University of Cagliari

PhD Degree in Neuroscience

Cycle XXXII

Neuroinflammation in Parkinson's Disease: role in neuropathology and L-DOPA-induced motor complications.

BIO/14 PHARMACOLOGY

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Final exam. Academic Year 2018 – 2019

Thesis defence: January-February 2020 Session

ABSTRACT

Background: Parkinson Disease (PD) is a neurodegenerative disorder characterized by the progressive dopaminergic loss in the Substantia Nigra (SN) and the presence of intracellular Lewy Bodies (LB) containing deposits of the protein α-synuclein (α-syn). Several studies identified the neuroinflammatory processes as important factors in the neuropathology of PD, involving mainly microglia cells. Indeed, in PD microglia lose their ability to autoregulate, sustaining a chronic pro-inflammatory environment in dopaminergic areas which exacerbates the neurodegenerative process. Moreover, recent pre-clinical studies have suggested a role of neuroinflammation in the pathophysiology of L-DOPA-induced dyskinesia, showing that the chronic administration of L-DOPA exacerbates the pre-existing inflammatory environment which may contribute to the altered neurotransmission associated with dyskinesia.

The main component of LB is α-synuclein in its fibrillar form, but recent evidence suggests that the most toxic strain of α-synuclein are small soluble α-synuclein oligomers. Moreover, structure-based features have been suggested to mediate the toxicity of highly toxic α-synuclein oligomers. While the central role of α-synuclein in PD is generally acknowledged, the mechanisms underlying the neurotoxicity of α-synuclein oligomers, and their pathological interaction with Central Nervous System (CNS) immune-cells within PD-related neuroinflammation is still largely unknown.

In the first part of our project (part I), we tested the neurotoxicity *in vivo* of α-synuclein oligomers previously recognized to hold a structure that confers high toxicity in *in vitro* models, and we focused on the inflammatory response elicited by these toxic α-synuclein oligomers and on their interaction with microglial cells.

Thereafter, in the second part of our project (part II) we addressed the role of neuroinflammation in L-DOPA-induced dyskinesia. L-DOPA therapy is the gold standard for PD, however long-term administration leads to the onset of L-DOPA-induced abnormal involuntary movements named dyskinesia. Recent studies have suggested that neuroinflammatory processes play a pivotal role in dyskinesia, and the proinflammatory cytokine Tumor Necrosis Factor (TNF)-α may be a key player being also involved in synaptic strength mechanisms and in angiogenesis, another important component in the neuropathology of L-DOPA-induced dyskinesia (LID). Here, we tested whether the immunomodulatory drugs thalidomide (TLD) and its more potent derivative 3,6-dithiothalidomide (DTT), which specifically inhibit the synthesis of TNF-α, were effective in alleviating the L-DOPA-induced dyskinetic outcome in a rat model of PD.

Methods I: α-synuclein oligomers (0,5 mg/ml) were unilaterally infused in the rat SN. One, three and five months after the infusion, rats were subjected to the Beam Challenging Test in order

evaluate motor performance. Then, brains were collected for immunohistochemical analysis of tyrosine hydroxylase in the SN, and measure of dopamine levels in striatal tissue by HPLC. Microglia reactivity, inflammatory markers and phosphorylated α-synuclein (p-αsyn) were analyzed in the SN pars compacta (SNpc) by confocal microscopy.
Methods II: Rats were stereotaxically infused with 6-OHDA into the left medial forebrain bundle. Three weeks after 6-OHDA infusion, rats received ten days treatment with L-DOPA plus benserazide (6 mg/kg each) and thalidomide (70 mg/kg) or 3,6'-dithiothalidomide (56 mg/kg), and Abnormal Involuntary Movements (AIMs) as well as contralateral turning were evaluated daily. Rats were euthanized 1 hour after the last L-DOPA injection, and levels of pro- and anti-inflammatory cytokines, microglia and angiogenesis markers were quantified in their striatum (Str) and SN pars reticulata (SNpr) to evaluate neuroinflammation and angiogenesis. We also evaluated GLUR1 levels in Str as a marker of post-synaptic changes.

Results I: oligomer infusion caused a gradual development of PD neuropathology. Microgliosis and increased levels of inflammatory cytokines were measured one month after oligomers-infusion, without any evidence of neurodegeneration and behavioral deficits. Notably, three months after the injection, rats displayed motor impairment, associated with 40% loss of dopaminergic neurons in the oligomers-treated SN, reaching a 50% cell loss after five months. Dopamine levels were significantly reduced by 40% in the striatum homolateral to α-synuclein infusion. An intense inflammatory response with reactive microglia and high levels of TNF-α immunoreactivity were detected in SNpc. Large deposits of p-αsyn were found within microglial cells three and five months post-infusion.
Results II: TLD and DTT significantly reduced the severity of AIMs while not affecting the contralateral turning. Both drugs inhibited the L-DOPA-induced microgliosis and excessive TNF-α in the Str and SNpr, while restoring control levels of the anti-inflammatory cytokine interleukine (IL)-10 at striatal level. DTT inhibited L-DOPA-induced angiogenesis in SNpr and Str. GLUR1 analysis revealed that L-DOPA-induced an overexpression of this GLUR1 subunit in the Str, that was restored to normal levels by DTT

Discussion I: we showed that particularly structured α-synuclein oligomers trigger a neurotoxic process accompanied by a neuroinflammatory response that precedes cell death, and by the development of PD-related motor symptoms. Reactive microglia acquired a proinflammatory phenotype, and the presence of deposits of p-αsyn suggest an intense phagocytosis of the protein. These data indicate that the local intracerebral infusion of α-synuclein oligomers model the α-

synuclein-induced neuropathology in PD, and suggest that the aberrant activation of microglial cells as a mechanism of α-synuclein oligomers-induced- neurotoxicity.

Discussion II: data from the second part of our study showed that the inhibition of TNF-α production by TLD and its analogue DTT attenuated the severity of LID by breaking the chronic inflammatory cycle induced by L-DOPA, restoring the cytokines to near physiological levels and inhibiting angiogenesis. TLD and the more recently synthetized analogues are FDA-approved drugs for several chronic inflammatory treatments. The present study suggest that thalidomide and more potent analogues may represent an effective therapeutic strategy to alleviate LID.

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ACRONYMS AND ABBREVIATIONS

AAV adeno-associated virus

ALP autophagy-lysosomal pathway

AMPA (S)-a-amino-3-hydroxy- 5-methyl-4-isoxazolepropionic acid

BBB blood–brain barrier

CNS Central Nervous System

COX cyclooxygenase

DARPP-32 DA- and cAMP-regulated phosphoprotein, Mr 32 kDa

DAT dopamine transporter

DTT 3,6-dithiothalidomide

ER Endoplasmic Reticulum

ERK extracellular signal-regulated kinases

GWAS genome-wide association studies

HLA human leucocyte antigen

IL interleukine

IMIDs immunomodulatory drugs

iNOS inducible nitric oxide synthase

KO knockout

LB Lewy Bodies

LID L-DOPA-induced dyskinesia

LPS lipopolysaccharide

LRRK2 leucine-rich repeat kinase 2

MHC-II major histocompatibility complex-II

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

MSNs medium spiny neurons

NF-kB Nuclear Factor kappaB

NMDA N-methyl-d-aspartate

NOX2 PHOX NADPH Oxidase

PD Parkinson Disease

PDGFB human platelet-derived growth factor subunit B

PFF preformed fibrils

PLD2 phospholipase D2

p-αsyn phosphorylated α-synuclein

p-αsyn phosphorylated α-synuclein

RNS reactive nitrogen species

ROS reactive oxygen species

SN Substantia Nigra

SNARE N-ethylmaleimide-sensitive factor attachment protein receptor

SNpc Substantia Nigra pars compacta

SNpr Substantia Nigra pars reticulata

TH Tyrosine Hydroxylase

Thy11 mouse thymus cell antigen

TLD thalidomide

TLRs toll-like receptors

TNF-α Tumor Necrosis Factor

UPS ubiquitin-proteasome system

VEGF Vascular Endothelial Growth Factor

VMAT2 vesicular transporter of monoamines

WT wild-type

α-syn α-synuclein

BACKGROUND

Parkinson's Disease (PD) is the second most common neurodegenerative disease, identified for the first time by James Parkinson in 1817 (Parkinson (2002). Neuropathological traits of PD are the progressive loss of dopaminergic neurons in the SN (Substantia Nigra) (Dauer & Przedborski 2003, Erkkinen et al 2018, Lees et al 2009) and the presence in the brain of LB (Lewy Bodies), which are deposits containing fibrillar α-synuclein (α-syn) (Baba et al 1998, Spillantini et al 1997) and other proteins such as ubiquitin, tau, parkin, heat shock proteins, oxidized/nitrated proteins, cytoskeletal proteins, proteasomal and lysosomal components and others (Xia et al 2008). Dopaminergic loss in the SN leads to motor dysfunctions, such as bradykinesia, resting tremors, rigidity and postural instability. Furthermore, PD patients show non-motor symptoms, such as anxiety, depression, sleep disorders and cognitive impairments (Chaudhuri & Odin 2010, Chaudhuri & Schapira 2009), which are probably related to the degeneration in other non-dopaminergic areas (Braak et al 2003)

The treatment with L-DOPA represents the gold standard for PD (Carlsson et al 1957, Rascol 2000). Unfortunately, long-term L-DOPA therapy leads to the onset of motor complications, such as motor fluctuations and L-DOPA-induced dyskinesia (LID) (Jankovic & Aguilar 2008). Conventionally, LID is a peak-dose dyskinesia which manifests with involuntary monophasic, dystonic, choreiform, and non-rhythmic movements of facial muscles, neck, limbs, and body axis, typically on the side of the body more severely affected by PD. The onset and intensity of dyskinetic movements involves several factors, such as the severity of dopaminergic degeneration and the L-DOPA dosage, administration route and regimen (Rascol et al 2015).

On the last two decades a growing amount of evidence suggested that neuroinflammation is a common trademark in different aspects of PD. While the role of a chronic and unremitting neuroinflammatory response in the neuropathology of PD is largely ascertained, the detrimental relationship with toxic forms of α-syn is becoming to be elucidated. Moreover, more recently studies point to the involvement of inflammatory mediators in the development of L-DOPA induced motor complications.

1. Neuroinflammation in PD neuropatholgy

An important histopathological marker of PD is neuroinflammation. In literature, lots of evidences show that neuroinflammation, and specifically microglia-mediated processes, play a key role in the development of PD (Hirsch & Hunot 2009, Hunot & Hirsch 2003, Joers et al 2017, Tansey & Goldberg 2010). Indeed, activated microglia, high levels of cytokines, Nuclear Factor kappaB (NF-kB) pathway activation, and oxidative damage have been reported in the cerebrospinal fluid (CSF) and brains of PD-affected patients, post-mortem PD brains at autopsy (Hirsch & Hunot 2009, Hunot & Hirsch 2003, McGeer & McGeer 1998) and in most experimental models of PD (Castano et al 1998, Czlonkowska et al 1996, Gao et al 2002, Herrera et al 2000, Kohutnicka et al 1998, Mogi et al 2000).

1.1 Microglial cells: the immunocompetent cells in our brain

Microglial cells represent the main immunocompetent macrophagic cells in the brain, in charge of immune surveillance and defense as well as maintenance of Central Nervous System (CNS) homeostasis (Streit 2002). The origin of microglial cells has been discussed for a long time: someone claimed that they derive from mononuclear cells in blood or perivascular cells and others believed that they derive from the neuroectoderm as the other glial cells (Davies et al 2013, Ginhoux et al 2010, Greter & Merad 2013, Mizutani et al 2012); but, the current hypothesis is that microglia derive from the yolk sac during embryogenesis (Alliot et al 1999, Ginhoux & Prinz 2015, Li & Barres 2018).

As a component of the immune innate system, microglia regularly assay the surrounding environment through surface receptors, searching for signals of external insults, such as pathogens (Davalos et al 2005, Lehnardt 2010, Nimmerjahn et al 2005), as well as internal signals due to damaged or dying cells (Bessis et al 2007, Hanisch & Kettenmann 2007). If some of these signals is detected, microglial cells activate to protect the CNS, solve the damage and to promote tissue repair (Goldmann & Prinz 2013, Minghetti & Levi 1998). Microglial activation entails rapid changes in cell morphology, represented by the transition from a stellate aspect, with small cell body and long ramified processes, to an ameboid aspect, with large cell body and short processes, and variations in phagocytic activity and release of cytotoxic and neuroprotective signaling molecules (Hanisch & Kettenmann 2007, Ransohoff & Perry 2009). As mentioned, microglial cells oversee phagocytosis in the brain. Similar to peripheral

macrophages, microglia-mediated phagocytosis includes the three steps “find-me”, “eat-me” and “digest-me” (Sierra et al 2013, Wolf et al 2017). Different classes of surface receptors directly recognize specific molecules, as phosphatidylserine and oligosaccharides, expressed in the surface of their targets (pathogens, dead cells or protein aggregates) and initiate the process. An important role in recognition of targets, including α-syn, is played by the toll-like receptors (TLRs) (Stefanova et al 2011).

Beyond the immune surveillance, microglial cells contribute to CNS homeostasis regulating neuronal proliferation and differentiation and affecting the formation, remodeling and deletion of synaptic connections in the healthy brain (Bialas & Stevens 2013, Blank & Prinz 2013, Hughes 2012, Lawson et al 1990, Perry et al 2007, Perry & O'Connor 2010, Tremblay et al 2010). In literature, there are clear evidences that microglia can influence neuronal membrane properties and synaptic connectivity releasing soluble signaling factors (Ferrini & De Koninck 2013, Lewitus et al 2016, Parkhurst et al 2013) or interacting with synaptic elements (Hong et al 2016, Schafer et al 2013, Sipe et al 2016, Tremblay et al 2010). Traditionally, cytokines released by microglia have been considered as a component of central immune system, but growing evidences are indicating that these microglia-released factors cover a prominent role as neuromodulatory molecules in the CNS (Marin & Kipnis 2013, Salter & Beggs 2014, York et al 2018). Of note, several studies identified Tumor Necrosis Factor (TNF)-α as an important regulator of neuronal function and synaptic plasticity (Beattie et al 2002, Leonoudakis et al 2004, Stellwagen & Malenka 2006). On one hand, microglia can sense neuronal activity and modulate synaptic function through the expression of numerous receptors for neurotransmitters and neuromodulators (Carta et al 2017, Pocock & Kettenmann 2007, Tremblay et al 2010); on the other hand, microglia release cytokines which can bind to receptors located in neurons or in the same microglial cells, providing paracrine as well as autocrine communication. Squarzoni et al., showed that during prenatal phase, microglia impact on synaptic circuitries in the forebrain (Squarzoni et al 2014); while the postnatal phase is characterized by a microglia-mediated pruning, necessary to remodel neural networks (Paolicelli et al 2011, Schafer et al 2013).

Conventionally, activated peripheral macrophages can assume two different phenotypes named M1 and M2 which correspond to a pro-inflammatory and to an anti-inflammatory profile, respectively (Mantovani et al 2004, Verreck et al 2004). Hence, the former is associated with

cytotoxicity and the other with repair and restoring (Mackaness 1962, Mills et al 2000). M1 activation state is associated with the production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12, and IL-1β and the upregulation of cell surface markers such as major histocompatibility complex-II (MHC-II) and CD86 (Martinez et al 2006, Nau et al 2002). M2 activation state includes three subtypes, named M2a, associated with the suppression of inflammation; M2b, related to phagocytosis; and M2c, implicated in tissue remodeling and immunoregulation (Biswas & Mantovani 2010, Edwards et al 2006, Martinez & Gordon 2014, Stout et al 2005). Each phenotype is activated by different stimuli: M2a is stimulated by IL-4, M2b by TLR ligands and M2c by the cytokine IL-10. However, following studies showed that this classification is not realistic, because different pathways overlap, indicating that the states of activation are dynamic and not so polarized (Martinez & Gordon 2014, Murray et al 2014, Zhu & Paul 2010). The duration of the inflammatory response depends on the stimulus; persistent inflammatory stimuli lead to a chronic state of inflammation.

When an insult occurs in the CNS, the immune response, can be acute or chronic. The acute response is transitory and during this process microglia proliferate, switch the morphology from resting to activated and act with the aim to limit the damage, promote the repair process as well as improve neuronal survival. When the insult persists, the response becomes chronic and microglial cells change the expression of surface markers toward a chronic activation profile. It is not clear if this response, named microgliosis, is protective and beneficial or if it is detrimental and worsen the degenerative process. In addition, whether in PD it is a pathogenic response or a disease-initiating factor is not understood (Joers et al 2017).

Similar to peripheral macrophages, microglia can acquire different phenotypes and accomplish specific effector functions depending on the type, intensity and duration of the stimulus (Olah et al 2011, Perry et al 2007, Tansey & Goldberg 2010). Once activated, microglia begin to synthesize various cytokines, growth factors and cell surface molecules. Based on similarities between microglia and macrophages, the microglial responses have been classified with the same M1 and M2 system utilized for macrophages. However, this system is oversimplified and, remarkably, microglial phenotypes are heterogeneous and can exist as a continuum of intermediates between pro- and anti-inflammatory functions. Furthermore, several evidences demonstrate that not always microglia and macrophages respond similarly to the same stimuli

(Melief et al 2012, Zeiner et al 2015). To make even more complex this scenarious, anatomical regional differences have been reported in microglia distribution and phenotypes. Indeed, microglia population is denser in the telencephalon and, within the same region, myelinated regions have more microglia density than no-myelinated regions (Lawson et al 1990, Mittelbronn et al 2001). Notably, within the mesencephalon, the area with the highest microglia density is the SN, especially in the pars reticulata (Lawson et al 1990, Perry 1998).

Moreover, gene expression analysis and immunohistochemical studies found an heterogeneity within the mature microglial population in different areas of the brain (de Haas et al 2008, Doorn et al 2015, Grabert et al 2016, Lawson et al 1990, Sharaf et al 2013, Yang et al 2013); and De Biase et al. demonstrated that local regulatory cues determine the region-specific phenotype of microglial cells (De Biase et al 2017). These differences between areas can contribute to regional vulnerability in a diseased state; for instance, the expression of TNF-α mRNA is higher in rat microglia isolated from the SN, as compared to microglia isolated from hippocampus (Doorn et al 2015), suggesting that a region-related susceptibility may exist. This idea is also supported by the different gene expression profile among young and aged microglia across brain regions (Grabert et al 2016).

1.2 The role of microglia in Parkinson's disease neuropathology

Markers of inflammation, including pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) and as well as excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, were found in the brain, CSF and blood of PD patients, and in experimental models of PD (Joers et al 2017, Lopez Gonzalez et al 2016, Mogi et al 2007, Sawada et al 2006). First evidences for a role of neuroinflammation in PD came from post-mortem studies, which revealed microglial and complement activation, T-lymphocyte infiltration, and increased levels of pro-inflammatory cytokines in the SN and striatum (Str) of PD patients compared to healthy controls (Hirsch & Hunot 2009, Hunot et al 1999, Loeffler et al 2006, McGeer et al 1988) and in patients accidentally exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al 1999). These microglial cells were localized near the remaining dopaminergic neurons and exhibited morphologies suggestive of activated and phagocytic cells, comparable to those ones reported in aging (Jyothi et al 2015). Furthermore, neuroimaging analysis

demonstrated microglial activation in the brainstem, basal ganglia, and frontotemporal cortex in PD patients (Edison et al 2013, Gerhard et al 2006).

