglial cells in the CNS are mirrored by equally diverse phenotypes. A classical model of pro-inflammatory/M1 *versus* an anti-inflammatory/M2 microglia has been extensively used. However, the complex and different functions of microglial cells can only be explained by the existence of varied and plastic microglial phenotypes mediated by distinct gene expression programs and a network of molecular pathways that relay environmental signals via signaling cascades (Guerau-de-Arellano y col., 2017). Therefore, M1 and M2 are just the extremes of a broad spectrum of phenotypes that cover the different functions of microglia. These different phenotypes can be achieved by stimulating microglial cells with different compounds. Hence, when stimulated with LPS and interferon (INF)-γ, macrophages/microglia has long been known as classically activated or M1 (Martínez et al., 2014), while when activated with IL-4 macrophages/microglia show an alternative activated phenotype or M2. In order to standardize the nomenclature and facilitate the communication of macrophage/microglia data, a novel nomenclature has been proposed in which the letter M is

followed by a parenthesis that includes the stimuli used for activation (Murray et al., 2014). The knowledge of the molecular programs that control the inflammatory phenotypes *versus* resolution provides a unique opportunity to find new targets that allow modulating these phenotypes and, therefore, controlling the excessively inflammatory responses that accompany neurodegenerative diseases. The knowledge of these molecular mechanisms is greatly advancing in recent years.

Functionally, M1 microglia is responsible for fighting infections, for which it adopts a clear pro-inflammatory phenotype with microbicidal, antigen-presenting and immune-enhancing functions. This type of microglia is characterized by the production of NO by the inducible nitric oxide synthase (iNOS, encoded by the *Nos2* gene; MacMicking et al., 1997; Arnold et al., 2014) and by the expression of inflammatory chemokines and cytokines, such as interleukin (IL)-6, IL-12, IL-1β, IL-23 and tumor necrosis factor (TNF)-α that attract new cells of the immune system to the site of infection (Mosser and Edwards, 2008; Murray et la., 2014). In the context of neurodegenerative disease this phenotype produces harmful effects in the neuronal population.

When neutrophils undergo apoptosis and microglia switch to a resolution/M2 phenotype, the initial acute inflammation evolves to a resolution phase (Serhan, 2014). This resolution/would healing phase is mediated by lipid mediators, such as classical eicosanoids, phospholipids and sphingolipids, endocannabinoids and specialized proresolving mediators (Chiurchiù and Maccarrone, 2016), that promote the switch to M2 (Bosurgi, 2017). Resolution/M2 microglia suppresses IL-12 secretion and induces the release of IL-10, transforming growth factor (TGB)-β, IL-1R antagonist and decoy IL-R II (Brancato y Albina, 2011). Besides, these microglial cells induce the expression of arginase-1 instead of iNOS, switching arginine metabolism from production of NO to ornithine, and also increase polyamines production for extracellular matrix and collagen synthesis (Gordon y Martínez, 2010). This phenotype promotes the neuroregeneration and tissue repair.

Uncontrolled M1 activated microglia can produce different substances that are considered neurotoxic, such as pro-inflammatory cytokines, TNF-α, NO, superoxide, and prostaglandin E2, and can produce neuronal death. In turn this neuronal damage cause further microglia activation, known as reactive microgliosis (presence of microglia in nervous tissue secondary to injury), leading to non-stop neuronal damage and death (Block and Hong 2005; Streit, et al. 1999). Thus, inflammatory response in the CNS is thought to favor damage more than reparative function and could explain most CNS pathologies. Therefore, in recent years, numerous therapeutic attempts have been aimed at alleviating it. However, at the moment, it does not seem that any drug has been useful to stop or control neuroinflammation.

In the case of DAergic neurons, inflammatory processes (proliferation and activation of microglia) could be a secondary event produced by degenerative processes that occur directly in DAergic neurons, but at the same time it could help the progression of degeneration. In addition, there is the possibility that inflammatory processes by themselves could be harmful to DAergic neurons in the nigrostriatal system (fig. 9).

There are several physiological/pathological inducers of neuroinflammation that could be involved in the degeneration of the DAergic system: neuronal injury (stroke or brain trauma) (Lees 1997; Tansey, et al. 2007), immunological challenges (viral or bacterial infections), other inflammatory syndromes (diabetes mellitus type 2, arthrosclerosis, rheumatoid arthritis, and multiple sclerosis), pro-inflammatory compounds (IgG, thrombin, LPS), and environmental toxins (like pesticides). Thus, different toxins, such as 6-OHDA, MPTP/MPP $^{+}$ or rotenone, induce an inflammatory process in addition to its specific toxic mechanisms. Many of these factors can increase the permeability of the blood brain barrier (BBB), thus allowing infiltrations of macrophages, lymphocytes and other toxins in to the brain (Tansey and Goldberg 2010), which could be involved in degeneration of the DAergic system.

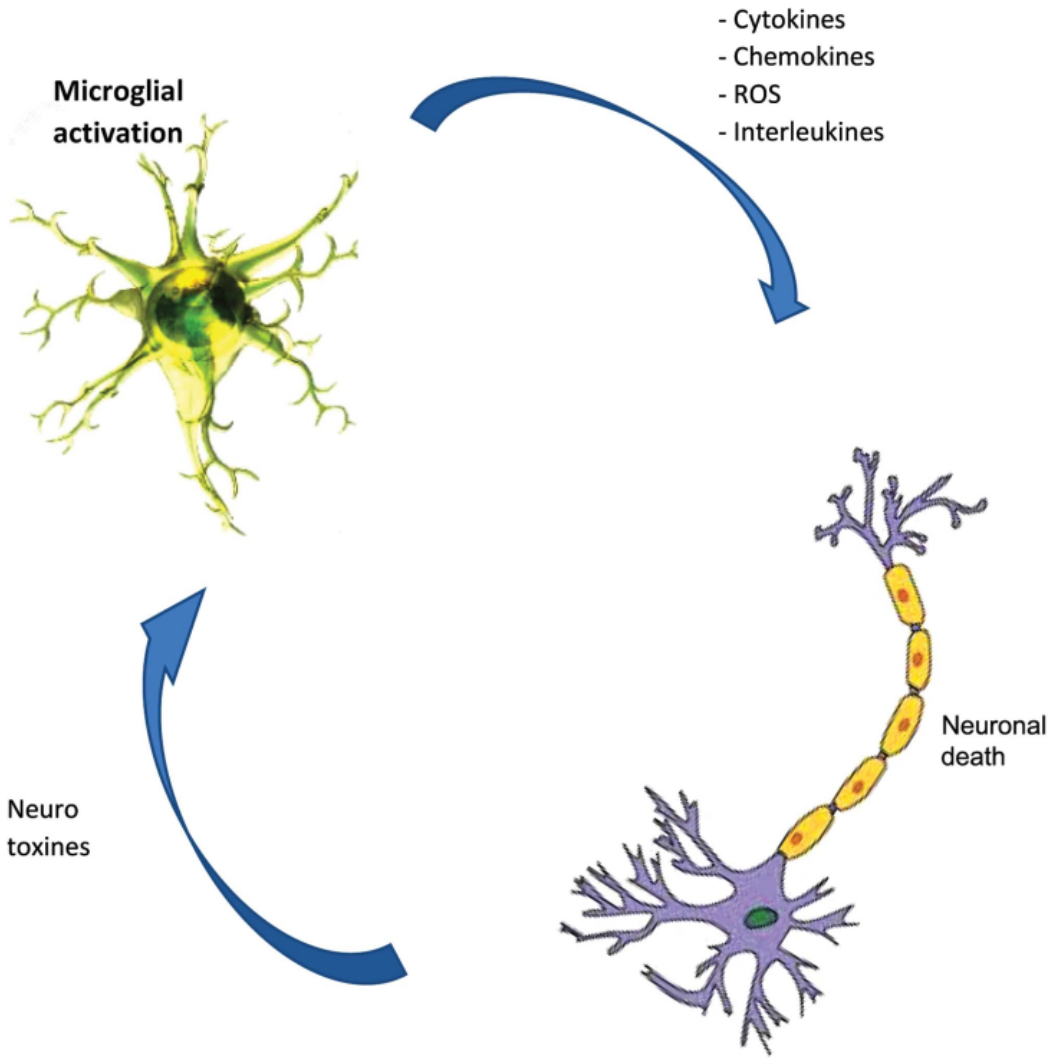

Figure 9. Relationship between microglial activation and neuronal death. Microglia can be a double player in inflammation releasing either neurotoxic or neuroprotector substances (Chen, et al. 2016b).

3. NEURODEGENERATION THEORIES

Neurodegenerative diseases are very complex pathologies that have as common characteristics the progressive character of the symptoms and the gradual degeneration of a part or parts of the nervous system that leads to physical and psychic incapacity. In the case of PD, as stated before, a number of key alterations occur in the SN, leading its degeneration.

Different theories try to elucidate the mechanisms involved in the degeneration of the DArgic system (Tomás-Camardiel y col., 2004):

A) *Accumulation of both intra and extracellular misfolded proteins.* An impairment in the protein quality control and clearance systems such as the ubiquitin-proteosome system (UPS) accounts for the formation of intracellular accumulations of proteins (Jellinger 2010). All this is known as proteinopathies, and is thought to play a major role in the neuronal dysfunction and death that characterizes the development of neurodegenerative diseases, such as Alzheimer's disease, PD, Huntington's, amyotrophic lateral sclerosis, and frontotemporal dementia (Abeliovich and Gitler 2016; Gitler, et al. 2017; Pierre, et al. 2009). If patients with PD have a malfunction of the UPS, the proteins that normally renew through this route will tend to aggregate and form inclusions.

B) *Oxidative stress.* The most important of neurodegeneration theories refers to oxidative stress as a cause of damage to neurons of the SN. This oxidative stress is triggered upon intracellular accumulation of reactive nitrogen species and reactive nitrogen oxygen (ROS) as a result of either reduction in the endogenous anti-oxidant capacity or the ROS overproduction (Lin and Beal 2006). During oxidative or respiratory explosion numerous enzymatic systems are activated among which are the NADPHox, iNOS and myeloperoxidase (MPO), which release into the medium toxic amounts of superoxide (O^{2-}), free radicals of NO (that cause the nitrosylation of certain proteins) or hypochlorous acid (HClO), a powerful oxidant, by converting amines to chloramines, phenols, etc., which in turn can damage macromolecules directly through the production of OH^{-}. The expression of these biocatalytic systems in SN is substantially higher in patients with PD (Fig. 10).

C) *Mitochondrial energy failure.* There are numerous data that point to the involvement of mitochondrial defects in the pathogenesis of PD (Beal, 2003). Although this does not seem to be the primary cause of the disease it could be an important factor in its development.

D) *Inflammation.* Since 1988, when McGeer et al. showed the presence of inflammatory markers in the CNS of PD patients, the implication of inflammatory events in the pathogenesis of this neurodegenerative disease was postulated (McGeer, et al. 1988a; McGeer, et al. 1988b). This was supported by the presence of activated microglia, accumulation of cytokines, activation of Nuclear Factor kappa β (NF-κβ) pathway, and oxidative damage to the proteins in the brains of PD individuals (McGeer et al., 1988; Hirsch and Hunot, 2009) and animal models (Gao, et al. 2002; Gao, et al. 2008; Herrera, et al. 2000). These authors showed the presence of T lymphocytes cytotoxic suppressors CD^+ and microglial cells with the activated major histocompatibility complex class II (MHC II), in the tissue of patients with PD. In addition, different inflammatory markers have been described in parkinsonian brains (Hunot and Hirsch 2003): (a) Significant proliferations of ameboid macrophages and reactive microglia (Hirsch, et al. 1998; McGeer, et al. 1988a); (b) Activated glial cells expressing different pro-inflammatory cytokines such as TNF-α, IL-1β and IFN-γ also appear in the SN of this type of patients (Hunot, et al. 1999), together with an expression of iNOS (Herrera, et al. 2000; Hunot, et al. 1997; Mogi, et al. 1998); c) Increases in the expression of IL-1β, IL-6 and TNF-α in the cerebrospinal fluid of patients with PD.

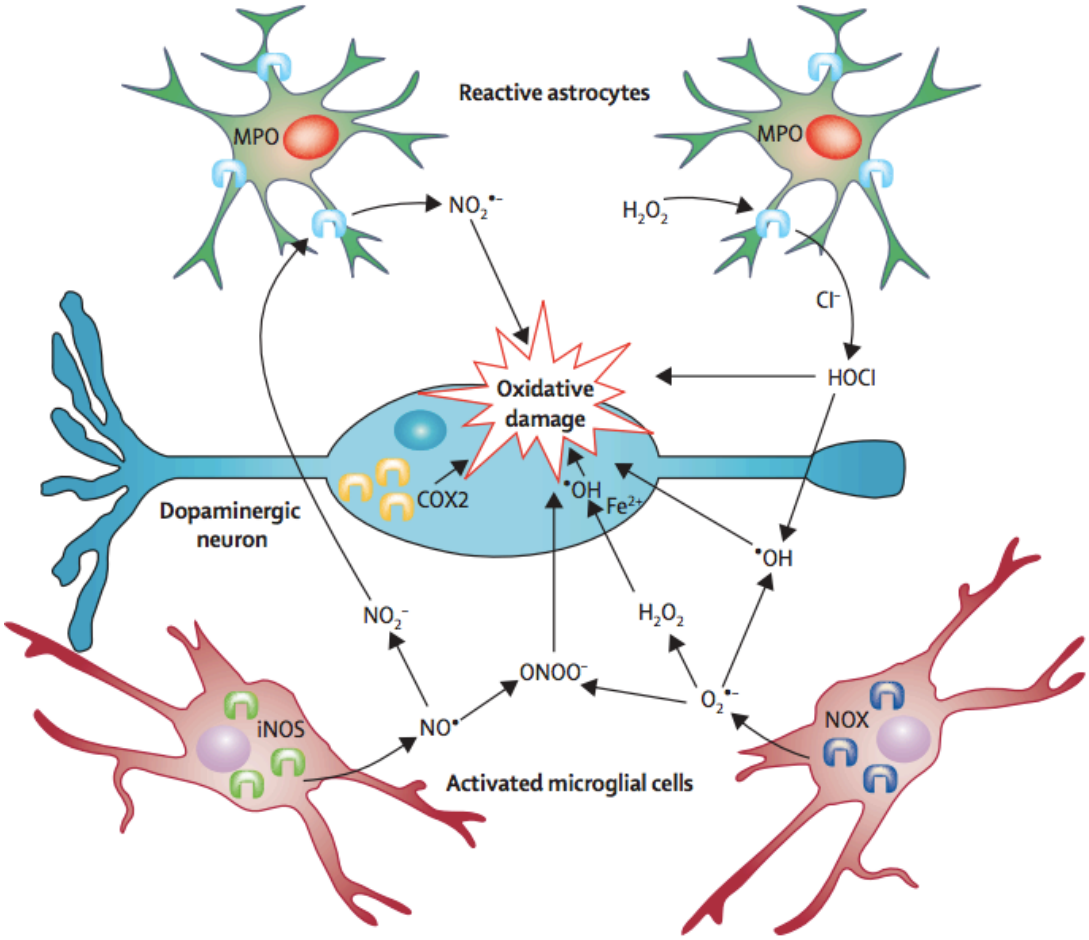

Figure 10. Schematic representation of the possible origins of oxidative damage in the dopaminergic neurons in PD (Hirsch and Hunot 2009).

4. ANIMAL MODELS

The techniques used to study neurodegenerative diseases are very different in nature. The generation of animal models for their study has meant a great advance in the knowledge of the mechanisms involved in the development of these diseases, as well as in the identification of therapeutic targets. In addition, they represent the most faithful way to reproduce the disease and are optimal for the global study of pathologies. A greater number of these animal models are

generated in higher mammals, whose nervous system is more similar to that of humans, and the development of pathology is more similar to that which occurs in patients.

Within the animal models we can distinguish those in which a genetic modification in a gene responsible for the disease is carried out (transgenic animals) and those based on the injection of neurotoxins that trigger some molecular mechanism suggested in the disease. In PD these models help to clarify some aspects of its pathogenesis, such as the role of oxidative stress, apoptosis, mitochondrial dysfunction, inflammation, protein deterioration and degradation pathways of the organelles and α-syn (Bové and Perier 2012).

4.1. Transgenic animals

Most of the animal models that are generated today are genetic models in which the disease reproduces by integrating in the genome of the animal or eliminating from it the mutations responsible for the pathology.

Recently, human genetic studies have identified a large number of genes related to familial PD (Fahn and Sulzer 2004). Transgenic models have been used to explore the functions of these PD-related genes.

Genes involved in regulating mitochondrial function were identified in families with rare inherited forms of PD (Clark, et al. 2006). The mitochondrial dysfunction may be of etiological importance in PD because it was found that PD patients had an increased number of midbrain DAergic neurons with respiratory chain deficits compared to non-PD patients (Harvey, et al. 2008). Hence, mitochondrial dysfunction may play an important role in PD. The manipulation of mitochondrial respiration genes (e.g. mitochondrial transcription factor A or ***FTMA***) has been used to create a conditional knockout animal model (K.O.) for FTMA in DAergic neurons, known as "MitoPark". This mice display several main features of PD based on an underlying deficiency of the respiratory chain in DAergic neurons of the midbrain (Fig. 11).

Apart from the *FTMA*, more genes related to familial PD have been studied in K.O. mice. **Parkina** is a protein related to the defense against oxidative stress. These K.O. mice were obtained by removing some of their exons, such as *PARK2*, thus generating animals exhibiting reduced mitochondrial respiration, and increased damage caused by oxidative stress. Another model is the animal K.O. for the ***DJ-1*** gene. This gene is related to processes that include oxidative stress and cellular transformation. Their mutations have been linked to early development of PD. In general, studies in mice with absence of *DJ-1* expression show deficits in motor functions and alterations of DA levels in the nigrostriatal pathway. Mutations in the ***PINK1*** gene are the second most frequent cause of inherited early-onset familial PD, This gene is purposed to prevent mitochondrial dysfunction. Thus, the K.O. mice study of *PINK1* showed that it can protect cells against oxidative stress, as *DJ-1* did (fig. 11(Dawson, et al. 2010; Gubellini and Kachidian 2015; Harvey, et al. 2008). Finally, a group of transgenic animals that overexpress ***α-syn*** has been described. Different strains of transgenic mice have been generated with wild and / or mutated α-syn human forms under different promoters. The result was an animal with a lower number of DAergic terminals, cytoplasmic inclusions of α-syn and ubiquitin, and nuclear deposits (Masliah, et al. 2000).

There is also the possibility of generating chemically induced animal models in cases where the cause of the disease is not of genetic origin or unknown.

4.2. Models based on neurotoxin injection

One of the characteristics of PD in all animal models induced by toxins is the neurodegeneration of the DAergic neurons of the SNc. Each model, however, has its own particularities, depending on the species involved and the toxin used. In other words, the death of DAergic cells is the cornerstone of these animal models, but the mechanism of action differs between them. Among the most used neurotoxins are 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and pro-inflammatory compounds (Fig. 11).

2.1.1. Injection of 6-OHDA

As stated above, the most important of neurodegeneration theories refer to oxidative stress as a cause of damage to CNS neurons. Oxidative stress happens when the balance between the oxidative events and the antioxidant defenses is corrupted due to loss of antioxidant enzymes or by increased production of oxidizing species (Bains and Shaw 1997). The most important oxidative stress PD animal model is based on the use of 6-OHDA as a specific DA-toxin. 6-OHDA destroys DAergic neurons by a mechanism mediated by ROS (Cohen and Heikkila 1974). It is important to know that DAergic neurons are susceptible to oxidative damage because of their high content of DA, iron accumulation that can participate in ROS formation, and reduced antioxidant capacity (Lull and Block 2010). The specificity of 6-OHDA neurotoxicity is associated with its reuptake by a specific transport mechanism of the DAergic neurons (the DA transporter; DAT) where it accumulates and oxidizes to produce hydrogen peroxide, superoxide and hydroxyl radicals (Cohen and Heikkila 1974; Graham 1978).

2.1.2. Injection of MPTP

There are numerous data pointing to the implication of mitochondrial defects in the pathogenesis of PD (Beal 2003), and the reduced activity of complex I in the brain, platelets, and skeletal muscle of PD patients (Parker, et al. 1989; Schapira, et al. 1990). Although this does not appear to be the primary cause of the disease, it could be an important factor in its development.

The animal model of PD due to mitochondrial energy failure which is more common and important, is based on the use of MPTP and its toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+), a compound whose main toxic effect is the inhibition of mitochondrial complex I activity (Gu, et al. 1998). Its selectivity over DAergic neurons seems to be due to its reuptake in the nerve terminals of the DA system through the above-mentioned DA reuptake system (Chiba, et al. 1985; Javitch, et al. 1985). MPP^+ reduces ATP synthesis, generates free electrons, and increases the amount of the

superoxide radical leading to lipid peroxidation, with its subsequent accumulation in the mitochondria (Bains and Shaw 1997).

Category	Model	Main pathogenic mechanisms	Usual administration or induction mode	Nigrostriatal degeneration	Progressivity	LB-like inclusions	Motor symptoms
Neurotoxic models	6-OHDA	Complex I inhibition	Injection in SNc or MFB	+++	–	–	+++
			Injection in striatum	++	+		++
	MPTP	Complex I inhibition	Systemic injection (single or repeated)	++	+	–	++/+++
	Paraquat®	Complex I inhibition, ROS production	Systemic injection (single or repeated)	++	+	+	+
	Maneb®	Impairment of glutamate and DA uptake	Systemic injection	+	+	–	+
			Associated with Paraquat	++	+	+	++
	Rotenone	Complex I inhibition, ROS production, etc.	Systemic (injection, oral, etc.) or brain injection	++	+	+	+
Genetic models	α-synuclein (PARK1, 4)	LB-like aggregate toxicity?	Overexpression, mutations, injection of aggregates	+	+	++	+
	Parkin (PARK2)	Loss of ubiquitin E3 ligase activity?	Mutations	+/–	+/–	+/–	+/–
	PINK1 (PARK6)	Mitochondrial insult?	KO	+/–	+/–	+/–	+/–
	DJ-1 (PARK7)	Oxidative stress, mitochondrial dysfunction?	KO	+/–	+/–	+/–	+/–
	LRRK2 (PARK8)	Loss of enzymatic activity?	Mutations, KO	+/–	+/–	+/–	+/–
Others	SHH, Nurr1, Pitx3, EN1	Impaired protein synthesis in DAergic neurons	KO	++	+/–	–	+/–
	MitoPark	Mitochondrial deficit	KO	++	++	+/–	+
	PDC	Acute EAATs blockade, excitotoxicity, oxidative stress	Injection in SNc	++	+++	–	+

6-OHDA: 6-hydroxydopamine; DA: dopamine; DAergic: dopaminergic; EAATs: excitatory amino acid transporters; EN1: engrailed 1; LB: Lewy body; MFB: medial forebrain bundle; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Nurr1: nuclear receptor related protein-1; PDC: L-trans-pyrrolidine-2,4-dicarboxylate; PINK1: PTEN-induced putative kinase 1; Pitx3: pituitary homeobox 3; ROS: reactive oxygen species; SHH: sonic hedgehog; SNc: *substantia nigra pars compacta*.

Figure 11. Main models of PD. The outcome of each model is rated with a 4-level scale:(-) abscent; (+) weak; (++) moderate; (+++) strong. Taken from (Gubellini and Kachidian 2015).

2.1.3. Injection of pro-inflammatory compounds

There are pro-inflammatory compounds capable of producing microglial activation along with an induction of inflammatory processes. Among these compounds are immunoglobulins G (IgGs), trisialoganglioside (GT1b), thrombin and lipopolysaccharide (LPS).

(A) Immunoglobulins G. Microglial cells can also be activated by IgGs from parkinsonian patients in the presence of DAergic cell membranes modified by low-doses of dopa-quinone or H_2O_2, but not in the presence of cholinergic cell membranes. This activation requires the FCγR microglial receptor. Although microglial activation leads to the release of several cytokines and ROS, only NO and H_2O_2 appear to mediate microglial-induced DAergic cellular damage (Le, et al. 2001).

(B) Trisialoganglioside. Gangliosides are sialic acid-containing glycosphingolipids, present in mammalian cell membranes, especially concentrated in the membranes of neuronal cells. Some of them have beneficial effects such as GD1a which protects DAergic neurons from neurotoxicity induced by MPTP (Date, et al. 1991); while others such as GT1b and GM1 causes apoptotic cell death of thymocytes (Zhou, et al. 1998).

Ryu et al. (2002) examined the neurotoxic effects of GT1b on DAergic neurons of the SN in rats and found that 7 days after GT1b injection, nigral neurons were dead, including DAergic neurons. In addition GT1b activated microglia was present in this structure. The neurotoxicity exerted by GT1b on the DAergic neurons of the SN is mediated, at least in part, by the release of NO from the microglial cells (Ryu, et al. 2002).

(C) Thrombin. Thrombin is a multifunctional serine protease rapidly produced from prothrombin at sites of BBB lesion and rupture, suggesting that it could easily enter the CNS. Previous studies in our laboratory (Carreno-Muller, et al. 2003) showed that the injection of different concentrations of thrombin in the rats SN produces a strong macrophage/microglial reaction in this structure, along with the induction of the expression of various cytokines and

mediators of inflammation. In addition, thrombin injection produces selective destruction of the SN DAergic neurons, leaving unaltered others neuronal phenotypes which are present in this structure (Carreno-Muller, et al. 2003).

5. LIPOPOLISACCHARIDE

LPS represents the main component of the outer membrane of Gram-negative bacteria and some cyanobacteria. It plays a key role in the development of infections and sepsis (Rietschel and Brade 1992; Schletter, et al. 1995). The LPS was discovered about 100 years ago by Richard Pfeiffer and, unlike the exotoxin secreted by the cholera bacteria, it is a heat-stable toxin (Rietschel and Cavaillon 2003).

Although there is a great variation in the composition of the LPS, they all show a common structure. They are mainly composed of three parts: lipid A, core, and specific O-chain. Lipid A is the toxic part of LPS and the hydrophobic component. When bacterial cells are lysed by the immune system, fragments of membrane containing lipid A are released into the circulation, causing fever, diarrhea, and possible fatal septic shock (Lerouge and Vanderleyden 2002). The polysaccharide and hydrophilic part comprises two regions: the O-specific chain or O antigen, and the central region or core. The O antigen is attached to the core and consists of repetitive units of oligosaccharide. The core, which consists of short chain of sugars, in turn is subdivided into an external core (formed by hexoses), through which it binds to the O antigen, and the internal core (formed by heptoses). Lipid A is generally composed of a phosphorylated disaccharide, linked to two D-glucosamines in position β1,6 and carrying a maximum of six or seven acyl residues. It is attached to the internal core by a residue called 2-keto-3-deoxyoctanoic acid (KDO) (Fig. 12).