Initially, activation of the immune central system was considered as an event secondary to the pathology; however, subsequent studies showed that neuroinflammatory response can contribute to pathogenic processes. Experiments performed with rodent models demonstrated that the suppression of microglial activation ameliorated dopaminergic cell death in the SN, suggesting that microglial activation may trigger the neurodegeneration (He et al 2001, Wu et al 2002). Microglial cells can also be activated by α-syn (Su et al 2008). Furthermore, genetic findings supported the involvement of neuroinflammatory responses in PD: genotyping studies associated the human leucocyte antigen (HLA) class II region (a key constituent of the immune system) and the risk of developing PD (Saiki et al 2010) and this finding has been confirmed with genome-wide association studies (GWAS), (Nalls et al 2014). Additionally, epidemiological surveys suggested an involvement of inflammatory mechanisms in the development of PD (Chen et al 2005, Chen et al 2003, McGeer & McGeer 1998): pro-inflammatory cytokines such as IL-6 and TNF-α have been correlated with PD, and particularly with non-motor symptoms (Chen et al 2008, Menza et al 2010). A minor risk of developing PD correlated with the regular assumption of nonsteroidal anti-inflammatory drugs (Gao et al 2011b), probably due to the inhibition of the cyclooxygenase (COX)-mediated oxidation of dopamine (Chen et al 2003, Samii et al 2009, Teismann et al 2003) and suppression of toxic mediators synthesis (Chen et al 2005, Chen et al 2003). However, other studies affirmed that the protective effect of these drugs is inconsistent (Hancock et al 2007, Hernan et al 2006). Other data showed that PD patients presenting a marked proinflammatory profile in the serum showed a faster progression of motor and cognitive symptoms (Williams-Gray et al 2016). Moreover, the neurodegenerative process occurs earlier and more severely in the lateral part than in the medial part of SN; a microarray study comparing PD vs healthy brain tissue found an increased expression of genes encoding pro-inflammatory cytokines and subunits of the mitochondrial electron transport chain, and a reduced expression of several glutathione-related genes, in the lateral part of the SN (Duke et al 2007), consistent with an involvement of neuroinflammatory-mediated oxidative stress and mitochondrial dysfunction in PD neuropathology.

A body of of evidence also came from studies on cells and animal models: Gayle et al. (Gayle et al 2002) showed that IL-1β and TNF-α mediate 50 per cent of lipopolysaccharide (LPS)-induced dopaminergic neuronal cell death in primary cultures of rat midbrain using antibodies against these two cytokines (Gayle et al 2002). Ferrari et al. displayed that chronic expression of IL-1β in the rat SN induced by adenovirus injection resulted in dopaminergic degeneration after three weeks (Ferrari et al 2006). *In vivo*, direct LPS administration into the SN or peripheral administration, activated microglial cells and led to release of pro-inflammatory factors that preceded the degeneration of nigrostriatal pathway (Castano et al 1998, Dutta et al 2008). Depending on the regimen and dose administered, LPS induced a rapid or delayed and progressive neurodegeneration (Arai et al 2004, Gao et al 2002, Herrera et al 2000, McCoy et al 2006, Tanaka et al 2013). Remarkably, results from the LPS model indicate that neuroinflammation may be both an initiating stimulus and a driving mechanism of progressive neurodegeneration.

All the most common models of PD reported signs of microglial activation. Acute administration of MPTP caused an early and transient microgliosis in the SN, which preceded neurodegeneration and disappeared after few weeks following the toxin injection. Chronic administration of MPTP, which better mimics the progressive neurodegeneration and symptoms development observed in PD neuropathology, caused an early microglial activation in the SN which preceded nigral cell loss and persisted at least six months after MPTP interruption (Rodriguez et al 2007, Schintu et al 2009).

Microglial activation in the SN and Striatum has been observed after 6-OHDA inoculation and, depending on the injection site, the activation appeared early, concurrent or delayed with respect to DA degeneration (Marinova-Mutafchieva et al 2009, Sanchez-Pernaute et al 2004, Walsh et al 2011). Generally, studies performed in toxin-based models suggested and early and persisting microglial activation with respect to dopaminergic degeneration (Armentero et al 2006, Maia et al 2012, Walsh et al 2011).

The involvement of neuroinflammation in PD neuropthology has also been demonstrated in α-syn-based models: α-syn overexpression induced a progressive increase of activated microglia and pro-inflammatory cytokine TNF-α in the Str and SN preceding neurodegeneration (Su et al 2008, Watson et al 2012). Several studies indicated that mutated α-syn can affect microglial

activation and modulate the microglial phenotype (Barkholt et al 2012, Gao et al 2011a, Sanchez-Guajardo et al 2010).

Studies based on genetic models strongly corroborated the role of neuroinflammatory process in PD and dopaminergic degeneration. Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are responsible for genetic and idiopathic PD (Healy et al 2008). LRRK2 knockout (KO) rats did not show midbrain degeneration after LPS infusion or α-syn overexpression, while LRRK2 overexpression exacerbated the advancement of the neuropathology in A53T α-syn transgenic mice (Daher et al 2014, Lin et al 2009). At the same time, LRRK2 levels were upregulated in activated microglia under pathological conditions, while LRRK2 ablation or inhibition blunted the inflammatory response induced by LPS stimulation (Kim et al 2012, Marker et al 2012, Moehle et al 2012). These evidences suggest that neuroinflammatory responses play a pivotal role in neurodegeneration in these models.

All together, evidences from human and animal models firmly support the involvement of microglia-mediated neuroinflammation in PD. However, PET imaging studies in human patients revealed that the progression of clinical symptoms does not always correlate with microglia activation (Gerhard et al 2006, Ouchi et al 2009). Similarly, both subacute and chronic MPTP treatment in monkey elicited the same level of microglia activation, which appeared independent from the lesion extent in the SN (Hurley et al 2003, Vazquez-Claverie et al 2009). These findings may suggest that the progressive degeneration of nigrostriatal pathway might not be solely ascribed to microglia proliferation, but rather to changes in microglia phenotype (Joers et al 2017). Hence, in PD microglia lose their ability to self-regulate, leading to a disbalance between pro- and anti-inflammatory phenotypes, in favor of a chronic pro-inflammatory reactive state that contributes to neurotoxicity and dopaminergic degeneration (Carta et al 2011, Hirsch & Hunot 2009).

1.3 Microglia phenotypes in PD

Aiming at clarifying whether and how microglia phenotypes change during the progression of PD, numerous studies investigated the expression of microglial surface markers and soluble factors, as chemokines and cytokines, in various PD models. Overall, results from these studies suggest that microglia phenotypes are regulated depending on the extent of dopaminergic damage/degeneration in their microenvironment. Since high levels of MHC-I, MHC-II, ICAM-I

and OX-42 have been detected in the early stages of the pathological process (Cebrian et al 2014, Kurkowska-Jastrzebska et al 1999, Marinova-Mutafchieva et al 2009, Yasuda et al 2007), the early stages of injury might be characterized by an antigen-presentation effector function, followed by the induction of a strong pro-inflammatory phenotype characterized by high levels of pro-inflammatory factors such as TNF-α, interferon-γ, IL-6 and iNOS (Barcia et al 2011, Bian et al 2009, Liberatore et al 1999, Lofrumento et al 2011, Nagatsu et al 2000, Pattarini et al 2007, Pisanu et al 2014, Yasuda et al 2007). Notably, TNF-α seems to play a pivotal role in driving the degeneration: indeed, inhibition of soluble TNF-α protected dopaminergic neurons from 6-OHDA toxicity (Harms et al 2011, McCoy et al 2006, McCoy & Tansey 2008), and transgenic mice with nonfunctional TNF-α receptors or lacking TNF-α were preserved from MPTP toxicity. The increase of pro-inflammatory markers is accompanied by a decrease in anti-inflammatory markers in the SN, including surface markers such as CD206, YM-1, Arg-1 and FIZZ-1, as well as cytokines (Pisanu et al 2014, Rojo et al 2010), confirming the hypothesis of a prevalence of the pro-inflammatory phenotype with respect to the anti-inflammatory phenotype.
Apparently, in the late stages of degeneration, microglial cells switch to a phagocytic phenotype, with high levels of CD68 expression (Cho et al 2006, Kurkowska-Jastrzebska et al 1999, Marinova-Mutafchieva et al 2009, Sanchez-Guajardo et al 2010). Increased levels of CD68 have been found in a microglia subpopulation located near dopaminergic dying neurons, leading to the suggestion that phagocytic mechanisms, which are upregulated in presence of strong tissue damage, may be responsible for microglia-mediated secondary neurodegeneration. However, the role of microglia-mediated phagocytosis in PD remains debated. In physiological conditions, phagocytic activity is beneficial, but a dysregulation of phagocytosis together with a prominent pro-inflammatory activity may become detrimental and contribute to neuronal death (Joers et al 2017).

Furthermore, while the upregulation of pro-inflammatory markers appeared early and persisted during the chronic MPTP administration in mice, it was accompanied by a delayed downregulation of anti-inflammatory markers concurrent with the onset of motor symptoms and extended neuronal loss in SN (Pisanu et al 2014). Hence, both pro- and anti-inflammatory phenotypes of microglia co-existed in the early phase of the disease, while a prevalence of pro-inflammatory microglia occurred in the late stages of degeneration.

Inflammatory cytokines, chemokines and enzymes are codified by genes which have the DNA binding site for the transcription factor NFkB in their promoter region, indicating that NFkB is an important player in the neuroinflammatory responses (Grinberg-Bleyer et al 2015, Hayden & Ghosh 2004, O'Neill & Kaltschmidt 1997, Tansey & Goldberg 2010). Glial cells in SN of PD patients and MPTP-treated monkeys and mice showed high levels of p65, a subunit of NFkB, in the nucleus and cytoplasm (Ghosh et al 2007, Hunot et al 1997, Mondal et al 2012), and KO mice for c-Rel, a regulator subunit of NFkB, develop an age-dependent progressive neurodegeneration accompanied by accumulation of aggregated α-synuclein with activation of microglial cells in the SN, supporting the involvement of NFkB-mediated microglial activation in PD (Baiguera et al 2012).

All together, current evidences suggest that manipulation of the immune system and particularly of microglia polarization represents a potential target for neuroprotective strategies (Lecca et al 2018, Martinez & Peplow 2018, Pisanu et al 2014, Sanchez-Guajardo et al 2013, Tansey & Goldberg 2010). A plethora of studies identified several drugs and compounds with anti-inflammatory and immunomodulatory effects which protected dopaminergic neurons from degeneration in animal models of PD and ameliorated motor deficits (Jing et al 2016, Wang et al 2015, Zhao et al 2017).

2. α-synuclein and PD

Aggregated α-syn has been identified as a component of LB in patients with both sporadic and familial forms of PD (Baba et al 1998, Spillantini et al 1997). Moreover, α-syn has been related to PD by genetic evidences, since several mutations in the SNCA gene (A53T, A30P, E46K, H50Q, G51D and A53E) have been associated with PD and dementia with Lewy bodies (Appel-Cresswell et al 2013, Kruger et al 1998, Lesage et al 2013, Pasanen et al 2014, Polymeropoulos et al 1997, Proukakis et al 2013, Zarranz et al 2004).

2.1 Structure and physiological function α-syn is a small protein of 140 amminoacids encoded by the gene SNCA (Shibasaki et al 1995) and highly expressed in neurons (Jakes et al 1994, Maroteaux et al 1988). The primary structure of α-syn can be divided in three main domains:

- the N-terminal domain (1-60), which is highly conserved and consists of a repeated sequence which gives alpha-helical propensity to this domain. Through this domain, α-syn can interact with membranes (Breydo et al 2012, Vamvaca et al 2009);
- the central domain (61-95), which is hydrophobic and seems to be the domain involved in α-syn aggregation when it acquires the β-sheet structure (Breydo et al 2012);
- the C-terminal domain (96-140), which is acidic and contains several phosphorylation sites on Tyr-125, 133, 136 and Ser-129. Through this domain, α-syn can interact with proteins (Burre et al 2012, Burre et al 2010, Woods et al 2007).

α-syn can undergo post-translational modifications as acetylation, serine and tyrosine phosphorylation, lysine ubiquitination and tyrosine nitration which affect the activity of the protein and may be relevant for its pathological function (Barrett & Timothy Greenamyre 2015, Oueslati et al 2010). In physiological conditions, only about the 4 per cent of α-syn is phosphorylated, while the 90 per cent of aggregated α-syn in LBs appears phosphorylated (Anderson et al 2006, Fujiwara et al 2002, Kahle et al 2002, Tenreiro et al 2014). The major site of α-syn phosphorylation is Ser-129 and notably, α-syn phosphorylated in Ser-129 (pS129 α-syn) has been found in LBs in the SN and other areas of patients suffering from PD and other synucleinopathies (Anderson et al 2006, Fujiwara et al 2002, Kahle et al 2002, Nishie et al 2004, Saito et al 2003, Waxman & Giasson 2008). Furthermore, pS129 α-syn has been identified in transgenic mice expressing human mutant A30P, A53T, or overexpressing wild-type (WT) α-syn (Freichel et al 2007, Kahle et al 2002, Wakamatsu et al 2007). Further studies showed that phosphorylation at Ser-129 promotes oligomer accumulation (Anderson et al 2006) and exacerbates the formation of inclusions (Smith et al 2005, Sugeno et al 2008). Another common site of phosphorylation is Ser-87, which has been associated with synucleinopathies (Paleologou et al 2010).

α-syn is naturally unfolded, but can acquire an α-helical conformation through the N-terminal when it interacts with phospholipidic membranes (Chandra et al 2003, Davidson et al 1998, Fauvet et al 2012, McLean et al 2000, Theillet et al 2016, Weinreb et al 1996).

Although the tertiary structure has not been clearly established yet, the physiological state of α-syn seems in a dynamic equilibrium between a monomer apt to aggregation (Burre et al 2013, Theillet et al 2016, Weinreb et al 1996) and a stable tetramer which resists aggregation (Bartels et al 2011, Wang et al 2011, Xu et al 2019). The different conformations adopted by α-syn have a lifespan that depends on intramolecular interactions between amino acid residues (Alam et al 2019).

The physiological function of α-syn is still under debate. According to α-syn localization in presynaptic terminals and its association with synaptic vesicles (Larsen et al 2006, Maroteaux et al 1988, Nemani et al 2010), this protein is thought to be involved in synaptic vesicle trafficking and release (Diao et al 2013). α-syn may play a prominent role in the cycling of synaptic vesicles in different ways, from modulating the vesicle pool size to mobilization and endocytosis (Bendor et al 2013, Vargas et al 2014). Indeed α-syn is able to interact with synaptic proteins at pre-synaptic level, such as the phospholipase D2 (PLD2) (Gorbatyuk et al 2010, Jenco et al 1998, Payton et al 2004, Rappley et al 2009) and the proteins of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-complex, promoting its assembly and leading to synaptic release of neurotransmitters (Burre et al 2010).

In the presynaptic compartment α-syn can also interact with the vesicular transporter of monoamines (VMAT2) (Butler et al 2015, Guo et al 2008, Swant et al 2011, Wersinger et al 2006), but this function is not well understood. It has been shown that in SH-SY5Y cell lines, overexpression of WT α-syn inhibits VMAT2 and increases the level of cytosolic dopamine (Guo et al 2008), while in vivo the absence of α-syn decreased the reuptake of dopamine at striatal level (Chadchankar et al 2011) and caused functional deficits in the nigrostriatal dopaminergic system (Abeliovich et al 2000). Furthermore, α-syn is able to inhibit the dopamine synthesis with a Tyrosine Hydroxylase (TH)-related mechanism (Baptista et al 2003, Perez et al 2002, Yu et al 2004). All these evidences support the hypothesis that α-syn plays a key role in the regulation of dopaminergic transmission at presynaptic level.

There are evidences displaying that α-syn can perform chaperone-activity; indeed, it shares structural and functional homology with a family of cytoplasmic chaperones and can bind these proteins affecting their activity inducing oxidative stress and neuronal death (Chandra et al 2005,

da Costa et al 2000, Kanda et al 2000, Ostrerova et al 1999). α-syn is also involved in apoptosis, by affecting the MAPK pathway (Iwata et al 2001, Menges et al 2017).

Besides the presynaptic terminal, α-syn has been identified in other cellular compartments, such as endoplasmic reticulum and Golgi apparatus (Cooper et al 2006, Thayanidhi et al 2010), mitochondria (Devi et al 2008, Li et al 2007, Nakamura 2013) and nucleus (Goncalves & Outeiro 2013, Kontopoulos et al 2006, Maroteaux et al 1988, Mori et al 2002). Inside the nucleus, α-syn can regulate the expression of genes related to DNA repair, increasing levels of phosphorylated p53 and decreasing levels of acetylated histone 3 (Paiva et al 2017). Notably, in condition of oxidative stress, the amount of α-syn localized in the nucleus increases and impacts on the expression of genes involved in metabolism and mitochondrial biogenesis (Siddiqui et al 2012). In this way, it may contribute to mitochondrial dysfunction. In mitochondria, α-syn is physiologically involved as a regulator of the respiratory chain and the synthesis of ATP by interacting with the ATP synthase subunit α (Ludtmann et al 2016).

2.2 Pathological α-syn. Pathological α-syn is misfolded with a β-sheet enriched structure and a strong propensity to aggregation. Several events may be responsible for the conformational changes and subsequent aggregation of α-syn in PD, including mutations, modifications of the environment, and as well as post-translational modifications (Barrett & Timothy Greenamyre 2015, Fujiwara et al 2002, Kruger et al 1998, Lesage et al 2013, Polymeropoulos et al 1997, Proukakis et al 2013, Singleton et al 2003, Uversky et al 2001, Zarranz et al 2004). The aberrant reshaping from α-helical to β-sheet structure is a remarkable pathological feature which α-syn shares with prion proteins (Pan et al 1993, Wood et al 1999, Yonetani et al 2009). α-syn aggregation occurs through a multistep process which follows a nucleation-dependent model, where monomers first assemble to generate the aggregation nuclei (Buell et al 2014). Monomers assemble to form oligomers, which are converted into protofibrils and then to mature amyloid fibrils. Finally, in the elongation phase monomers are added at the fibrils terminal resulting in rapid growth (Buell et al 2014, Invernizzi et al 2012). Importantly, a dynamic equilibrium exists between all the different conformations, and in the PD brain different species and states of aggregation of α-syn have been detected, including unfolded monomers, soluble oligomers, protofibrils, and high molecular weight insoluble fibrils (Baba et al 1998).

Although LBs contain α-syn in its fibrillar form, recent evidence suggested that the toxic species is represented by small soluble oligomers (Karpinar et al 2009, Winner et al 2011), while LBs

may represent a protective mechanism by sequestering the toxic oligomers from the cytoplasm (Bengoa-Vergniory et al 2017, Bucciantini et al 2002, Muchowski 2002, Soto & Estrada 2008). Notably, LBs have been found in the brain of neurologically healthy subjects (Frigerio et al 2011, Parkkinen et al 2005) and the amount of LBs in the PD brain did not reflect the severity of symptoms such as dementia and cognitive deficits (Colosimo et al 2003, Parkkinen et al 2008). Ultimately, patients affected by familial forms of PD displayed neurodegeneration without LBs accumulation (Cookson et al 2008, Gaig et al 2007).

Oligomers toxicity was first suggested by their presence in degenerating areas in the PD brain (Sharon et al 2003, Tofaris et al 2003), and their toxicity has been demonstrated both *in vitro* and *in vivo*. α-syn oligomers induced cell death *in vitro* through various mechanisms including inhibition of tubulin polymerization, mitochondrial dysfunction, morphological alterations as well as calcium dysregulation (Chen et al 2007, Danzer et al 2007, Nasstrom et al 2011, Theillet et al 2016). Of note, α-syn mutants generated with a resistance to form fibrils and a major propensity to form oligomers have toxic effects in cell lines, rat primary neurons and in dopaminergic neurons of C. Elegans and Drosophila (Karpinar et al 2009).

Winner et al. elegantly demonstrated for the first time *in vivo* that oligomers are the main toxic α-syn species (Lo Bianco et al 2002), by using a rat model of synucleinopathies based on the injection of lentivirus expressing α-syn mutants which differed on their ability to generate fibrils. Their data showed that the expression of the oligomer-prone mutants E35K and E57K caused a higher loss of dopaminergic neurons as compared to the overexpression of WT α-syn. Conversely, the expression of fibril-prone variants only induced a mild dopaminergic loss, supporting the hypothesis that oligomer-forming mutants are the most toxic forms. Interestingly, Winner et al. showed that α-syn oligomers might interact and potentially damage the lipidic membranes (Winner et al 2011).

A wide variety of α-syn oligomers exist, which may differ in molecular weight, β-sheet content and tertiary structure which determine hydrophobic properties. Generally, the term oligomer includes all α-syn aggregates which have not acquired a fibrillary structure. Pieri et al. showed that large stable α-syn oligomers with an elongated shape seed the aggregation of soluble α-syn more efficiently as compared to low-molecular weight α-syn oligomers *in vitro* (Pieri et al 2016). SNCA mutations bring to the formation of oligomers with variable structure, with the A30P mutant generating annular pore-like oligomers, while A53T mutant tubular oligomers (Lashuel et

al 2002). To this regard, Danzer et al. showed that small annular oligomers affect calcium signaling and cause caspase activation and cell death *in vitro* but have low seeding activity, while larger oligomers do not have these toxic effects but show seeding properties (Danzer et al 2007).
More recently, Fusco et al., characterized two α-syn oligomers with different structure-based toxicity. These namely type A and type B shared structural and biological properties with other forms of α-syn oligomers previously identified, obtained from non-toxic and toxic α-syn variants (Cremades et al 2012, Fusco et al 2017). They showed that the oligomer toxicity was strictly dependent on their structure, and particularly on the presence of a core β-sheet-folded region together with a dynamic and accessible N-terminal region, which conferred toxicity to the oligomer type B, while were absent in the no-toxic oligomer A.
Fusco et al. further demonstrated that type A and B oligomers have different ability to damage lipidic membranes *in vitro*. Upon incubation with lipidic vesicles, both oligomers bound to membrane, however type B displayed a more stable binding and was able to insert within the membrane by the rigid core region. This resulted in oligomer-B induced toxicity when incubated with human neuroblastoma SH-SY5Y cells and rat primary cortical neurons (Fusco et al 2017). Interestingly, neurotoxic effects were similar to cells carrying a triplication in the SNCA gene (Angelova et al 2016, Deas et al 2016, Devine et al 2011).

2.3 α-syn oligomers-mediated toxicity. Although several evidences suggest that soluble oligomers of α-syn are the main neurotoxic strain, the underlying mechanisms of neurotoxicity are still largely unknown. A main gap in this regard is the poor availability of animal models of synucleinopathies that replicate cellular and behavioral changes and the progressive nature of the neurodegeneration with a pathologically relevant mechanism (Villar-Pique et al 2016). Toxic mechanisms may involve both a direct interaction with neuronal membranes or subcellular organelles or the interaction with glial cells, such as microglia and astroglia, as discussed below.
2.3.1 Synaptic impairment. On account of the key role that α-syn has in the SNARE-complex assembly at synaptic level, it has been proposed that oligomers may affect its function generating a synaptotoxic effect (Choi et al 2013). Diogenes et al. demonstrated that oligomers can impact in a negative way on neuronal signaling; they observed a suppression of long-term potentiation when exposing hippocampal slices to oligomers but not to monomers and fibrils (Diogenes et al 2012). Moreover, the toxic effect might depend on the N-methyl-d-aspartate (NMDA) receptors

since their blockade inhibited it (Diogenes et al 2012). Oligomers have been shown to decrease the excitability of pyramidal neurons (Kaufmann et al 2016). *In vivo* experiments have shown that expression of the oligomer-prone variant E57K induced a strong synaptic and dendritic loss and a decrease in synapsin I and synaptic vesicles, suggesting that oligomers may affect synaptic activity through the disruption of presynaptic vesicles (Rockenstein et al 2014). Recently, Wegrzynowicz et al. reported that dopaminergic dysfunction was associated with an increased formation of oligomers in the synaptic compartment and that pharmacologically modulating the aggregation of α-syn the dopaminergic release was restored (Wegrzynowicz et al 2019).

2.3.2 Toxicity against structural elements of the cells. One of the most common hypotheses to explain the toxic effect of oligomeric species of α-syn is the disruption of the membrane integrity and consequent loss of cell homeostasis. Toxic oligomers have the ability to permeabilize the lipidic bilayer, leading to an increase in the influx of ions into the cytoplasm (Danzer et al 2007, Lashuel et al 2002, van Rooijen et al 2010, Volles & Lansbury 2002, Volles et al 2001). Indeed, α-syn oligomers may cause cytotoxicity increasing the calcium influx (Danzer et al 2007), while another study showed that depleting calcium from the extracellular space counteracted the oligomer-induced cell death (Angelova et al 2016). Hydrophobic oligomers may insert into the membrane and promote the flux of hydrophilic molecules (Stockl et al 2012, van Rooijen et al 2010). Moreover, tubular-shaped oligomers may integrate into membranes inducing the pore-formation and acting as pathological membrane channels (Tosatto et al 2012, Volles & Lansbury 2002).

α-syn oligomers may also stabilize pre-existing membrane defects and accelerate membrane disruption (Chaudhary et al 2016), and may affect membrane properties such as input resistance, reducing neuronal excitability (Kaufmann et al 2016).

Notably, targeting the interplay between membranes and oligomers seems to improve deficits and protect from neurotoxicity (Wrasidlo et al 2016, Ysselstein et al 2017).

Toxic effects of α-syn have been observed in several cellular compartments, including the cytoskeleton where oligomers inhibit tubulin polymerization (Chen et al 2007), and the Endoplasmic Reticulum (ER), where aggregates accumulation leads to oxidative stress (Colla et al 2012).

All these evidences indicate that α-syn oligomers disrupt lipidic membranes and damage subcellular components, exerting a toxic effect on the cell.

2.3.3 Clearance impairment. Cells can exert a quality control and delete misfolded proteins mainly through two systems of clearance: autophagy-lysosomal pathway (ALP), which is directly responsible for oligomers deletion, and the ubiquitin-proteasome system (UPS). Both systems are impaired in synucleinopathies (Xilouri et al 2013). Lysosomal depletion and decreased lysosomal markers have been detected in nigral neurons positive for α-syn inclusions (Chu et al 2009, Dehay et al 2010). Lysosomal and autophagy malfunctioning dysfunction can trigger aggregation and accumulation of pathologic α-syn, that in turn can affect the ALP (Cuervo et al 2004, Tanik et al 2013, Winslow et al 2010, Xilouri et al 2009).

Reduced proteasome activities and structural alteration have been recorded in PD indicating UPS dysfunction and α-syn pathology may favor such proteasomal impairment (Furukawa et al 2002, McNaught & Jenner 2001, McNaught et al 2002). *In vitro*, overexpression of α-syn blocked proteasomal activity (Outeiro & Lindquist 2003, Tanaka et al 2001) and induced accumulation of ubiquitin-positive deposits and cellular damage (Stefanis et al 2001). Several evidences suggested that α-syn can inhibit UPS by the steric blocking of the proteasome machinery (Lindersson et al 2004, Snyder et al 2003). The 20S proteasome component was detected in LBs and in a-syn aggregates *in vitro* (Lindersson et al 2004, Tanik et al 2013). Notably, another component of the proteasome, the 26S, was isolated together with α-syn oligomers (Emmanouilidou et al 2010b).

2.3.4 Mitochondrial dysfunction. Generally, mitochondrial dysfunction has been reported as a distinctive trait of synucleinopathies (Nakamura 2013). Dopaminergic neurons are more sensitive to mitochondrial dysfunction than others, probably because of their high energy needs and elevated oxidative stress (Ryan et al 2015).

a-syn can fragment mitochondria through the N-terminal region, alter their permeability and membrane potential, increase levels of ROS and alter mitochondrial autophagy (Chen et al 2015a, Nakamura et al 2011, Sarafian et al 2013, Shen et al 2014). Particularly, a-syn aggregates have been found in the mitochondria of SN and Striatum from PD patients and correlated with complex I dysfunction (Devi et al 2008). Moreover, Plotegher et al demonstrated *in vitro* that oligomeric species altered mitochondria morphology (Plotegher et al 2014).

a-syn aggregates have been identified in the mitochondrial membranes of neuroblastoma cells expressing variants A53T and A30P and showing reduced mitochondrial transmembrane potentials and impaired respiratory activity (Parihar et al 2009). Importantly, oligomers but not

the other α-syn strains, can inhibit the mitochondrial protein influx, which was found impaired in PD brains (Di Maio et al 2016). A recent study showed that oligomers disrupt axonal mitochondrial transport and axonal integrity in human neurons derived from a Parkinson's disease patient affected by α-syn gene duplication (Prots et al 2018). The inhibition of oligomers synthesis restored the axonal impairment (Prots et al 2018).

a-syn oligomers have been identified not only in neuronal mitochondria, but also in astrocytic mitochondria. Astrocytes cover an important role in sequestering a-syn oligomers from the extracellular space and digesting them via the lysosomal pathway. Prolonged exposure to oligomers provoked incomplete digestion and deposition of the aggregates by astrocytes, leading to mitochondria damage (Lindstrom et al 2017).

2.3.5 Neuroinflammation. α-syn is released from neurons in a calcium-dependent manner (Emmanouilidou et al 2016, Yamada & Iwatsubo 2018) and may spread in the brain. In this context, microglia function might be extremely relevant. Firstly, a-syn is a chemoattractant for microglia: *in vitro* studies showed that a-syn induced microglia migration by up-regulating the adhesion molecule CD44 and cell surface protease membrane-type 1 matrix metalloproteinase MT1-MMP (Kim et al 2009). Moreover, interaction between a-syn and CD11b activate PHOX NADPH Oxidase (NOX2), leading to an increase in H_2O_2 levels, which stimulate microglia migration (Wang et al 2015). According to this, post-mortem studies found activated microglia in close contact with neurons showing α-syn deposits (Croisier et al 2005).

Several studies demonstrated that pathologic α-syn stimulates proinflammatory responses in microglial cells, with elevated production of cytokines such as IL-1β, IL-6, and TNF-a (Klegeris et al 2008, Lee et al 2010, Lee et al 2009, Su et al 2008), increased COX-2 and iNOS (Lee et al 2009, Su et al 2009, Su et al 2008), production of free radicals (Lee et al 2010, Su et al 2008). Microglia stimulation preceded the neuronal death (Barkholt et al 2012, Emmer et al 2011, Gomez-Isla et al 2003, Miller et al 2007, Sanchez-Guajardo et al 2010, Su et al 2009, Su et al 2008, Theodore et al 2008, Tofaris et al 2006, Watson et al 2012).

In literature, there are evidences that oligomers of α-syn can activate proinflammatory responses in vitro and in vivo, by TLRs and MAP kinase activation (Kim et al 2013, Wilms et al 2009). Moreover, oligomeric preparations activate glial cells and stimulate the production of ROS (Zhang et al 2005).

One of the main candidates as mediator of α-syn effects on microglia is represented by TLRs, which seems to be involved both in inflammatory responses and phagocytosis (Janda et al 2018, Kim et al 2012, Stefanova et al 2011). In fact, oligomeric α-syn activate microglial cells by stimulating TLR2, leading to the synthesis of inflammatory mediators (Kim et al 2013), while activation of TLR4 would activate α-syn phagocytosis (Stefanova et al 2011). The interaction between TLRs and oligomeric α-syn may activate the traslocation of NFkB, leading to an increase in TNF- α and IL-1β levels (Daniele et al 2015, Fellner et al 2013).

Human and rodent studies found high levels of both the TLR2 and TLR4 associated with α-syn deposition, in peripheral immune cells and in the Striatum as well as SN microglia (Doorn et al 2014, Drouin-Ouellet et al 2014), suggesting that α-syn may activate both pathways in PD. Microglial cells are mainly in charge for clearing the extracellular α-syn in the brain by phagocytosis (Ferreira & Romero-Ramos 2018, Lee et al 2008b).

This process seems to depend upon levels and type of α-syn (Lee et al 2008b, Park et al 2008) although results are sometime controversial. Microglia incubated with A53T mutant α-syn displayed a pro-inflammatory profile which was associated with impaired phagocytic function (Rojanathammanee et al 2011). In contrast, Roodvelt et al., 2010 showed that both WT and A53T α-syn promoted phagocytosis in microglial cells, while the A30P and E46K α-syn induced opposite effect. These studies clearly highlighted the importance of the different α-syn variants on the induced microglial phenotype and aberrant phagocytosis. Interestingly, WT α-syn was associated with a mild inflammatory response, with presence of pro-inflammatory as well as phagocytic microglia, suggesting the coexistence of mixed phenotypes (Roodveldt et al 2010).

Interestingly, microglia from mice lacking α-syn displayed an exaggerated response to LPS, with activated morphology, increased production of pro-inflammatory cytokines and expression of CD68, impaired phagocytic function, suggesting that physiological levels of α-syn may prevent inflammation and promote phagocytosis (Austin et al 2006).

A study from Park et al., clearly showed that α-syn conformation impacts on microglia phagocytic activity, demonstrating that monomeric α-syn stimulated while oligomeric α-syn inhibited both basal and LPS-stimulated phagocytosis (Park et al 2008). Additionally, an increased microglial phagocytic activity was observed after exposure to soluble or fibrillar α-syn, together with increased production of ROS and pro-inflammatory cytokines, confirming that microglia may acquire mixed phenotypes in pathological conditions (Fellner et al 2013).

Importantly, aging is a factor involved in the interaction between α-syn and microglia, since microglia-mediated phagocytosis of oligomeric α-syn decreases with age (Bliederhaeuser et al 2016). According to this, telomerase shortening, normally associated with aging, exacerbated α-syn pathology and altered microglial response (Scheffold et al 2016).

Therefore, these studies indicate a specificity of function for α-syn conformations. Of note, the coexistence of multiple α-syn conformations has been detected in extracellular fluids of PD patients, with prevalence of oligomeric α-syn which is uniquely formed in the pathological state (Majbour et al 2016, Tokuda et al 2010).

2.4 Spreading and prion-like behavior of α-synuclein

Recent evidence suggests that α-syn pathology can spread cell-to-cell. This implies that aberrant species can induce misfolding and aggregation of monomeric α-syn and α-syn oligomers have been implicated on this seeding effect. First evidences for cell-to-cell propagation emerged about twenty years ago, when researchers found the presence of LBs in fetal dopaminergic graft implanted in a PD brain 15 years earlier as an experimental therapeutic strategy. Here, pathologic α-syn spread from the endogenous to the implanted tissue (Kordower et al 2008, Li et al 2008). These studies, in addition to the Braak staging hypothesis, were considered strong proof of the prion-like spreading of α-syn pathology in the brain (Olanow & Prusiner 2009). Subsequent studies confirmed that α-syn can propagate from the host to the graft tissue (Angot et al 2012, Desplats et al 2009, Reyes et al 2014).

Further studies *in vitro* and *in vivo* further suggested that the oligomeric forms may play a pivotal role in the spreading process, inducing intracellular α-syn aggregation and promoting spreading (Danzer et al 2009, Hansen et al 2011). Interestingly, human-derived material led to disease in different species. The ability to transmit between different species is one of the chief features of prion proteins, suggesting that pathologic α-syn can acquire prion-like properties. Moreover, as prion proteins, aberrant α-syn is able to induce misfolded conformations in the endogenous proteins (Breydo et al 2012, Guo et al 2013, Peelaerts et al 2015).

Generally, abnormal α-syn within the cell is degraded by the ALP and/or UPS systems, depending on the conformation (Shin et al 2005). When the degradation fails, α-syn may deposit in intracellular inclusions or be released in the extra-cellular space. Once released, α-syn can spread among other cells.

Several mechanisms have been proposed to explain the cell-to-cell propagation, including passive diffusion (Ahn et al 2006, Chandra et al 2003, Grozdanov & Danzer 2018), membrane pores (Stockl et al 2013), exosomal transport (Emmanouilidou et al 2010a), tunneling nanotubes (Abounit et al 2016a, Abounit et al 2016b, Dieriks et al 2017), and the possibility of transport through carrier proteins (Sung et al 2001, Yang et al 2017). Passive release occurs by diffusion or leakage through the damaged cell membrane. Diffusion is only possible for monomeric species, not aggregated strains (Ahn et al 2006, Lee et al 2008a), can be bidirectional and probably requires an unidentified translocator (Lee et al 2005, Lee et al 2008a). Moreover, α-syn may cross membranes by pore-like structures, which may act as non-selective channels for the release (Hoogerheide et al 2017, Stockl et al 2013, Vasili et al 2019). In addition, mechanisms of exocytosis (Lee et al 2005) or via exosomes from the cell soma or from the synaptic button (Emmanouilidou et al 2010a), have been reported (Yamada & Iwatsubo 2018, Yang et al 2017). α-syn mutants and oligomers seem to be more prone to exosomal release than the WT form (Danzer et al 2012), supporting the hypothesis that oligomeric strains may mainly drive the propagation of α-syn pathology. Finally, α-syn can spread among cells by tunneling nanotubes, which are membranous bridges that connect the cytoplasmic compartments of cells (Abounit & Zurzolo 2012, Dieriks et al 2017).

Factors including intracellular concentration and conformation of the protein seem to determine the release process and function (Grozdanov & Danzer 2018). For instance, the release of monomers or small protein oligomers may have a physiological function for improvement of vesicle recycling or to protect from protein aggregation (Chandra et al 2005). Conversely, release of aggregated forms may have the aim to prevent neurotoxicity and recruit other cell types for clearance, as astrocytes and microglia (Grozdanov & Danzer 2018).

The uptake from the extracellular space plays a pivotal role in cell-to-cell propagation of pathologic α-syn and results in toxicity and seeding of aggregation of endogenous α-syn. Similar to release, the uptake of α-syn from the extracellular space can be passive or occur by diffusion or endocytosis, either pinocytosis and phagocytosis (Lee et al 2005, Mao et al 2016). While all mammalian cells can perform pinocytosis, only professional phagocytes such as microglia perform phagocytosis. Inside the cell, the endosome/phagosome should be driven to the phagolysosome for degradation. However, failure in the degradation process leads to the release

of α-syn in the cytoplasm, where it may directly seed the aggregation of the α-syn from the physiological pool (Flavin et al 2017, Freeman et al 2013, Grozdanov & Danzer 2018).

2.5 α-synuclein-based models of PD

In the last decade much effort has been spent in order to develop preclinical models of PD with neuropathological relevance, focusing on the pivotal role of α-syn aggregates on neurotoxic mechanisms. Most information about α-syn aggregation and toxicity came first from cellular models, which are useful to study the involvement of single pathways in pathological processes, but clearly cannot reproduce the *in vivo* physiology, which involves a crosstalk between different cell types and also the involvement of the surrounding tissue and vasculature (Alberio et al 2012, Astashkina et al 2012). Thereafter, different strategies have been used to reproduce α-syn pathology in *in vivo*:

- transgenic models;
- viral vector delivery;
- exogenous α-syn injection.

2.5.1 Transgenic models. Most of the transgenic models overexpress human WT α-syn (modelling SNCA multiplications), or human A53T, A30P mutant α-syn (modelling SNCA missense mutations), and two models overexpress mouse α-syn (Koprich et al 2017, Rieker et al 2011). In the most common models, transgene expression is controlled by the human platelet-derived growth factor subunit B (PDGFB) promoter, mouse thymus cell antigen 1 (Thy1) promoter and Th promoter (Fleming et al 2004, Manning-Bog et al 2003, Matsuoka et al 2001, Richfield et al 2002), using mostly transgenic mouse strains, but also rats (Nuber et al 2013). Generally, rats display some advantages as compared to mice, since they are able to perform more complex behavioral tasks and their brain is easier to image (Dehay et al 2016).

Some α-syn transgenic models exhibit representative features of PD such as α-syn deposits, nigrostriatal dysfunction, motor phenotype and non-motor features, but some of these models display minimal expression of the transgene in SN but often fail to reproduce DA degeneration (Daher et al 2009, Gispert et al 2003, Janezic et al 2013, Matsuoka et al 2001, Richfield et al 2002, Wakamatsu et al 2008, Yavich et al 2005). Interestingly, transgenic models without SN cell death show anomalies at striatal level, such as reduced TH immunoreactivity, decreased

dopamine levels or release, and increased dopamine transporter (DAT) expression, which are markers of an early nigrostriatal dysfunction (Clark et al 2010, Daher et al 2009, Hansen et al 2013, Kim et al 2015, Kurz et al 2010, Richfield et al 2002, Tofaris et al 2006). Motor impairment has been variably described regardless of the presence of α-syn inclusions or nigrostriatal dysfunction (Gomez-Isla et al 2003). Moreover, only a mild inflammatory response has been described in transgenic models (Sekiyama et al 2012).