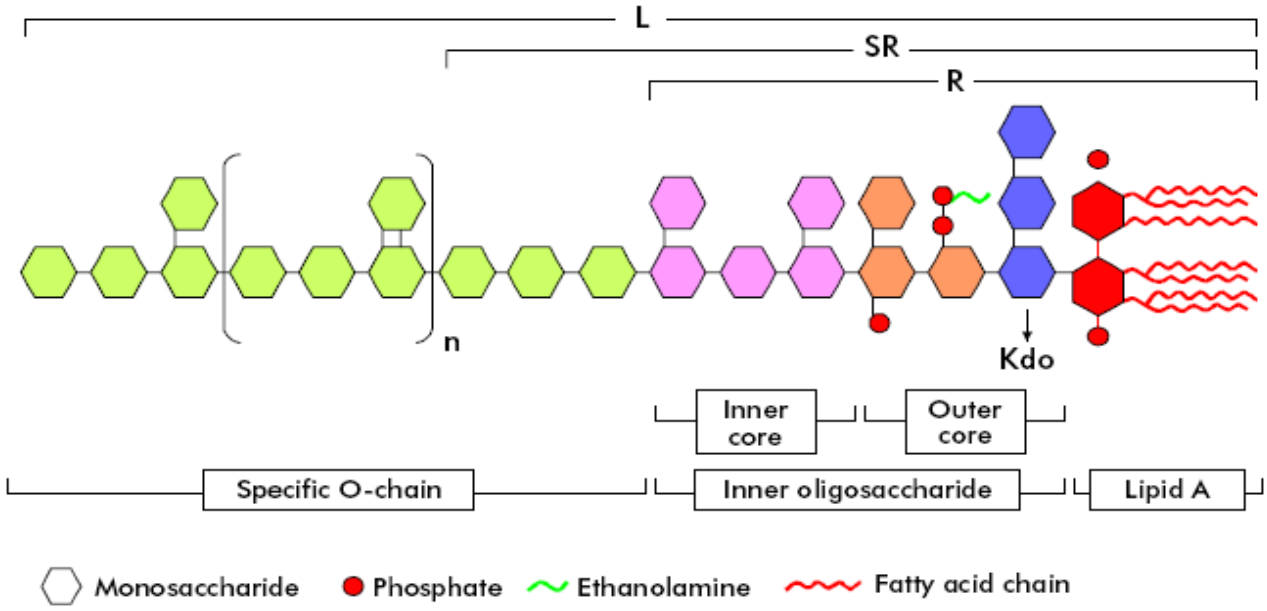

Figure 12. **General chemical structure of bacterial lipopolysaccharide (Pupo and Hardy 2009).**

5.1. Interaction between LPS and soluble membrane proteins

A prerequisite for the recognition of LPS by the innate immunity cells and thus activate them is its interaction with specific LPS-binding molecules on the surface of the target cells. Several LPS binding structures have been described, but physiological relevance has only been demonstrated for some of them (Fig. 13).

The activation starts when LPS binds to a 60 kDa serum glycoprotein called LPS-binding protein (LBP), which in turn catalyzes the transfer of LPS to membrane-bound CD14 (mCD14) or soluble (sCD14) (Triantafilou and Triantafilou 2002). CD14 is the most important cell surface protein related to LPS binding and cell activation (Tanaka, et al. 2000). CD14 is a 55 kDa glycoprotein that exists as a membrane protein (CD14m) anchored to a glycosylphosphatidylinositol (GPI) in monocytic cells, epithelial cells, polymorphonuclear leukocytes and some B lymphocytes (Gu, et al.

1998). The binding of LPS to CD14m in monocytes is necessary for the stimulation of these cells, which elicits strong pro-inflammatory responses causing production and release of immune mediators.

As mentioned before, LPS binding to the CD14 receptor is strongly increased by LBP (Staal and Sonsalla 2000; Tanaka, et al. 2000). During the acute phase response, the concentration of LBP increases from 5-10 μg/ml to 200 μg/ml (Staal and Sonsalla 2000). LBP reduces the concentration of LPS required for the activation of monocytes by forming LBP-LPS complexes, which are recognized by CD14 (Fig. 13).

In addition to CD14m, there are different types of soluble CD14 (48, 53 and 55 kDa) present in concentrations around 2-6 μg/ml in serum (Frey, et al. 1992), and which are released by monocytes or secreted by free forms of GPI (McNaught and Olanow 2003). It is known that LPS is capable of binding directly to the soluble forms of CD14 (sCD14), a process highly facilitated by LBP, although LBP is not present in the LPS-sCD14 complexes. The LPS-sCD14 complexes can activate some mCD14-negative cells, such as endothelial cells, fibroblasts and smooth muscle cells, to produce cytokines (Frey, et al. 1992; Haziot, et al. 1993; Pugin, et al. 1993). Thus, it has been postulated the existence of a specific receptor for the LPS-sCD14 complexes that would be expressed in these cells and that would mediate the activation by LPS of cells lacking CD14. In conclusion, CD14 as a soluble membrane receptor would play an extremely important role in the molecular recognition of LPS in several cells. However, it appears that additional membrane molecules are required for cellular activation (Rietschel, et al. 1996).

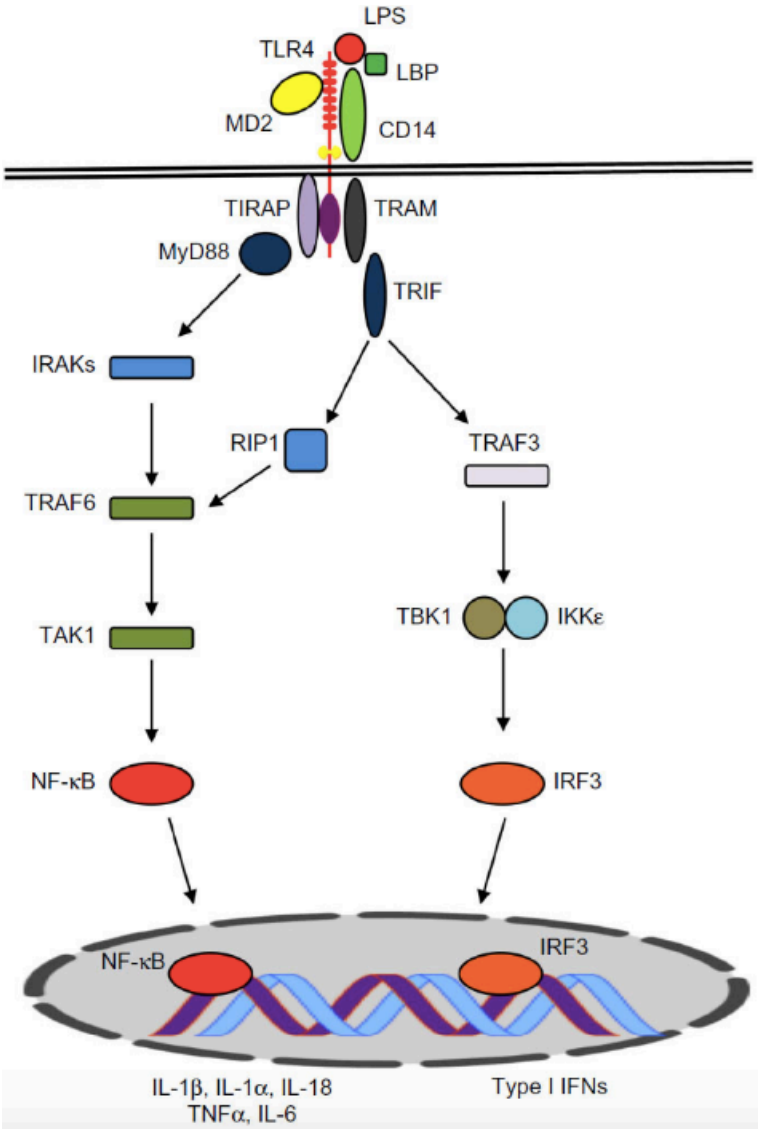

Figure 13. Schematic view of the LPS signaling pathway through TLR4 (Diamond, et al. 2015).

A family of proteins called Toll-like receptors (TLRs) has been identified in humans and mice. These are transmembrane proteins with an extracellular domain with leucine-rich repeats (LRR) and a cytoplasmic receptor Toll/IL-1 (TIR) with a domain of great structural similarity to the IL-1 receptor (Anderson, et al. 2000; Fukata and Abreu 2012; O'Neill and Greene 1998; Shah 2008). There is evidence that these TLRs are the most important pathogen recognition molecules and they are used in the start of the signaling cascades in response to infections with different classes of pathogens (Lester and Li 2014).

Both of LBP and CD14 are considered key accessory molecules for the efficient transfer of LPS to the Toll like receptor (TLR)4-MD2 (MD2 is an accessory molecule required by the TLR4 to capture its ligand, LPS) and increasing the sensitivity of LPS detection (Hailman, et al. 1994; Ryu, et al. 2017).

5.2. Transduction of signal induced by LPS

Once the LPS stimulate the target cells a series of intracellular events occurs. The most important and best characterized is the activation of the NFκB, which plays a central role in the regulation of inflammatory and immune responses (Ghosh, et al. 1998; May and Ghosh 1998). NFκB represents a group of structurally related proteins, including p65, p50, p52, RelB and c-Rel. Under normal conditions, NFκB is sequestered in the cytosol as an inactivated homo or heterodimeric form, with non-covalent interactions with its inhibition proteins, called IκBs. After stimulation with the appropriate agonist, IκB is phosphorylated, ubiquitinated and degraded by the proteasome (Israel 2010). Thus, NFκB is released and translocated to the nucleus initiating gene expression phenomena. The target genes of NFκB include those that code for cytokines, chemokines, adhesion molecules, acute phase proteins, antimicrobial peptides, iNOS and cyclooxygenase 2 (COX-2). Together, these mediators provide immediate protection to the host and induce the development of the adaptive immune response (Ghosh, et al. 1998; May and Ghosh 1998).

In recent years there has been a significant expansion of our knowledge about the signaling mechanisms by which LPS induces the activation of NFκB. In fact, several of the molecular components involved in this process have been discovered (Fig. 13 and 14). The TLR4 is a primary transmembrane signaling receptor for the LPS. Once bound to CD14, LPS must be transferred to TLR4, resulting in the homodimerization of TLR4 and a conformational change of its cytosolic domain (Kagan 2017). Subsequently, an adaptor protein called MyD88 is recruited on the receptor,

which is followed by the interaction with the kinase associated with IL-1 receptor (IRAK)-1, -2 or -M. The IRAK dissociates from the receptor complex and recruits TNF receptor-associated factor 6 (TRAF6), resulting in the assembly and activation of the IKKα/β/γ complex and the subsequent phosphorylation and degradation of IκB and translocation to the nucleus of NFκB (Kawai, et al. 1999; Lu, et al. 2008; Rosadini and Kagan 2017).

The mechanism by which TRAF6 activates IKKs is not fully understood, although there is evidence that show that TRAF6 could interact with additional molecules that would help to activate kinase proteins in cascade, leading to phosphorylation and activation of IKKs. Three TRAF6 proteins have been identified, named evolutionarily conserved signaling intermediate in Toll pathways (ECSIT), TAK-1 binding proteins (kinase activated by transforming growth factor β) -2 (TAB2), and TRAF6 binding protein (Fig. 14) (Wi, et al. 2014).

There are other signal transduction pathways of the LPS. In several *in vitro* studies with monocytes and fibroblasts, G proteins and small G proteins have been implicated in the activation of tyrosine kinases (TK) (Cattaneo, et al. 2014; Mayeux 1997; Tanke, et al. 1991), phospholipase C (PLC) and (A2) (Chang, et al. 1990; Fleming, et al. 1996), as well as calcium modulated protein (calmodulin) (Mattson and Rydel 1996; Nakano, et al. 1993). Also sphingomyelitis has been considered another second messenger, which would hydrolyze sphingomyelin into ceramide, which would activate different protein phosphatases and protein kinase (PK), as the PK activated by ceramide (Joseph, et al. 1994; Liu, et al. 1994), which could intervene in the LPS signal by activating or inhibiting various enzymes such as PK C, phospholipases PLC and PLA_2, and COX-2 (Hayakawa, et al. 1996; Liu, et al. 1999).

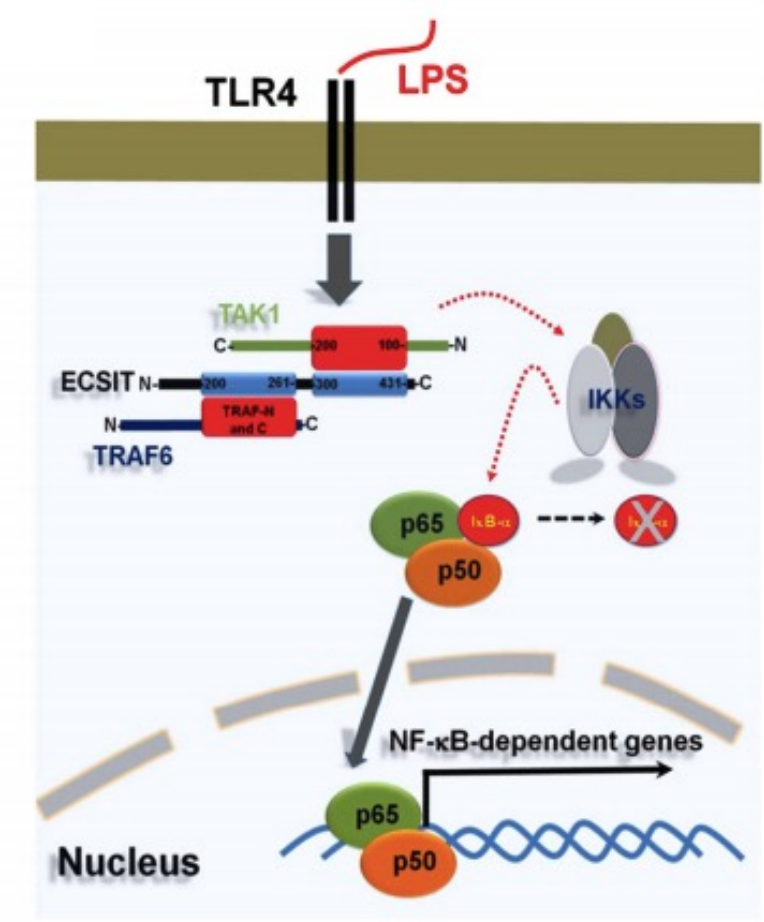

Figure 14. **Molecules implicated in the transduction of signal induced by LPS** (Wi, et al. 2014).

Another way is formed by PKs, in which several groups are involved: the serine-threonine PK A and C (Kozak, et al. 1997) and a large set of TK (Ruetten and Thiemermann 1997). The set of mitogen-activated TKs, MAP kinases (MAPKs), also participate to a large extent in the intracellular signals of the LPS.

5.3. Mitogen activated protein kinase

This large family consists of PK that phosphorylates serine-threonine and tyrosine-threonine residues. They act in cascade form, in which signal is spread by sequential phosphorylation and activation of kinases (Plotnikov, et al. 2011). According to different *in vitro* studies performed on macrophages, leukocytes, endothelial cells, smooth muscle cells and other cell types (Baydoun, et al. 1999; Downey and Han 1998), there are at least four subgroups of MAPK, of which it has been described that three are related to the responses induced by the LPS. One of them is formed by kinases regulated by extracellular signals (ERK). ERK1/2 is responsible for proliferation and differentiation, neuronal plasticity, and apoptosis. The other pathways related to MAPKs include the set of proteins that make up the PK subfamily of transcription factor c-Jun, called JNK. JNK is a stress-associated MAPK pathway that can be activated by inflammatory cytokines, oxidative stress, and UV radiation. It phosphorylates a large number of nuclear and cytoplasmic substrates, which in turn regulates many genes involved in cellular processes such as apoptosis and neuronal and immunological activities (Haeusgen, et al. 2009; Rincon and Davis 2009; Yoon and Seger 2006). Dysregulation of JNK has an important role in a number of neurodegenerative diseases, mainly Alzheimer's disease, PD, amyotrophic lateral sclerosis, and other diseases such as diabetes and cancer.

P38 MAPK pathway is known as a stress-activated protein kinase pathway. It plays a crucial role in regulating the response with cytokines after stimulation with LPS. In fact, it is known that a specific inhibitor of these kinases, SB203580, completely prevents the release of cytokines after stimulation of monocytes with LPS (Lee, et al. 1994). For this role in inflammation induction, it is considered a therapeutic target for inflammatory and autoimmune diseases. P38 also has an important role in the management of immunological effects, cellular and apoptotic aging, and cell cycle checkpoints. Its importance in such processes gives an overview about the pathological manifestations resulting

from its dysregulation. It also plays a role in cardiovascular and neurodegenerative diseases, diabetes, and cancer (Plotnikov, et al. 2011).

Other important pathway is the PI3K/AKT, which is an intracellular signaling pathway involved in cell cycle regulation. PI3K phosphorylates and thus activates AKT. AKT is a Ser/Thr kinase, which regulates cell survival, proliferation, migration, and differentiation through the mTorc1 pathway, p53 pathway, Forkhead O family (FOXO) transcription factors and other substrates. AKT activation is considered the main output of growth factor-induced PI3K signaling (Dinsmore and Soriano 2018).

5.4. LPS as an inductor of the inflammasome components expression

Inflammasomes are cytosolic multiprotein complexes in the CNS that enable autocatalytic activation of inflammatory caspases upon different stimuli, resulting in the production of pro-inflammatory cytokines including IL-18 and IL-1β (Lamkanfi and Dixit 2012). These multiprotein complexes constitute a part of the innate immune response, functioning as intracellular sensors and responding to infectious agents, pathogenic stimuli, and host-derived danger signals associated with neurological diseases. Inflammasomes consist of three components: caspase-1, a cytosolic pattern-recognition receptor, and an adapter protein enabling interaction between them. Upon activation, the cytosolic receptors, including members of the NOD-like receptor (NLR) family (Nucleotide-binding oligomerisation domain receptors), undergo oligomerisation results in the assembly of the inflammasome complex and increase in pro-IL-1β and NLRP3 levels. This acts as a recruitment and activation way for pro-caspase-1. Activation of caspase-1 leads to the cleavage and release of IL-1β and IL-18, resulting in inflammatory reactions. Both the ligands and their cognate receptors are expressed in the CNS, where they modulate homeostatic and neuroinflammatory processes (Fig. 15) (Lamkanfi and Dixit 2012; Machado, et al. 2016; Schroder and Tschopp 2010; Walsh, et al. 2014).

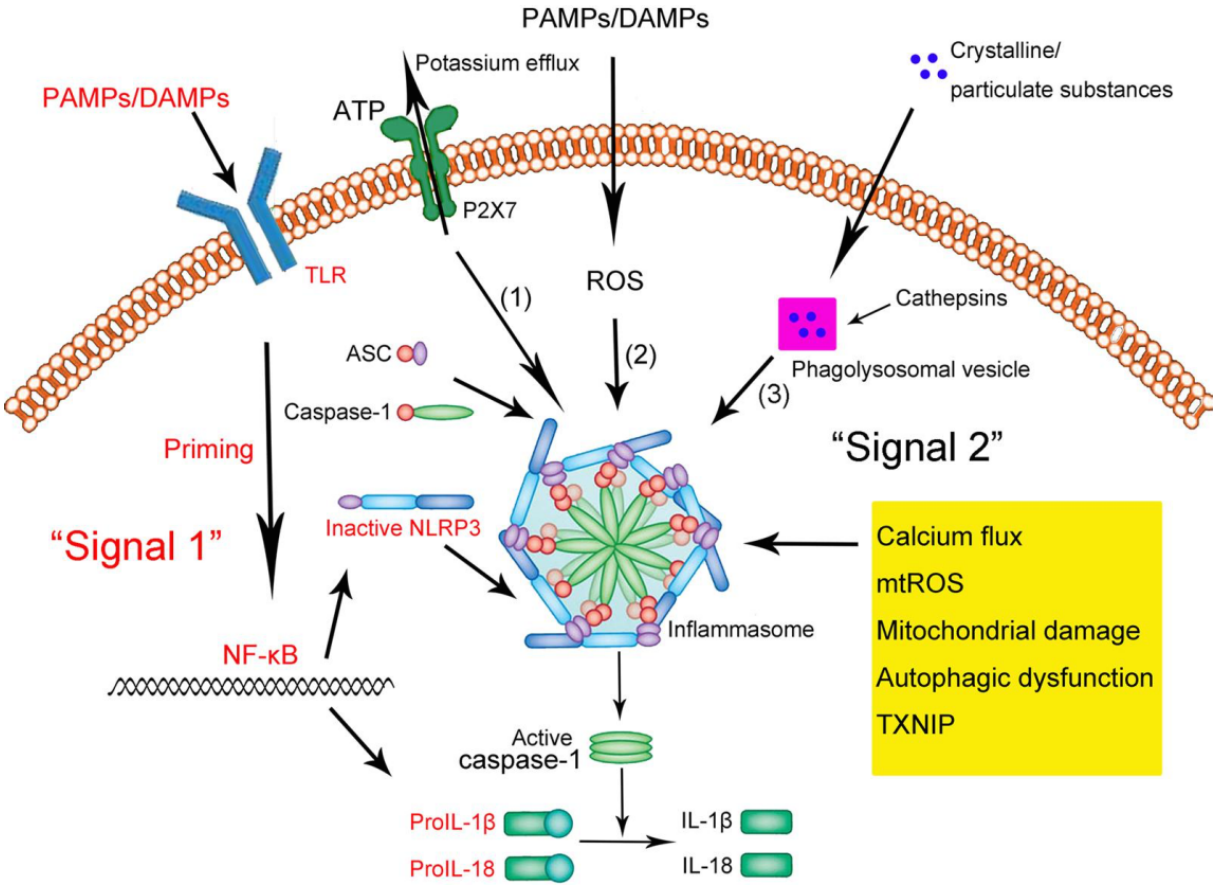

Figure 15. Schematic representation of inflammasome activation process (Shao, et al. 2015).

ROS is produced in response to initiating factors, triggering the inflammasome mediated inflammatory cascade. Components oligomerization results in inflammasome assembly. As a consequence it activates IL-1β and IL-18 through caspase-1, which causes oxidative DNA damage. Alongside with inflammation, they cause pyroptosis (Harijith, et al. 2014).

6. THE LPS AS A MODEL OF THE PD

As already mentioned, and due to the importance of neuroinflammation role in the pathology of PD, there are several models that allow us to study the molecular mechanisms involved in

neuroinflammation and eventually to improve the therapeutic strategy (Hoban, et al. 2013; Liu and Bing 2011). Among all of them the most used is based on the intranigral injection of LPS.

Inflammatory processes, including microglial activation, appear to be important in the development and progression of several degenerative neuronal diseases, including PD (Akiyama and McGeer 1989; McGeer, et al. 1988b). The kinetics of the inflammatory reaction following intracerebral administration of LPS has already been described in the CNS of mice (Andersson, et al. 1992) and rats (Bourdiol, et al. 1991; Montero-Menei, et al. 1996; Szczepanik, et al. 1996). Similarly, previous studies in our research group tried to determine if the glial activation following a single injection of LPS in the SN could affect the viability of the DAergic neurons *in vivo* and if this effect was selective for this type of neurons. The model they described would allow to determine the degree of glial activation involvement in the progressive nigral degeneration which is a hallmark of PD. Besides, it can give an idea about the degree of involvement of the glia derived factors, such as cytokines or the deprivation of neurotrophic factors, which are classically associated with neuronal death *in vivo*.

These studies revealed that the injection of 2 μg of LPS in the SN of the rat produced a series of events in this brain structure (Castano, et al. 1998). Four days after LPS injection, degeneration of the DAergic system was observed, which was revealed by the significant decrease in DA levels both in the SN and in the striatum. While DA levels decreased, there was an increase in homovanilic acid (HVA)/DA and DOPAC/DA (indicators of the metabolic exchange of DA) values at all times, in SN and striatum, which can give a clue that the DAergic damage induced by LPS injection increases the synaptic transmission of DA. The decrease in DA levels is also consistent with the decrease in the activity of tyrosine hydroxylase (TH, the initial and rate-limiting enzyme of catecholamine biosynthesis), especially in the striatum. This DAergic neurons damage was also revealed by the loss of TH-positive neurons in the SN.

The neurodegeneration found is in agreement with previous studies with LPS. In 1992 Andersson et al. described that intrahypocampal injection of 2 μg LPS caused the loss of pyramidal cells of the CA1 layer of the hippocampus in mice (Andersson, et al. 1992). In addition, a slight but significant loss of NMDA neuronal receptors has been described after a LPS single injection into the striatum (Bourdiol, et al. 1991).

These findings were also confirmed by Gao et al. through chronic intranigral infusion of LPS which produced delayed death of DAergic neurons, resembling what is found in PD. Microglial activation under these conditions, reached a plateau 2 weeks earlier than the appearance of degenerative events on the DAergic system (Gao, et al. 2002).

The efficacy of LPS as an immune activator in the CNS was shown by the increase in the number of OX-42 positive-cells and the morphological changes of microglia. Two days after the injection of LPS, the microglial cells in the damaged SN already show an activated morphology. This result is in agreement with previous studies that show that LPS is a powerful stimulant of microglia (Andersson, et al. 1992; Castano, et al. 1998; Lee, et al. 1993; Montero-Menei, et al. 1996).

The inflammatory reaction induced by the injection of LPS seems to affect the different neuronal types in different ways. *In vitro* studies have shown that DAergic neurons are twice sensitive to the toxic effects of LPS than TH negative neurons (Bronstein, et al. 1995; Machado, et al. 2011). Our group showed in previous studies that DAergic neurons are sensitive to LPS only when it has been injected in the SN. The different biochemical and immunohistochemical parameters studied showed that injection of LPS in other locations such as in the medial forebrain bundle (MFB) or in the striatum, do not affect the DAergic system, except for an increase in DA and its metabolite DOPAC levels in the striatum after 15 days after LPS injection into this structure, which could be due to an increase in the production of growth factors in response to LPS.