2.5.2 Virus-based models. First virus models employed lentivirus to overexpress α-syn into the SN (Lo Bianco et al 2002, Winner et al 2011), however adeno-associated virus (AAV) allow to target more specifically neurons (Decressac et al 2012, Low & Aebischer 2012, Ulusoy et al 2010), becoming the most common vector for virus-based models which cause the overexpression of WT or A53T α-syn (Kirik et al 2002). These models exhibit progressive dopaminergic neurodegeneration with presence of α-syn inclusions and aggregates within neurons and motor symptoms (Decressac et al 2012). However, the AAV–α-syn models also present limitations. In fact, high expression of the protein, about four- to five fold above the normal and much higher than in human disease, is required to induce a dopaminergic cell loss (Decressac et al 2012). Moreover, the inflammatory response, which is a hallmark of Parkinson's disease, is transient and usually of modest magnitude (Chung et al 2009, Sanchez-Guajardo et al 2010, Theodore et al 2008).

2.5.3 Exogenous α-syn injection. These models are based on the unilateral injection of preformed fibrils (PFFs) of α-syn or human LB-containing homogenate into the SN, leading to a seeding process from the injection site (Abdelmotilib et al 2017, Luk et al 2012a, Mougenot et al 2011). The spreading process may involve endogenous α-syn and lead to extensive diffusion of pathological α-syn (as demonstrated by pSer129 immunoreactivity) in both hemispheres (Luk et al 2012a, Mougenot et al 2011). A significant spreading of pathological α-syn was detected 1-month post-injection and increased after 3 months (Luk et al 2012a, Luk et al 2012b, Masuda-Suzukake et al 2013). Dopaminergic neuron loss (Luk et al 2012a) as well as decrease in striatal dopamine levels were observed after six months (Paumier et al 2015).

These models are useful to evaluate approaches interfering with α-syn accumulation and aggregation. While brain-extract models show high variability, synthetic preparations, such as PFF, allow a more standardized model. Limitations of these models may be the long time to develop the neuropathology the motor symptomatology.

On the last years, the AAV-induced human α-syn expression has been combined with the injection of PFF in SN, leading to formation of α-syn aggregates, dopaminergic degeneration associated with motor deficits and a prominent inflammatory reaction (Thakur et al 2017). Moreover, in order to reproduce the non-motor symptomatology of PD, a similar model has been recently developed by injecting the AAV and the fibrils into the prefrontal cortex, leading to significant behavioral deficits in working memory, attention, and inhibitory control (Espa et al 2019). Despite the long and complex methodological procedure, the combined AAV/PFF-based approach has been successful in reproducing both neuropathological and symptomatic features of PD .

3. Neuroinflammation in LID

As mentioned above, recent evidence point to a physiopathological role of neuroinflammation in the development of LID. The physiopathology of dyskinesia is complex and includes neuronal and non-neuronal mechanisms which have been extensively characterized and investigated in the rat model of LID. In the rat bearing a unilateral lesion of the nigrostriatal pathway obtained by infusion of the neurotoxin 6-OHDA, repeated L-DOPA treatment induces abnormal involuntary movements named AIMs, which are considered a valid preclinical model of LID (Lundblad et al 2002). Classical neuronal mechanisms involve basal ganglia circuits, where L-DOPA affects signal transduction and neurotransmission. The nigrostriatal degeneration and the lack of dopamine lead to a hypersensitization of D1 receptors in the dorsolateral striatum and to a subsequent up-regulation of the cyclic adenosine monophosphate (cAMP) pathway (Feyder et al 2011, Picconi et al 2003, Santini et al 2007), up-regulation of the cAMP dependent proteins protein kinase A (PKA) and DA- and cAMP-regulated phosphoprotein, Mr 32 kDa (DARPP-32). Consequently, other signalling cascades are activated including the extracellular signal-regulated kinases (ERK) associated with gene expression regulation (Fasano et al 2010, Santini et al 2009). These maladaptive responses to L-DOPA impact on the expression and composition of synaptic receptors, affecting the excitability of medium spiny neurons (MSNs). Particularly, modifications in receptor subunits and post-translational changes of NMDA and the (S)-a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor drive abnormal synaptic plasticity at corticostriatal synapses (Ba et al 2006, Gardoni et al 2006, Santini et al

2007, Silverdale et al 2010). These pathological changes in MSN are exacerbated by the pulsatile stimulation of D1 receptors. Moreover, as the dopaminergic degeneration progresses, a more consistent amount of L-DOPA is metabolized by serotonergic neurons, which do not have the machinery to modulate the release of dopamine properly. This process strongly aggravates the intermittent release of DA and pulsatile stimulation of DA post-synaptic receptors, which play a key role in the onset of dyskinesia (Carta & Bezard 2011).

During the last decade growing evidence suggested a role of neuroinflammation in the development of LID. Although the mechanism underlying L-DOPA-induced inflammatory response are still elusive, the increased dopamine metabolism and associated oxidative load may likely contribute (Carta et al 2017). As discussed above, microglia-mediated responses play a pivotal role in the neurodegenerative process associated with PD and, moreover, data from preclinical studies support the involvement of neuroinflammatory responses in the motor complications associated with L-DOPA therapy. Results from rodent models of PD reported an inflammatory response to a L-DOPA dyskinetic treatment in the DA-denervated striatum, which is the main area where the modifications related to dyskinesia occur (Barnum et al 2008, Bortolanza et al 2015a, Bortolanza et al 2015b, Mulas et al 2016). Mulas et al., comparing the motor complications and neuroinflammation induced by a pulsatile or continuous administration of L-DOPA in 6-OHDA injected rats, found that the pharmacokinetic and delivery mode of L-DOPA, which are critical factors for the onset of LID, are also relevant for the development of inflammatory responses. Indeed, increased levels of markes of inflammation, such as OX-42 and TNF-α, have been detected in the dorsolateral striatum of pulsatile L-DOPA treated dyskinetic rats, while no signs of inflammation have been found in the dorsolateral striatum of rats treated with continuous L-DOPA which did not exhibit dyskinetic movements (Mulas et al 2016). Additionally, the administration of anti-inflammatory compounds such as corticosterone, nitric oxide synthase inhibitors and PPAR-γ agonists, significantly attenuates the dyskinetic outcome (Barnum et al 2008, Bortolanza et al 2015a, Bortolanza et al 2015b, Martinez et al 2015), while administration of LPS worsen the intensity of LID in hemiparkinsonian rats (Mulas et al 2016), corroborating the link between LID and neuroinflammation. Therefore, LID have been related to an increased inflammatory response in the CNS but not peripherally (Mulas et al 2016), according with the results from Barnum et al., who demonstrated that intrastriatal administration of an IL-1β receptor antagonist attenuated dyskinetic movements (Barnum et al 2008).

All these data suggest that a local action of cytokines may affect the onset of dyskinesia. Hence, preclinical evidence suggest that the neuroinflammatory mechanisms contributing to LID involve microglia-secreted cytokines. As said above, an intense crosstalk between neurons and microglial cells exists and chronically activated microglia can participate in the development of synaptic maladaptive responses driving the onset of LID. Specifically relevant to LID is the interplay between glutamatergic system and microglia. Clark et al. showed that the activation of CX3CR1 receptor located in microglia leads to the release of IL-1β, which acts on postsynaptic NMDA and ultimately brings to an increased release of neurotransmitter (Clark et al 2015). Notably, besides being an important player in immune response, the pro-inflammatory cytokine TNF-α can regulate synaptic plasticity interacting with the glutamatergic system (Balosso et al 2005, Balosso et al 2009, Beattie et al 2002) by both TNFR1 and the TNFR2 receptors, which mediate opposite effects on neuronal excitability (Balosso et al 2005). Interestingly, the application of cerebrospinal fluid from multiple sclerosis patients, containing pathological levels of pro-inflammatory cytokines (IL-1β and TNF-α), enhanced glutamate transmission in corticostriatal slices (Rossi et al 2012a, Rossi et al 2012b). The study from Beattie et al. showed that TNF-α promoted synaptic efficacy in hippocampal slices by increasing AMPA receptors expression in neuron surface, while blocking TNF-α caused the opposite effect. These data indicate that the persistent presence of TNF-α is necessary to preserve synaptic function at excitatory synapses (Beattie et al 2002).

Differently, a reduction in synaptic strength due to the TNF-α-mediated modulation of AMPA receptor trafficking has been observed in the MSNs (Lewitus et al 2014). Lewitus et al. observed decreased levels of surface AMPA receptors and reduced phosphorylation of DARPP32 in mouse striatal slices after exposure to TNF-α and proposed that TNF-α might play an adaptive role during the early stages of striatal dysfunction, inducing the internalization of AMPA receptor and, in this way, reducing synaptic strength at corticostriatal level (Lewitus et al 2014).

Generally, available data indicate that the TNF-α-mediated control of synaptic strength is region specific and dependent on the type of the synapse and especially on the type of post-synaptic element which can drive different responses after TNF-α stimulation. Moreover, data corroborate the involvement of TNF-α in a fine regulation of glutamatergic synapses.

Clinical and preclinical studies showed that dysfunction of plasticity at corticostriatal synapses and alterations in the AMPA receptor are strongly associated with LID (Calabresi et al 2010,

Calon et al 2003, Kobylecki et al 2010, Ouattara et al 2010, Picconi et al 2003). According to these findings, the blockade of AMPA receptor attenuates dyskinetic movements in MPTP-treated monkeys (Konitsiotis et al 2000).

Among the different AMPA receptor subunits, GLUR1 is considered relevant for the TNF-α mediated strengthening of synapses (Centonze et al 2010, Stellwagen et al 2005). Of note, not only the up-regulated expression but also the phosphorylation state of GLUR1 subunit seems to correlate with dyskinesia (Santini et al 2007). Indeed, LID is associated with an abnormal activation of the PKA/DARPP32 pathway, which in turn induces excessive GLUR1 phosphorylation and enhances glutamate transmission (Santini et al 2007).

These clues suggest that chronic elevated levels of TNF-α in the L-DOPA treated striatum might play a primary role in dysfunctional synaptic plasticity by modulating AMPA receptors and specifically GLUR1 subunit, contributing to LID.

4. Angiogenesis

Another important recognized mechanism in dyskinesia neuropathology is represented by angiogenesis. The proangiogenic activity of L-DOPA has been elegantly characterized in the parkinsonian brain and in animal models of LID (Janelidze et al 2015, Lerner et al 2017, Munoz et al 2014, Ohlin et al 2012, Teema et al 2016, Westin et al 2006). Microvascular changes, endothelial proliferation and increased levels of Vascular Endothelial Growth Factor (VEGF) have been reported in striatum and its output nucleus, especially in the Substantia Nigra pars reticulata (SNpr) of L-DOPA treated dyskinetic rats (Ohlin et al 2011, Westin et al 2006). Angiogenic activity and up-regulation of VEGF have also been observed post-mortem in brains from dyskinetic PD patients (Ohlin et al 2011). Of note, treatment with antiangiogenic compounds such as vandetanib and candesartan reduced the intensity of AIMs (Munoz et al 2014, Ohlin et al 2011).

In the adult brain, angiogenic processes can occur to supply local increased metabolic needs (Swain et al 2003). Since an increased energy use is thought to contribute to dyskinesia (Konradi et al 2004), L-DOPA-induced angiogenesis can be seen as a plastic response to provide oxygen and nutrients to the affected brain regions. Nevertheless, the extent of this angiogenic response is positively correlated with the severity of the dyskinetic movements induced by the treatment

(Westin et al., 2006), suggesting that the angiogenesis in this situation represents a maladaptive response (Lindgren et al 2009).

Angiogenesis and neuroinflammation are two events strictly related and modulate each other (Szade et al 2015). The majority of angiogenic processes are related to inflammation both in physiological conditions and pathological situations, such as in tumor growth or cardiovascular diseases. According to this, treatment with anti-inflammatory drugs inhibit blood vessel formation (Monnier et al 2005). Inflammatory stimuli can activate endothelial cells and increase microvascular permeability, leading to extravasation of immune cells, which release proangiogenic factors such as VEGF, TNF-α, basic FGF, IL-8, or insulin-like growth factor 1, and activate angiogenic responses (Bates et al 2002, Muller 2014, Sunderkotter et al 1994, Szade et al 2015).

5. TLD and derivatives

Despite many drugs are under evaluation in clinical trials for the treatment of LID, amantadine is currently the only drug in use to attenuate these motor complications following L-DOPA long-term treatment (Fox et al 2011). Therefore, LIDs treatment is an unmet urgent therapeutic need (Johnston et al 2018). To this regard, repurposing drugs represent an useful strategy to shorten the testing of new therapies. Indeed, repurposing FDA-approved drugs which are already in use for other pathologies, and have past pharmacokinetic and toxicological tests in human, can be a faster and less costly approach to move drugs to clinical testing in PD (Johnston et al 2018). Of note, amantadine itself represents a repurposed drug for LID (Rajput et al 1998, Verhagen Metman et al 1998).

The infamous drug thalidomide (TLD) belongs to the class of immunomodulatory drugs (IMIDs) and is now recognized as a compound with interesting therapeutic properties. Indeed, considering its immunomodulatory and antiangiogenic capacities, TLD is a good candidate for the treatment of many inflammatory diseases and cancer (D'Amato et al 1994, Franks et al 2004, Millrine & Kishimoto 2017). Moreover, safer and more potent TLD derivatives have been recently synthetized and are under investigation for neurological disorders (Frankola et al 2011).

TLD and derivatives act inhibiting the synthesis of TNF-α by increasing the degradation of TNFα-mRNA. This dampens the inflammatory cascade by preventing the activation of NF-kB and by decreasing the synthesis of factors involved in inflammatory processes (Deng et al 2013,

Mercurio et al 2017, Moreira et al 1993, Sampaio et al 1991, Tweedie et al 2011). Moreover, recent studies established that the antiangiogenic properties of TLD are also based on TNF-α inhibition (Mercurio et al 2017, Zhu et al 2018).

As said, more potent and selective derivatives have been synthesized (Frankola et al 2011). Among them, 3,6 dithiothalidomide (DTT) is a TLD derivative with higher selectivity and more potent TNF-α-inhibiting activity than the parent compound, while maintaining comparable blood–brain barrier (BBB) permeability (Tweedie et al 2011). Previous studies reported that DTT can attenuate memory deficits and the synthesis of inflammatory mediators in *in vitro* and *in vivo* models of Alzheimer's Disease (Russo et al 2012, Tweedie et al 2012).

AIM

Microglia-mediated neuroinflammatory processes play a pivotal role in the neuropathology of PD and in the physiopathology of LID. We further addressed both these issues in the present study, in order to better investigate the interplay between neuroinflammation and α-syn as a neuropathological mechanism of PD, and in order to investigate the potential use of immunomodulatory drugs to treat LIDs.

Project I

α-syn pathology represents an important hallmark of PD. As reported, several studies are indicating that the most toxic α-syn specie is represented by oligomers, which can exert their toxic effects in different ways. Among these effects, the interaction with the immune central system seems to be relevant. Particularly, it is conceivable that α-syn oligomers affect the effector function of microglial cells, which are the immunocompetent cells of our brain and in charge for phagocytosis of α-syn oligomers.

The study from Fusco et al., identified a toxic oligomeric specie relevant for PD (Fusco et al, 2017). *In vitro* experiments showed that these oligomers strongly interact with biological membranes, disrupting their integrity. Based on these evidences, we evaluated the neurotoxic effects of α-syn oligomers *in vivo*, in order to evaluate if they can reproduce the neuropathologic and symptomatic traits of PD and in order to study the α-syn-induced neuroinflammatory response and specifically the interaction between α-syn oligomers and microglia.

Firstly, we performed a dose finding experiment testing different doses of α-syn oligomers. Once established the optimal dose, we investigated at progressive time points the behavioral and neurotoxic effects induced by the inoculation of α-syn oligomers in the rat SNc. Moreover, we investigated the neuroinflammatory response to α-syn oligomers in the SN focusing on microglial cells. Finally, we assessed the presence of pathologic phosphorylated α-syn and its interaction with microglia.

Project II

Besides the classical neuronal mechanisms, LID involves non-conventional mechanisms such as neuroinflammation and angiogenesis. Both these events involve TNF-α, which is a potent pro-inflammatory cytokine but also affects neuronal excitability. As said, repurposing FDA-approved drugs can accelerate the process toward clinical testing.

Here, we hypothesized that TLD and DTT, which are immunomodulatory drugs and inhibitors of TNF-α, may attenuate dyskinetic movements in a rat model of PD. Hence, we tested the efficacy of these drugs in ameliorating AIMs induced by a chronic treatment with L-DOPA in 6-OHDA injected rats. Moreover, we investigated the effect of TLD and DTT on L-DOPA-induced neuroinflammation and angiogenesis in the SNpr and Str. Finally, based on the evidence that LID involves changes in glutamatergic receptor subunits, which in turn are under TNF-α modulation, we assessed levels of GLUR1 subunit in the dorsolateral Str.

METHODS

All experimental procedures met the guidelines and protocols approved by the European Community (2010/63 UE L 276 20/10/2010), by the Ethical Commission for Animal Care and Use at the University of Cagliari and Italian Ministry of Health (pr. # 1293/2015-PR).

1. Methods I

1.1 Oligomer synthesis

Oligomers were synthetized from α-syn previously expressed and purified in E. coli using the plasmid pT7-7 encoding for the protein (Aprile et al 2017, Fusco et al 2017). All α-syn fractions containing the monomeric protein were pooled together and concentrated by using Vivaspin filter devices (Sartorius Stedim Biotech, Gottingen, Germany). The protein purity was analyzed by SDS-PAGE and the protein concentration was determined spectrophotometrically.

One week before the intranigral injection, oligomeric species were prepared. Shortly, 6 mg of lyophilized protein was resuspended in PBS buffer at a pH of 7.4 and at a concentration of 12 mg/ml. The solution was filtered passed through a 0.22 μm cut-off filter and subsequently incubated at 37 °C for 24 h in stationary mode and without agitation in order to avoid acceleration of fibril formation (Chen et al 2015b). Despite this precaution, small numbers of fibrillar species were formed during this incubation period and removed by ultracentrifugation for 1 h at 90,000 rpm (using a TLA-120.2 Beckman rotor; 288,000g). The excess of monomers and small oligomers in the sample was then removed by means of several filtration steps using 100 kDa cutoff membranes, which resulted in the enrichment of the oligomeric species (Fusco et al., 2017)

1.2 Animals and stereotaxical surgery

In order to find the proper volume and concentration of α-syn oligomers, male Sprague Dawley rats weighting 275-300g were deeply anesthetized with Fentanyl (3 mg/Kg) and injected with 5, 10 and 20 μl of fluorescent oligomers at two different concentrations (1 mg/ml and 0,50 mg/ml). Three days after the surgery, animals were transcardially perfused with PFA 4% and brains

collected and vibratome cut in 40 µm slices. Slices were observed with a Fluorescence microscope (Zeiss, 5X) to detect any mechanical damage, excessive diffusion in the SN or along the injector-trace, or large deposits of aggregates that may reduce their ability to interact with membranes. Considering all these factors, we chose a final volume of 5 µl with 0,5 mg/ml concentration.

Rats (n=96) were stereotaxically injected in the left SNc (anteroposterior: -5,4; mediolateral: -1,9; dorsoventral: -7,2) with the established dose of oligomer (n=48) or vehicle (n=48). In order to test the toxicity of α-syn oligomers at three different time points after infusion, animals were randomly divided in groups of 16 for each time point (8 vehicle + 8 oligomer).

1.3 Beam Challenging Test

After one, three and five months post-surgery, rats were tested by the Beam Challenging Test to assess motor deficits, adapted from a protocol previously developed (Drucker-Colin & Garcia-Hernandez 1991). The testing apparatus consisted of 2 m-long wooden beam placed between a starting platform and the home cage with an slope of 15°. The beam width was 15, 10 and 5 mm. Rats were trained for three days to walk along the beam. The test day rats were videotaped. Rats were placed at the lower end of the beam and allowed to traverse the full length to reach to home cage, and the number of stepping errors was counted. The same procedure was repeated for the three different width beams. When the animal was not able to complete the task in 120 s or if it fell off the beam, the error score was assigned.

1.4 Immunohistochemistry

After the behavioural assessments, rats were anesthetized and transcardially perfused with 4% paraformaldehyde. Brains were post-fixed and 40 µm thick coronal sections were vibratome-cut. Midbrain sections (Bregma -4,4 mm) were pre-incubated with a blocking solution with normal serum/BSA and then immunoreacted with primary antibodies for single or double immunolabelling: polyclonal rabbit anti-TH (1:1000, Millipore); goat anti Iba-1 (1:1000; Novus Biologicals); polyclonal rabbit anti-TNF-α (1:500, Novus Biologicals); phosphorylated α-syn

(1:800, Abcam). Control slices were incubated without primary antibodies, and all slices were thereafter incubated with the appropriate fluorochrome-conjugated secondary antibodies. TH-positive cells were visualized using the classic avidin-peroxidase complex (ABC, Vector, UK) protocol was applied, using 3,30- diaminobenzidine (Sigma) as a chromogen. For fluorescence visualization of Iba-1 a two-step indirect labeling protocol was used, while a three-step detection was performed to increase the signal of TNF-α and phosphorylated α-syn, as previously described (Mulas et al 2016).

1.5 Stereological counting of TH immunoreactivity

All immunohistochemical reactions were analyzed by an operator blind to experimental groups, and different from the experimenter that performed the behavioral tests and histology. TH-immunoreactive neurons were counted bilaterally in the SNc as previously described (Lecca et al 2015). A dedicated software was used (Stereologer, System Planning and Analysis, Inc., VA) linked to a motorized stage on the BX-60 Olympus light microscope (Olympus). The total number of TH-stained cells was estimated by means of Optical Fractionator method, which combines the optical dissector with the fractionator sampling scheme, giving a direct estimation of the number of 3-D objects unbiased by shape, size and orientation (Mouton et al 2002). A systematic random sampling of cells within the area of interest was achieved by "Stereologer" program. Equidistant counting frames (frame area=50μm2) were obtained. Sampling fraction was delimited at low power and cells were sampled with a ×40 oil immersion objective through a defined depth with a 2 μm guard zone. The coefficient of error (CE) for each estimation and animal ranged from 0.05 to 0.1.

1.6 Microscopy analysis

Qualitative and quantitative analysis for markers of neuroinflammation was performed using a spinning disk confocal microscope (Crisel Instruments) with a 63X oil objective. Qualitative analysis for p-αsyn was perfomed using a laser scanning confocal microscope Zeiss with a 63X oil objective. Each frame was acquired eight times and then averaged to obtain noise-free

images. Surface rendering, maximum intensity, colocalization, and simulated fluorescence process algorithms were used (ImageJ and Imaris 7.0).

Volumes occupied by Iba-1 and TNF-α/Iba-1 colocalization were determined. For colocalization analysis, a colocalization channel was automatically generated by Imaris 7.3. A stack was obtained from each dataset (20–40 images). In the resulting stacks, ten regions of interest in each acquired area and for each animal (x =700 μm; y = 700 μm; z = 40 μm) were randomly chosen, and volume of the elements of interest was calculated. Values were expressed as a volume. The uninjected sides of brains were utilized as inner controls across studies.

1.7 HPLC

Striatal tissue was sonicated in 250 μl of 0.2 M perchloric acid, then centrifuged at 9391×g for 15 min at 4°C. Supernatant was filtered (0.6 μm) and diluted to a ratio 1:200. Twenty microliters were injected into an HPLC apparatus, equipped with a reverse-phase column (LC-18 DB, 15 cm, 5 μm particle size; Supelco, Milano, Italy) and coulometric detector (ESA Coulochem IIm, Bedford) to quantitate DA. Electrodes were set at +150 mV (oxidation) and −200 mV (reduction). The mobile phase (in mM: CH3COONa, 0.23 M; Citric acid, 0.15 M; Na2EDTA, 100 mg/ml; pH: 5.5) was pumped (Jasco Europe, Italy) at 1 ml/min flow rate. The assay sensitivity for DA was 5 and 10 fmol/sample, respectively.

1.8 Phagocytosis assay

The murine microglial cell line MMGT12 was used. Cells were cultured in DF culture medium comprising DMEM/F12 (1:1, vol/vol), supplemented with 10% fetal bovine serum (FBS) without antibiotics, grown in humidified atmosphere of 5% CO2 at 37°C, harvested and seeded twice a week. MMGT12 cells were seeded on 24-well plates at the density 5 x 104 cells per well, incubated in DF culture medium with α-syn oligomers (0.6 μm) for 6, 24 or 48 hours. The whole experiment was repeated three times, with an N=3 of independent samples in each experimental group. Upon verification of the homogeneity of the data, results from three experiments were pooled and statistical analysis was performed with a final N of 9 samples for experimental group.

Cells were washed in PBS, trypsinized in 0.05% trypsin for 3 min and re-plated in the same wells, using the same medium for one hour. Fluoresbrite Carboxy YG 6.0 micron Microspheres (Polysciences Inc., Warrington, PA, USA, cat#18141) were resuspended in PBS with 5.5 mM glucose, 1.5 mM Magnesium and 1 mM Calcium, and pre-opsonized by addition of 50% FBS and incubation for 30 min in 5% CO2 at 37 °C. 12 x 106 pre-opsonized microspheres were resuspended in 12 ml of DF culture medium without FBS, distributed on cells (around 10 beads/cell) and incubated for 2 hours. Cells were washed with PBS, trypsinized for 5 min and collected with 0.5 ml of culture medium in conical tubes (for flow cytometry), centrifuged at 1200 rpm for 5 min, PBS washed and centrifuged for 3 min. Cell pellet was suspended in 0.3 ml PBS containing 1% FBS. Cells were acquired in the green channel (502 nm) by FACSCanto II (BD Biosciences, Erenbodgem, Belgium). The percentage of green cells was determined on the single cell population by BD FACSDiva software.

1.9 Statistical analysis

Statistical analysis was carried out by Statistica 8 (Stat Soft Inc., Tulsa, OK, US). Behavioral, himmunohistochemical and HPLC data were statistically compared by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test, while in vitro data were compared by t-test. Level of significance was set at $p<0.05$.

2. Methods II

2.1 Drugs

L-DOPA methyl ester and benserazide (Sigma Aldrich, Milan) were dissolved in saline and administered subcutaneously (s.c.). TLD (70 mg/kg body weight, Sigma Aldrich, Milan) and DTT (56 mg/kg body weight) synthesized and chemically characterized to >99% purity, were suspended in 0.5 % carboxymethylcellulose and administered intraperitoneally (i.p.). The drug dose for DTT was chosen based on prior *in vivo* studies in rodents where drug benefits were observed using this dose (Belarbi et al 2012, Gabbita et al 2012, Yoon et al 2013, Zhu et al 2003). Due to the more potent anti-inflammatory actions of DTT over TLD, a higher TLD dose was used in order to compare similar activities. A pilot dose-finding study revealed that doses higher than 100 mg/kg acutely induced some sedation in the rats, while the dose of 70 mg/kg was devoid of such effect, as also confirmed by the contralateral turning behavior recorded in rats treated with TLD in association with L-DOPA. Therefore, the dose of 70 mg/kg was used for all further studies. Rats receiving these drug doses in the chronic regimen did not show signs of sufferance or altered general behaviors, except for some abdominal discomfort in the first 5 min after i.p. TLD administration. DTT is not an FDA approved compound and has not been tested in the clinic. TLD is currently a first line treatment for multiple myeloma with escalating doses from 200 mg/d to 400 mg/d (Kropff et al 2012), and similar doses were used in a clinical trial in Alzheimer disease patients (Breitkreutz et al 2007, Decourt et al 2017). Therefore, the TLD dose used in the present study, although on the high side, owns a clinical translatability significance.

2.2 Animals and pharmacological treatments

In order to achieve a full nigrostriatal lesion, male Sprague Dawley rats (Harlan, Italy) weighting 275-300 g were deeply anesthetized with fentanyl (3 mg/kg i.p.), and stereotaxically injected with 6-OHDA into the left medial forebrain bundle, as previously described (Fenu et al 2016, Mulas et al 2016).

The cylinder test and TH (tyrosine-hydroxylase) immunohistochemistry in the Substantia Nigra pars compacta (SNc) were performed to assess dopamine depletion. Only animals showing an

asymmetry score (n. of affected limb wall touches/n. of unaffected limb touches) <0.25 and a nigrostriatal degeneration above 95% were included in the experiments (representative SNpc lesion in Fig. 1A).

Three weeks after 6-OHDA lesioning rats were treated for ten days as follows: vehicle (Veh) rats received saline plus 0.5% carboxymethylcellulose (n=11), Veh+DOPA rats received vehicle and 6 mg/kg each of L-DOPA+benserazide(n=16), TLD+DOPA rats received TLD (70 mg/kg) and L-DOPA+benserazide (6 mg/kg each) (n=11), DTT+DOPA rats received DTT (56 mg/kg)and L-DOPA+benserazide (6mg/kg) (n=7), TLD+Veh and DTT+Veh rats received TLD (70 mg/kg) or DTT (56 mg/kg), respectively, and vehicle (n=4). TLD and DTT were administered daily 30 minutes before L-DOPA. The ten days administration regimen of L-DOPA is a widely used protocol for assessing LIDs in rats, and was chosen based on previous studies showing that after a daily drug treatment for 7-8 days the severity of AIMs reaches a plateau, as shown in supp. fig 1. In a second experiment TLD was tested in the expression of AIMs. One group of rats received daily for fifteen days vehicle+L-DOPA+benserazide (Veh+DOPA, 6 mg/kg each, N=5), one other group received the same treatment for 10 days, followed by daily TLD (70 mg/kg) + L-DOPA+benserazide (N= 5) for additional 5 days.

2.3 Behavioral studies

Limb and axial abnormal involuntary movements (AIMs) were recorded as a rodent model of LID daily during the 10 days treatment, together with contralateral turning behavior. One hour before drugs administration, rats were placed in separate cages. Individual dyskinetic behaviors were recorded for 1 min every 20 min, over a 120 min period. Dyskinetic behaviors were classified into three subtypes: limb AIMs (horizontal purposeless movement of the anterior limb contralateral to the lesion side), axial AIMs (dystonic posture of neck and trunk, toward the side contralateral to the lesion), and contralateral turning (tight turns toward the side contralateral to the lesion). The seconds spent by the animal doing each individual AIM and the number of contralateral turns over the 1 min monitoring period were assessed.

2.4 Immunohistochemistry

On the last day of pharmacological treatments, rats were anesthetized and transcardially perfused with 4% paraformaldehyde 1 hour after L-DOPA administration, when rats show the peak of AIMs response. Brains were post-fixed and 40 μm thick coronal sections were vibratome-cut. Striatal (Bregma +1,28 mm) or midbrain sections (Bregma -4,4 mm) were pre-incubated with a blocking solution with normal serum/BSA and then immunoreacted with primary antibodies for single or double immunolabelling: polyclonal rabbit anti-TH (1:1000, Millipore); monoclonal mouse anti-GluR1 (1:1000, Novus Biologicals); monoclonal mouse anti-OX-42 (1:400, Serotec-Oxford); polyclonal rabbit anti-TNF-α (1:500, Novus Biologicals); polyclonal rabbit anti-IL-10 (1:200, Abbiotec); polyclonal rabbit anti-GFAP (1:1000, Novus Biologicals); monoclonal mouse anti-vimentin (1:1000, Novus Biologicals) and monoclonal mouse anti-VEGF (1:200, Novus Biologicals). Control slices were incubated without primary antibodies, and all slices were thereafter incubated with the appropriate fluorochrome-conjugated secondary antibodies. For fluorescence visualization of TH, OX-42, GFAP and vimentin a two-step indirect labeling protocol was used, while a three-step detection was performed to increase the signal of cytokines.

2.5 Confocal microscopy analysis

Qualitative and quantitative analysis for GLUR1 expression, all markers of neuroinflammation and angiogenesis immunoreactivity (IR) was performed using a Leica 4D confocal laser scanning microscope, equipped with an argon-krypton laser. Confocal images were generated using PL Fluotar, 40 oil (na. 1.00) lens, as previously described (Mulas et al 2016). Each frame was acquired eight times and then averaged to obtain noise-free images. Surface rendering, maximum intensity, colocalization, and simulated fluorescence process algorithms were used (ImageJ and Imaris 7.0).

Volumes occupied by GLUR1, cytokines/OX-42 colocalization, vimentin and VEGF were determined. For colocalization analysis, a colocalization channel was automatically generated by Imaris 7.3. A stack was obtained from each dataset (20–40 images). In the resulting stacks, ten regions of interest in each acquired area and for each animal (x = 200 μm; y = 200 μm; z = 20

μm) were randomly chosen, and volume of the elements of interest was calculated. Values were expressed as a volume. The unlesioned side of Veh-treated brains was utilized as the inner control across studies.

2.6 ELISA assay

For multiplex enzyme-linked immunosorbent assay (ELISA) the tissues were homogenized in a Tris based lysis buffer with 3 x protease and phosphatase inhibitors (Halt Protease & Phosphatase inhibitor Single-Use Cocktail, Thermo Scientific), then the samples were centrifuged (10,000g, 4oC, 10 minutes) and the supernatant placed into a new set of tubes. The protein concentrations were measured by use of the bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay kit, Thermo Scientific). Equal protein loading concentrations (150 μg/well) were assessed in duplicate on the Mesoscale Discovery (MSD) V-PLEX Proinflammatory Panel 2 for rat. The protocol suggested by manufacturer was used. Samples were briefly centrifuged (10,000g, 4oC, 60 s) to remove any particulate material, and a multiplex standard and tissues were added to the plate. The plates were incubated for 2 hr, washed, and incubated for2 hr with a multiplex detection antibody solution. After washing, the Read Buffer was added and the electrochemiluminescence (ECL) signal levels were measured (MESO QuickPlex SQ 120). The MSD Discovery Workbench software was used to determine the tissue cytokine protein levels by comparing the rat tissue ECL signals to those of the appropriate protein standard curve. Protein concentrations were expressed as pg/ 150 μg of tissue. Data points were assessed to identify any statistical outliers (Grubb's Test), if any were identified they were excluded from further analysis. Data are expressed as mean±SEM.

2.7 Statistical analysis

All data were statistically analyzed by Statistica 8 (Stat Soft Inc., Tulsa, OK, US). Data from behavioral studies (AIMs and contralateral turning) were analyzed by Two-way ANOVA with repeated measures or One-way ANOVA, followed by Newman-Keuls post-hoc tests. Linear regression was calculated to assess development of AIMs. Immunohistochemistry data were

analyzed by One-way ANOVA followed by Tukey's HSD post-hoc test. Significance was set at $p<0.05$.

RESULTS

1. Results I

We firstly investigated the effect produced by the α-syn oligomer infusion into the SN in terms of motor impairment, neurodegeneration and neuroinflammation in the SN.

In a preliminary study, in order to select the optimal oligomer dose and concentration that did not induce mechanical damage in the injection site, local deposits or diffused staining, we performed a dose-finding experiment by using fluorescent α-syn oligomers. Results showed that an oligomer dose of 1 mg/ml induced a mechanical damage to the tissue when injected in infusion volumes of 10 and 20 µl, and large fluorescent deposits in the infusion site when the infusion volume was 5 µl (Fig.1), according with the notion that highly concentrated α-syn oligomers rapidly aggregate and precipitate. In contrast, the dose of 0.5 mg/ml in 5 µl was void of damaging effects, did not form large local deposits and did not diffuse in the tissue and along the injector-trace (Fig. 1). This dose was therefore used for all subsequent studies.

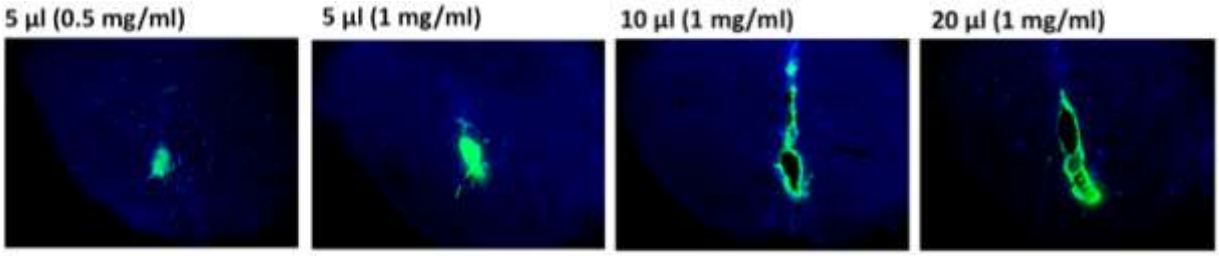


Fig.1 Representative images of the SNpc three days after the infusion of different concentrations/volumes of fluorescent α-syn oligomers.

1.1 The intranigral infusion of α-syn oligomers induced progressive motor deficits.

Motor performance was evaluated at progressive time points by mean of the beam challenging walking test. One month post-infusion of α-syn oligomers, rats did not display any motor impairment as compared with vehicle-infused rats in any of the three beams used (15-10-5 mm widths) (Fig. 2A). Conversely, three and five months post-infusion, α-syn oligomer-injected rats displayed a significant increase of errors per step when traversing the beams of 10 and 5 mm width as compared with vehicle-injected rats ($p<0.05$ by Tukey's post-hoc test; Fig. 2A). When

comparing the errors made in the same beam at progressive time points, we found a significant increase in the number of errors made by rats injected 5 months earlier as compared to rats injected 1 month earlier, suggesting that the development of motor deficits was progressive ($p<0.05$ by Tukey's post-hoc test; Fig.2B).

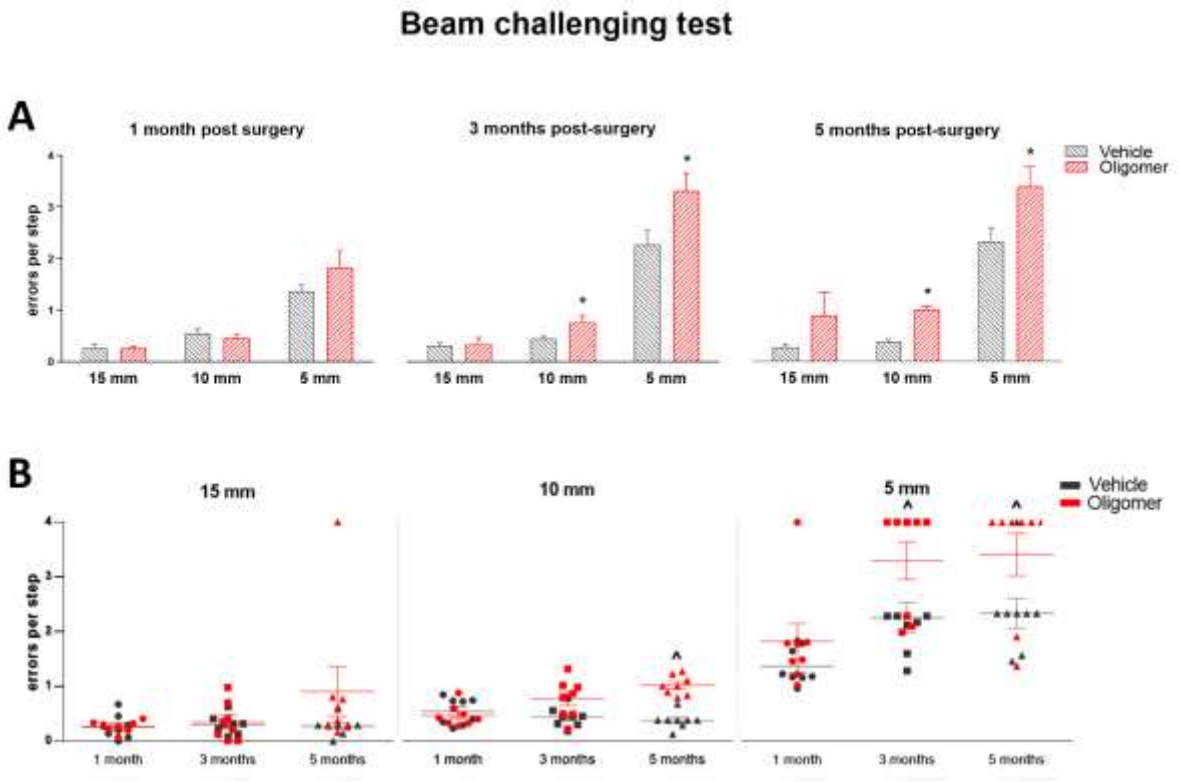


Fig.2. α-syn oligomer-infused rats developed progressive motor impairment. Motor deficits measured in Vehicle and Oligomer-infused animals in the three different beam widths (15, 10 and 5 mm) at each time point (one, three and five months post-infusion) (A). Scatter plots displaying the development of motor impairments through the different time points in the different beam widths (B). Motor impairment is expressed as errors per step. Values represent the mean ± SEM. *p<0.05 vs Vehicle, ^p<0.05 vs 1 month by Tukey post-hoc test (n=8).