This different inflammatory reaction between the SN and the striatum was also clear in the characteristic disappearance of the astrocytes at the site of the LPS injection, so upon LPS injection into the striatum, the area lacking astrocytes was very narrow and located around the injection tract, and strong clusters of macrophage were absent. The loss of astrocytes had already been described in previous studies when damage was done to the CNS, as with the injection of excitotoxins (Jorgensen, et al. 1993; Ross and Ebner 1990) and with the injection of 6-OHDA in the MFB (Stromberg, et al. 1986). However, some authors have not observed loss of astrocytes when neurotoxins were injected into the striatum (Bjorklund, et al. 1986; Herrera, et al. 2000; Isacson, et al. 1987). The disappearance of the astrocytes may allow the opening of the BBB along with a massive reaction of microglia and macrophages and leukocyte recruitment. The swelling of the astrocytes with disorganization of the cycle has been described in areas of loss of BBB, but it is not possible to conclude whether the swelling of the astrocytes is the cause or consequence of the opening of the BBB (Kimelberg and Ransom, 1986). After the opening of the BBB, as in the case of neuronal death, the massive increase in serum components, such as Glu could be responsible for the swelling of astrocytes (Kimelberg and Ransom 1986). However, it is important to bear in mind that in *in vitro* studies LPS causes alterations in the structure and function of astrocytes, through the release of pro-inflammatory cytokines (Hu et al., 1999).

In contrast to its effect on the DAergic system, LPS did not affected other neuronal types studied. These studies showed that LPS did not induce damage to GABAergic neurons after LPS injection either into the SN or the striatum. On the other hand, the serotoninergic system showed temporary damage (Herrera, et al. 2000); serotonin levels decreased from day 4 onwards but returned to normal values on day 15 in the striatum and on 21 in the SN, while DA levels did not recover during the whole studied period. There was an increase in serotonin neurotransmission that could be indicated by the increase in 5-hydroxyindoleacetic acid levels on day 2. Alterations in 5-hydroxyindoleacetic acid levels disappeared from day 15 onwards, reinforcing the idea that

treatment with LPS fundamentally affects the DAergic system. Likewise, LPS injection into the dorsal raphe did not produce any change in the serotonergic cells immunostaining.

Thus, it seems that the neuronal damage induced by LPS, at least in the studied areas, is specific to DAergic neurons and is triggered in the neuronal body of the SN. The degeneration must be produced by the inflammation induced by the LPS.

In addition, the previous works of our group not only show that the damage induced by the LPS is specific for DAergic neurons but also that this damage is not reversed during the period of time studied which was 1 year. Measurements of DA concentration, TH activity and immunohistochemical analysis 1 year after a single injection of LPS in the SN, revealed the loss of around 50-60% of DAergic markers. Although there are some variations in the results with HPLC, the study of the different indexes of replacement seems to indicate the absence of significant changes in the replacement of the DA after LPS injection, indicating the possibility of compensatory mechanism rather than a cellular recovery as a reason for these changes. Thus, it can be assumed that the damage in the DAergic system induced by LPS is permanent.

In conclusion, these studies showed that a single LPS injection produces a strong inflammatory response in the SN, which leads to the damage of DAergic neurons without affecting other neuronal types. The neurodegeneration is triggered in the neuronal body, which is consistent with Patt et al. that suggest that in PD the lesion begins in the SN. The damage is permanent, at least in the period of time studied (1 year), and is accompanied by damage in the DAergic terminals in the striatum.

Nowadays it is not clear whether neuroinflammation is a cause or consequence of the disease (Tieu 2011). What is clear is that either if it is developed in the early or late stages of the disease, helps to the progression of the disease. For this, the interest in the search for anti-inflammatory treatments that, at least, delay the progression.

7. METFORMIN

7.1. Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder of multiple aetiologies characterized by chronic hyperglycaemia with disturbances of carbohydrate, lipid and protein metabolism resulting from defects in insulin secretion, insulin action, or both (Association 2010).

Type 2 DM is a progressive disease starting with an insulin resistance. It is the most frequent form of diabetes in people aged over 40 years. It is also known as adult-onset diabetes, although its incidence is increasing greatly in adolescents and even pre-adolescents with obesity. In this type of diabetes the ability to produce insulin does not disappear but the body has a resistance to this hormone. In early stages of the disease, the amount of insulin produced by the pancreas is normal or high. Over time the production of insulin by the pancreas may decrease. People with diabetes do not die because of the disease, but because of the complications, since they are an increased risk of cardiovascular, peripheral vascular and cerebrovascular disease.

The long-term effects of DM include:

- Progressive development of macrovascular complications potentially leading to heart attack and stroke,
- Retinopathy with potential blindness,
- Nephropathy that may lead to renal failure,
- Neuropathy with risk of foot ulcers, amputation, and Charcot joints (a progressive degeneration of weight bearing joints also called neuropathic osteoarthropathy),
- Autonomic dysfunctions, including sexual dysfunction,
- In its most severe forms, ketoacidosis (high concentrations of ketone bodies) or a non-ketotic hyperosmolar state may develop and lead to coma and, in absence of effective treatment, death,

- Neurodegeneration (Calcutt, et al. 2009).

7.2. Neurodegeneration and diabetes mellitus interrelation

PD and DM type 2 are two prevalent diseases among the aging population nowadays. Lately, research has revealed that they are interrelated by convergent molecular and biological pathways. In PD and DM inflammation, endoplasmic reticulum stress, mitochondrial dysfunction, and altered glucose metabolism are disrupted (Santiago and Potashkin 2013a).

Recent studies have revealed that the CNS is directly involved in type 2 DM as part of a mechanism associated with hypothalamic inflammation that causes insulin resistance. Strong association was found between glucose intolerance and hypothalamic inflammation as a result of upregulation of pro-inflammatory NFκB pathway following endoplasmic reticulum stress. Other factors that activate NFκB pathway are elevated oxidative stress and ROS in the hypothalamus resulting in hyperglycemia and insulin resistance (Drougard, et al. 2014). Poor diabetes control could have a long-term negative impact on brain deterioration. The research found in this field provides an explanation about the prevalence of cognitive deterioration in patients with metabolic disorders, establishing a direct link between insulin resistance and neurodegenerative diseases. Likewise, epidemiological studies reveal the greater risk of neurodegeneration in people with diabetes, such as in AD and PD, due to the fact that neuronal insulin resistance and metabolic overload in diabetic patients render neurons more sensitive to neuronal inflammation and death (de la Monte, et al. 2009; Han, et al. 2016).

Regarding PD, recent epidemiological studies have shown an association between DM and the risk of suffering from PD (Sun, et al. 2012; Wahlqvist, et al. 2012). In a meta-analysis of population-based cohort studies done in 2016, Yue et al showed that DM is associated with increased PD risk by around 38% (Yue, et al. 2016). This was also confirmed by Pegano et al. (Pagano, et al. 2018).

The probability of getting PD in DM patients increases with age. Therefore, researchers found that both PD and DM are age-related, and that they share similar pathways of mitochondrial dysfunction (Santiago and Potashkin 2013b; Yang, et al. 2017) and have a common feature of hippocampal volume loss. It was reported that PD might predispose to DM because of mutations of mitochondria-associated proteins, such as DJ-1 and Parkin, which have been implicated in familial PD. These alterations may promote mitochondrial dysfunction in beta islet cells, thereby leading to T1D development.

Inflammation appears to play an important role in both DM and PD. In DM, CNS neuronal loss is focused in the hippocampus, while in PD neurodegeneration is centered in the SN; since midbrain DAergic neurons have some projections into the posterior hippocampus, it is possible that DA neuronal loss in PD with DM could start in the hippocampus and progress to the midbrain (Miller, et al. 2017).

Recently the neuronal protein named amyloid precursor protein (APP), which was previously linked to neurodegeneration and insulin regulation, has been identified and described as a potential biomarker for PD in T2DM patients. It was found that APP expression is upregulated in PD, which suggests that increased expression of APP in blood may alter the neurodegenerative phenotype in type 2 DM (Santiago and Potashkin 2013a).

7.3. Treatment of diabetes mellitus

At the beginning of diabetes, it can be usually controlled by changing lifestyle that includes weight loss, a normal or hypocaloric healthy diet and daily physical exercise. Exercise reduces the need for insulin even when all other factors remain the same, since working muscle has some ability to take up glucose without insulin help. The capillary blood glucose monitoring is very important whether insulin is being used as a treatment or not. If these methods (diet and exercise) do not help achieve

good metabolic control, oral antidiabetic drugs will be used as initial therapy. Normally these drugs cannot give long-term control, thus insulin will be added to maintain glycemic control.

Most of the antidiabetic drugs are orally administrated. Selection of the optimal compound depends mainly on the nature of diabetes, age and patients situation. There are different types of oral antidiabetics that can be classified depending on their mechanism of action:

Secretagogues: Sulfonylureas and Meglitinides (repaglinide)

Sensitizers: Biguanides (metformin) and thiazolidinedione (rosiglitazone and pioglitazone)

Alpha-glucosidase inhibitors and peptide analogs: (acarbose)

7.4. Metformin and its history

Commercially named metformin hydrochloride, is a member of the biguanide class of oral antihyperglycemic agents (3- (diaminomethylidene) -1,1-dimethylguanidine). Its empirical formula is C4H11N5 * HCl. It is a white to off-white, crystalline powder, which is easily soluble in water and practically insoluble in acetone, ether and chloroform, and whose structural formula is shown in Fig. 16.

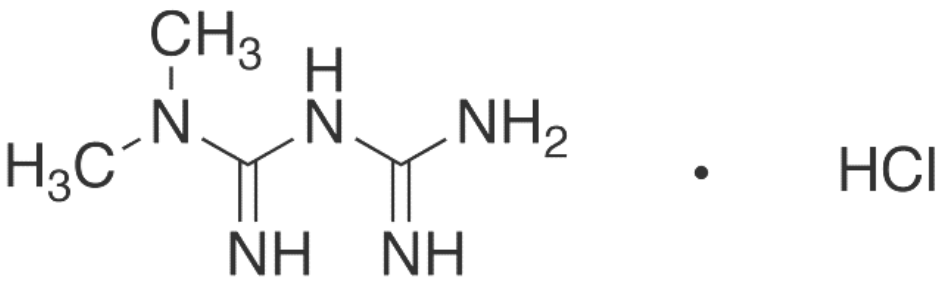

Figure 16. Chemical structure of Metformin.

Discovered in 1922, metformin was accepted clinically for the treatment of hyperglycemia in England in 1958, in Canada in 1972, and in the USA in 1995, for its antihyperglycaemic effect by reducing hepatic glucose production and decreasing insulin resistance, as well as fasting plasma insulin levels. Insulin resistance has been the central mechanism underlying various pathologies including DM type2, by decreasing this resistance. Hence, metformin imparts its beneficial role in a broad spectrum of diseases associated with insulin resistance (Viollet, et al. 2009). In addition, it has therapeutic potential in other conditions among which include diabetic nephropathy, cardiovascular diseases, polycystic ovarian disease and has even been investigated as antiviral and anticancer (Ben Sahra, et al. 2010; Rotella, et al. 2006).

In fact, today, it is the most widely prescribed treatment to treat hyperglycemia in individuals with DM type 2 and is recommended as first-line oral therapy. Its beneficial effects are shown by acting on different organs such as liver and intestine involving complex mechanisms in which mitochondria is its main target (Foretz and Viollet 2015). It lowers blood glucose levels by suppressing hepatic glucose output, increasing insulin-mediated glucose disposal and intestinal glucose use, and decreasing fatty acid oxidation metformin is effective in reducing high blood glucose levels as many other antidiabetics but, unlike many of them, by itself, does not produce hypoglycemia. It also reduces insulin levels, inflammation and thrombosis, and the risks of metabolic syndrome and diabetes in healthy persons (Li, et al. 2015).

It also has beneficial effects on circulating lipids linked to cardiovascular diseases (Wu, et al. 1990). More interestingly it has shown neuroprotective effects. Some recent analyses have shown a reduction in PD risk in DM type2 when used in combination with sulfonylurea (Bayliss, et al. 2016; Wahlqvist, et al. 2012).

7.5. Metformin mechanism of action

The main action of metformin is to decrease glucose concentrations without causing hypoglycemia. This is mainly achieved by inhibiting gluconeogenesis thus decreasing hepatic glucose production (Natali and Ferrannini 2006). This inhibitory action on hepatic gluconeogenesis could be due to either reduction in the hepatic uptake of gluconeogenic substrates or changes in the enzyme activities (Argaud, et al. 1993; Radziuk, et al. 1997). It exerts its direct metabolic action on the liver (Fig. 17), apparently due to the predominant expression of organic cation transporters 1 (OCT 1) in hepatocytes, which in turn facilitates the cellular entry of metformin, due to its high dissociation constant. Under physiological conditions it exists in a positively charged protonated form and can only cross the plasma membrane by passive diffusion (Shu, et al. 2007).

The interest in the therapeutic use of metformin has been increased since the recognition of its pleiotropic actions in various tissues, which are affected by insulin resistance and /or hyperinsulinemia (Palomba, et al. 2009). This improvement in insulin sensitivity is associated with its positive effect on the expression of insulin receptors (Gunton, et al. 2003). According to Zhou et al. (Zhou, et al. 2001), the pleiotropic actions of metformin are intimately associated with the activation of AMP-activated protein kinase (AMPK), an important protein kinase enzyme in the body energy balance signaling and a master regulator of metabolism (Gruzman, et al. 2009). It acts as a fuel gauge monitoring systemic and cellular energy status controlling cellular functions under restrictive energy conditions (Galdieri, et al. 2016). It responds to metabolic requirments either by stimulating energy production including glucose and lipid catabolism or by inhibiting energy consuming pathways. It is known that impairment of AMPK activity can induce insulin resistance (Salminen, et al. 2011a).

AMPK is a heterotrimeric protein consisting of an α catalytic subunit and two β and γ regulatory subunits, where each subunit has at least two isoforms. AMPK is activated by an increase in the AMP/ATP ratio and/or intracellular ADP/ATP resulting from the imbalance between consumption

and production of ATP. This activation involves the binding of AMP to the regulatory sites of the γ subunit. This causes conformational changes that allosterically activate the enzyme and inhibit the dephosphorylation of Thr172 within the α subunit (activation of AMPK requires phosphorylation of Thr172 by kinases such as LKB1 and CaMKKβ). Once AMPK is activated by ATP depletion, it inhibits the pathways that consume ATP and stimulates alternative pathways for the regeneration of ATP (Carling 2004); that is, it changes the cells from an anabolic state (ATP consuming) to a catabolic one (ATP producing), closing the synthetic pathways that consume ATP and restoring the energy balance. As a result, the synthesis of glucose, lipids, and protein, and cell growth are inhibited while the oxidation of the fatty acids and the uptake of glucose are stimulated (Fig. 17).

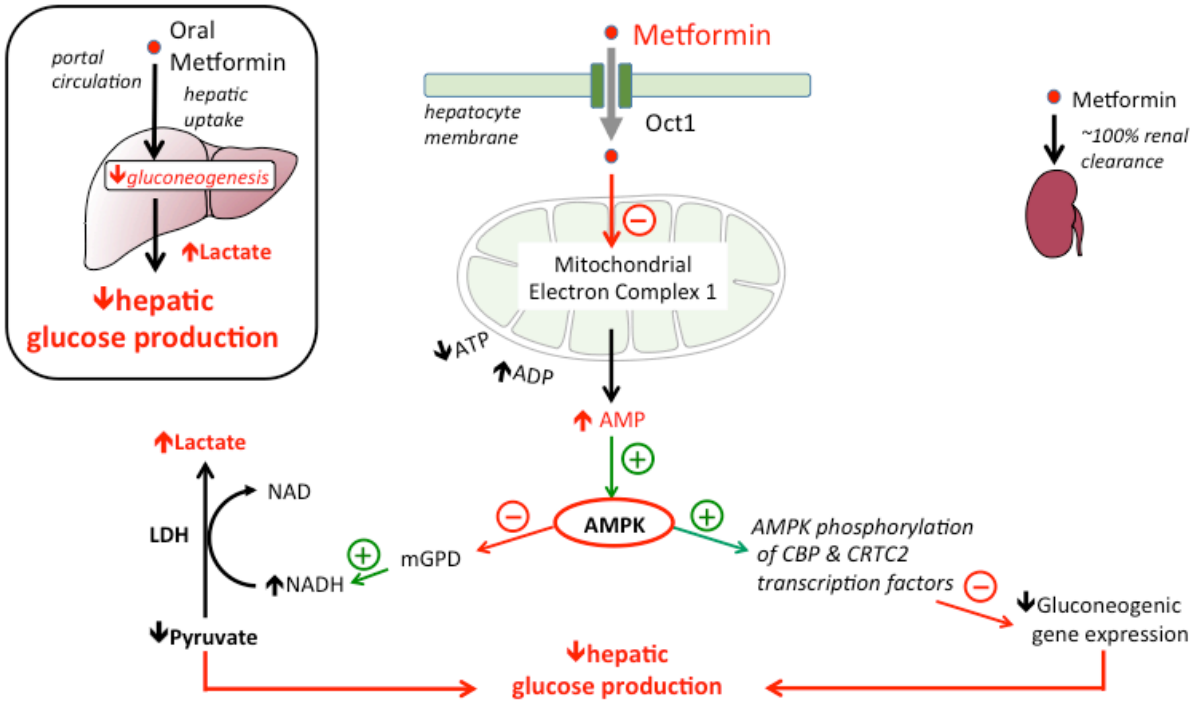

Figure 17. Mechanism of action of metformin (He and Wondisford 2015).

But the activation of AMPK by metformin does not seem to occur directly, but is secondary to its effect on the mitochondria, which is the drug primary target. As reported in early 2000, metformin induces specific inhibition of mitochondrial respiratory chain complex I (Fig. 18). This was initially observed in perfused livers and hepatocytes isolated from rodents (El-Mir, et al. 2000; Owen, et al. 2000), but later also in other tissues, such as skeletal muscle (Brunmair, et al. 2004), endothelial cells (Detaille, et al. 2005), pancreatic beta cells (Hinke, et al. 2007) and neurons (El-Mir, et al. 2008).

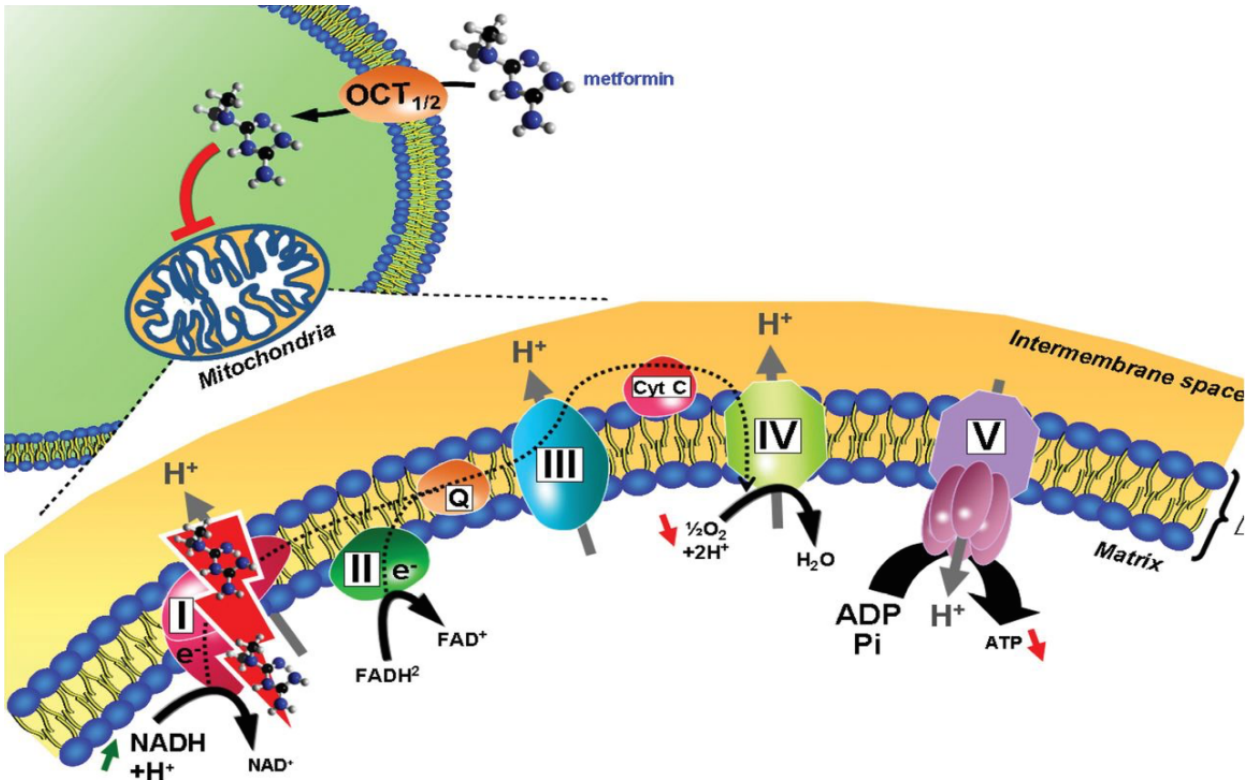

Figure 18. Metformin primary target is the mitochondrial respiratory chain complex 1.

(Viollet, et al. 2012).

Although the exact mechanism by which metformin inhibits complex I of the respiratory chain remains unknown, it has been shown that this effect does not require AMPK. Therefore, the activation of AMPK by metformin in the liver, and probably in other tissues, is the direct consequence of a transient reduction in the cellular energy state induced by the specific inhibition of the respiratory chain complex I by the drug (Stephenne, et al. 2011), which results in low oxygen consumption by the cells and an increase in the NADH/NAD ratio.

The β-oxidation of the fatty acids is carried out by the enzyme L-3 hydroxy acyl CoA dehydrogenase, which uses NAD as a cofactor. When there is an increase in the NADH/NAD ratio, the β-oxidation of the fatty acids is blocked, increasing the AMP/ATP ratio and resulting in the activation of AMPK (Aljada and Mousa 2012) (Fig. 19).

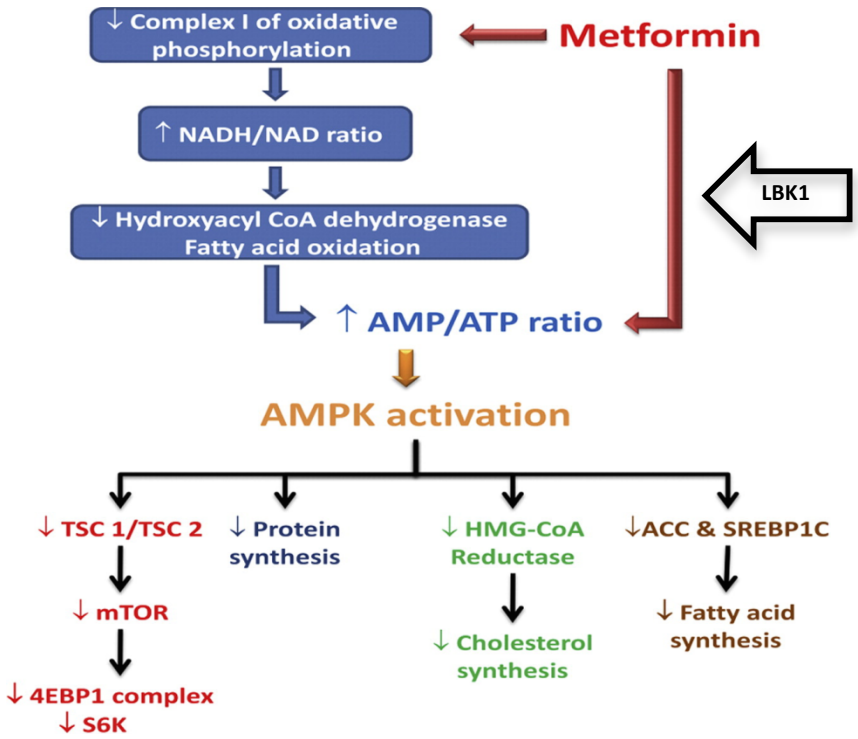

Figure 19. Metformin interrelation with AMPK (Aljada and Mousa 2012).

The demonstration that complex I of the respiratory chain and not AMPK is the main target of metformin was reinforced by showing that the metabolic effect of the drug is preserved in the liver of mice deficient in AMPK, so that other mechanism (s) independent of AMPK could be involved.

Changes in AMPK signaling are associated with human health, and implicated in T2DM, inflammatory disorders, stroke and neurodegenerative diseases (Salminen, et al. 2011b).

7.6. Anti-inflammatory effects of metformin

As it has already been demonstrated in different studies, inflammation seems to be implicated in several pathological mechanisms, such as, for example, those that lead to the death of DAergic neurons in PD. Several results from clinical and experimental studies suggest that metformin, in addition to its hypoglycaemic action, may attenuate both peripheral and central inflammation. The anti-inflammatory potential of metformin has been studied in particular in experimental models of peripheral inflammation. It has been shown that it attenuates the pro-inflammatory response in endothelial cells (Isoda, et al. 2006), decreases the proliferation of smooth muscle cells of human aorta (Li, et al. 2005) and reduces the activation of macrophages (Mamputu, et al. 2003).

Isoda et al. (Isoda, et al. 2006) investigated these anti-inflammatory effects in cells of the human vascular smooth muscle, where they observed that metformin exerts an anti-inflammatory. On the other hand, in endothelial cells, the activity of AMPK is associated with the phosphorylation and activation of endothelial nitric oxide synthase (eNOS), which results in an anti-inflammatory action in the vascular wall (Zou, et al. 2002).

Anti-inflammatory effects of metformin have also been described in human monocytes in which a reduction in the production of TNF and tissue factor is observed through the inhibition of ERK1/2-Egr.1 pathway (Arai, et al. 2010). Recent studies have also shown that metformin significantly reduces inflammatory cytokines such as TNF-α, monocyte chemoattractant protein 1 (MCP-1), IL-1β, macrophage inflammatory proteins (MIP-1α), IL-6, leptin, and IL-18 in uveitis induced by

endotoxin in rats (Kalariya, et al. 2012). Similarly, a recent study showed upon metformin treatment a reduction in macrophage infiltration and reduction in TNF-α, MCP-1 and leptin in adipose tissue of high-fat fed rats with insulin resistance (Lu, et al. 2016a).

Another study showed that metformin treatment inhibited the cardiac expression of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in endotoxin-induced acute myocarditis mice through the AMPK activation (Liu, et al. 2017a).

Jing et al. showed similar findings concerning metformin anti-inflammatory role in obese mice and palmitate induced activation of macrophages. *In vivo* and *in vitro* studies showed the ability of metformin to reduce the pro-inflammatory cytokines, TNF-α and IL-6, and alteration in macrophages polarization, decreasing the pro-inflammatory M1 macrophage proportion while increasing the anti-inflammatory M2 proportion (Jing, et al. 2018). Likewise, the therapeutic benefits of metformin have been described in endotoxin-induced hepatic injury, which suggests its pharmacological potency in inflammation disorders.

Other data supporting the anti-inflammatory effect of metformin were obtained by Koh et al. (2014) to find that metformin suppresses the activation of NFκB in intestinal epithelial cells and amyloid colitis in mice, suggesting that metformin could be used as a potential therapeutic agent for the treatment of inflammatory bowel diseases (Koh, et al. 2014).