1.2 The α-syn oligomers infusion reduced TH+ neurons in SNpc and reduced striatal dopamine levels.

Stereological analysis of TH IR in the SNpc showed that one month post-infusion of α-syn oligomers the density of TH+ neurons was similar to controls (Fig.3). Conversely, the density of TH+ neurons decreased after three (up to 40%) and five (up to 50%) months post-infusion in the

α-syn oligomers-infused SNpc as compared with the contralateral SNpc and with the vehicle-infused SNpc ($p<0.001$ vs all others groups; Fig.3).

As shown in Fig.4. the dopaminergic cell loss was significantly higher three months post-infusion as compared with one month post-infusion. Moreover, the dopaminergic loss was significantly higher five months post-infusion as compared with one and three months, suggesting that the neurodegenerative process was progressive ($p<0.001$ vs all others groups of the same time point; Fig. 4).

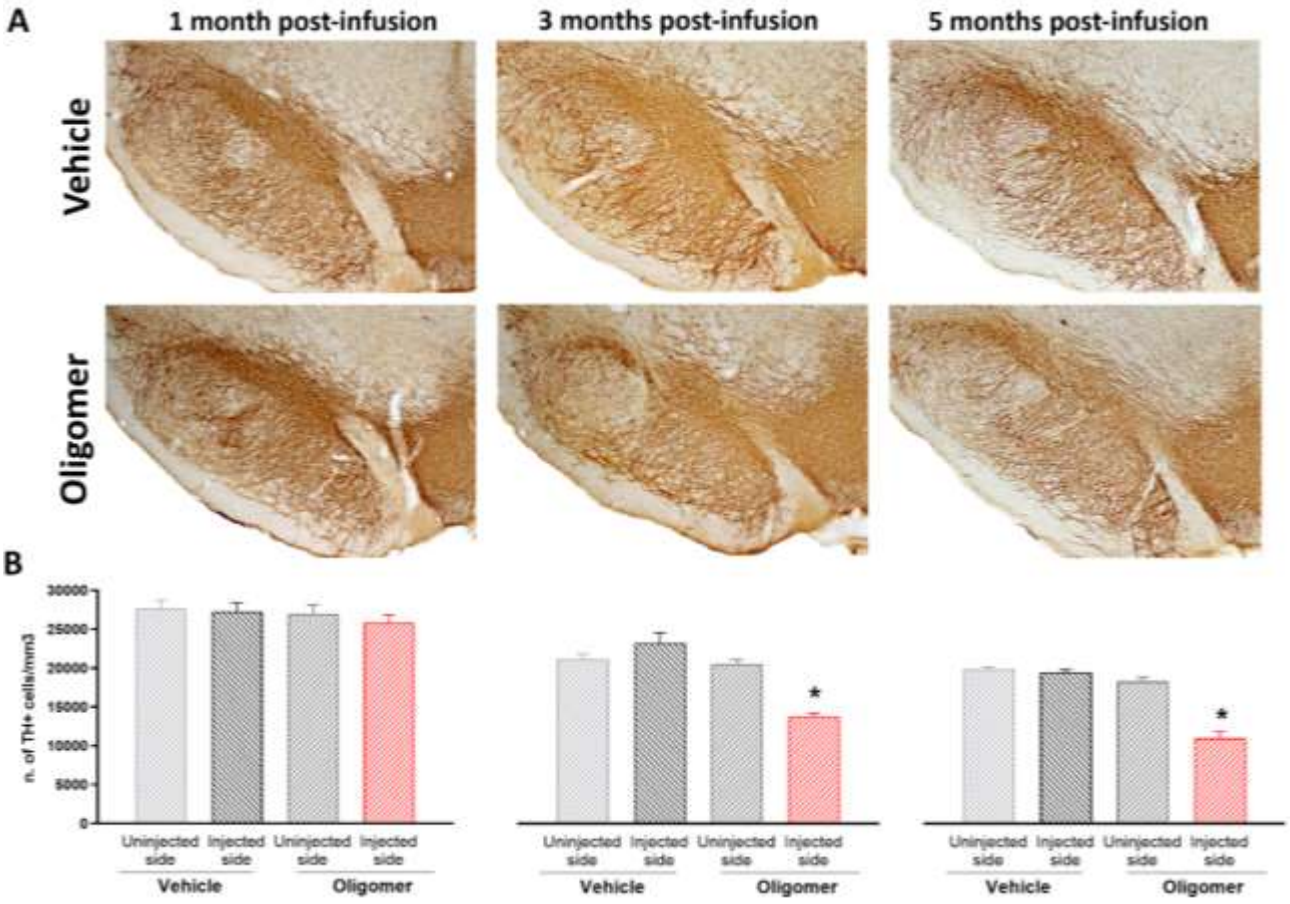


Fig. 3. The α-syn oligomers infusion induced dopaminergic loss in SNpc. Representative images of the TH IR in the vehicle- or α-syn oligomers-injected SNpc; magnification 5X (A). Total number of TH+ cells/mm^3 measured by stereological counting one, three and five months after the α-syn oligomers infusion (B). Values represent the mean ± SEM. $p<0.001$ vs all other groups of the same time point by Tukey's post-hoc test (n=8).

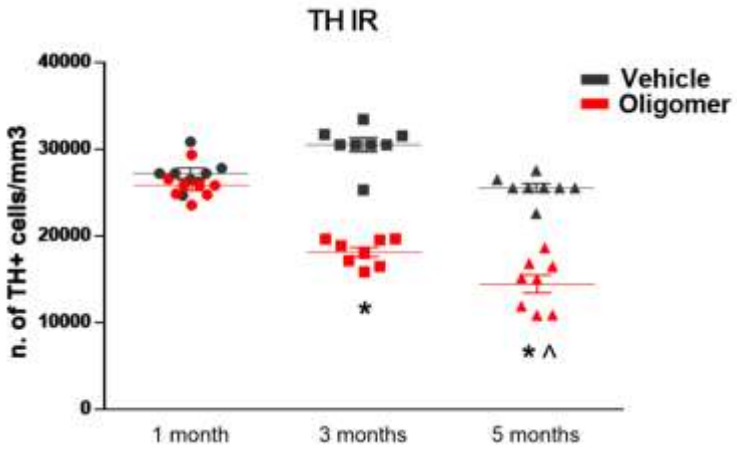


Fig. 4. The dopaminergic loss increased progressively. Scatter plots showing the total number of TH+ cells/mm3 measured by stereological counting in the vehicle- or α-syn oligomers-injected SNpc one, three and five months after the α-syn oligomers infusion. Values represent the mean ± SEM. *p<0.001 vs 1 month post-infusion, ^p<0.001 vs 3 months post-infusion by Tukey's post-hoc test (n=8).

1.3 The infusion of α-syn oligomers in SNpc caused a decrease in striatal dopamine content.

HPLC measurement of striatal dopamine levels was performed three months post-infusion and showed a significant reduction in the striatum homolateral to the oligomer-infusion as compared with the contralateral side as well as with the striatum of vehicle-infused rats (p<0.05 vs all other groups, Fig. 5).

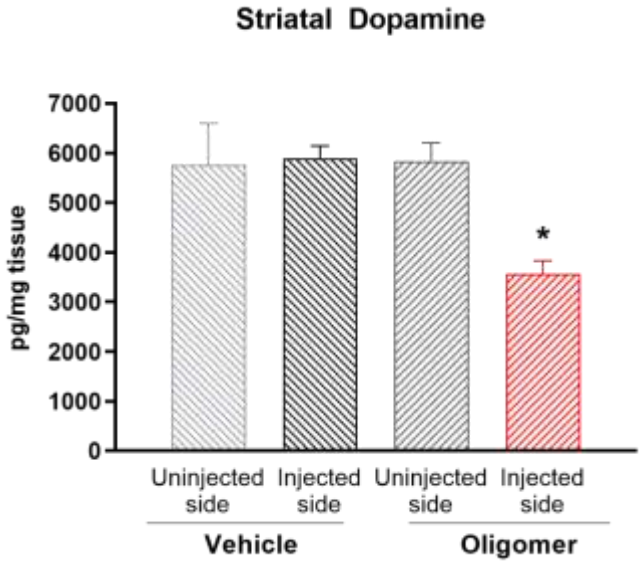


Fig. 5. Dopamine content decreased in the Str homolateral to the α-syn oligomers infusion. The graph shows levels of striatal dopamine three months after α-syn oligomer infusion, expressed as pg/mg of tissue. Values represent the mean ± SEM. *p<0.05 vs all other groups by Tukey post-hoc test.

1.4 The infusion of α-syn oligomers in SNpc induced prolonged microglial reactivity and increase of TNF-α in the SNpc.

Iba-1 IR. Our results showed that one month post-infusion of α-syn oligomers Iba-1 IR was significantly higher in the injected SNpc as compared with the uninjected SNpc (#p<0.05 by Tukey's post-hoc test; Fig. 6C). In contrast, a slight but not significant increase in Iba-1 IR occurred in the vehicle-injected SNpc as compared to the contralateral SNpc. Three months post-infusion of α-syn oligomers the Iba-1 IR was further increased with respect to Iba-1 levels measured 1 month post-oligomers infusion (^p<0.0001 vs oligomer-injected side by Tukey's post-hoc test, Fig. 6C).

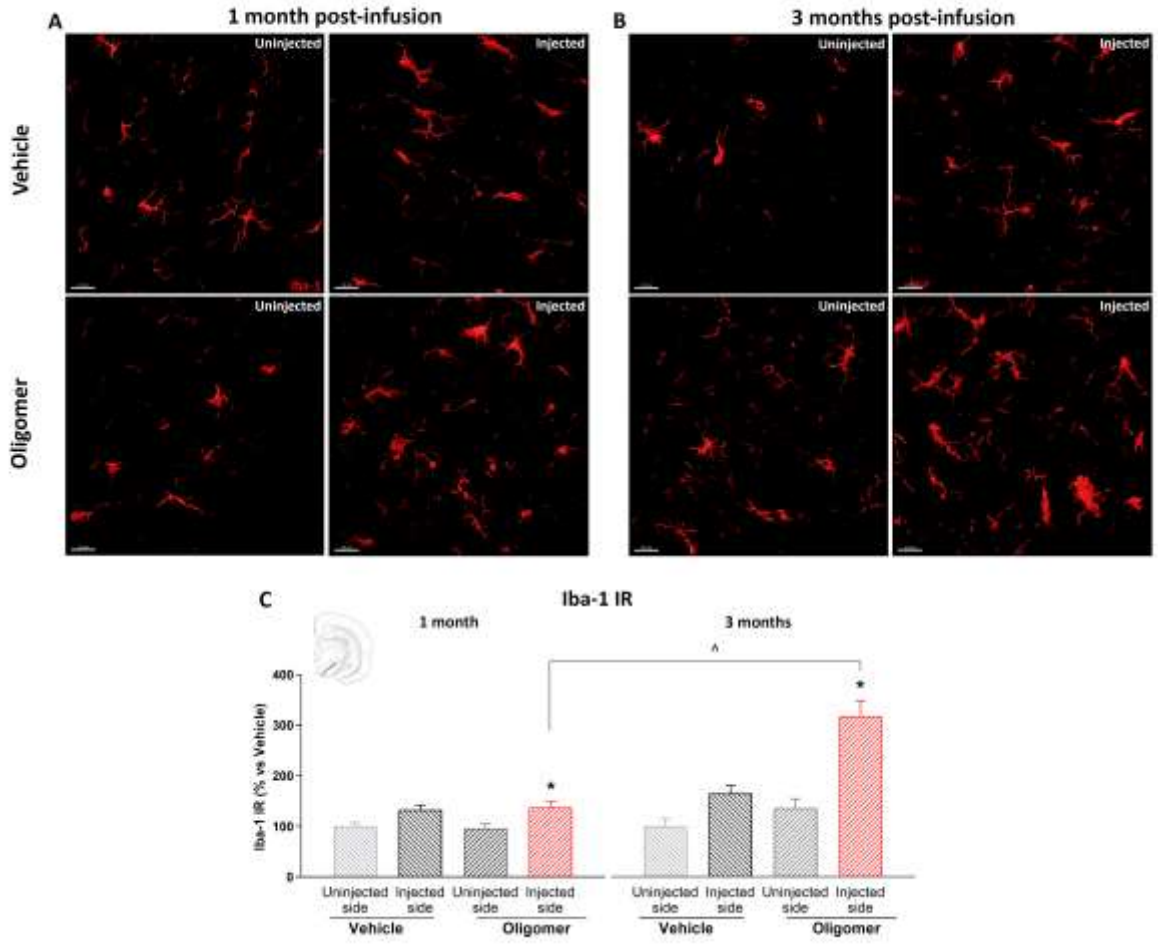


Fig. 6 The α-syn oligomers infusion induced an increase in Iba-1 IR one month post-infusion which was further exacerbated three months post-infusion. Representative images of Iba-1 (red) and colocalized TNF-α (yellow) IR in SNpc one month (A) and three months (B) post-infusion. Magnification 63X. Iba-1 IR levels in SNpc are expressed as % vs Vehicle one and three months post-infusion. #p<0.05 vs uninjected sides; * p<0.0001 vs all other groups of the same time point; ^p<0.0001 vs oligomer-injected side by Tukey post-hoc test (Fig. 6C).

TNF- α IR. Infusion of the α-syn oligomers into the SNpc induced an increase of TNF-α levels colocalized with Iba-1-positive cells as compared to controls, that was already significant after 1 month, and remained stable 3 months after the oligomers infusion (*p<0.001 vs all other groups).

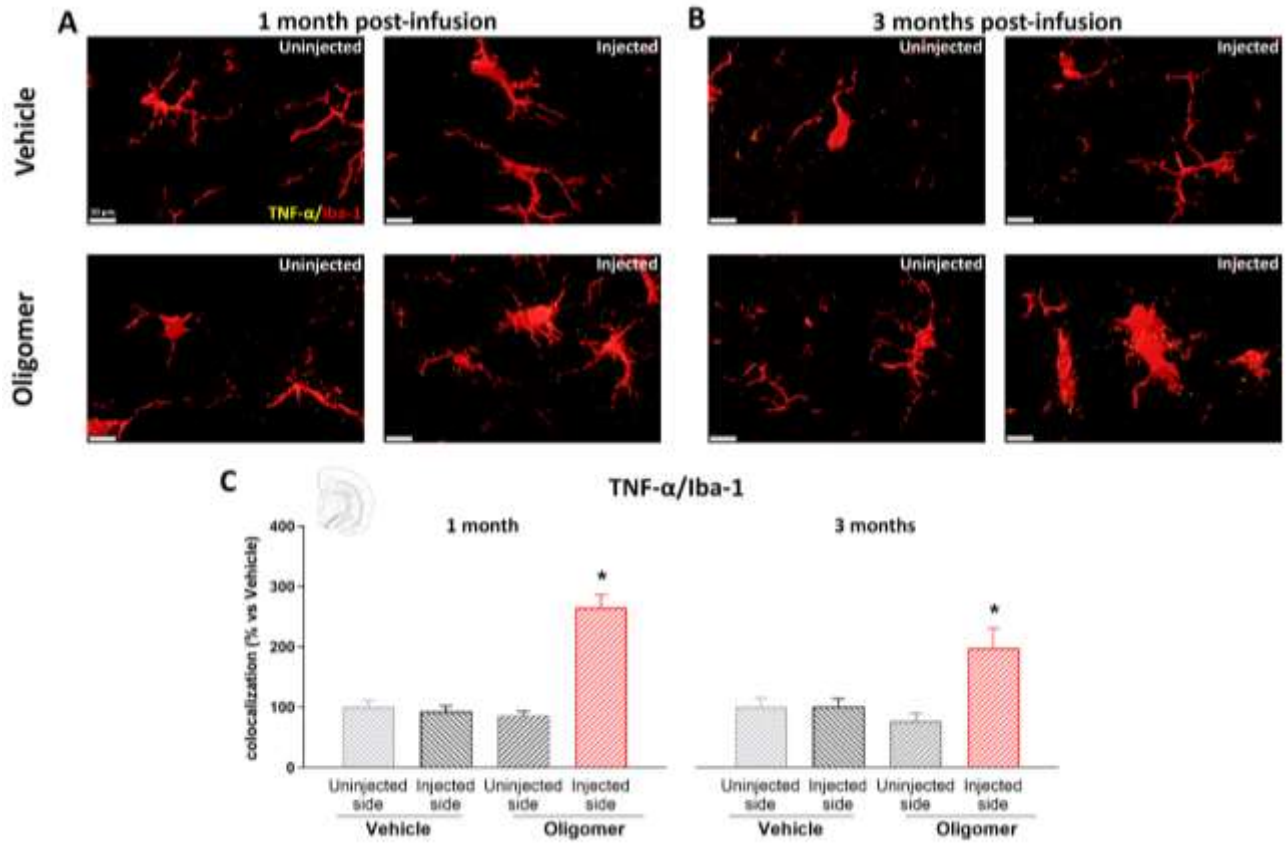


Fig. 7 The α-syn oligomers infusion in SNpc induced an increase of TNF-α colocalized with Iba-1 positive cells in the SNpc. Representative images of TNF-α (yellow) colocalized with Iba-1 positive cells (red) in SNpc one month (A) and three months (B) post-infusion. Magnification 63X. Volume of colocalized TNF-α with Iba-1 expressed as % vs Vehicle one and three months post-infusion. Values represent the mean ± SEM. *p<0.001 vs all other groups at the same time point (C).

1.5 Microglial cells in the oligomer-injected SNpc were positive for phosphorylated α-synuclein.

Pathologic p-αsyn was almost absent in control groups (Uninjected and Injected Vehicle, Uninjected Oligomer, Fig.8). Conversely, 1 month post- infusion of αsyn oligomers microglial cells displayed few and small deposits of p-αsyn. Moreover, microglia exhibited an activated morphology with enlarged cell body and numerous ramified processes. Three and five months post-infusion of αsyn oligomers, Iba-1 positive cells displayed large deposits of p-αsyn inside the cell body, and microgliaacquired a round-shaped phagocytic morphology (Fig.8).