Cameron et al. in 2016 concluded that the anti-inflammtory activity of metformin is exerted irrespective of the diabetes status, where metformin specifically blunted secretin of pro-inflammatory cytokines in macrophages without inhibiting M1/M2 activation or differentiation (Cameron, et al. 2016).

Vasamsetti et al. showed the ability of metformin to inhibit monocyte-to-macrophage differentiation in THP-1 cells, a human monocytic leukemia cell line, which can be considered a novel mechanism of metformin anti-inflammatory activity. This inhibition was AMPK dependent,

in a way by reducing STAT3 activity due to AMPK activation, thus reducing the production of pro-inflammatory cytokines. This inhibition of monocytes-macrophage differentiation by metformin could be a promising treatment in cases of vascular injury, atherosclerosis, cancer, and insulin resistance (Vasamsetti, et al. 2015) (Fig. 20).

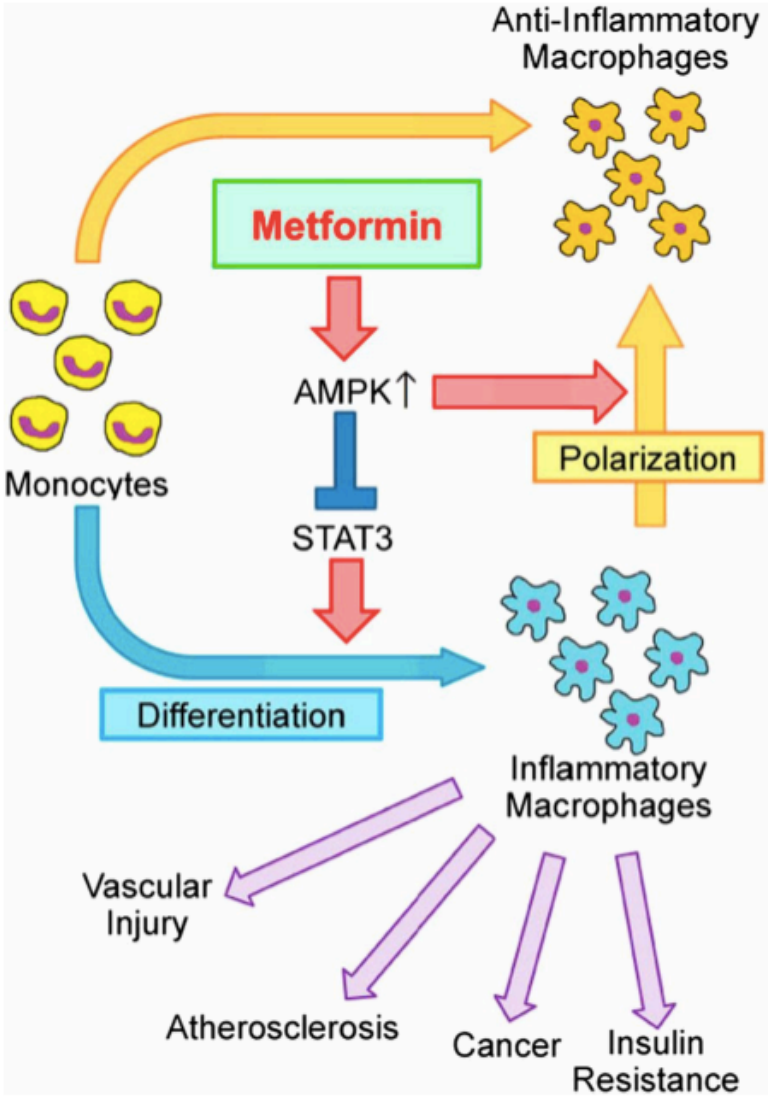

Figure 20. Anti-inflammatory effect of metformin through macrophage-targeting mechanism (Hattori, et al. 2015).

Thus the anti-inflammatory effect of metformin seems to be mediated by AMPK. When activated by metformin, AMPK inhibits NFκB signaling and, therefore, inflammation, since NFκB is the key inducer of inflammatory responses (Fig. 21). AMPK has several phosphorylation targets, but it seems to indirectly suppress NFκB signaling through its subsequent mediators, for example, SIRT1, FoxO, peroxisome proliferator-activated receptor γ coactivator 1-alpha (PGC-1α), which can thereafter repress the expression of inflammatory factors (Salminen, et al. 2011a).

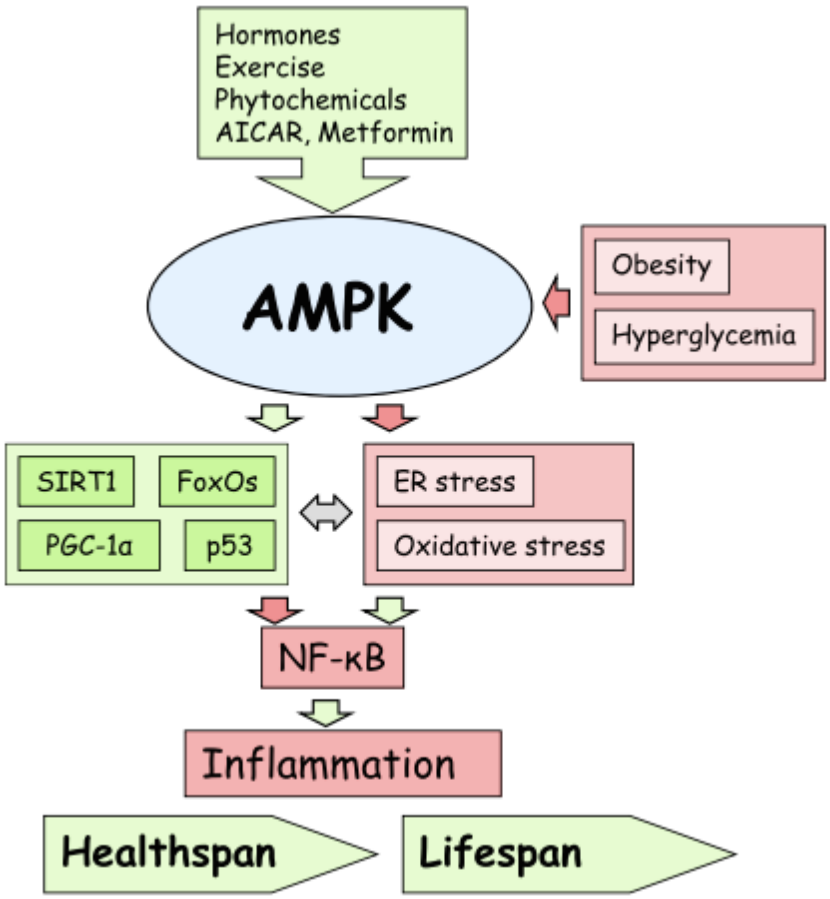

Figure 21. Schematic illustration of the functional relation of AMPK linked to the inhibition of NFκB signaling and inflammation suppression. Green arrows represent the activating pathways while red arrows are the inhibitory ones (Salminen, et al. 2011a).

It should be noted that there are other upstream activators of AMPK, such as CaMKKβ; Thus, AMPK activates the NFκB system via protein kinase Cδ and p38MAPK signaling (Bair, et al. 2009; Labuzek, et al. 2010b; Salminen, et al. 2011a). It is also known that some of the biological responses to metformin are not limited to the activation of AMPK but are mediated by other pathways independent of AMPK, including the inhibition of different intracellular targets such as p70S6K1 (Towler and Hardie 2007), p38 MAPK and protein kinase C (PKC) (Saeedi, et al. 2008).

It seems reasonable to consider that the activation of AMPK by metformin can affect not only peripheral tissues but also the brain, because it has been shown that the drug crosses the BBB and has been found to accumulate in the brain of rodents (Wilcock and Bailey 1994). Consequently, metformin is recognized as a drug that acts directly on the CNS and has been tested in several experimental models of neurodegeneration and neuroinflammation. Thus, for example, it prolongs the survival time in models of transgenic mice of Huntington's disease (Ma, et al. 2007), attenuates the experimental induction of autoimmune encephalomyelitis (Nath, et al. 2009), decreases migration and invasion of glioma cells U87 and LN229 (Beckner, et al. 2005) and exhibits neuroprotective effects against apoptosis induced by etoposide in primary cortical neurons (El-Mir, et al. 2008).

Chung et al 2017 provided a new evidence that metformin, through AMPK activation, has a protective effect against diabetic glycosylation end product (AGE) and amyloid beta (Aβ) induced inflammatory responses in human neuronal stem cells (hNSCs) (Chung, et al. 2017).

There is also evidence that metformin can modulate the pro-inflammatory response induced by LPS in rat primary microglia, both by mechanisms dependent on AMPK, that regulate the release of TNF-α, and by mechanisms independent of AMPK, that affect the release of IL-1β, IL-6, IL-10 and TGF-β, and that due to its inhibitory effect of the mitrochondrial respiratory chain complex I,

metformin inhibits the production of free radicals such as NO and ROS, increasing cell viability (Labuzek, et al. 2010b).

A recent study showed that metabolic effects of metformin when administrated intermittently in old mice were associated with an overall improvement in health. These improvements were manifested as reduction in body weight and liver steatosis (Alfaras, et al. 2017).

All these data suggest that metformin could be used as an adjuvant treatment in neurodegenerative pathologies, such as PD.

II. OBJETIVES

PD is the second most common neurodegenerative disease after Alzheimer´s disease that affects the elderly. It is characterized by the degeneration of DAergic neurons in the SNc of the brain.

The loss of these specific neurons results mainly in motor symptoms such as tremor, movement slowness and muscle stiffness. These symptoms may also be accompanied by non-motor symptoms. The etiology of PD is still ambiguous, although many risk factors were revealed and theories were established lately.

Cumulative evidence of neuroinflammation as an underlying process in many cases of PD has been accumulating in which microglia, the immune cells of the brain, are believed to be the main player. Since metformin has an established anti-inflammatory activity, the objective of this research is to study its anti-inflammatory effect on the neuroinflammation situation accompanying PD, which is responsible for the death of dopaminergic neurons of the SNc.

In vitro studies will be done using murine microglial BV2 cell-line stimulated by the pro-inflammogen LPS. Real-time RT-PCR technique will be used to check metformin effect on activated microglia phenotype, through measuring the release of different cytokines and chemokines such as TNF-α, IL-1β, iNOS, and C-X-C motif chemokine ligand 10 (CXCL10) after stimulation of BV2 cells with LPS, or arginase and IL-10 after stimulation with IL-4.

Also metformin effect on the oxidative stress will be examined through measuring the expression of NADPH oxidase subunits ($p22^{phox}$, $p47^{phox}$, $gp91^{phox}$) using RT-PCR and reactive oxygen species (ROS) production.

Since inflammasome is involved in inflammatory responses, the ability of metformin treatment to decrease inflammasome activation after LPS treatment in BV2 cells will be examined. This will be done using Western blot and ELISA for the measurement of IL-1β production.

Once the anti-inflammatory effect of metformin is proven *in vitro*, *in vivo* studies will be done. The aim is to examine metformin ability to protect the DAergic neurons of the SNc after inducing a strong inflammatory situation using an animal model of PD based on the intranigral injection of the bacterial inflammogen LPS.

Activation of SN microglia will be assessed using immunohistochemistry of morphological marker of reactive microglia such as OX-6. For Release of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and other chemokines, RT-PCR techniques will be carried out.

Metformin effect on the activated microglia phenotype will be assessed by double immunofluorescence using IKKβ and Iba-1 for the classical pro-inflammatory M1 phenotype, while arginase and Iba-1 markers will be used for the alternative anti-inflammatory M2 phenotype examination.

To complete our understanding of the protective effect of metformin on the inflammatory situation accompanying PD, astroglia status should be studied.

Activation of MAPKs will be determined by Western blot analysis using specific antibodies against the phosphorylated and non-phosphorylated forms of JNK, p38 and AKT. Finally integrity of the nigro-striatal DAergic neurons will be evaluated at the cellular level by immunohistochemistry against tyrosine TH, as an index of DAergic neurons.

All these data will allow us to study the effect of metformin treatment on the DAergic neurons of the SN after damage induced by LPS either *in vitro* or *in vivo*.

III. MATERIALS AND METHODS

1. MATERIALS

0.45µm nitrocellulose membrane	Bio-Rad Laboratories, Hercules, CA (USA)
30% Acrylamide solution	Bio-Rad Laboratories, Hercules, CA (USA)
Amonium persulfate	Sigma Aldrich, San Luis, MO (USA)
Anti- AKT	Santa Cruz Biotechnology, Dallas (USA)
Anti-arginase	Santa Cruz Biotechnology, Dallas (USA)
Anti-GAPDH	Santa Cruz Biotechnology, Dallas (USA)
Anti-GFAP	Chemicon International Inc, (USA)
Anti-Iba-1	Wako, Osaka, Japan
Anti-IKKβ	Santa Cruz Biotechnology, Dallas (USA)
Anti-JNK	Santa Cruz Biotechnology, Dallas (USA)
Anti-p38	Santa Cruz Biotechnology, Dallas (USA)
Anti-TH	Sigma Aldrich, San Luis, MO (USA)
ATP	Sigma Aldrich, San Luis, MO (USA)
Biotinylated anti-IgG	Vector (UK)
CellROX® Deep Red Reagent	Invitrogen, Thermo Fischer Scientific (USA)
Clarity™ and Clarity Max™ western ECL	Bio-Rad Laboratories, Hercules, CA (USA)
DAB	Sigma Aldrich, San Luis, MO (USA)
DMEM high glucose	Invitrogen, Carlsbad, CA (USA)
DMSO	Sigma Aldrich, San Luis, MO (USA)
DPX mountant	VWR (USA)
ExtraAvidin®-Peroxidase	Sigma Aldrich, San Luis, MO (USA)
Heat-inactivated fetal bovine serum (FBS)	Sigma Aldrich, San Luis, MO (USA)

Histolemon	Carlo Erba®, France
Hydrogen peroxide	Sigma Aldrich, San Luis, MO (USA)
Ibidi® plates	Invitrogen, Carlsbad, CA (USA)
IL-4	Sigma Aldrich, San Luis, MO (USA)
LPS from Escherichia coli serotype 026: B6	Sigma Aldrich, San Luis, MO (USA)
Metformin	Dianben® 1000mg, MERK, (USA)
Monastral Blue inert tracer	Sigma Aldrich, San Luis, MO (USA)
Mouse IL1β ELISA Ready-SET-GO	Invitrogen, Thermo Fischer Scientific (USA)
OX-6	Serotec, Oxford, (UK)
Paraformaldehyde	Carlo Erba®, France
PBS	Sigma Aldrich, San Luis, MO (USA)
Pierce™ BCA protein assay kit	Thermo Fischer Scientific (USA)
Protease inhibitor	Sigma Aldrich, San Luis, MO (USA)
Revert Aid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific (USA).
RIPA buffer	Sigma Aldrich, San Luis, MO (USA)
RNeasy® kit	QIAGEN, Gmbh, Hilden (Germany)
SDS	Sigma Aldrich, San Luis, MO (USA)
SensiFASTTM SYBR NO-ROX KIT	Bioline,(USA)
Streptomycin/penicillin	Biowest, Nuaillé (France)
TBS	Sigma Aldrich, San Luis, MO (USA)
TEMED	Bio-Rad Laboratories, Hercules, CA (USA)
Tris	Sigma Aldrich, San Luis, MO (USA)
TRIsure™	Bioline, (USA)

Trypan blue	Sigma Aldrich, San Luis, MO (USA)
Trypsin-EDTA	Biowest, Nuaillé, France
TWEEN 20	Sigma Aldrich, San Luis, MO (USA)
Trypan blue	Sigma Aldrich, San Luis, MO (USA)
DMSO	(Sigma Aldrich, San Luis, MO, USA)

2. METHODS

1. Cell culture

For all cell culture experiments, murine microglial BV2 cell line was used. These cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, San Luis, MO, USA), streptomycin (100 mg/ml), and penicillin (100 IU/ml) (Biowest, Nuaillé, France), under 100% humidity and 5% CO_2. Experiments were performed in reduced 5% FBS media. The medium was changed every 3 days and allowed to grow up to 80% confluence. All experiments were carried out in 3-20 passage cells. The passing and treatment of cells were always carried out in a laminar flow hood.

2.1.1. Thawing cells

The thawing of the vials to recover the conserved cells was done as fast as possible, according to the following protocol: Vials were placed in a 37°C bath until the cell suspension was thawed. Then the thawed cell suspension was transferred to a centrifuge tube with 9 ml of culture medium preheated to 37 ºC. Cells were centrifuged at low speed (300 xg) for 5 min at 4 ºC, the precipitate resuspended in preheated culture medium and seeded in a flask (Sarstedt AG & Co, Germany).

2.1.2. Passing cells

The separation of the crops was carried out when their growth reached a 80% confluence, by the following procedure. Cells were washed with phosphate buffer (PBS) 1X, deattached from the flasks by adding enough 1X trypsin-EDTA (Biowest, Nuaillé, France) to cover the crop surface, and incubated for 5 min at 37 ºC. To stop the trypsinization, pre-heated culture medium at 37 ºC was added. Then cells were collected, centrifuged at 500 xg, for 5 minutes at room temperature, and the precipitate resuspended in a desired volume and reseeded. To determine the number of living cells, a Neubauer chamber and Trypan blue (Sigma Aldrich, San Luis, MO, USA) were used. Trypan blue is a colloid that is introduced inside the cells that have ruptured membranes, thus dead cells will

have blue color while white cells are the viable ones. The used procedure was as follows. Cell suspension was homogenized and 10 µL transferred into an eppendorf tube where an equal volume of a 0.4% solution of Trypan blue was added. The compartment of the Neubauer chamber was filled with 10 µL of the suspension and an inverted microscope (Leica DMIL) was used to count the number of cells. The number of cells/ml was calculated using the following formulas:

Cells/ml = mean of the number of cells counted in the four grids × 10^4 × 2 (Dilution factor)

Total cells = (Cells / ml) × original volume of cell suspension.

2.1.3. Conservation of cells

For the purpose of conserving the cells, the following protocol was followed. 10 ml of a cell suspension was collected and centrifuged at 500 xg for 5 min at room temperature. Then the precipitate was resuspended in 5-10 ml of freezing medium consisting of 10% (w/v) dimethyl sulfoxide (DMSO) (Sigma Aldrich, San Luis, MO, USA), as a cryoprotectant, and 90% DMEM supplemented with 10% FBS. The cell suspension was aliquotted in sterile freezing cryogenic vials. Since DMSO is toxic at room temperature, it was convenient to proceed quickly to the cooling of the vials. However, freezing must be carried out slowly. For this purpose, the vials were put in a MR. Frosty™ (ThermoFischer) freezing box containing isopropanol to allow gradual decrease of temperature and kept at -80 ºC for two days, then moved to liquid nitrogen for long storage.

2. Animals and surgery

Male albino Wistar rats (200–270 g) were used for these studies. The rats were kept at constant room temperature of 22 ± 1 ºC and relative humidity of 60% with a 12-h light–dark cycle with free access to food and water. Rats were anaesthetized with isoflurane and positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA) to conform to the brain atlas of Paxinos and Watson (1986). Injections into the SN were made 5.5 mm posterior, 1.8 mm lateral and 8.3 mm ventral to the bregma point at day 1.

Experiments were carried out in accordance with the Guidelines of the European Union Directive (2010/63/EU) and Spanish regulations (BOE 34/11370-421, 2013) for the use of laboratory animals; the study was approved by the Scientific Committee of the University of Seville.

3. Treatment

2.8.1. *In vitro* treatment

For testing the effect of metformin on the pro- and anti-inflammatory phenotypes of microglial cells, BV2 cells were treated with LPS (pro-inflammatory phenotype inducer, 1 µg/ml, Sigma-Aldrich, San Luis, MO, USA) or IL-4 (anti-inflammatory phenotype inducer, 20 ng/ml, Sigma-Aldrich, San Luis, MO, USA), with and without metformin (1 mM, Dianben® 1000mg, MERK) for 12 h (Fig. 22 A). The effect on production of ROS was measured by treating cells with LPS and/or metformin for 24 h (Fig. 22 B). The effect of metformin on inflammasome activation was tested by Western Blot and ELISA, cells were treated with LPS and/or metformin for 3 h then ATP (1 mM, Sigma-Aldrich, San Luis, MO, USA) was added for 1 h as the second signal for inflammasome activation (Martinon et al., 2002) (Fig. 22C). At least four experiment were done for each technique.

2.8.1. *In vivo* treatment

Animals were divided into four groups according to two different variables (Fig. 23): solution administered orally by a plastic enteral feeding tube (tap water or 150 mg/kg of metformin dissolved in tap water, 4 ml/kg) and product injected in the SN (2 µl of saline solution or 2 µg of LPS dissolved in 2 µl of saline solution). In group I (the control group), animals received a single intranigral injection of saline solution (Monastral Blue inert tracer, 1% in saline solution; Sigma-Aldrich, St Louis, MO, USA) the first day.

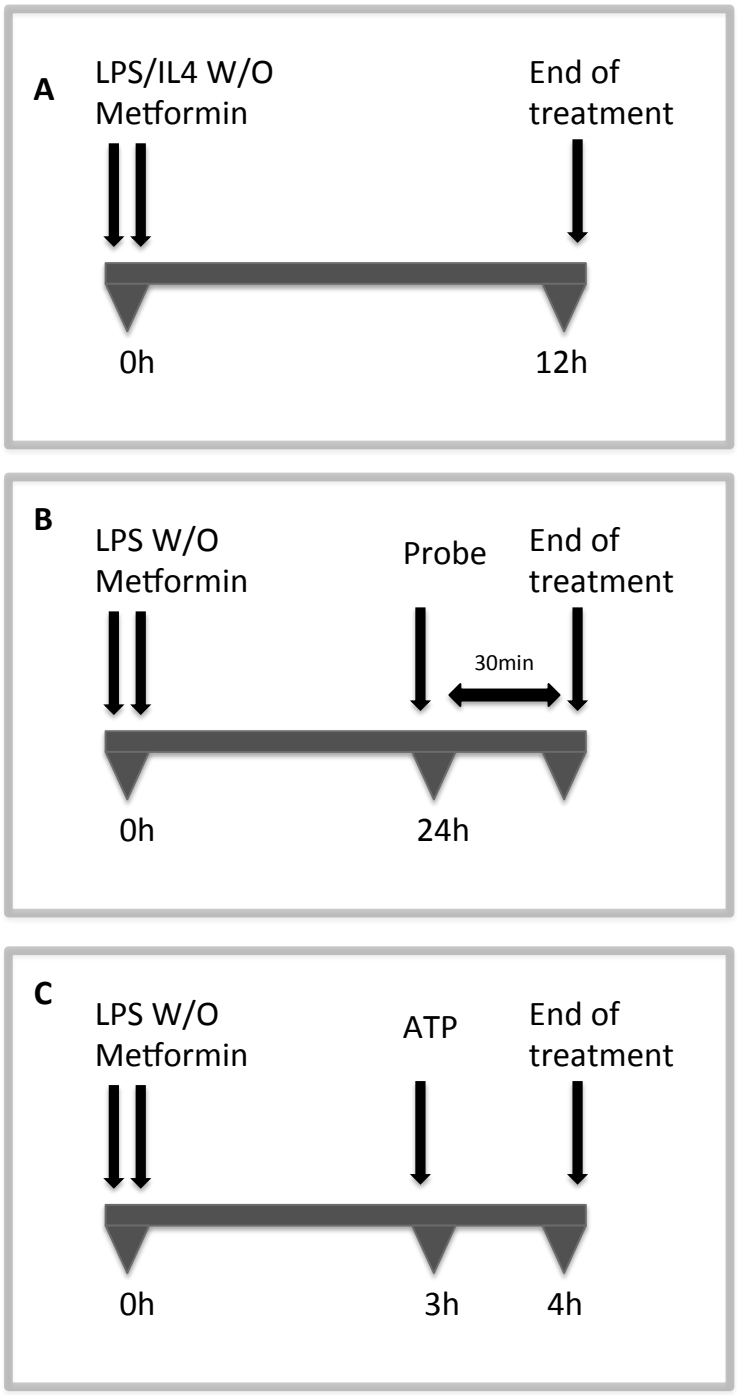

Figure 22. Time course of different *in vitro* treatments. A. Real-time PCR treatment, **B.** ROS measurement treatment, **C.** Inflammasome activity treatment. (LPS W/O with or without metformin)

Twice a day, animals received 4 ml/kg of tap water through the plastic tube for seven days. Group II received a single intranigral injection of saline the first day and two daily doses of metformin dissolved in tap water for seven days. Group III received a single intranigral injection of LPS (from

E.coli, serotype 026:B6; Sigma Aldrich, San Luis, MO, USA) the first day and 4 ml/kg of tap water through the plastic tube twice a day for seven days. Group IV received a single intranigral injection of LPS the first day and two daily doses of metformin dissolved in tap water for seven days. The first dose of metformin or water was administrated 1 h before intranigral injection. All animals were sacrificed seven days after the initiation of treatment, unless otherwise stated. At least four animals were used for each group.