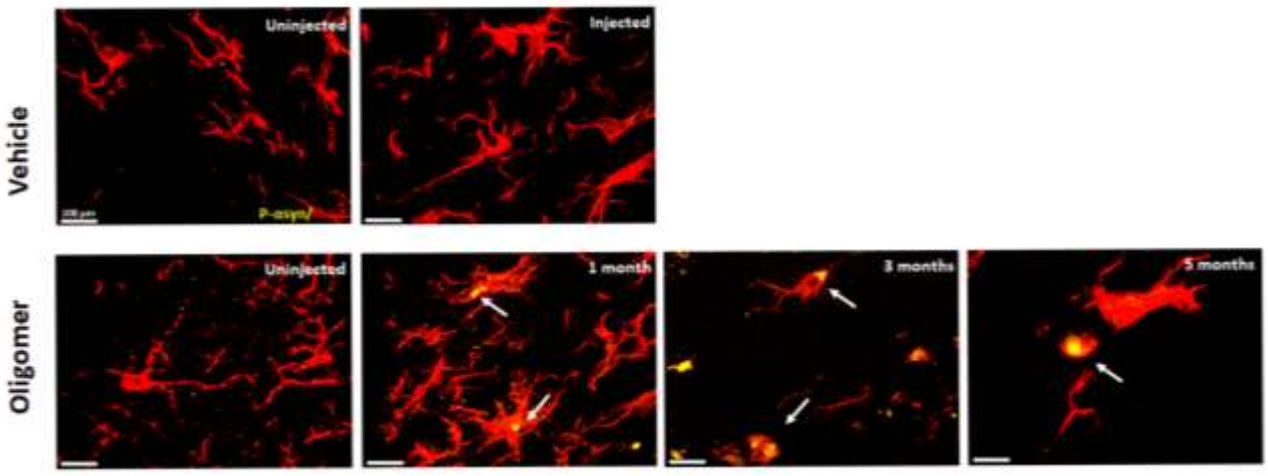
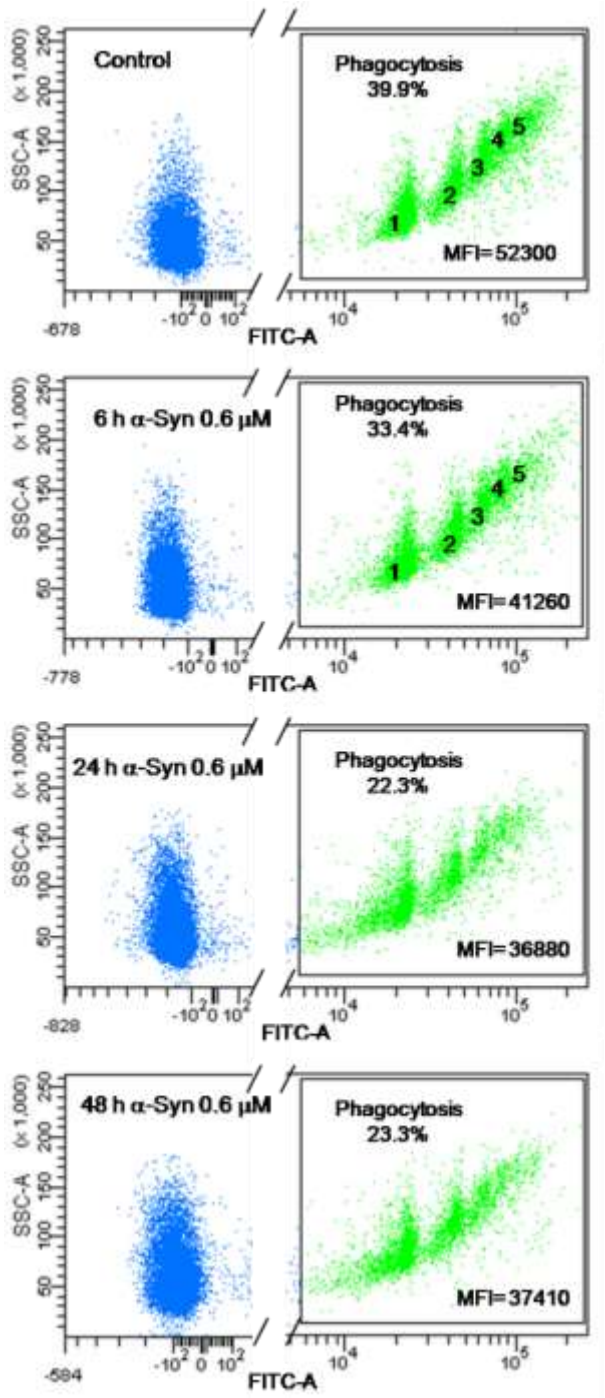


Fig. 8 Iba-1 positive cells showed p-*α*syn deposits inside the cell body. Representative confocal images of p-αsyn (yellow) colocalized with Iba-1 positive cells (red) in SNpc. Magnification 63X.

1.6 Pre-exposure to α-syn oligomers reduced the phagocytic activity of microglia *in vitro*.

Since a main function of microglia is the clearance of harmful molecules from the microenvironment, we evaluated whether the infusion of α-syn oligomers caused any alteration of the phagocytic function of cultured microglia. The fluorescent beads phagocytosis assay showed that the phagocytic activity of MMGT12 microglia was unchanged after exposure to α-syn oligomers for 6 hours. Conversely, exposure of microglia to α-syn oligomers for 24 and 48 hours significantly decreased the phagocytosis of fluorescent beads as compared to unexposed microglia (Fig. 9).

A



B

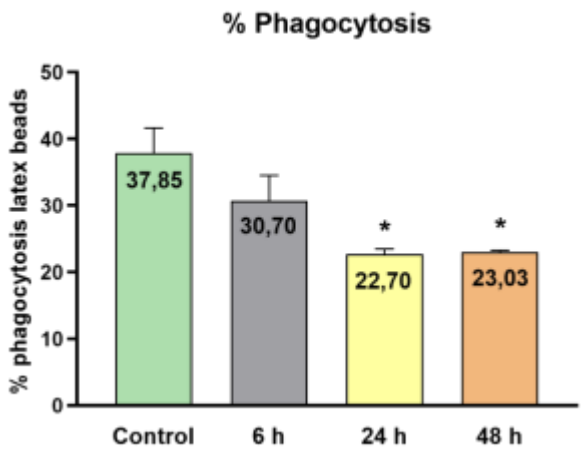


Fig. 9 The exposure to α-syn oligomers decreased the phagocytosis of fluorescent latex beads by MMGT12. Representative flow-cytometry dot-plots (green fluorescence vs side-scatter (SSC-A)) of MMGT12 cells pre-exposed for 6, 24 and 48 hrs to α-syn oligomers. After incubation with α-syn oligomers microglia were incubated with green-fluorescent latex beads (A). The graph shows the percentage of phagocytosis after exposure to α-syn oligomers. Data are expressed as absolute % phagocytosis. Values represent the mean ± SD. *p<0.001 vs Control group by t-test.

2. Results II

Recent studies have shown an involvement of neuroinflammation not only in the neurophatology of PD, ma also in the physiopathology of L-DOPA-induced dyskinesia. Based on these evidence, we addressed the ability of immunomodulatory drugs TLD and DTT to counteract the development of L-DOPA-induced AIMs and neuroinflammation in the 6-OHDA PD model.

2.1 Motor responses

Our results showed that L-DOPA treatment induced a gradual development of limb and axial AIMs, whereas rats treated with TLD and DTT exhibited a significantly lower dyskinetic response to L- DOPA (Fig. 10B). Two-way ANOVA revealed a main treatment effect ($F_{2,21} = 16.206$, $P < 0.0001$), a main time effect ($F_{8,168} = 26.478$, $P < 0.0001$), and a significant treatment/time interaction ($F_{16,168} = 3.743$, $P < 0.0001$). The antidyskinetic effect of IMiDs was confirmed by linear regression analysis, which revealed a significant difference between the L-DOPA and TLD/DTT slopes, indicating a different propensity to develop a sensitized behavioral response ($F = 4.446$, $P < 0.01$; Fig. 10B). TLD and DTT significantly attenuated the severity of limb and axial AIMs in response to L- DOPA within the 120 minutes of recording, while not changing the duration of L- DOPA effect ($P < 0.01$; Fig. 10C). Importantly, TLD and DTT did not affect the severity of contralateral turning, suggesting that IMiDs did not affect L- DOPA therapeutic efficacy (Fig. 10D). Total limb ($F_{3,21} = 23,353$, $P < 0.0001$) and axial ($F_{3,21} = 15,758$, $P < 0.0001$) AIMs measured for the whole treatment were reduced by IMiDs, whereas the total turning response was unaffected (Fig. 10E, F). Interestingly, TLD was more efficient in reducing axial AIM ($P < 0.001$), whereas DTT was more effective in limb AIM ($P < 0.001$; Fig. 10E). Neither TLD nor DTT administered alone induced a motor response (data not shown). In contrast, when TLD was administered to rats with established AIMs, it failed to reduce AIMs severity (Fig.11).

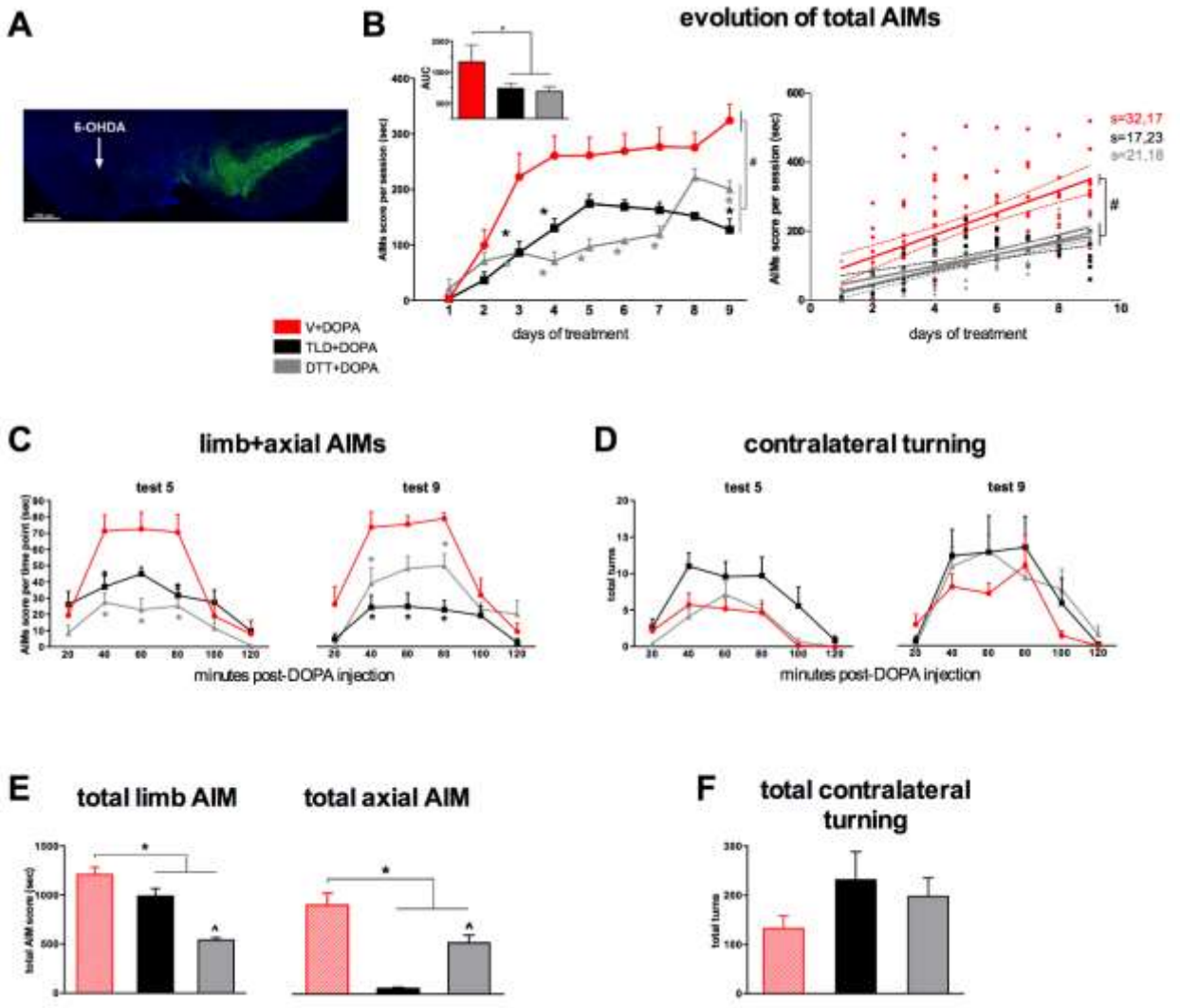


Fig. 10. TLD and DTT reduced the development of LID in hemiparkinsonian rats. Representative image of the 6-OHDA-infused SNc. (A) Development of AIMs during 10 days of treatment, score is shown as total seconds spent in AIMs in daily session, and linear regression of AIM development during 10 days of treatment (S, slope). (B) Development of AIMs (C) and contralateral turning (D) post-L-DOPA injection during daily sessions (days 5 and 9); total limb and axial AIM score (E) and contralateral turns (F) in the whole treatment. Values represent the mean ± SEM. #P < 0.0001 for main treatment effect (B); *P < 0.05 versus V + dopa (B, D, F, I); ^P < 0.01 vs TLD + dopa (F).

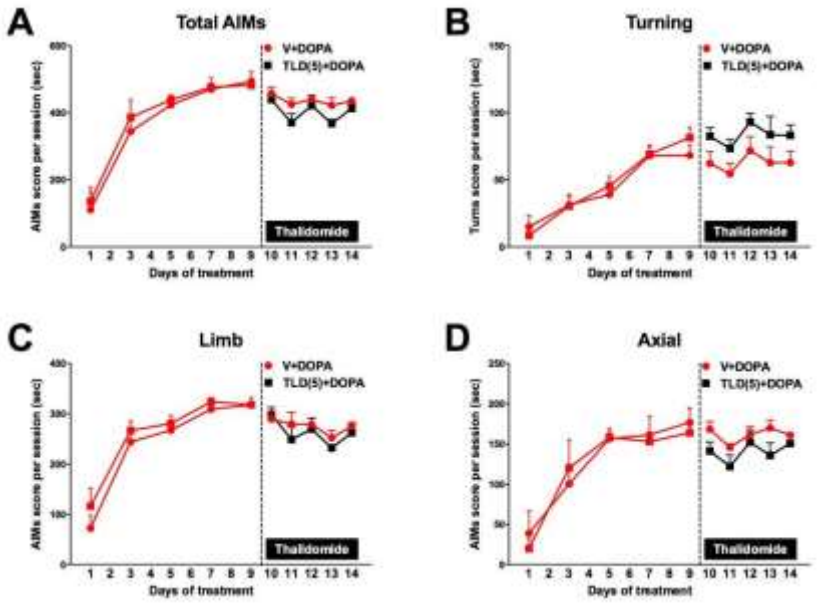


Fig. 11. Development of AIMs and contralateral turning behavior during 10 days L-DOPA treatment and last 5 days TLD co-administration, showing that TLD did not modify LID expression. Total AIMs score is shown as the sum of total seconds spent in limb and axial AIMs in each day session of 120 minutes, $F(4,32)=0,86$, $p=0,50$ (A); contralateral turning score is shown as number of turns per session, $F(4,32)=0,02$, $p=0,99$ (B); limb AIM score is shown as total second spent in limb AIM in each session, $F(4,32)=0,90$ $p=0,47$ (C); axial AIM score is shown as total second spent in axial AIM in each session, $F(4,32)=0,97$ $p=0,43$ (D); Values represent the mean ± SEM. N=5 for each group. Two-way ANOVA for repeated measures was done for the 5 days of TLD and L-DOPA of co-administration.

2.2 Neuroinflammation

The generation of LID was associated with a marked proinflammatory response in microglia in the lesioned dorsolateral striatum (Str) and SNpr, which was significantly attenuated by treatment with IMiDs.

OX-42 IR. OX-42 was analyzed as a classical marker of reactive microglia in the Str and SNpr. The 6-OHDA lesion induced an increase of OX-42 IR that was significant in the Str only ($P < 0.0001$), and that was exacerbated by the chronic L-DOPA treatment in both areas ($P < 0.0001$; Fig. 12B ,C). Both TLD and DTT significantly reduced the OX-42 IR ($P < 0.0001$) induced by L-DOPA, restoring control levels in the Str and SNpr (Fig. 12B, C). In the unlesioned contralateral Str, L-DOPA induced a nonsignificant increase in OX-42 IR, whereas TLD and DTT significantly reduced OX-42 IR (Fig. 12C). We also analyzed levels of the proinflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10, colocalized with

OX-42, to investigate changes in microglial phenotype. L-DOPA caused an imbalance in the microglial content of both cytokines that was restored by both IMiDs (Figs. 12 and 13).

TNF-α. Dopamine depletion induced a slight, but not significant rise of TNF-α IR colocalized with OX-42 in both Str and SNpr, whereas chronic L-DOPA caused a large increase in TNF-α ($P < 0.0001$), indicating the augmentation of proinflammatory microglia (Fig. 12B, C). A slight but significant increase in TNF-α was induced by L-DOPA in the unlesioned Str (Fig. 12C). The coadministration of TLD or DTT with L-DOPA fully restored TNF-α/OX-42 colocalization to control values, in agreement with their mechanism of action and indicating a reduction of proinflammatory microglia (Fig. B, C).

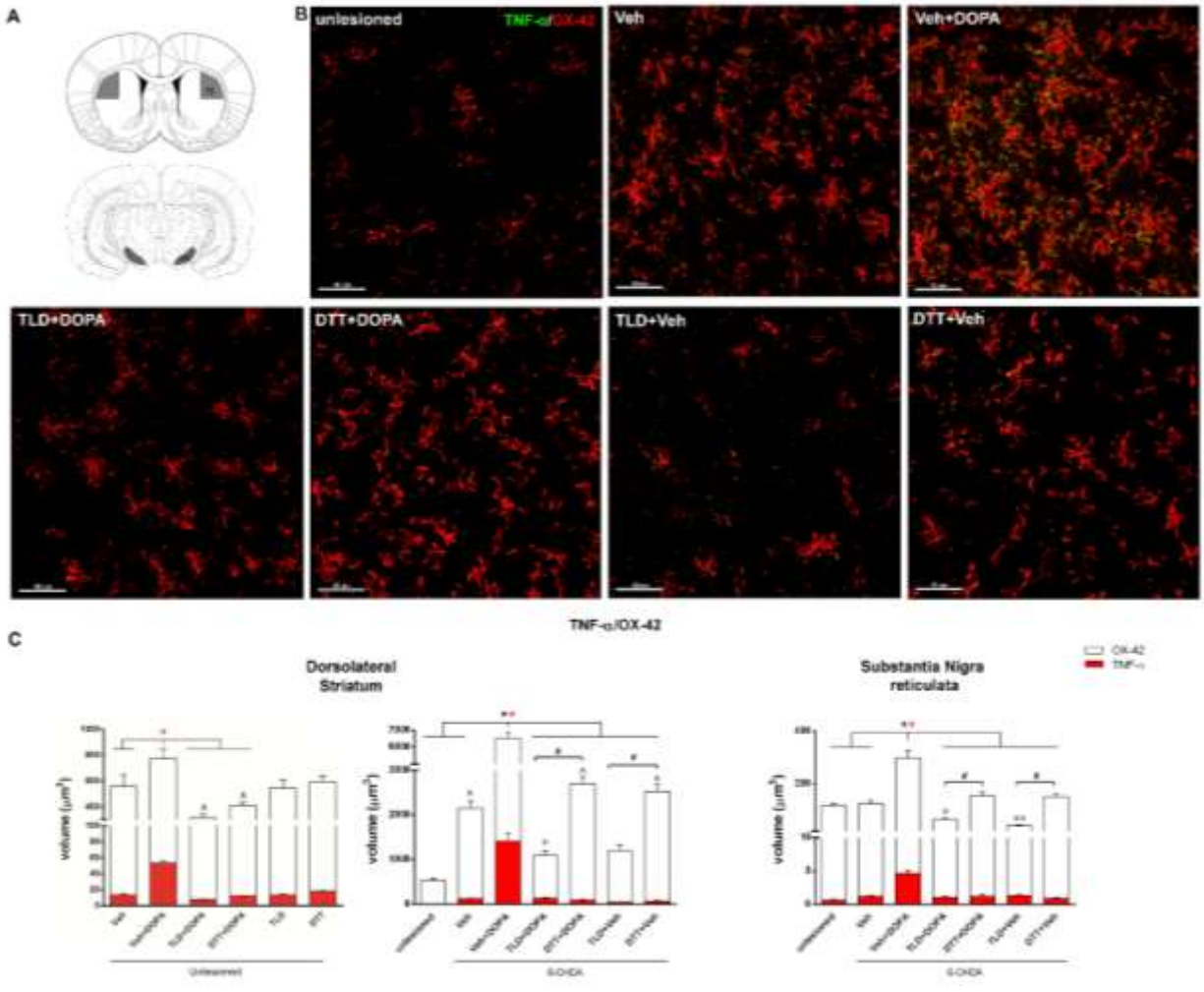
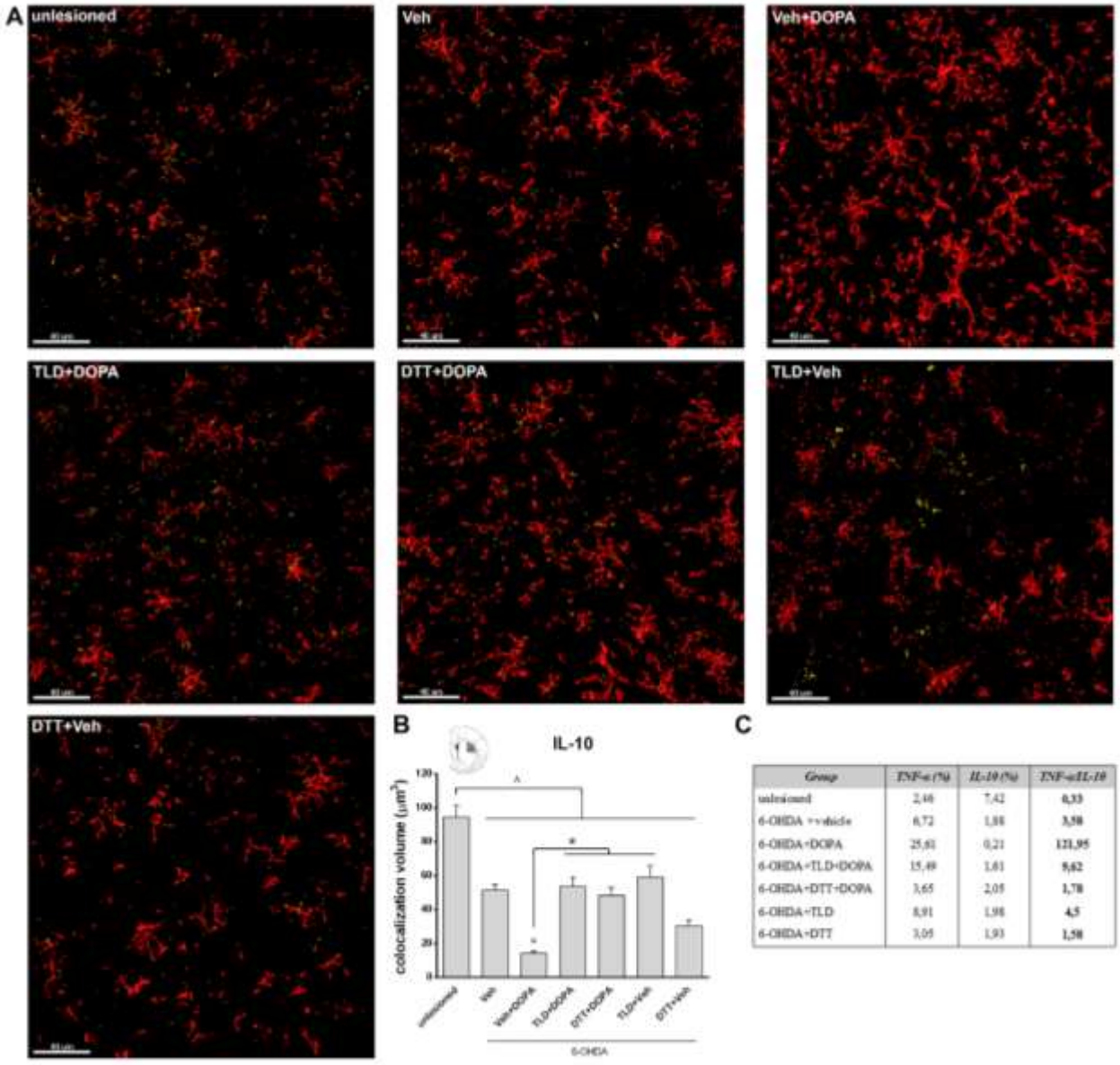