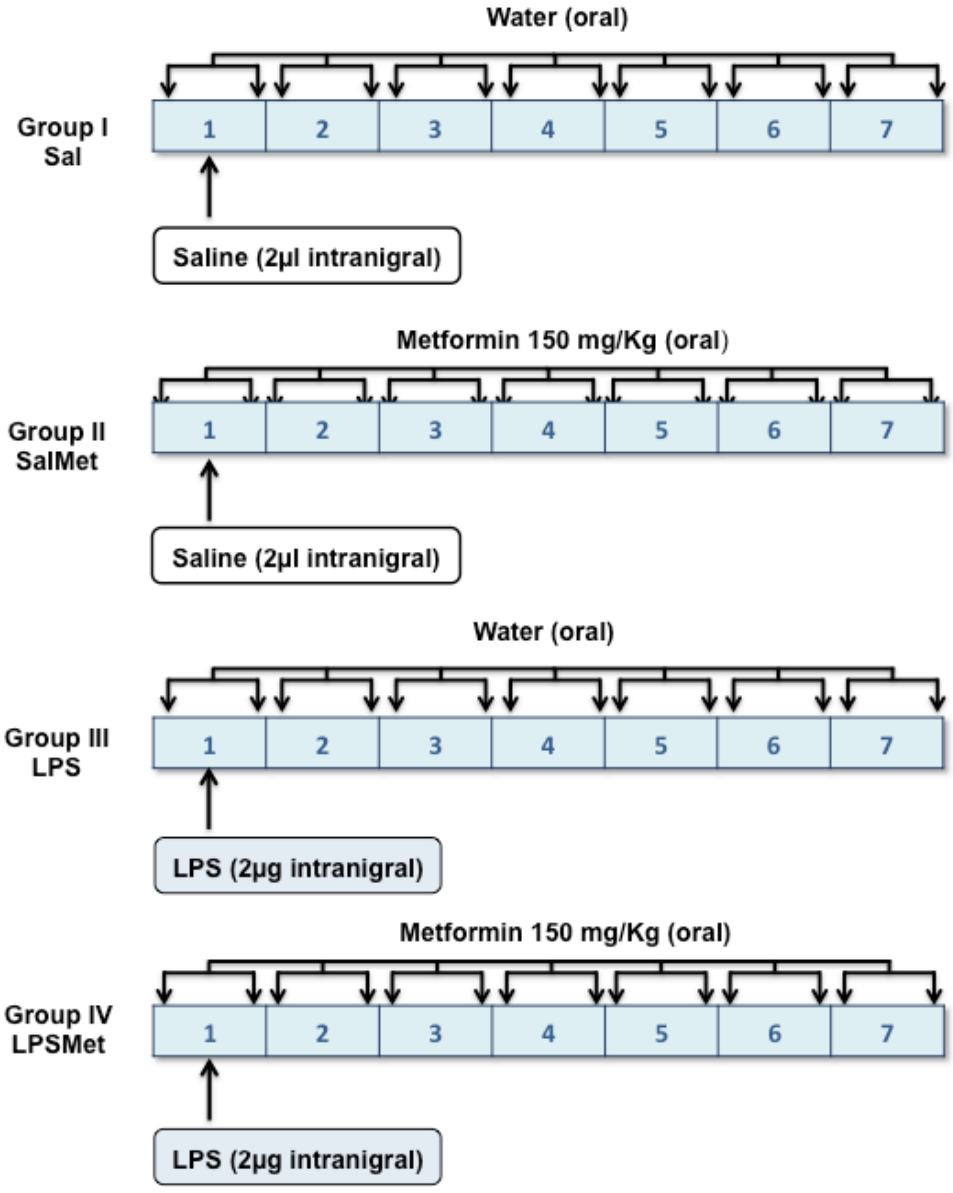

Figure 23. Schematic representation of the experimental design: groups and treatments *in vivo*. Tap water or metformin were administrated orally, twice a day for 7 days to the corresponding groups. Saline solution or LPS were injected intranigrally to the corresponding groups the first day of the experiment, before tap water or metformin were administered.

4. Immunohistological evaluation: Tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), and OX-6

Animals used for immunohistochemistry completed 7 days of treatment with water/metformin. One week after the surgical procedure in the SN, animals were perfused through the heart under deep anesthesia (isofluorane) with 150-200 ml of 4% paraformaldehyde in phosphate buffer, pH 7.4. The brains were removed and then cryoprotected serially in sucrose dissolved in phosphate-buffered saline (PBS), pH 7.4, first (24 h) in 10% sucrose, next (24 h) in 20% sucrose, and finally in 30% sucrose until they sank (2–5 days). Brains were then frozen in isopentane at -20 ºC, and sections of 20 μm thickness for each were cut on a cryostat at -20 ºC, and mounted in gelatine-coated slides. Primary antibodies used were rabbit-derived anti-tyrosine hydroxylase (anti-TH, Sigma, 1:300), mouse-derived antiglial fibrillary acidic protein (anti-GFAP; Chemicon International Inc, USA.; 1: 300) and mouse-derived OX-6 (Serotec, Oxford, UK; 1: 200). OX-6 is directed against a monomorphic determinant of the rat MHCII antigens, expressed by activated microglia but not for the resting cells. Incubations and washes for all the antibodies were in Tris-buffered saline (TBS), pH 7.4. All work was performed at room temperature.

Sections were washed and then treated with 0.3% hydrogen peroxide in methanol for 15 min, washed again, and incubated in a solution containing TBS and 1% horse (for GFAP and OX-6) or goat (for TH) serum for 60 min in a humid chamber. Slides were drained and further incubated with the primary antibody in TBS containing 1% horse serum/goat and 0.25% Triton-X-100 for 24 h. Sections were then incubated for 2 h with biotinylated horse anti-mouse (for GFAP and OX-6) or biotinylated goat anti-rabbit (for TH) IgG (Vector, 1:200). The secondary antibody was diluted in TBS containing 0.25% Triton-X-100, and its addition was preceded by three 10-min rinses in TBS. Sections were then incubated with ExtrAvidin®-Peroxidase solution (Sigma Aldrich, San Luis, MO, USA, 1:100) for 1h. The peroxidase was visualized with a standard diaminobenzidine/hydrogen reaction for 5 min.

5. Immunohistochemistry data analysis

Analysis were made in a bounded region of the SN with a length of 300 μm in the anterior-posterior axis centred at the point of injection (5.5 mm with respect to bregma), that is, between 5.35 and 5.65 mm with respect to bregma. In each case, five sections per animal were used, with random starting point and systematically distributed through the anterior-posterior axis of the analyzed region. For the measurement of areas lacking GFAP immunoreactivity, the AnalySIS imaging software (Soft Imaging System GmbH, Münster, Germany) coupled to a Polaroid DMC camera (Polaroid, Cambridge, MA) attached to a Leika light microscope (Leika Mikroskopie, Wetzlar, Germany) was used. For counting cells showing OX-6 immunoreactivity, a systematic sampling of the area occupied by the OX-6 positive cells in each section was made from a random starting point with a grid adjusted to count five fields per section. An unbiased counting frame of known area (40 x 25 μm = 1000 μm^2) was superimposed on the tissue section image under a 100X oil immersion objective. The different types of OX-6-positive cells (displaying different shapes depending on their activation state) were counted as a whole and expressed as cells per mm^2. The number of TH-positive neurons in the SN was estimated using a fractionator sampling design (Gundersen et al. 1988). Counts were made at regular predetermined intervals (x = 150 μm and y = 200 μm) within each section. An unbiased counting frame of known area (40 × 25 μm = 1000 μm^2) was superimposed on the tissue section image under a 100× oil immersion objective. Therefore, the area sampling fraction is 1000/(150 × 200) = 0.033. The entire z-dimension of each section was sampled; hence, the section thickness sampling fraction was 1. In all animals, 20-μm sections, each 100 μm apart, were analyzed; thus, the fraction of sections sampled was 20/100 = 0.20. The number of neurons in the analyzed region was estimated by multiplying the number of neurons counted within the sample regions by the reciprocals of the area sampling fraction and the fraction of section sampled.

6. Immunofluorescence

Animals were perfused and sections were prepared as described above. Incubations and washes for all the antibodies were in PBS, pH 7.4. All work was carried out at room temperature.

For double-labelling of Iba-1 with IκB kinase β (IKKβ) or aginase, sections were blocked with PBS containing 1% goat serum (Vector, for Iba-1) and rabbit serum (Invitrogen, for IKKβ or arginase) for 1 h. The slides were washed three times in PBS and then incubated overnight at 4 ºC with either rabbit-derived anti-Iba-1 (1:300; Wako), goat-derived anti-IKKβ (1:300; Santa Cruz Biotechnology) and goat-derived anti-arginase (1:50; Santa Cruz Biotechnology), diluted in PBS containing 1% goat/rabbit serum and 0.25% Triton X-100. Sections were incubated with goat anti-rabbit secondary antibody conjugated to Alexa Fluor® 594 (1:200, for Iba-1; Invitrogen) and rabbit anti-goat secondary antibody conjugated to fluorescein (1:200, for IKKβ and arginase; Vector) for 1 h at 22 ± 1 ºC in the dark and its addition was preceded by three 10-min rinses in PBS. As a control, another set of experiments was performed where the sections were only incubated with the Iba-1 antibody and then visualized with both filters. No signal was detected when Iba-1 alone with fluoresceine filter was used (photomicrograph not shown). The same was true with IKKβ or arginase when Alexa Fluor® 594 filter was used.

Fluorescence images were acquired using a confocal laser scanning microscope (Zeiss LSM 7 DUO) and processed using the associated software package (ZEN 2010).

7. Real-time RT-PCR

BV2 cells were seeded at a concentration of 1×10^5 cells/well in 12-well plate (Sarstedt AG & Co, Germany) and then treated with LPS (1μg/ml) or IL-4 (20 ng/ml), with and without metformin (1 mM). Treatments were conducted for 12 h. At the end of the treatment, the medium was removed and cells were washed twice with PBS. Then cells were collected in TRIsure™ (Bioline, USA) and RNA was extracted from cells according to the following protocol:

Cells were lysed directly in the wells by adding 800 µl of TRIsure per well, the cell lysate was pipetted several times to ensure sufficient cell disruption. Samples were incubated for 5 minutes at room temperature, then 0.16 ml of chloroform was added, tubes were capped securely and the sample was shook vigorously by hand for 15 seconds. Afterwards, samples were incubated for 3 minutes at room temperature, and centrifuged at 12,000 x g for 15 minutes at 4 ºC. The sample separated into a pale green, organic phase, an interphase, and a colorless upper aqueous phase; the aqueous phase was collected because it contained the RNA which was precipitated by mixing with 0.4 ml cold isopropyl alcohol. Samples were incubated for 10 minutes at room temperature then centrifuged at 12,000 ×g for 10 min at 4 ºC. The supernatant was removed and the pellet was washed once with 75% ethanol. Samples were vortexed and centrifuged at 7500 ×g for 5 minutes at 4 ºC. The pellet was air-dried and dissolved in 30-50 µl PCR water by pipetting the solution up and down. Finally RNA was quantified using a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using 1 µg of extracted RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) in 20 µl reaction volume as described by the manufacturer.

Animals used for RT-PCR followed the procedure described above but were sacrificed by decapitation 6 hours after LPS/saline injection in the SN, thus they only received a single dose of metformin/water orally 1 hour before the LPS/saline injection. SN was dissected from each rat, snap frozen in liquid nitrogen and stored at -80 ºC. Total RNA was extracted from the rat SN of different treatments using RNeasy® kit (QIAGEN,Gmbh, Hilden, Germany). cDNA was synthesized from 1 µg of total RNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) in 20 µL reaction volume as described by the manufacturer.

Real-time PCR was performed using 5 µL SensiFASTTM SYBR NO-ROX KIT (Bioline, USA), 0.4 µL of each primer (stock concentration was 10 µM) and 4.2 µL cDNA to get to a final reaction volume of 10 µL for 384 well-plate. Controls were carried out without cDNA. Amplification was

run in a Lightcycler® 480 Instrument II (Roche, Basel, Switzerland) thermal cycler at 95 ºC for 2 minutes followed by 40 cycles consisting of a denaturation phase for 5 seconds at 95 ºC, followed by a second phase of hybridization at 65 ºC for 10 seconds, and a final phase of elongation at 72 ºC for 20 seconds. The process was terminated by a final step of 7 minutes at 72 ºC. Analysis confirmed a single PCR product. β-actin served as reference gene and was used for samples normalization. The cycle at which each sample crossed a fluorescence threshold (Ct value) was determined, and the triplicate values for each cDNA were averaged. The primer sequences for IL-1β, TNF-α, arginase, IL-10, IL-6, CX3C chemokine receptor 1 (CX3CR1), CD200, MCP-1, NAPDH subunits (P22, P47, and P91) and β-actin are shown in Table 1 and 2. All primers were purchased from Sigma Aldrich (San Luis, MO, USA).

Table 1. Primers used for RT-PCR *in vitro*.

mRNA	**Primers**
IL-1β	F: 5′-TTGACGGACCCCAAAAGATG-3′ R: 5′-AGAAGGTGCTCATGTCCTCA-3′
TNF-α	F: 5′-AGCCCACGTCGTAGCAAACCACCAA-3′ R: 5′-AACACCCATTCCCTTCACAGAGCAAT-3′
iNOS	F: 5′-CTTTGCCACGGACGAGAC-3′ R: 5′-TCATTGTACTCTGAGGGCTGAC-3′
Arginase	F: 5′- TCA CCT GAG CTT TGA TGT CG -3′ R: 5′- CTG AAA GGA GCC CTG TCT TG -3′
IL-10	F: 5′- CCAAGCCTTATCGGAAATGA -3′ R: 5′- TTTTCACAGGGGAGAAATCG -3′
CXCL10	F: 5'- AAGCATGTGGAGGTGCGAC-3′ R: 5'- CTAGGGAGGACAAGGAGGGT-3′
β-actin	F: 5′-CCA CAC CCG CCA CCA GTT CG-3′ R: 5′-CCC ATT CCC ACC ATC ACA CC-3′

Table 2. Primers used for RT-PCR *in vivo*

mRNA	Primers
TNF-α	F: 5′-TACTGAACTTCGGGGTGATTGGTCC-3′ R: 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′
IL-6	F: 5′-AAAATCTGCTCTGGTCTTCTGG-3′ R: 5′-GGTTTGCCGAGTAGACCTCA-3′
IL-1β	F: 5′-CAGGATGAGGACATGAGCACC-3′ R: 5′-CTCTGCAGACTCAAACTCCAC-3′
iNOS	F: 5′-CCTCCTCCACCCTACCAAGT-3′ R: 5′-CACCCAAAGTGCTTCAGTCA-3′
CD200	F: 5′-TGTTCCGCTGATTGTTGGC-3′ R: 5′- ATGGACACATTACGGTTGCC-3′
CX3CR1	F: 5′-GGC CTT GTC TGA TCT GCT GTT TG-3′ R: 5′- AAT GCT GAT GAC GGT GAT GAA GAA-3′
MCP-1	F: 5′-AGCATCCACGTGCTGTCTC-3′ R: 5′-GATCATCTTGCCAGTGAATGAG-3′
β-actin	F: 5′-TGTGATGGTGGGAATGGGTCAG-3′ R: 5′-TTTGATGTCACGCACGATTTCC-3′
$p22^{phox}$	F: 5′-GAATTCCGATGGGCAGATCGA-3′ R: 5′-GGAQTCCCGTCACACGACCTCA-3′
$p47^{phox}$	F: 5′-ATTTGGAGCCCTTGACAG-3′ R: 5′-GATGGTTACATACGGTTCACCTG-3′
$gp91^{phox}$	F:5′-GCACAGCCAGTAGAAGTAGATCTTT-3′ R: 5′-GCTGGGATTGGAGTCACG-3′

8. Western blot

2.8.1. Protein extraction from BV2 cells

In order to obtain proteins from BV2 cell line, cells were seeded in 6-well plates (Sarstedt AG & Co, Germany) at a seeding concentration of 200 × 10^3 cells/well and left for 24 h at 37 ºC to reach the required confluence. Then cells were treated for 4 h as previously described (*In vitro* treatment section) and collected in 50 µl RIPA buffer (Sigma-Aldrich) with the aid of a scraper after being washed three times with ice-cold PBS. Cells lysate was sonicated (10% amplitude, 10 s, on ice) using a probe sonicator (Hielscher Ultrasound Technology). All steps were carried out on ice. Protein concentrations were determined using Pierce™ BCA protein assay kit (Thermo Fischer Scientific, USA).

2.8.1. Protein extraction from SN

Animals used for Western blot followed the procedure described above (*In vivo* treatment section) and were sacrificed by decapitation seven days after LPS/saline injection in the SN. Proteins were extracted from the SN of rats brains, through the addition of 350 µl RIPA buffer along with 3.5 µl protease inhibitor to the SN followed by sonication (10% amplitude, 10 s, on ice). Afterwards, samples were heated in the thermoblock for 5 min at 37 ºC, left 20 min on ice and centrifuged (4 ºC, 13000 ×*g*, 15 min). The clear supernatant which contained the protein was collected and quantified with Pierce™ BCA protein assay kit (Thermo Fischer Scientific, USA).

2.1.1. Western blot analysis

Proteins (40 µg) were separated on 10% SDS-PAGE gel, subjected to electrophoresis at 150 V, and then transferred using Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, Hercules, CA,

USA) onto 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) for 7 min.

After blocking with 5% skimmed milk diluted in TBS-Tween (TBST) (0.05% Tween 20 in TBS) for 1 h, membranes were incubated with a primary antibody against phosphorylated and non-phosphorylated forms of the MAPKs family proteins P38 and JNK (Santa Cruz Biotechnology) for *in vivo* experiments and against IL-1β (R&D Systems, Minneapolis, USA) for *in vitro* experiment. Antibodies of non-phosphorylated forms of MAPKs and the housekeeping GAPDH were used at a dilution of 1:1000 in 5% skimmed milk dissolved in TBST, while the antibodies of the phosphorylated forms of MAPKs and IL-1β were used at a dilution of 1:500 in 5% skimmed milk dissolved in TBST.

Membranes were incubated with these antibodies for 24 h at 4 ºC. Membranes were washed 4 times (10 min each) with TBST, then incubated for 1 h with the secondary antibody (1:5000) in 5% skimmed milk dissolved in TBST against the specific primary antibody at room temperature. Another 4 washes with TBST (10 min each) were done before visualizing the bands with Clarity™ and Clarity Max™ western ECL Blotting Substrates (Bio-rad, Laboratories, Hercules, CA, USA) using Amersham Imager 600 Imagers (general electric, USA). These images were later analyzed using the ImageJ software.

9. Production of reactive oxygen species

The level of oxidative stress produced in viable BV2 cell cultures was determined by seeding 35 × 10^3 cells/well in Ibidi® plates (Invitrogen, Carlsbad, CA, USA), followed by incubation for 24 h at 37 ºC. Then, cells were treated with metformin (1 mM) and/or LPS (1 µg/ml) for 24 hours At the end of the treatment a membrane permeable reagent (CellROX® Deep Red Reagent) was added to each well. This reagent is a fluorogenic probe that exhibits fluorescence signal in the presence of ROS in the cell cytoplasm.

The test was performed following the manufacturer's instructions. The reactive was added to the treated cells at a final concentration of 5 mM, and incubated at 37 ºC for 30 min. Subsequently, the media was removed and cells were washed with PBS three times. Confocal microscope (Zeiss LSM 7 DUO) was employed to acquire images of the treated cells. Images of five fields per condition were captured. These images were later analyzed using the ImageJ software. In each image the total number of cells was calculated, and the area integrated intensity for fluorescent and non-fluorescent cells was measured. Consequently, the following formula was used to compute the corrected total cell fluorescence (CTCF).

CTCF = Integrated density – (Area of selected cell × Mean fluorescence of background readings)

10. Enzyme-linked immunosorbent assay

BV2 cell cultures were seeded in 24-well plates at a concentration of 75×10^3 cells/well. Then, cells were treated with metformin (1 mM) and/or LPS (1 μg/ml) and incubated at 37 ºC for 3 hours. Afterwards, ATP (1mM) was added to each for 1 h. At the end of the treatment, the supernatant was collected and centrifuged at 400xg , 5 min, 4 ºC to remove cell debris. After centrifugation, the supernatant was stored at -80 ºC for subsequent use. 100 μl of the supernatant was used for the measurement of IL-1β production by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (Invitrogen, Mouse IL1β ELISA Ready-SET-GO! Thermo Fisher Scientific). All standards and samples were run in duplicates. Samples were diluted in a proportion of 1:1 in ELISA/ELISASPOT Diluent (1×), except LPS containing samples, which were diluted in a proportion of 1:2.

11. Statistical analysis

Results are expressed as mean ± SD. Means were compared by One-Way ANOVA followed by the LSD test for *post hoc* multiple range comparisons. An alpha level of 0.05 was used. The Statgraphics Plus 3.0 statistical package was used for the analyses.

IV. RESULTS

1. *IN-VITRO* EXPERIMENTS

1.1. Metformin treatment reduces microglial activation *in vitro*

Activated microglia can be categorized into the classically pro-inflammatory and neurotoxic M1 phenotype, and the alternative activated anti-inflammatory M2 phenotype involved in the resolution of inflammation, phagocytosis and tissue repair. Depending on the inductor of their activation, when microglia are activated they produce different cytokines and pro or anti-inflammatory substances with different actions. When LPS activates microglia, these cells produce pro-inflammatory cytokines such as IL-6, TNFα and IL-1β, and oxidative metabolites like nitric oxide; while IL-4 treatment induces microglia activation toward M2 phenotype, expressing lower levels of these previously mentioned markers, but higher levels of IL-10, IL-4, and arginase (Fig. 24) (Kim, et al. 2016).

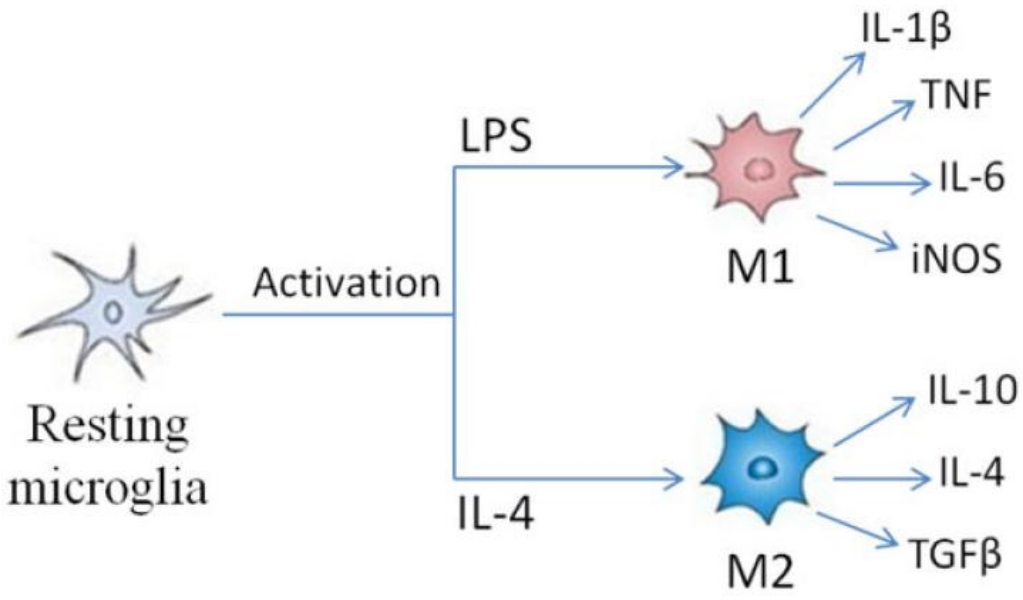

Figure 24. Microglia phenotypes. Activated microglia has two activation phenotypes: classically activated (M1) and alternatively activated (M2). Taken from(Liu, et al. 2017b).

In this work, BV2 cells were treated with LPS to induce a M1 phenotype, or with IL-4 to induce a M2 phenotype. When BV2 cells were treated with LPS, RT- PCR analysis showed a strong induction of the four pro-inflammatory mediators studied, ranging from 348% for TNF-α to 1438% for IL-1β with respect to the control ($p < 0.001$; Fig. 25). Metformin treatment reduced the expression levels of the mRNA of IL-1β (56 ± 11.5% with respect to the LPS; $p < 0.001$; Fig. 25A). No statistical difference was found in the amounts of iNOS, TNF-α, and CXCL10 mRNAs when metformin was added to LPS-treated cells. When BV2 cells were treated with IL-4 a strong induction of arginase was observed (20000-fold compared to the control group, $p < 0.001$; Fig. 25D). This induction was decreased by 37.7% by metformin treatment ($p < 0.001$; Fig. 25E). However no statistical difference was found concerning the levels IL-10 mRNA when metformin was added to IL-4 treated cells (Fig. 25F).

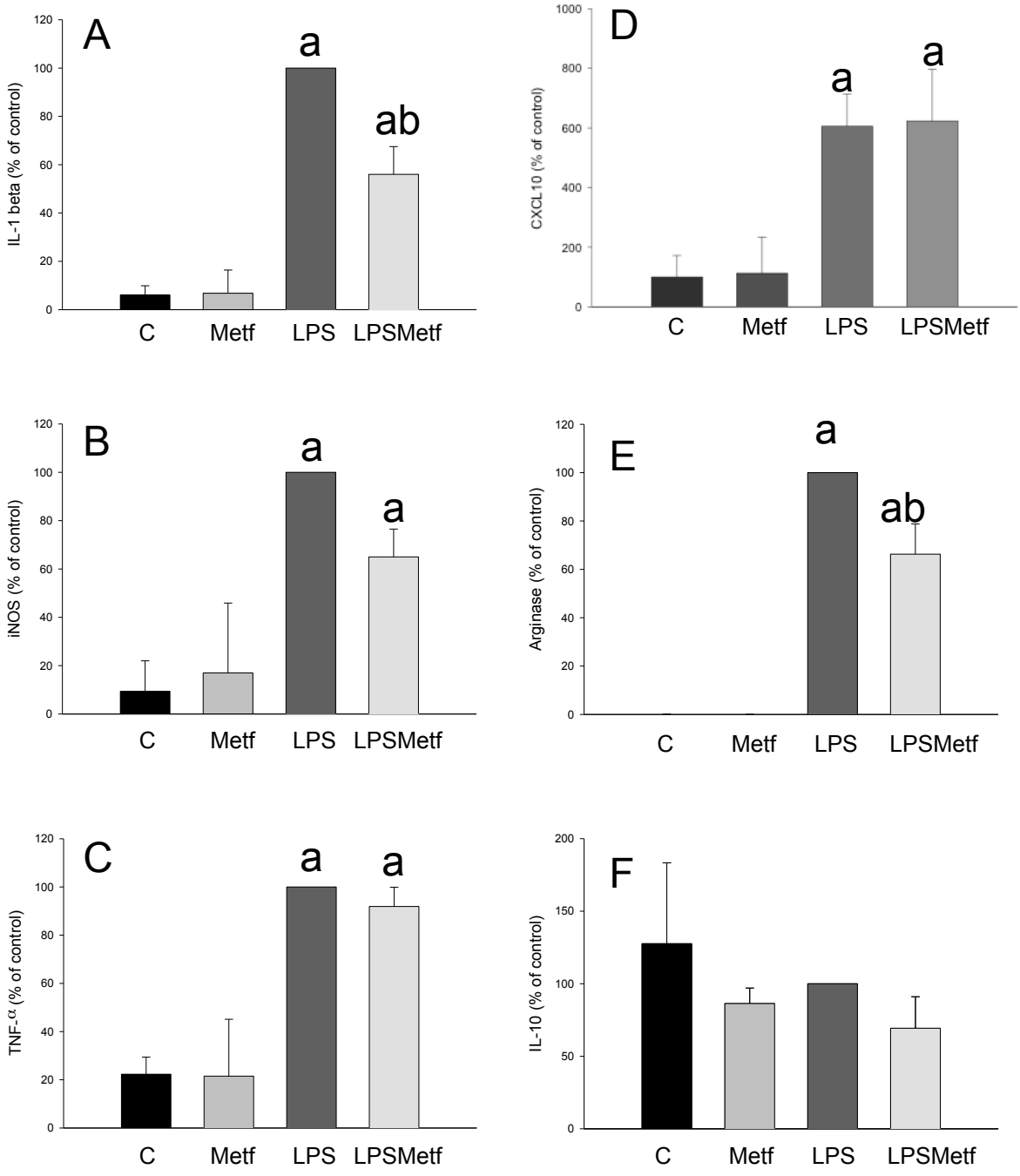

Figure 25. Quantification of mRNA expression levels of different pro and anti-inflammatory markers in BV2 cell-line. BV2 cells were treated with LPS or IL-4 for 12 hours. Total RNA was extracted from the cells to measure the change in the expression levels of mRNA of (A) IL-1β, (B) iNOS, (C) TNFα, and (D) CXCL10, when the used treatment was LPS; (E) Arginase and (F) IL-10, when IL-4 was the treatment. Results are mean ± SD of at least 3 independent experiments, normalized to β-actin and expressed as percentage relative to the LPS/IL4 group. Statistical significance (one-way ANOVA followed by the LSD post hoc test for multiple comparisons): a,

compared with the control group; b, compared with the LPS/IL4 group; $p<0.001$. (C, control cells without treatment; Metf, cells treated with metformin; LPS, cells treated with LPS; LPSMetf, cells treated with LPS and metformin).

1.2. Metformin treatment prevented the induction of NADPH oxidase by LPS

In microglial cells, ROS are mainly derived from NADPH oxidase (Block 2008; Gao, et al. 2012). NAPDH oxidase have seven described homologs which differ in their structure, expression levels in different tissues, and their activation mechanisms. NAPDH oxidase is a multi-subunit enzyme complex, consists of the membrane bound cytochrome b558 ($p22^{phox}$ and the heme binding enzymatic subunit $gp91^{phox}$), cytosolic subunits ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) and the Rac G-protein (Block 2008; Dohi, et al. 2010). During stimulation by a pathogenic trigger, the cytosolic subunits and Rac associate with the membrane-bound subunits resulting in activation of the complex (Block 2008; Gao, et al. 2012). Therefore, to study the effect of metformin on the oxidative stress induced by LPS treatment, the expression levels of the $p22^{phox}$, $p47^{phox}$ and $gp91^{phox}$ subunits of the NADPH oxidase enzyme were measured using RT-PCR. In our experimental conditions LPS treatment increased the expression levels of $p22^{phox}$, $gp91^{phox}$ and $p47^{phox}$ subunits (251.1± 74.2, 249.7 ± 96.4 and 524.8 ± 222.7 % respect to the control respectively, Fig 26A, B and C, $p < 0.05$), whereas metformin reduced this elevation in the expression level of $p22^{phox}$, and $gp91^{phox}$ subunits caused by LPS treatment (173.2 ± 21.7 and 308.7 ± 136.9, $p < 0.05$, Fig 26A and C), no effect was observed in the expression level of $p47^{phox}$ subunit upon metformin treatment (Fig. 26B). These results indicate that metformin inhibited NADPH oxidative activation, which mediate the production of ROS, which is involved in microglia activation by LPS and the resultant inflammatory process. ROS is produced by NAPDH-dependent one-electron reduction of oxygen to superoxide, and its production is central to many inflammatory diseases, acting as both signalling molecule and inflammatory mediator.

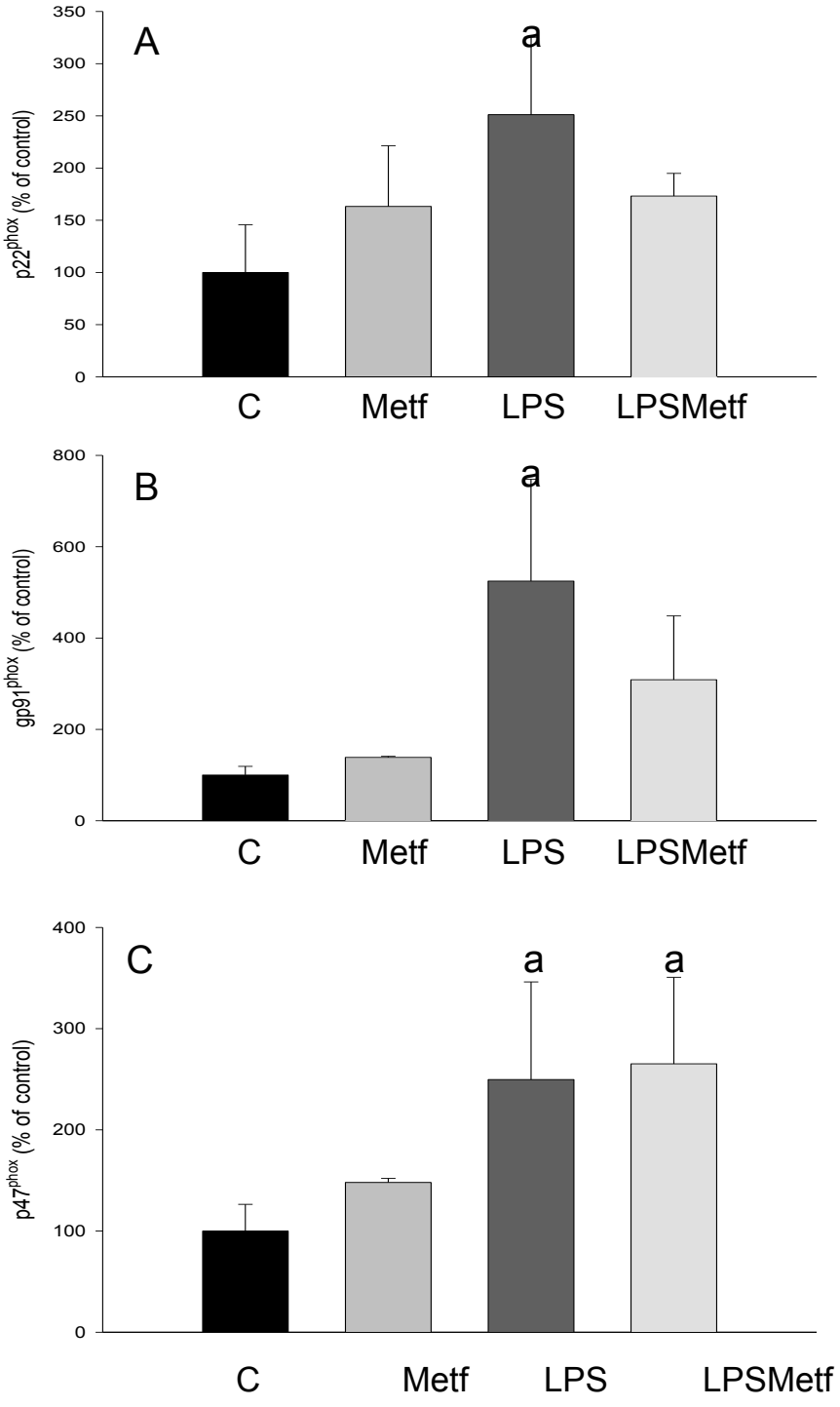

Figure 26. Effect of Metformin on the expression levels of NADPH oxidase subunits. Expression levels of (A) $p22^{phox}$, (B) $p91^{phox}$ and (C) $p47^{phox}$ mRNAs were measured by RT-PCR in BV2 cells from the different treatments assayed (C, control cells without treatment; Metf, cells treated with metformin; LPS, cells treated with LPS; LPSMetf, cells treated with LPS and metformin). Results are mean ± SD of at least 3 independent experiments, normalized to β-actin and expressed as percentage relative to the control group. Statistical significance (one-way ANOVA followed by the LSD post hoc test for multiple comparisons): a, compared with the control group; $p<0.05$.

1.3. Metformin treatment decreases the production of ROS

Oxidative stress has an important role in the degeneration of the DAergic neurons occurring in PD. During oxidative stress there is a state of disequilibrium between the levels of produced ROS and their clearance. Activated microglia are source of superoxide and nitric oxide, which contribute to the oxidative stress in the brain. Neurons are sensitive to oxidative stress due to their membrane composition of high polyunsaturated fatty acid content, high oxygen consumption and weak antioxidant defense (Liu, et al. 2017c).

Taking in consideration that the main source of ROS in microglial cells is the NADPH oxidase as well as metformin effect on the expression of this enzyme, we next sought to measure the level of oxidative stress in BV2 cells after LPS treatment and the effect of metformin on ROS production after LPS treatment. Using a fluorogenic probe that exhibits fluorescence signal in the presence of ROS in the cell cytoplasm, a two-fold increase of ROS production in BV2 cells after treatment with LPS (197.1 ± 31.6% with respect to the control, $p < 0.001$; Fig 27C and E) was observed. Whereas metformin treatment decreased ROS production in LPS-treated BV2 cells by approximately 30% (1388 ± 9.7% respect to the control, $p < 0.001$; Fig 27D and E).

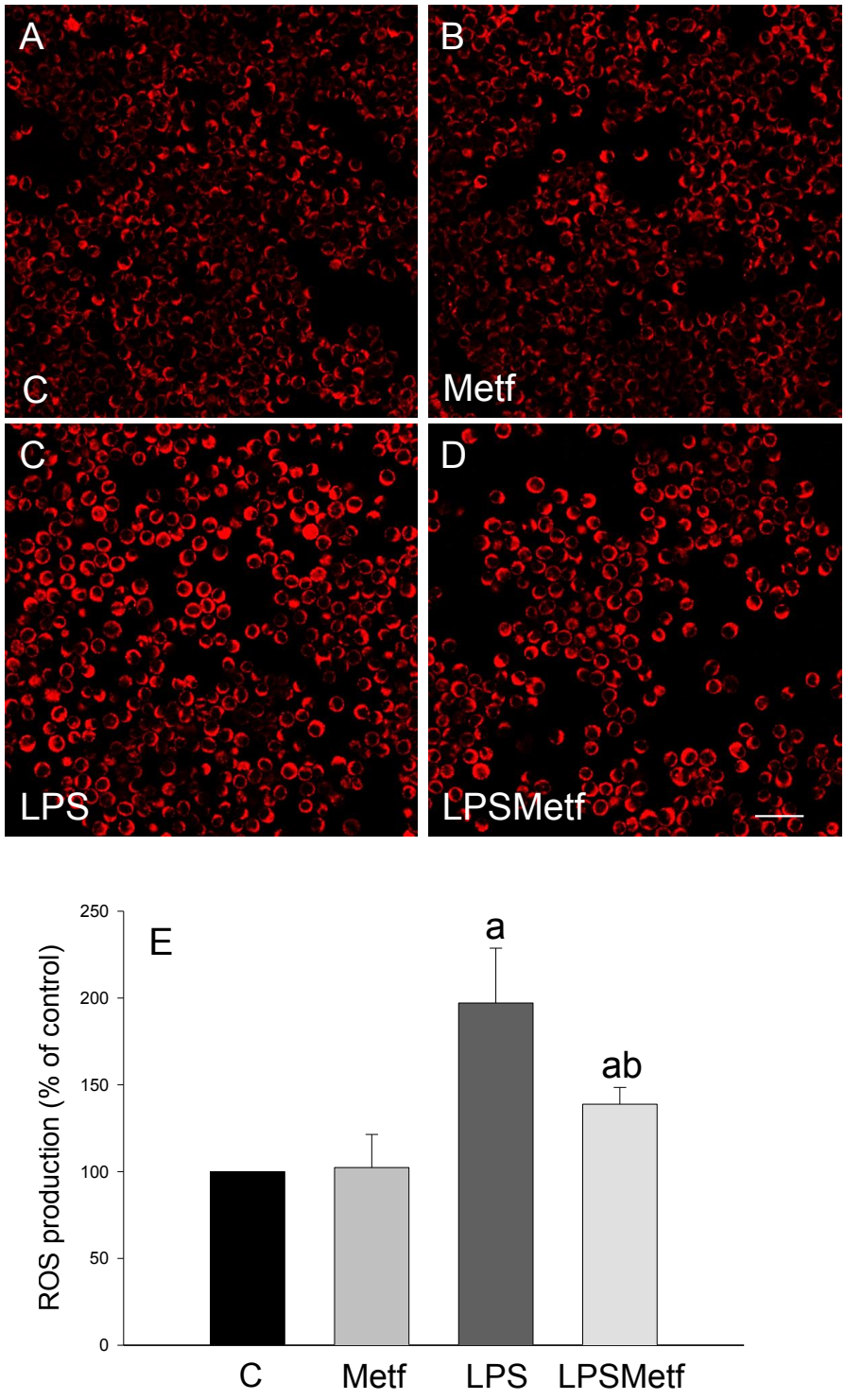

Figure 27. Effect of LPS and metformin on ROS production in BV2 cells. ROS were measured 24 h after the different treatments assayed: (A) Control; (B) Metformin; (C) LPS; (D) LPS plus metformin. (E) Quantification of ROS production. ROS production was measured after adding CellROX flurogenic probe. Images of different conditions were captured using confocal microscope. Results are mean ± SD of at least 3 independent experiments, expressed as percentage of control. Statistical significance (one-way ANOVA followed by the LSD post hoc test for multiple comparisons): a, compared with the control group; b, compared with the LPS group; $p<0.001$. Scale bar, 50 µm.

1.4. Metformin treatment decreases the activation of the inflammasome

The NLRP3 inflammasome, mainly located in microglia, is one of the most intensively investigated inflammasomes which controls disease progression and inflammatory responses (Yang, et al. 2014a)

As stated in the introduction section, assembly of the inflammasome triggers proteolytic cleavage of procaspase-1 into active caspase-1 converting the pro-IL-1β into mature IL-1β alongside with other cytokines. Inflammasome activation in macrophages requires a two-signal model. In this model LPS provide the first signal (priming) which induce NLRP3 and pro-IL-1β expression through activation of NF-κB; the second signal (activation) is triggered by ATP. NLRP3 induces the modulation of pro-IL-1β into IL-1β through a caspase1 dependent mechanism, leading to the release of this cytokine to the extracellular medium.

Therefore, the objective of this set of experiments was to explore the ability of metformin treatment to decrease inflammasome activation after LPS/ATP treatment in BV2 cells. This can be detected through measuring IL-1β levels. LPS/ATP treatment increases the amount of both extracellular and intracellular IL-1β, measures by ELISA and Western blot, respectively (Fig. 28 and B), whereas metformin treatment decreased the expression levels of IL-1β to 50% ($p < 0.001$; Fig 28 A and B).

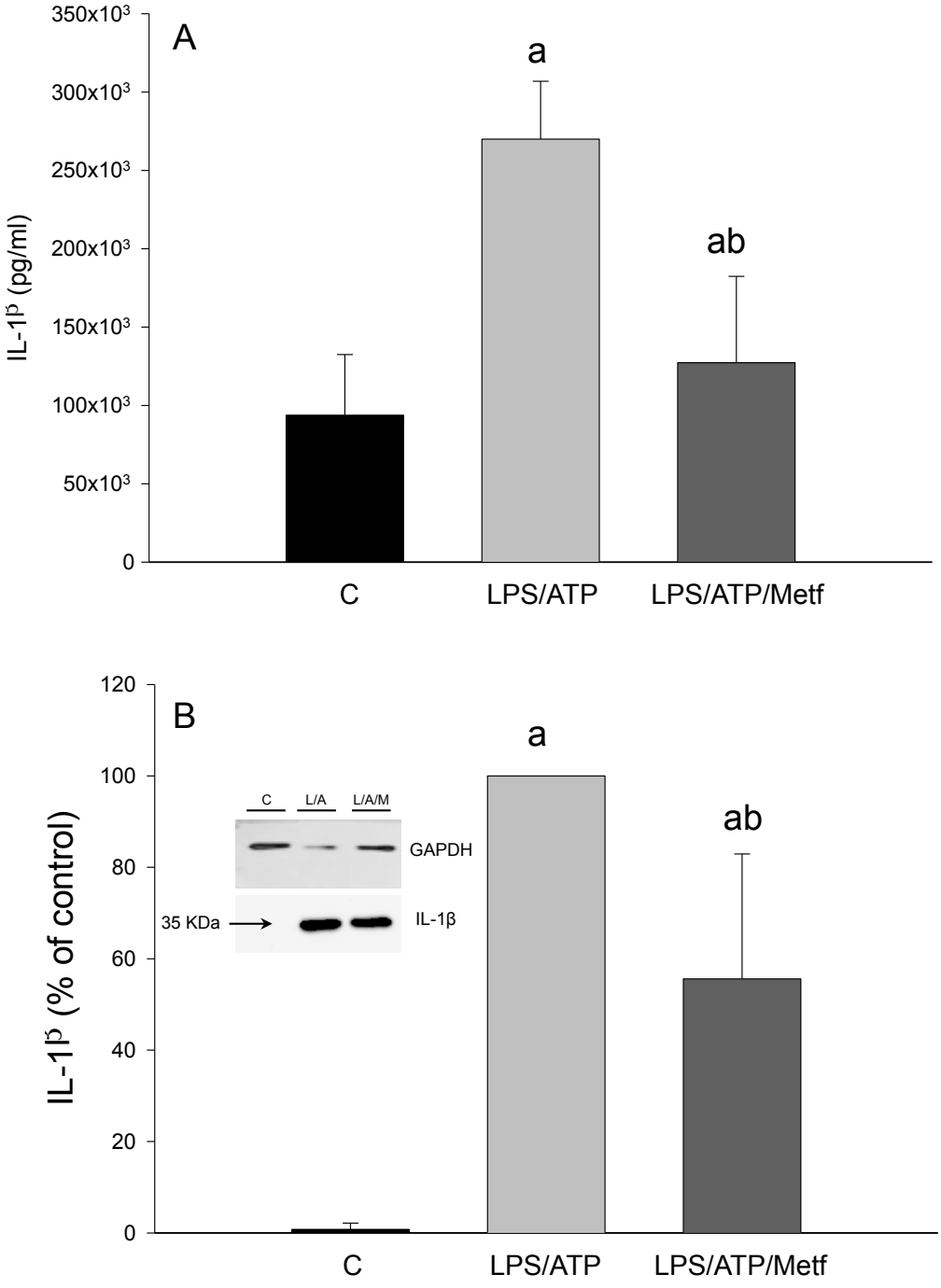

Figure 28. Effect of metformin on inflammasome activation in BV2 cells. (A) Extracellular IL-1β measured by ELISA. (B) Intracellular IL-1β measured by Western blot. Results are mean ± SD of at least 3 independent experiments, expressed as pg/ml (A) and percentage of control cells (B); intracellular amounts are normalized to GAPDH expression. Statistical significance (one-way ANOVA followed by the LSD post hoc test for multiple comparisons): a, compared with the control group; b, compared with the LPS/ATP group; $p < 0.001$. C, control group; LPS/ATP, cells treated with LPs and ATP; LPS/ATP/Metf, cells treated with a combination of LPS, ATP and metformin.

2. *In-vivo* experiments

2.1. Metformin treatment decreased the number of activated microglial cells *in vivo*

Once proven that metformin treatment was capable of reducing microglial activation *in vitro*, the next step was to study, at the morphological and molecular level, the activation of microglial cells in the SN after LPS injection *in vivo*. Microglial cells become activated (that is, change their morphology from resting, resident ramified microglia with two or three fine processes surveying constantly the surrounding environment, to round cells resembling tissue macrophages) and proliferate when challenged (Nimmerjahn, et al. 2005). As stated before in the introduction, activation of microglia during inflammatory response induces the expression of the MHCII, which is normally undetectable in microglia in normal CNS (Redwine, et al. 2001).

To assess the activation of microglia in the SN, immunohistochemistry in which MHCII was stained using a marker of reactive microglia (OX-6) was performed. This allowed the evaluation of metformin effect on induced neuroinflammation after intranigral injection of LPS.

Examination of the OX-6 immunoreactivity revealed that only mild immunoreactivity could be observed in both control and metformin-treated groups around the injection tract (132.6 ± 84.3 and 94, 4 ± 131.1 cells/mm^2; Fig. 29A, B and E). This indicates that metformin did not produce any added effect to the saline injection itself.

In contrast, the LPS-treated group showed a higher number of OX-6 cells representing a clear microglial activation almost of 10 times higher than the control group (1141.9 ± 207.8 cells / mm^2, $p<0.01$; Fig 29C and E.). However, metformin treatment reduced microglial activation induced by LPS to almost the half (584.2 ± 230.1 cell /mm^2, $p<0.01$; Fig 29D and E). Thus metformin was able to cause a significant decrease in the inflammatory response caused by LPS injection in the SN.

2.2. Metformin treatment does not protect from the LPS-induced damage to the astroglial population

Glial fibrilar acid protein (GFAP) is a protein expressed by several cell types of the CNS including astrocytes and its increase is indicative of astrocyte activation, which is often termed reactive gliosis. Astrogliosis is a characteristic change in the morphology and function of astrocytes seen in many neurological disorders such as neurodegenerative diseases, and it is considered as a response to CNS insults. Activated astrocytes present some morphologic characteristics, such as altered gene expression, upregulation of GFAP, and cellular hypertrophy and proliferation (hyperplasia) (Montgomery 1994; Oliveira, et al. 2016).

Our group has previously shown that the intranigral injection of LPS induces the loss of astroglial cells through a mechanism not fully elucidated yet (Castano, et al. 1998; Castaño, et al. 2002b; Herrera, et al. 2000). To examine the status of astroglia in different groups, immunostaining of GFAP was performed. The findings revealed that there is a slight astrogliosis around the injection site of vehicle in control and metformin treated animals without loss of GFAP immunostaining (Fig 29F, G and J). However, astroglia disappeared around the injection site of LPS, with an area absent of GFAP-positive structures (2.30 ± 1.48 mm^2; $p < 0.05$ compared with control animals) but surrounded by hyper-reactive astrocytes (Fig. 29H and J). Metformin treatment failed to prevent the loss of astrocytes after LPS injection (2.78 ±1.48 mm^2; Fig. 29I and J).

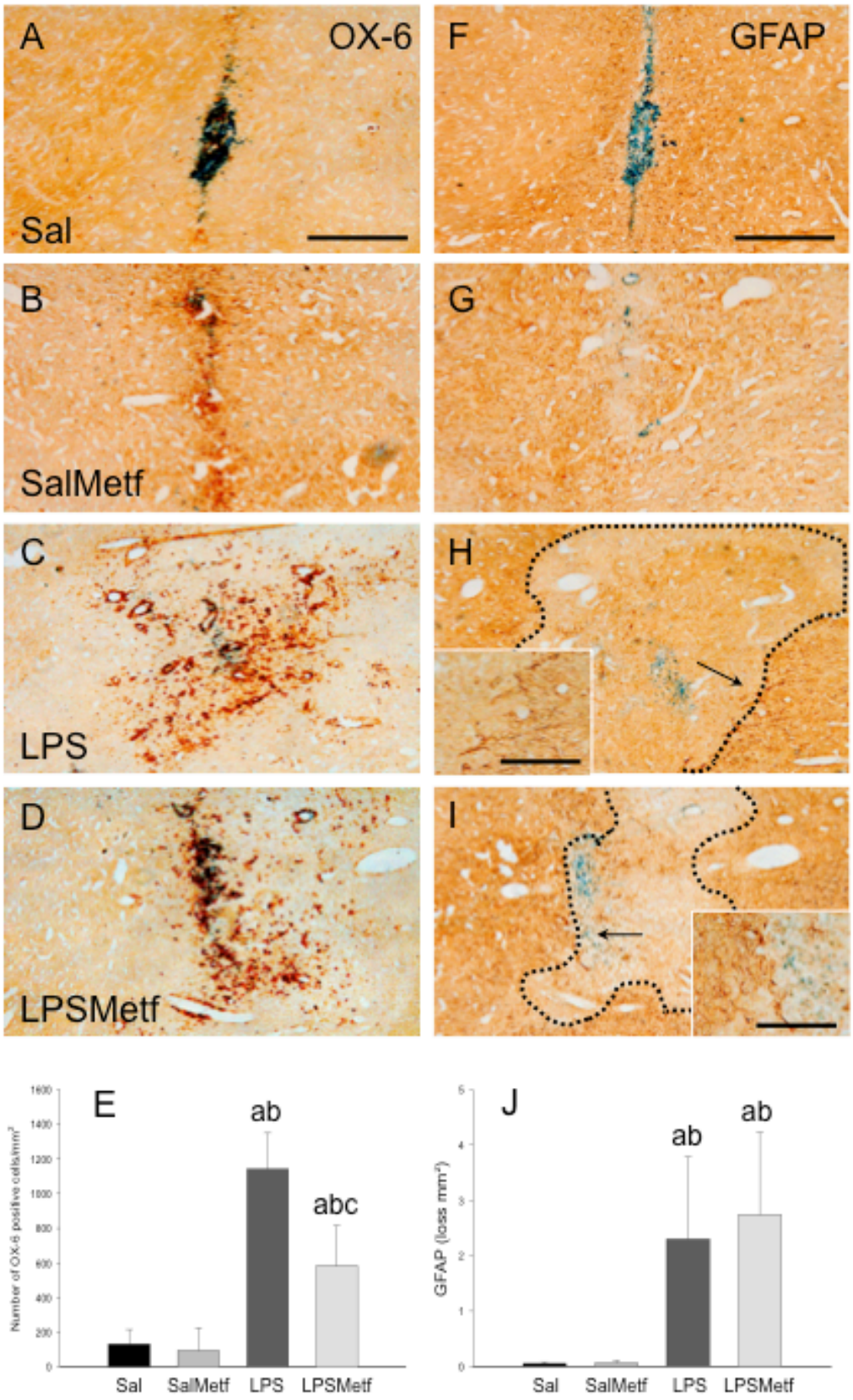

Figure 29. Effect of metformin and LPS on microglia and astroglia, evaluated by immunohistochemistry with the OX-6 (microglia; panels A-D) and the anti-GFAP antibodies

(astroglia, panels F-I). (A and F) Animals injected with saline solution in the SN plus oral administration of water; (B and G), animals injected with saline solution in the SN plus oral administration of metformin; (C and H), animals injected with LPS in the SN plus oral administration of water; (D and I) animals injected with LPS in the SN plus oral administration of metformin. The activation of microglia induced by LPS (C) was partially prevented by metformin (D). GFAP immunohistochemistry showed an important loss of astroglia induced by LPS (H) that was not prevented by metformin (I). Metformin alone had no effect on either microglia (B) or astroglia (G). The inserts in panels H and I show a high magnification of the zones indicated by arrows. (E) Quantification of the density of OX-6 positive cells. Results are mean ±SD of at least 4 independent experiments, expressed as number of cells per mm^2. (J) Quantification of the areas lacking GFAP immunostaining. Results are mean ± SD of at least 4 independent experiments, expressed as mm^2. Statistical significance (one-way ANOVA followed by the LSD post hoc test for multiple comparisons): a, compared with the control group; b, compared with the SalMetf group; c, compared with the LPS group; $p < 0.01$ for panel E and $p < 0.05$ for panel J. Scale bars: A-I, 500 μm; inserts, 200 μm.

2.3. LPS-activated microglial cells express a M1 phenotype that is reduced by metformin

The classical M1 microglia phenotype is featured by the production of different pro-inflammatory cytokines, contributing to the amplification of the pro-inflammatory responses. IKKβ is one of the key signaling molecules of the classical M1 microglia phenotype. Activated IKKβ phosphorylates Iκβ and triggers its degradation by proteasomes, which results in translocation of NF-κβ to the nucleus and the induction of NF-κβ dependent transcription of a wide range of immune and inflammatory genes (Mieczkowski, et al. 2015). The alternative M2 microglia phenotype executes anti-inflammatory effects and promote tissue repair. Arginase is known as a marker for M2 phenotype of microglia, which is anti-inflammatory, and it can downregulate the production of nitric oxide (Munder 2009).

The aim was to study the effect of metformin on the phenotype of microglial cells challenged by LPS, Thus a double immunohistochemistry using a specific microglia marker (Iba-1) and either a

pro-inflammatory (IKKβ) or an anti-inflammatory (arginase) marker was performed. Iba-1, is a calcium binding protein that is specifically expressed in microglia and is up regulated during microglia activation.

LPS injection is known to evoke M1 microglial activation, thus after LPS treatment most of microglial cells were co-localized with IKKβ (Fig.30A-C), whereas only scare co-localization appeared with arginase (Fig. 30G-I). Thus microglial cells activated with LPS displayed a pro-inflammatory phenotype. When animals were treated orally with metformin, most microglial cells expressed IKKB (Fig. 30J-L), whereas no co-localization appeared with arginase (Fig. 30J-L). Therefore, LPS also induces a pro-inflammatory phenotype even in metformin-treated animals.

Arginase (panels H and K) is not induced in Iba-1-labelled microglial cells, except for a few ones (G, H, J and K; merge panels I and L) in the animals treated with LPS and LPsMetf. Images in the inserts in panels C, F, I and L are high-magnification photographs of the small white boxes in their respective panels. Scale bars: A-L, 200 μm; insert, 25 μm.

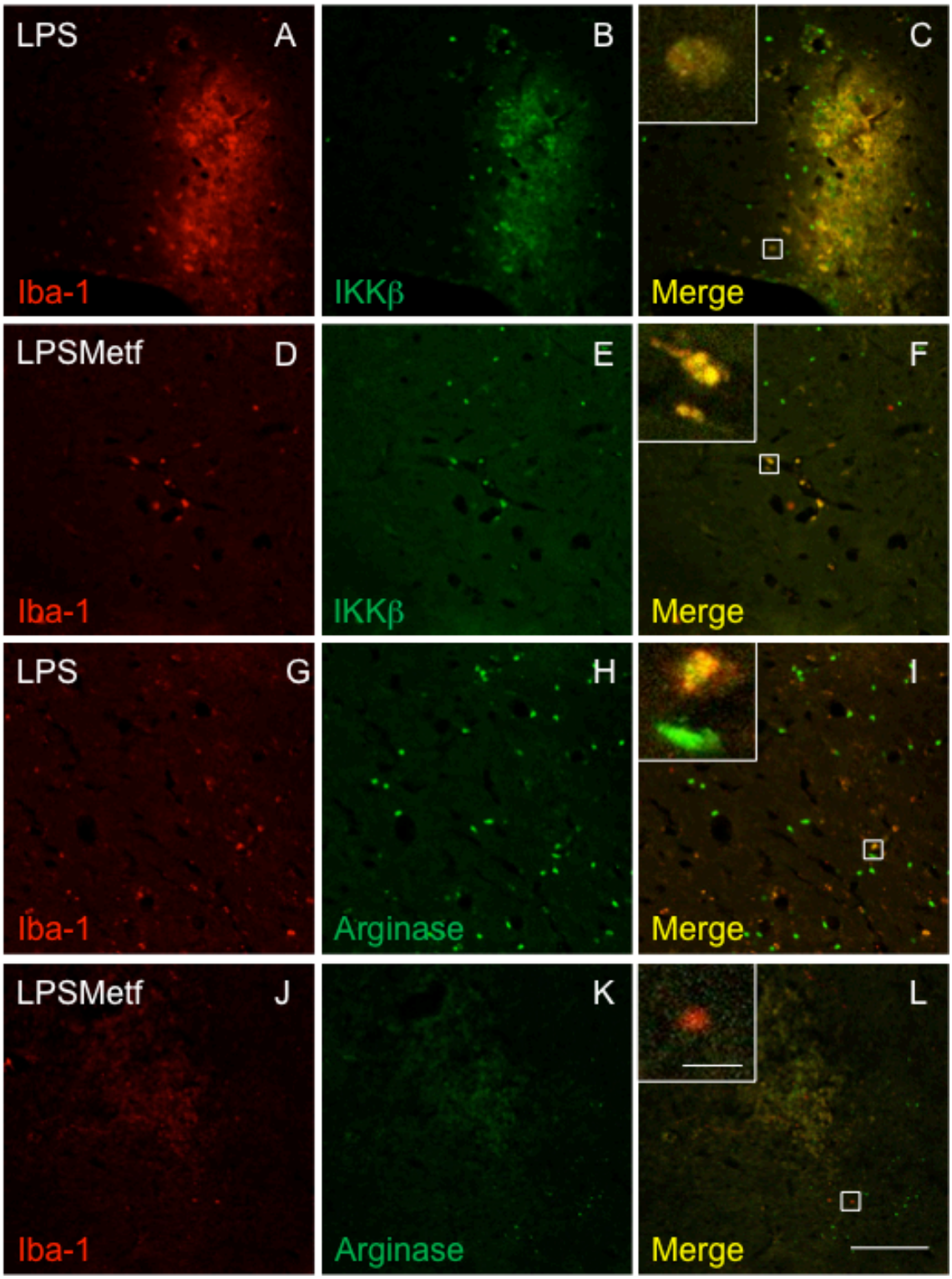

Figure 30. Effect of metformin and LPS on Iba-1, IKKβ and arginase immunostaining in the SN after LPS (panels A-C and G-I) and LPSMetf (panels D-F and J-L) treatments. Iba-1 immunofluorescence in panels A and D, and IKKβ immunofluorescence in panels B and E show an induction of IKKβ in Iba-1-labelled microglial cells (merge panels C and F).

2.4. Metformin treatment decreases the expression levels of several inflammatory mediators after LPS injection *in vivo*

Activated microglia produces pro-inflammatory cytokines and several anti-inflammatory mediators. To examine metformin effect on these different cytokines, mRNA levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and iNOS) in the SN were measured after treatment as described above. Beside the afore mentioned cytokines the expression level of CD200, CX3CR1, and MCP1 were quantified. Analysis by real-time PCR showed that the mRNAs for the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were induced in the SN up to 6 hours after LPS injection (496.3% ± 53 and 243, 3 ± 96% compared to the control group, respectively; $p < .0.01$ for TNF-α and IL-1β; Fig.318A, B, C). Moreover, LPS treatment induced the expression of MCP-1 (369 ± 87% with respect to the control; $p < 0.001$; Fig. 31E).

CD200 and CX3CR1 have shown to have inhibitory actions on brain microglia. Hence, we decided to study these molecules in our experimental conditions to seek further explanations of how metformin induces an attenuated effect after LPS treatment. Our PCR analysis showed that the expression of CD200 and CX3CR1 mRNAs decrease in respect to the control group (40 ± 2.45% and 71 ± 12.7%, respectively; $p < 0.001$ for CD200 and $p < 0.05$ for CX3CR1; Fig. 31F and G).

Metformin significantly reduced the LPS-induced increase in the mRNA levels of inflammatory markers compared to the control group (322.94 ± 67.6%; 38.1 ± 13.5% and 122.47 ± 18.76 for TNF-α, IL-1β and IL-6 of control values, respectively; $p < 0.01$; Fig. 31A, B and C). On the contrary, metformin treatment increased the levels of MCP-1, CD200 and CX3CR1 mRNAs (619.7 ± 59.2%, $p < 0.001$; 68 ± 10.50%, $p < 0.001$; and 110 ± 12.09% of the control values, $p < 0.05$, respectively; Fig. 31E, F and G).

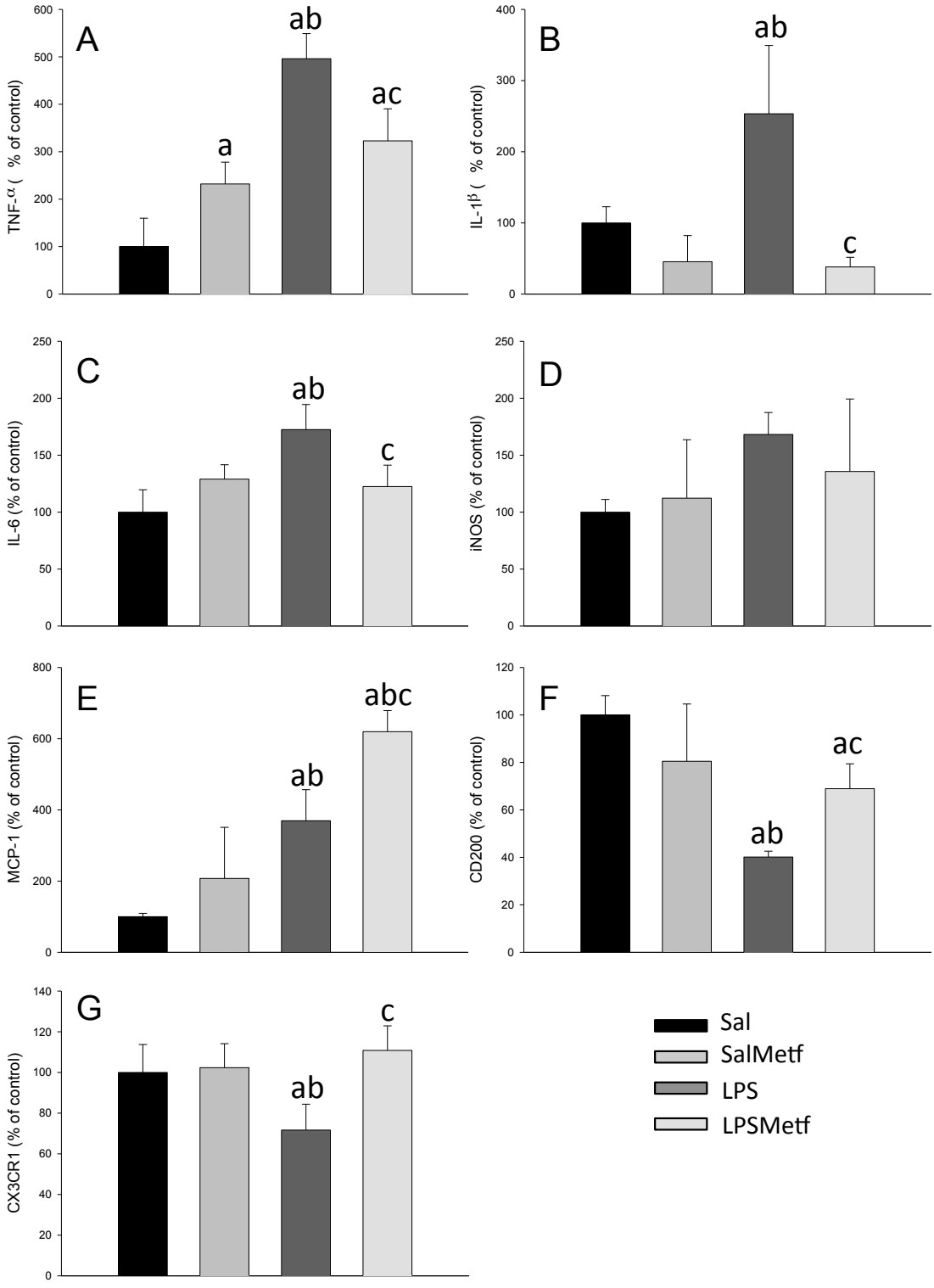

Figure 31. Effect of metformin and LPS on the expression of TNF-α (A), IL-1β (B), IL-6 (C), iNOS (D), MCP-1 (E), CD200 (F) and CX3CR1 (G) mRNAs in the SN of rats from the different treatments assayed, measured by RT-PCR. Sal, animals injected with saline solution in the SN plus oral administration of water; Metf, animals injected with saline solution in the SN plus

oral administration of metformin; LPS, animals injected with LPS in the SN plus oral administration of water; LPSMetf, animals injected with LPS in the SN plus oral administration of metformin. Results are mean ± SD of at least four independents experiments, normalized to β-actin and expressed as percentage relative to the control (Sal) group. Statistical significance (one-way ANOVA followed by the LSD post hoc test for multiple comparisons): a, compared with the Sal group; b, compared with the Sal/Metf group; c, compared with the LPS group; $p < 0.01$.

2.5. Metformin effects on microglial activation are mediated by MAPK

The MAPK signaling pathway plays a vital role in microglial activation (Zhao, et al. 2017) Metformin is known as an activator of AMPK, where AMPK is involved as an upstream element of MAPKs signaling pathways in different cell types (Wu, et al. 2011). Thus, the involvement of JNK, p38 in the anti-inflammatory role of metformin was examined by Western blot analysis. Specific antibodies against the phosphorylated and non-phosphorylated forms of JNK and p38 were used.

As expected, LPS treatment increased the ratio P-JNK/JNK (183.68 ± 42.82%; $p<0.001$ compared with control animals; Fig. 32A), whereas metformin treatment abolished this effect (39.93 ± 21.98%). Metformin treatment also reduced the ratio P-p38/p38 (57.89 ± 29.16%; $p < 0.05$ compared with control animals; Fig. 32B).

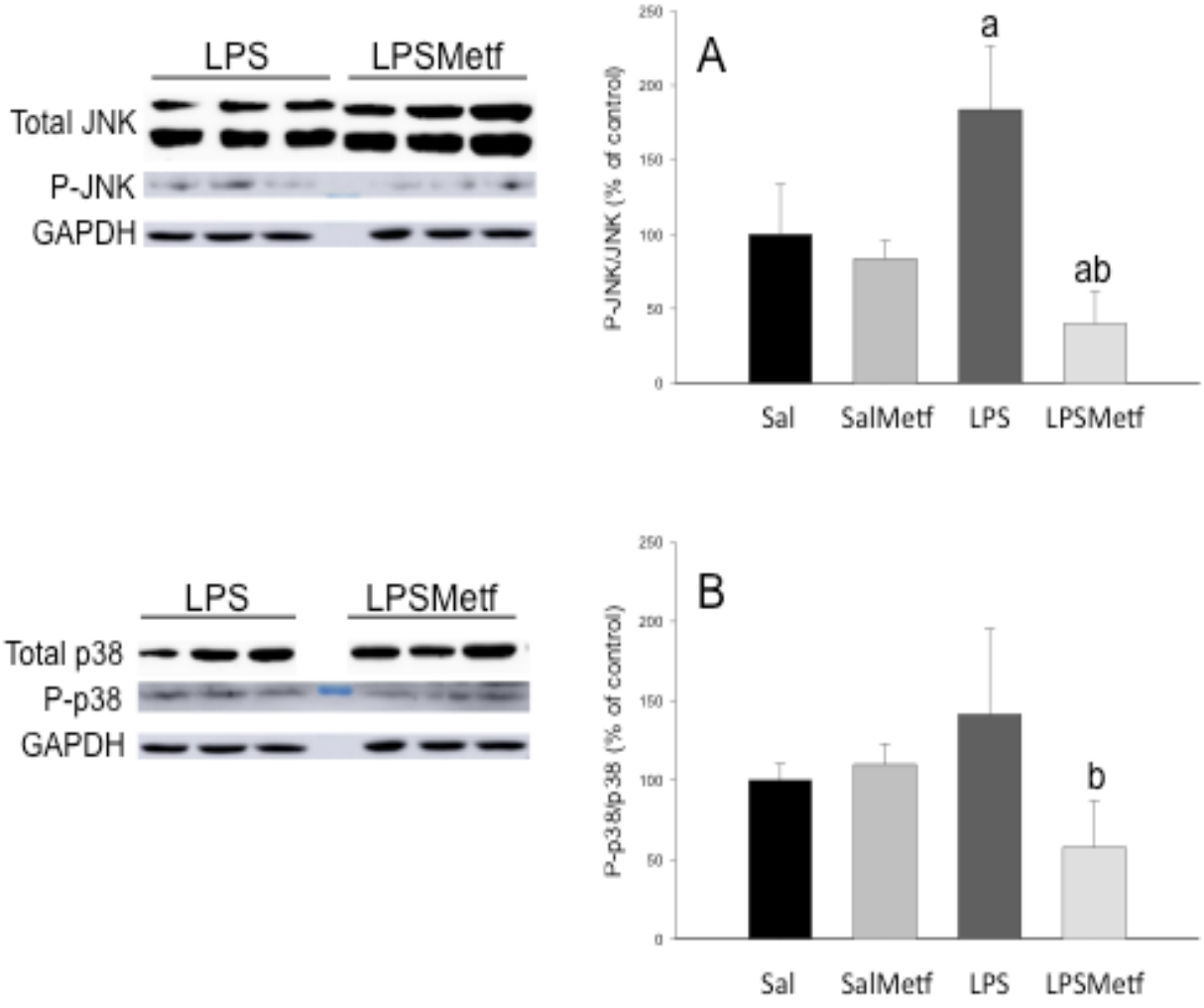

Figure 32. Effect of metformin on the expression of JNK, P-JNK, p38, and P-p38 proteins in the SN. Proteins from the SN of rats under the different treatments assayed were separated by electrophoresis, transferred to nitrocellulose membranes and stained using anti-JNK, anti-P-JNK, anti-p38 and anti-P-p38 antibodies. Total optical density of each band was calculated. Results are mean ± SD of at least 4 independent experiments, expressed as P-JNK/JNK (A) and P-p38/p38 (B) intensity ratios (normalized to GAPDH expression) relative to the control (Sal) group. Statistical significance (one-way ANOVA followed by the LSD post hoc test for multiple comparisons): a, compared with the control group; b, compared with the LPS group; $p < 0.01$. Bands corresponding to the Sal and SalMetf treatments can be seen in Figure 33. (Sal, animals injected with saline solution in the SN plus oral administration of water; Metf, animals injected with saline solution in the SN plus oral administration of metformin; LPS, animals injected with LPS in the SN plus oral administration of water; LPSMetf, animals injected with LPS in the SN plus oral administration of metformin).

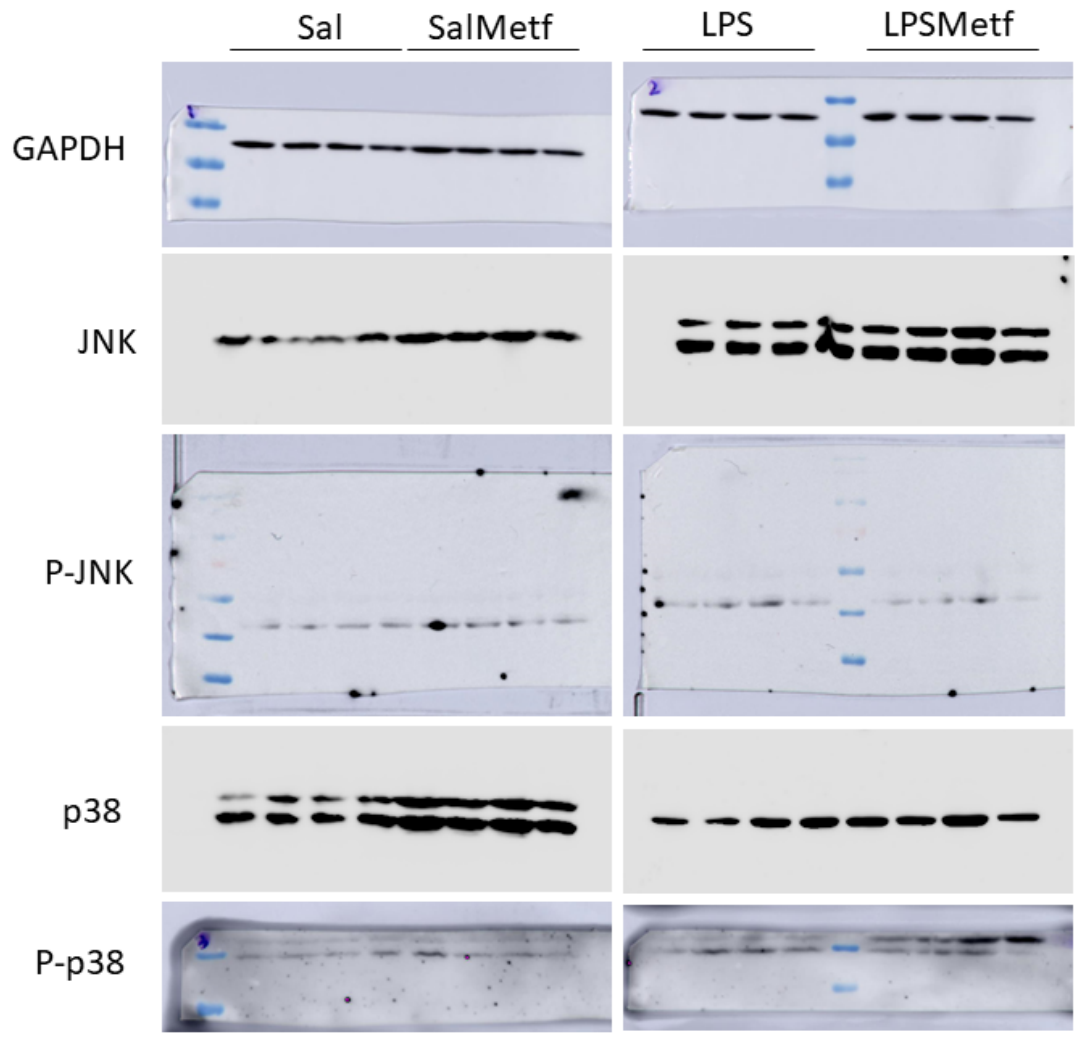

Figure 33. Effect of metformin on the expression of JNK, P-JNK, p38, and P-p38 proteins in the SN. Bands corresponding to the Sal and SalMetf treatments

2.6. Effect of metformin and LPS on the dopaminergic system

Herrera et al. reported that a single intranigral injection of LPS results in microglial activation and strong inflammatory response in SN causing a specific damage to the DAergic neurons without

affecting other neuronal types. These findings suggest that microglial activation may cause a decrease in TH immunoreactivity (Herrera, et al. 2000). Same findings were published by Gao et al in 2002 (Gao, et al. 2002).

Therefore, the integrity of the nigro-striatal DAergic neurons was evaluated at the cellular level by immunohistochemistry against TH, which is the rate-limiting enzyme in the synthesis of DA and widely used as an index of DAergic neurons.

Oral administration of metformin (Fig. 34B and E) had no effect on the number of DAergic neurons with respect to the control group (2882±685 and 2294±530 for control and metformin, respectively; Fig.34 A, B and E). Intranigral administration of LPS reduced the number of neurons to 66.1% of control values (Fig 34C and E; $p < 0.01$). Interestingly, metformin not only failed to protect against the LPS-induced death of DAergic neurons, but even exacerbated the damage (29.6% of control value; Fig 34D and E).

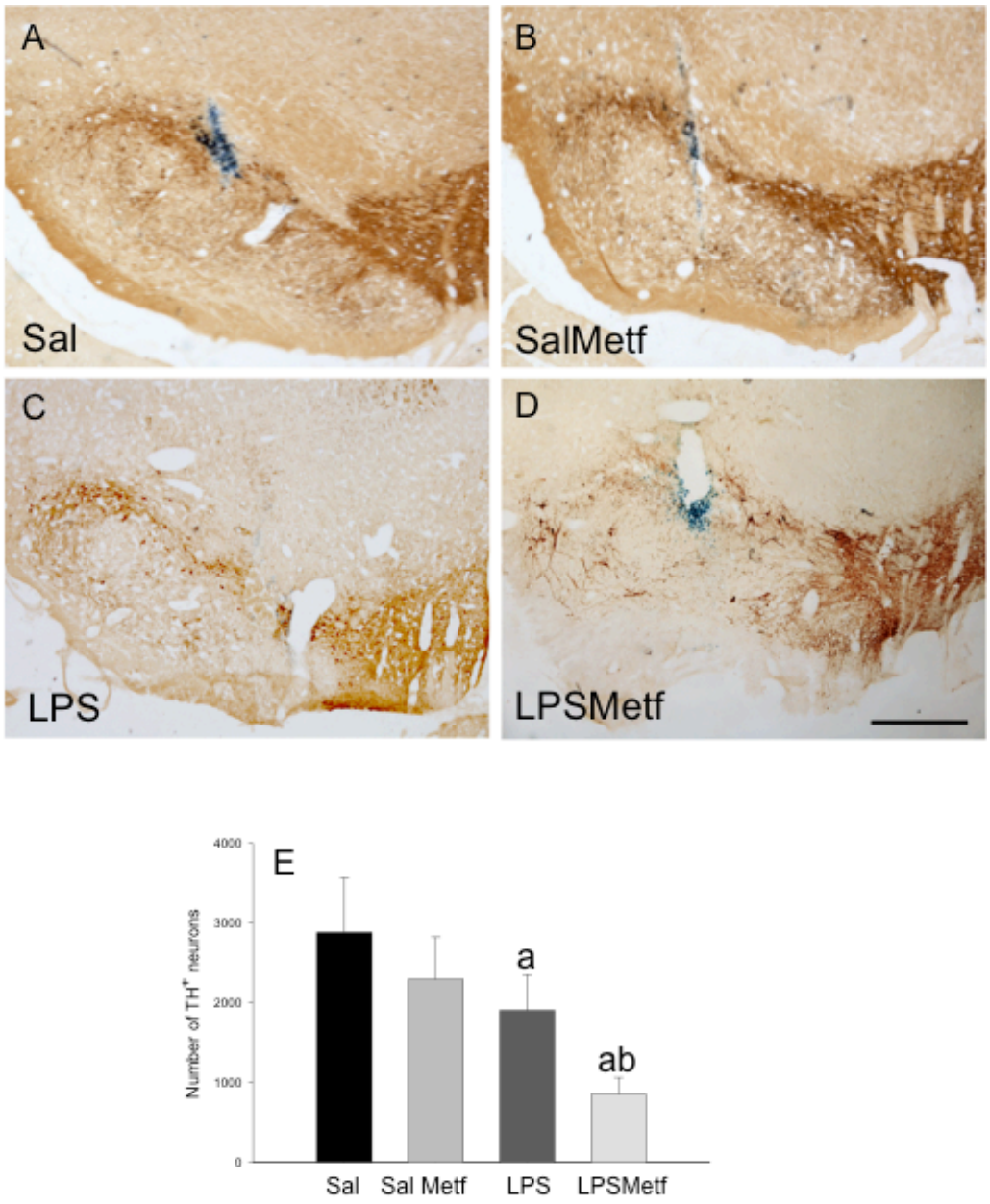

Figure 34. Effect of metformin and LPS on dopaminergic neurons. (A) Coronal section showing that TH immunreactivity was not affected by the injection of saline solution in the SN of rats . (B) Coronal section showing the TH immunreactivity was not affected after vehicle injection in the SN of rats receiving metformin treatment twice daily. (C) Coronal section showing a decrease in TH immunoreactivity around the injection area after the injection of 2 µg of LPS in comparison with the control group. (D) Coronal section showing that the loss of TH immunoreactivity induced by LPS was increased in the animals treated orally with metformin. Scale bar: 500 µm. (E) Quantification of the number of TH-positive cells of the four treatment groups. Results are expressed as mean ± SD of 4 independent experiments, expressed as TH-positive cells within the bounded area of the SN. Statistical significance (one-way ANOVA followed by the LSD post hoc test for multiple comparisons): a, compared with the control group; b, compared with the LPS group; $p < 0.01$.

V. DISCUSSION

Neurodegenerative disorders such as PD outstrip clinicians and researchers in two decades, the approximate time during which the processes leading to the neuronal death that causes the recognizable symptoms of the disease are silently developing. PD, the second-most common neurodegenerative disorder, affects mainly the dopaminergic neurons of the SN. Unfortunately the damage caused by PD to the neuronal system is irreversible, resulting in gradual decline in motor and cognitive abilities. This irreversibility of PD and likewise diseases remains a huge challenge for scientists in terms of first illustrating the underlying causes of the disease, and later understanding the mechanisms involved in its progression, making their main objective to develop key articulated points for therapeutic intervention, which can improve patient's health and thus quality of life. Available PD treatments are mainly medications that alleviate its symptoms, and a small proportion of PD patients require surgical procedures at developed stages. Nowadays, the primary treatment of the disease, based on the use of L-DOPA and other substances to increase the dopaminergic signal in the nigro-striatal pathway (Aminoff 2015), is symptomatic and does not stop the progress of the disease. Considering that a satisfactory method to replace the neurons lost during the progression of the disease has not yet been developed, it is of vital importance to investigate and develop strategies that modify the processes leading to neuronal death, such as oxidative and inflammatory processes.

Many neurodegenerative disorders are associated with neuroinflammation, which is a key factor in PD progression. When the inflammation becomes chronic, it causes microglia and astrocytes to release of pro-inflammatory cytokines, which in turn exacerbates the degeneration of DAergic neurons in the SN. Other immune cells from the periphery are also involved in the pathogenesis of PD. All of these factors combined highlight the importance of understanding the inflammatory role and mechanisms, and the need for exploring substances that show anti-inflammatory activity and therefore could delay the progression of the disease (Cayero-Otero, et al. 2018) .

Microglia, the major cell type involved in CNS inflammatory processes, have been usefully hired in disclosing its involvement in neurodegenerative diseases. Despite the huge research that has been

carried out during the last three decades to illustrate the mechanisms of neuroinflammation, including epidemiological and genetic studies, there is still little known about such condition. In neuroinflammation there is an interplay between the immune system, neuronal cells, protein aggregation, genetic mutation, and environmental external factors.

In this study, we have used metformin, a first-choice drug for newly diagnosed type 2 diabetes. Recently metformin has shown to exhibit anti-inflammatory effects and attenuate both peripheral and central inflammation. In this study we have delineated the effect of metformin on microglia polarization and inflammasome activation and its potential *in vivo* neuroprotective effects over the nigral dopaminergic system under conditions of neuroinflammation.

Metformin decreases the inflammatory response *in vitro*

The diverse functions of microglial cells in the CNS are mirrored by equally diverse phenotypes. A classical model of pro-inflammatory versus an anti-inflammatory microglia polarization states has been extensively used. However, the complex and different functions of microglial cells can only be explained by the existence of varied and plastic microglial phenotypes mediated by distinct gene expression programs. These different phenotypes can be achieved by stimulating microglial cells with different compounds. Hence, when activated with LPS, microglia have a pro-inflammatory phenotype, characterized by the expression of pro-inflammatory cytokines such as IL-1β, TNF-α and iNOS. When stimulated with IL-4, microglial cells switch to an anti-inflammatory phenotype, characterized by the expression of resolution genes, such as arginase. Keeping this view in mind, we firstly studied the effect of metformin treatment on the LPS/IL-4 induced activation of BV2 cells. BV-2 cells, the most frequently used substitute for primary microglia, were derived from raf/myc-immortalised murine neonatal microglia. They have been used in different fields, for pharmacological studies (Lund, et al. 2005), phagocytosis studies (Hirt and Leist 2003) and many important immunological discoveries in at least 200 publications. In relation to neurodegeneration

studies, BV-2 resembles primary microglia in expressing functional NADPH oxidase, an enzyme frequently implicated in microglia-triggered neuronal damage (Wu, et al. 2006; Yang, et al. 2007).

Our results showed that metformin was able to decrease the switch of microglial BV2 cells towards both previously mentioned opposite polarization states; it reduced both the LPS-induced expression of the pro-inflammatory mediator IL-1β and the IL-4-induced expression of the anti-inflammatory mediator arginase. The absence of induction of IL-10 (usually achieved by the stimulation of microglia with LPS/IC) corroborates the correct degree of polarization in our model. These results expand those from other authors (Gu, et al. 2014; Isoda, et al. 2006; Labuzek, et al. 2010a), confirming the overall anti-inflammatory effect of metformin *in vitro*, but going further showing metformin as an immunosuppressant of microglia. This overall microglia immunosupression would prevent the beneficial and detrimental roles ascribed to microglia biology.

Metformin anti-inflammatory activity was specific since no effect was observed on other pro-inflammatory cytokines such as TNF-α in LPS activated microglia. This finding is contradicted with another study that showed metformin ability to decrease TNF-α levels in a mouse model of endotoxaemia.

TNF-α plays an important role in PD in both early and late stages. Several studies of TNF receptors (TNFR 1 and TNFR2) show that TNFR1 is expressed in nigrostriatal DA neurons, while TNFR2 is expressed mainly in active cells of the immune system such as microglia, and also in DAergic and hippocampal neurons (Bernardino, et al. 2005; Marchetti and Abbracchio 2005). TNF-α levels in human adult brains are generally very low, normally produced by neurons (D., et al. 1993) (Breder, et al. 1993). On the other hand, high levels of TNF-α were found in the brain and cerebral spinal fluid of PD patients, and elevated TNFR1 levels were measured in their SN (Boka, et al. 1994). The SN DAergic neurons are highly sensitive to TNF-α (Aloe and Fiore 1997), since it can activate the microglia, which would enhance the inflammatory response, carrying out the overproduction of

ROS, NO and superoxide radicals that enhance the formation of peroxynitrites (Beckman, et al. 1993). This microglial activation in the SN creates a microenvironment of oxidative stress mediated through NADPHox activation that strengthens damage to DAergic neurons (Dong, et al. 2015; Tansey, et al. 2007)

Oxidative stress is known to be a crucial factor in the pathogenesis of neurodegenerative diseases. LPS can provoke the release of ROS, contributing to cellular damage. Therefore, we examined the ability of metformin treatment to decrease ROS levels in LPS-stimulated microglial cells. We took advantage of a fluorogenic probe to demonstrate that metformin treatment caused a clear decrease in ROS production in LPS-activated BV2 cells. This result is in agreement with the finding of Algire et al, 2012(Algire, et al. 2012).

NADPH oxidases (NOX) are a family of enzymes involvedd in phagocytic bactericidal and fungicidal activities mediated through ROS production. NOX enzymes, mainly NOX2 (also known as $gp91^{phox}$), are also involved in microglia activation. When microglial $gp91^{phox}$ is activated, several ROS including superoxide anion ($O2^{\cdot -}$), hydroxyl radical ($OH^{\cdot -}$), lipid hydroperoxides, and byproducts (e.g., H_2O_2), are generated. In turn, these ROS can directly damage neurons through extracellular pathways which can induce further ROS generation and the expression of pro-inflammatory genes such as IL-1β, TNFα, and iNOS (Huang, et al. 2018a; Huang, et al. 2018b).Since NADPH oxidase enzyme is the most important source of intracellular ROS, we studied the impact of metformin treatment on the activation of this enzymatic complex. Again, our results showed that metformin treatment affected the expression of NADPH enzymes associated with oxidative stress; it decreased the levels of the $p22^{phox}$ and $gp91^{phox}$ subunits of the NADPH oxidase.

The NAPDH enzyme subunits, the primary source of ROS, are located in the mitochondria. Metformin is believed to act in the mitochondrial respiratory chain, which explains the decrease in ROS production through its effect on NAPDH.

Metformin is also known to act on central metabolism and several major signaling pathways. The anti-inflammatory properties of metformin can be mediated by several routes, one of which is controlled by the NLRP3 inflammasome. Microglial cells constantly survey the proximal environment through the pattern-recognition receptors that they express, including Toll-like receptors and NOD-like receptors (NLRs)(Walsh, et al. 2014). Activated microglia produce a variety of inflammatory cytokines that contribute to the degeneration of DAergic neurons. Among these inflammatory cues, IL-1β has been found to be essential for the initiation and progress of PD (Koprich, et al. 2008; Masliah, et al. 2005). IL-1β is an important pro-inflammatory mediator, generated at the site of immunological injury in order to control the ongoing incident through the recruitment of specialized cells to the injury site. Elevated expression of IL-1β has been observed in the microglia surrounding Lewy bodies in PD patients as well as in animal models (Hirsch, et al. 2005; Koprich, et al. 2008; Stone, et al. 2009). Several pro-inflammatory stimuli provoke the inactive IL-1β pro-form expression, resulting in IL-1β maturation and secretion. It has been found that the protease caspase-1 is critically involved in inflammatory responses due to its pivotal role in regulating the cleavage of the inactive precursor pro-IL-1β and pro-IL-18 to matured IL-1β and IL-18 in the cytosol by different stimuli (Dinarello 2007a). The activity of caspase-1 is highly controlled by cytosolic multiprotein complexes called ‘inflammasomes’, which are composed of the nod-like receptor protein (NLRP) family, adaptor protein ASC and pro-inflammatory precursor pro-caspase-1(Dinarello 2007b). Inflammasomes that contains NLRP3 are highly expressed in microglia and essential to the neuroinflammation process (Heneka, et al. 2013). NLRP3 inflammasome is one of the most intensively investigated inflammasomes, due to its role in controlling disease progression and inflammatory responses(Yang, et al. 2014b) and its activation

has been detected in a number of neurodegenerative diseases, including AD, PD and amyotrophic lateral sclerosis (ALS)(Heneka, et al. 2014).

It is known that LPS treatment is able to activate the NLRP3 inflammasome, inducing the release of IL-1β (Martinon, et al. 2002). Metformin has shown to decrease the NLRP3 inflammasome activation in several tissues, including adipocytes (Li, et al. 2016), blood mononuclear cells (Bullon, et al. 2016) and monocyte-derived macrophages (Lee, et al. 2013). However, the effects of metformin on the NLRP3 inflammasome activation in microglial cells have never been studied. Therefore, we sought to investigate the ability of metformin treatment to inhibit the NLRP3 inflammasome and hence to decrease the inflammation induced by LPS. Our results showed that metformin treatment in BV2 cells decreased the levels of both intra- and extra-cellular IL-1β induced by LPS, pointing up that inhibition of the NLRP3 inflammasome could be one of the possible mechanisms by which metformin exerts its anti-inflammatory effects in microglial cells. It is known that ROS can activate the NLRP3 inflammasome (Heid, et al. 2013); therefore, metformin could inhibit NADPH oxidase in our experimental conditions, leading to a decrease in ROS production that in turn could decrease the NLRP3 inflammasome. Finally, this inhibition would lead to the decrease in the synthesis and release of IL-1β.

Metformin decreases inflammation *in vivo*

Once we confirmed the anti-inflammatory effects of metformin in LPS-stimulated BV2 cells and some of their possible mechanisms of action *in vitro*, we moved on to explore whether metformin treatment would also exhibit anti-inflammatory activity in an *in vivo* model of PD. To date, several studies have shown the anti-inflammatory effects of metformin on different conditions and tissues. Most of these studies have been done in cell cultures, but only a few have investigated the effect of this commonly used drug *in vivo*. Some of this research has demonstrated the anti-inflammatory effect of metformin in the SN, the most affected structure in PD. A significant number of these

studies explore the possible protective effect of metformin on DAergic neurons after treatment with MPTP (Bayliss, et al. 2016; Ismaiel, et al. 2016; Lu, et al. 2016b; Patil, et al. 2014a). Here, we study for the first time the effect of metformin on the *in vivo* model of PD based on the injection of LPS within the SN. We have chosen this model for several reasons: i) it is a well-established and extensively used model of PD; ii) inflammation is the main mechanism of cell death involved; iii) the effect of metformin on this model of PD is not well documented in the literature.

Therefore, we firstly measured microglial activation after the injection of LPS within the SN. OX-6 immunostaining and PCR revealed that metformin decreased the number of activated microglial cells and the expression levels of several pro-inflammatory cytokines and mediators. In this sense, metformin treatment decreased the mRNA levels of TNF-α, IL-6 and especially IL-1β. These results corroborate the *in vitro* results, showing that IL-1β is especially downregulated by metformin.

To shed more light into this issue, we also studied the mRNA expression of CD200, a surface molecule expressed on the microglial cell membrane that keeps the microglia in a quiescent state when it interacts with its receptor CD200R, and the mRNA expression of CX3CR1, also known as fractalkine receptor, which has been associated with inhibitory actions on brain microglia. Microglia-deficient of CX3CR (Kierdorf and Prinz 2013), as well as MCP-1 (a potent chemokine involved in different neurological disorders) (Conductier, et al. 2010), has an over-activated phenotype. As expected, intranigral LPS injection led to a significant down-regulation of CD200 and CX3CR1 mRNA expression in the ventral mesencephalon. However, metformin treatment turned the down-regulation of CD200 and CX3CR1 expression induced by LPS into a significant up-regulation of these microglia regulators, thus pointing up metformin as a molecule responsible for inactivating microglia. This finiding suggest a beneficial role of metformin treatment in neurodegenerative disease.

Inflammatory processes start by the attraction of certain immune cells to the site of inflammation. Chemokines are the main cells responsible for creating a concentration gradient inducing inflammatory cells from the circulation to the injury site.

MCP-1 is one of the main chemokines which regulates microglia movement, and the most potent at activating signal transduction pathways leading to monocyte transmigration (Sozzani, et al. 1994). On the contrary to several authors have demonstrated that metformin can damp the induction of this chemokine by LPS (Chen, et al. 2018b; Han, et al. 2018), our results showed that metformin treatment increased the mRNA levels of MCP-1 in the SN in our experimental conditions. These findings suggest that metformin may not a protective effect against MCP-1.

As stated above, microglial cells can adopt different phenotypes, named traditionally M1 (a classical pro-inflammatory phenotype), an initial defense mechanism characterized by the production of inflammatory cytokines and reactive oxygen species, and M2 (an alternative anti-inflammatory phenotype) that has a repairing role after the inflammation. Several studies have proposed that metformin could exert its anti-inflammatory actions inducing a shift in microglia towards a M2 phenotype. This modulation has been suggested as a novel promising therapeutic strategy in neurodegenerative diseases (Pena-Altamira, et al. 2017)

Although our PCR data from BV2 cells have indicated that metformin decreased both the M1 and M2 phenotypes *in vitro*, we sought to corroborate these results *in vivo*. We took advantage of co-localization techniques and found that almost all Iba-1 microglial cells (a general microglia marker) co-localized with the M1 marker IKKβ, whereas none of them co-localized with the M2 marker arginase in both the LPS and LPS+metformin groups. Hence, our results showed again that metformin treatment decreased both the classical and alternative activation forms of microglia in our experimental conditions.

One of the possible mechanisms by which metformin could decrease inflammatory mediators is related to the activation of MAPKs and the PI3/Akt pathways. The mitogen-activated protein kinases (MAPKs) in mammals include c-Jun NH2-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK). These enzymes are serine-threonine protein kinases that regulate various cellular activities including proliferation, differentiation, apoptosis or survival, inflammation, and innate immunity. The compromised MAPK signaling pathways contribute to the pathology of diverse human diseases including cancer and neurodegenerative disorders such as AD, PD, and amyotrophic lateral sclerosis (Kim and Choi 2015). Therefore, in a next step we analyzed the phosphorylated and total amounts of JNK and p38 MAPK and found that metformin was able to damp the increase of the activated state of this proteins induced by LPS, metformin suppressed the phosphorylation of the MAP kinases studied here, p38 and JNK. These results in microglial cells agree with those found in models such as the human monocytic leukemia cell line THP-1 (Chen, et al. 2018b), the rat cardiac myoblast H9c2 cells (Chen, et al. 2018a), and colitis mice models.

The PI3K/AKT pathway also regulates key proteins such as mTOR, FOXO and BAD, leading to multiple biological effects (Manning and Cantley 2007).

Therefore, we also measured the levels of AKT and found that, as expected, LPS induced a high decrease in the levels of this protein, whereas metformin treatment increased it. PI3K phosphorylation has an important function as an upstream activator of Akt. PI3K/Akt pathway plays a crucial role in the regulation of inflammatory mediators, in inflammatory cell recruitment, and in immune cell function (Hawkins and Stephens 2015). In accordance with our results, Liu et al., (2018) (Guimei, et al. 2018) has shown that metformin is able to decrease levels of IL-6 and TNF-α and increase Akt/PI3K expression in High-Fat Diet/Streptozotocin-Induced Type 2 Diabetes in Rats. These authors suggested that Akt/PI3K pathway possibly regulate the effects of metformin on inflammatory cytokines. Moreover, Tang et al., (2017) (Tang, et al. 2017) showed that

metformin ameliorates sepsis-induced brain injury by inhibiting apoptosis, oxidative stress and neuroinflammation via the PI3K/Akt signaling pathway.

In brief, metformin was able to prevent, totally or partially, most of the alterations induced by LPS. Thus, microglial activation (to both M1 and M2 phenotypes), expression of NADPH, production of ROS, activation of the inflammasome or production of pro-inflammatory cytokines induced by LPS were reduced or avoided by metformin. These anti-inflammatory actions of metformin seem to be mediated by the activation of the AMPK signaling, an energy sensing which mediates down-regulation of the NF-κB pathway. Since metformin is an inhibitor of complex I of the mitochondrial respiratory chain, it increases the AMP/ATP or ADP/ATP ratios, which activates AMPK after binding of AMP or ADP (El-Mir, et al. 2000; Owen, et al. 2000) with the subsequent down-regulation of the NF-κB pathway.

The results discussed so far support the use of metformin to prevent the inflammation associated with PD. However, metformin has also shown negative aspects that must be carefully considered before its possible use in this disease. Thus, metformin does not reduce the initial damage induced by LPS to astrocytes, as demonstrated by the loss of GFAP staining at the injection site of the endotoxin. The loss of GFAP staining in the SN after the injection of LPS is characteristic in this model. This loss was avoided by the treatment with minocycline (Tomas-Camardiel, et al. 2004), but not with dexamethasone (Castaño, et al. 2002a) which seems to suggest the existence of more than one mechanism able to induce the disappearance of astrocytes after exposure to LPS. The early loss of astrocytes is followed by an astrogliosis that repopulates the area initially affected (Herrera, et al. 2000). This transitory disappearance of astrocytes alters the BBB in the SN, perhaps facilitating the arrival from the blood of elements that can contribute to damaging this structure.

Furthermore, the loss of dopaminergic neurons from the SN is a key feature of PD. In previous works, our group demonstrated that the injection of LPS in this structure, besides provoking a

strong pro-inflammatory response of the microglia, it induces the specific death of nigral dopaminergic neurons (Castano, et al. 1998; Herrera, et al. 2000) and that this harmful effect could be avoided with the use of several anti-inflammatory substances such as dexamethasone (Castaño, et al. 2002b) and of other substances not categorized as anti-inflammatory compounds nevertheless exerted an anti-inflammatory effect, such as minocycline (Tomás-Camardiel et al., 2004) or simvastatin (Hernandez-Romero, et al. 2008). Metformin, however, despite the aforementioned anti-inflammatory effects, fails to prevent the death of the dopaminergic neurons induced by LPS. Interestingly, the protective effect of dexamethasone against an inflammatory process induced in the SN is not general, but depends on the inflammatory agent used; thus, in spite of avoiding the dopaminergic damage induced by LPS, dexamethasone did not prevent the death of the dopaminergic neurons of the SN induced by the injection of thrombin into the striatum (Herrera, et al. 2008). In this case, dexamethasone not only failed to prevent the damage induced by thrombin but increased it by inducing the expression of the MAO B enzyme, which acts in the catabolism of DA producing DOPAC and H_2O_2.

In consistence with the results presented here, in a previous work (Ismaiel, et al. 2016), it was shown that metformin, in spite of exhibiting strong *in vivo* anti-inflammatory properties, was unable to protect the damage induced by MPTP to the nigrostriatal dopaminergic system in mice; in fact, metformin reinforced the loss of DA and its metabolite DOPAC induced by MPTP. Other authors, however, have described a protective effect of metformin against MPTP on dopaminergic neurons in mice, which is supported by reduced brain oxidative stress, and TH-positive dopaminergic neurons (Lu, et al. 2016b; Patil, et al. 2014b). This discrepancy is possibly due to experimental differences in the dosage and MPTP exposure. Since MPTP and metformin are both inhibitors of complex I of the respiratory chain, they could be competing for their access to the complex and have some mutual influence on mitochondrial function. It can be postulated that in those previous studies metformin was not able to restore damaged neurons but reduced the harmful effects of

MPTP. In this sense, the use of metformin in other animal models of PD, such as the LPS model used in the present work, would allow a better study of its potential usefulness.

If metformin is not able to avoid the dopaminergic damage induced by LPS in this experimental design, despite the anti-inflammatory effects demonstrated, it may be because it is exerting some harmful effect on the dopaminergic neurons independent of the inflammatory processes induced by the single injection of LPS. It is known that metformin is a weak inhibitor of complex I of the mitochondrial respiratory chain (El-Mir, et al. 2000; Owen, et al. 2000) and complex I deficiency has been long associated with mitochondrial dysfunction and PD risk (Schapira and Jenner 2011); Thus, despite its anti-inflammatory effects, metformin could be producing an alteration in the ability of mitochondria to produce the enormous amount of energy necessary for the functioning of the dopaminergic neurons, inducing a mitochondrial oxidant stress that could be fatal in the long term. The question that arises is why metformin should produce such a specific effect on a particular neuronal phenotype if it is administered orally. The key to explain this specific effect could be in the complex axonal arborization of these neurons (Hunn, et al. 2015). Thus, dopaminergic neurons seem to have a number of synapses and an unmyelinated axonal arborization that would greatly exceed those of other neuronal phenotypes (Pissadaki and Bolam 2013) making them very sensitive to any energy imbalance that they may suffer (Bolam and Pissadaki 2012; Surmeier, et al. 2017), regardless of how small it might be. Thus, treatment with metformin, despite the benefit of its anti-inflammatory action, could be producing an alteration in the ability of mitochondria to produce the enormous amount of energy necessary for the functioning of these neurons, inducing a mitochondrial oxidant stress that could be fatal in the long term.

Another important factor that should be taken into consideration is the dose being used of metformin. Isoda et al. 2006 showed that the ability of metformin to inhibit proinflammatory responses in human vascular wall cells depends largely on the dose being used.

Should we use metformin for the prevention and treatment of Parkinson's disease?

Over the last 40 years, metformin has revolutionized the treatment of type-2 diabetes worldwide and is still the most influential oral antidiabetic drug today. The key role of insulin in nutrient sensing which balances growth and proliferation with life extending conservation makes metformin an interesting drug.

Type-2 diabetes is a disease with high prevalence nowadays and seems to be a risk factor for some neurodegenerative diseases such as AD (Cooper, et al. 2015; Monette, et al. 2014; Palta, et al. 2014; Vincent and Hall 2015) and PD (D'Amelio, et al. 2009; Driver, et al. 2008; Gang, et al. 2007; Miyake, et al. 2010; Schernhammer, et al. 2011; Simon, et al. 2007; Xu, et al. 2011). Until now, only a few studies have assessed the effect of metformin on this neurodegenerative disease and the results are conflicting. While some of these studies have shown beneficial effects on AD (Allard, et al. 2016; Lennox, et al. 2014; Pintana, et al. 2012), and PD (Katila, et al. 2017; Lu, et al. 2016a; Patil, et al. 2014b) others, including ours, show harmful effects or no effect (Chen, et al. 2016a; Ismaiel, et al. 2016; Kuan, et al. 2017; Thangthaeng, et al. 2017). Trials using metformin to treat or protect against neurodegenerative diseases in humans and animals have also produced mostly conflicting results. Therefore, the data shows either positive, no or even detrimental effects of metformin on neurodegenerative processes in cell cultures, animals and humans. These contradictory results may be due to differences in the experimental design and to the multiple biological pathways in which metformin exerts influence. Precisely because of this complexity, it is possible that this compound cannot be used as a common treatment for different neurodegenerative disorders. In fact, it is possible that the patient's risk profile determines the type of effect produced by metformin (Wang, et al. 2017). Therefore, it will be necessary to assess very carefully the use of metformin for the treatment of diabetes in patients who may suffer from Alzheimer's or Parkinson's diseases. If metformin was to be used in the treatment of this type of disease, it would first be necessary to study in depth the most appropriate dosage in relation to the type of neurodegenerative

process that takes place. One of the major contributions of metformin is that it can help to understand the complex molecular mechanisms that underlie neurodegenerative processes, which would allow applying more specific treatments.

VI. CONCLUSION

1- Mteformin treatment is able to decrease the immune associated alterations in *in vitro* and in *in vivo* PD model based on the intranigral injection of LPS.
2- Metformin attenuates the oxidative stress accompanying neuroinflammation in PD.
3- Inflammasome activity is reduced upon metformin treatment, which suggests that it could be a proposed route for metformin anti-inflammatory activity.
4- Although metformin treatment is able to reduce the pro and anti-inflammatory forms of activated microglia, it could not protect against LPS induced damage to the astrocytes.
5- It was not able to protect against the death of DAergic neurons of the SN. On the contrary, Metformin exacerbated the damage.
6- We can conclude that the use of metformin treatment in diabetic patients should be done with caution due to the increased susceptibility of developing PD.
7- Metformin can be considered a risk factor for developing PD in diabetic patients.

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