Fig.12. TLD and DTT reduced L-DOPA-induced microgliosis and microglial TNF-α content in the dopamine-depleted Str and SNpr. Representative confocal images showing TNF-α (yellow) in OX-42 (red)-positive cells in the Str (A); total volume occupied by OX-42 and colocalized with TNF-α (red columns) in the Str (unlesioned and lesioned sides) and SNpr (C). Values represent the mean ± SEM. *P < 0.001; #P < 0.05; ^P < 0.001 versus unlesioned; °P < 0.05 versus Veh (One-way ANOVA followed by Tukey HSD post hoc test).

IL-10. In contrast to TNF-α, the 6-OHDA lesion significantly reduced levels of the anti-inflammatory cytokine IL-10 in microglia compared with the unlesioned Str (Fig. 13B, C; P < 0.0001). Moreover, chronic L-DOPA administration further reduced IL-10/OX-42 colocalization below 6-OHDA levels (P < 0.0001; Fig. 13B), whereas the coadministration of either TLD or DTT restored IL-10 to 6-OHDA values (Fig. 13B). Therefore, repeated L-DOPA treatment dampened anti-inflammatory microglia, whereas both IMiDs significantly attenuated such effect.

Results shown in Figure 13C compare the % volume of striatal OX-42-positive cells colocalized with TNF-α or IL-10, and the related proportion is shown as a ratio to highlight overall imbalance in the microglia phenotype. Under physiological conditions microglia containing IL-10 prevailed over microglia containing TNF-α, whereas dopamine depletion inverted this ratio from 0.33 to 3.57. L-DOPA further exacerbated this imbalance by elevating the ratio to 121.95, inducing a dramatic increase in proinflammatory microglia over anti-inflammatory. Of note, both IMiDs restored the ratio to near control values (9.62 and 1.78 for TLD and DTT, respectively); however, DTT was more effective than TLD, a feature in agreement with the drug's greater potency in lowering TNF-α levels (Fig. 13C).



Group	TNF-α (%)	IL-10 (%)	TNF-α/IL-10
unlesioned	2,46	7,42	0,33
6-OHDA +vehicle	8,72	1,88	3,58
6-OHDA+DOPA	25,61	0,21	121,95
6-OHDA+TLD+DOPA	15,49	1,61	9,62
6-OHDA+DTT+DOPA	3,65	2,05	1,78
6-OHDA+TLD	8,91	1,98	4,5
6-OHDA+DTT	3,05	1,93	1,58

Fig. 13. TLD and DTT reverted L-dopa-induced decrease of IL-10 content in the dopamine-depleted Str. Representative confocal images showing IL-10 (yellow) in OX-42 (red)-positive cells (A); volume of OX-42 colocalized with IL-10 (B). Total OX-42-occupied volume is shown in Figure 2. TNF-α/IL-10 volume ratio expressed as % versus OX-42 volume (C). Values represent the mean SEM.^*P < 0.0001; °P < .0001 versus Veh. DL, dorsolateral striatum.

In a separate experiment we extended the investigation of inflammatory markers by multiplex ELISA, and found that several cytokines were expressed at higher levels in L-DOPA-treated rats compared with the unlesioned side of vehicle-treated rats (IL-6, IL-1β, IL-5, CXCL1, IL-10), whereas TLD reduced IL-6 and IL-1β levels compared with unlesioned-treated rats (Table 1).

		Str	*SN*
IL-6	**Unlesioned VEH**	451,7±39,5	354,4±76,6
	VEH	537,9±78,2	290,2±21,0
	VEH-DOPA	707,7±48,4 *	618,11±37,5*^
	TLD+DOPA	392,8±57,4@	445,78±34,0@
IL-1β	**Unlesioned VEH**	47,9±2,4	42,7±5,5
	VEH	66,1±2,6	36,8±2,4
	VEH-DOPA	77,4±6,4*	49,4±3,2
	TLD+DOPA	49,5±3,9@	46,5±1,9
IL-4	**Unlesioned VEH**	0,59±0,1	0,51±0,1
	VEH	0,54±0,01	0,31±0,1
	VEH-DOPA	0,50±0,1	0,50±0,1
	TLD+DOPA	0,55±0,03	0,48±0,04
IL-5	**Unlesioned VEH**	8,2±0,5	7,4±0,7
	VEH	9,4±0,1	7,1±0,3
	VEH-DOPA	10,1±0,4*	8,1±0,3
	TLD+DOPA	10,1±0,5*	8,6±0,4
IL-13	**Unlesioned VEH**	0,81±0,1	0,62±0,1
	VEH	0,99±0,11	0,69±0,1
	VEH-DOPA	0,98±0,1	0,84±0,1
	TLD+DOPA	0,86±0,05	0,84±0,02
INF-γ	**Unlesioned VEH**	25,0±2,1	23,8±2,6
	VEH	27,0±1,4	23,9±2,4
	VEH-DOPA	23,0±1,7	20,9±2,1
	TLD+DOPA	24,7±1,1	27,9±2,1
CXCL1	**Unlesioned VEH**	0,76±3,2	0,99±0,02
	VEH	1,6±0.1*	1,33±0,1
	VEH-DOPA	1,13±0,1*	1,57±0,2*
	TLD+DOPA	1,15±0,05*	1,69±0,1*
TNF-α	**Unlesioned VEH**	0,42±0,01	0,45±0,02
	VEH	0,50±0,1	0,49±0,1
	VEH-DOPA	0,48±0,1	0,48±0,1
	TLD+DOPA	0,68±0,1	0,61±0,1
IL-10	**Unlesioned VEH**	3,20±0,4	3,1±0,5

VEH	5,4±0,5*	3,7±0,4
VEH-DOPA	5,7±0,8*	4,6±0,8
TLD+DOPA	5,6±0,4*	4,9±0,3

Tab 1. Cytokine changes in the Str and SN of dopamine-depleted rats after 10 days treatment with vehicle, L-DOPA or co-administration of TLD and L-DOPA. The levels of cytokines were analyzed by multiplex ELISA. Unlesioned VEH refers to the unlesioned side of unilaterally-lesioned rats treated with vehicle. * $p < 0.05$ vs unlesioned vehicle; ^ $p < 0.05$ vs vehicle; @ $p< 0.05$ vs VEH-DOPA.

2.3 Angiogenesis

As elevations in TNF-α protein and angiogenesis are coupled, and as the development of dyskinesia is linked with abnormal angiogenesis occurring mainly within the SNpr (Distler et al 2003, Janelidze et al 2015, Lerner et al 2017, Ohlin et al 2012), we investigated new vessel formation in this area and in the Str by analyzing vimentin and VEGF as markers of angiogenesis (Lai et al 2013). The astroglial marker GFAP showed that astrocytes enwrapped vessels expressing different levels of vimentin and VEGF, depending whether they were preexisting or newly formed vessels (arrows in Fig. 14A and Fig. 15A, respectively). We found a slight, not significant increase in both vimentin and VEGF expression after the 6-OHDA lesion, compared with the unlesioned hemisphere, which was in line with the changes observed in TNF-α protein (Fig. 14 and 15). Chronic L-DOPA dramatically increased the formation of new vessels, as clearly shown by the increased vimentin and VEGF expression in Figure 14 and 15 ($P < 0.0001$). DTT prevented L-DOPA-induced angiogenesis, in line with the restoration of physiological TNF-α protein levels and with the significant attenuation of AIMs (Fig. 14 and 15; $P < 0.0001$).

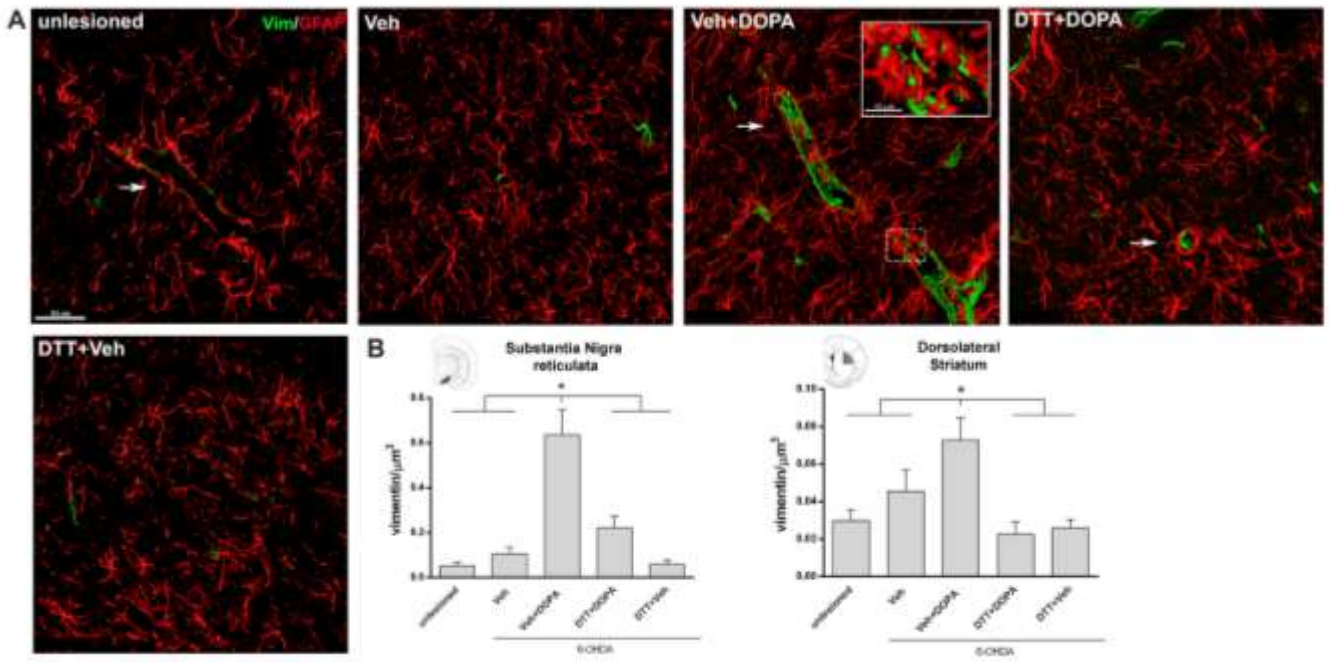


Fig. 14. DTT prevented L-DOPA-induced increase of vimentin IR in the SNpr and Str. Representative confocal images showing GFAP (red) and vimentin (green, A) in the SNpr, and total volume occupied by vimentin and in SNpr and Str (B). Values represent the mean SEM. *P < 0.0001.

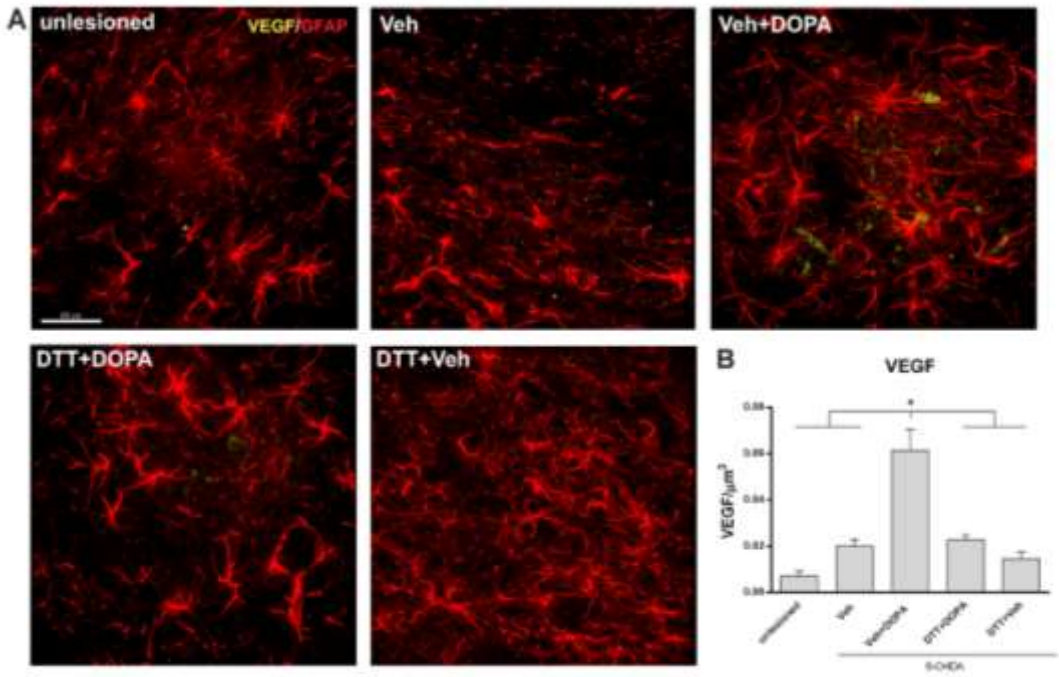


Fig. 15. DTT prevented L-DOPA-induced VEGF increase in the SNpr. Representative confocal images showing GFAP (red) and VEGF (yellow, A) in the SNpr, and total volume occupied by VEGF colocalized with GFAP in SNpr (B). Values represent the mean ± SEM. *P < 0.0001.

2.4 Striatal GLUR1 Protein Levels

Changes in GLUR1 protein levels were investigated in the Str as a postsynaptic neuronal event that is under TNF-α regulation. The confocal analysis showed that GLUR1 IR remained unchanged after the 6-OHDA lesion (Fig. 16A, B). Moreover, L-DOPA induced a significant increase in striatal GLUR1 protein, whereas the administration of DTT in association with L-DOPA restored control levels (P < 0.001; Fig. 16A, B). DTT administered alone did not change striatal GLUR1 levels.

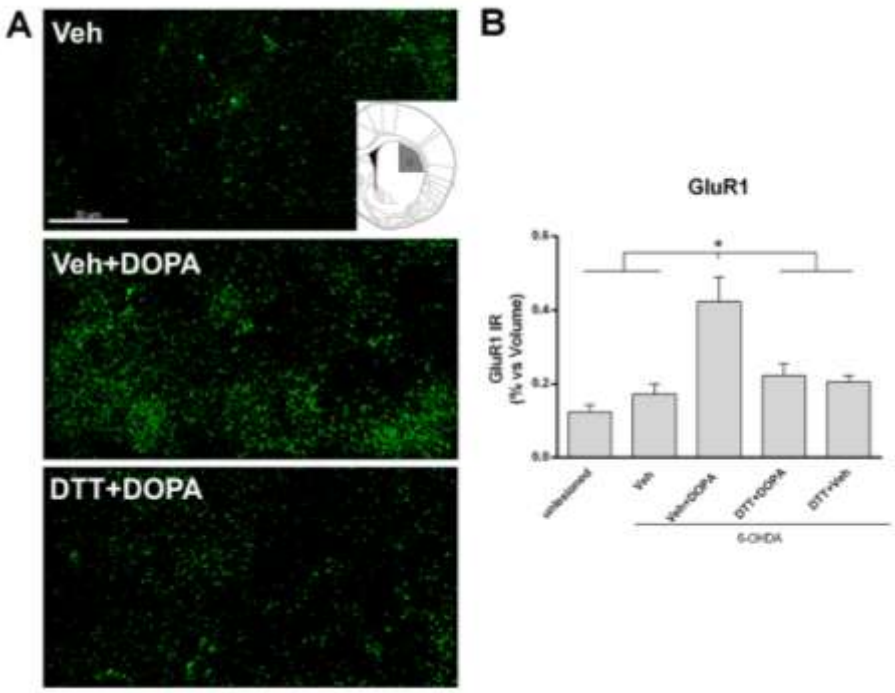


Fig. 16. DTT restored control levels of GluR1 in Str. Representative confocal images of GLUR1 protein expression in the dopamine-depleted Str (A) and GLUR1 levels expressed as % of the analyzed volume (B).

DISCUSSION

In the present studies we aimed at investigating the involvement of neuroinflammation in the α-syn-induced neuropathology of PD as well as its involvement in the physiopathology of L-DOPA-induced dyskinesia.

Discussion I

In order to investigate the first issue, i.e. the interrelationship between α-syn and neuroinflammatory response in PD, we exploited α-syn oligomers whose neurotoxicity has been recently demonstrated *in vitro* (Fusco et al 2017). Several studies have indicated that the most pathologically relevant form of α-syn are oligomeric soluble aggregates (Fortuna et al 2017, Fusco et al 2017). Moreover, previous studies have demonstrated that α-syn oligomers holding different tertiary structure induced different toxicities, suggesting a structure-based toxicity (Fusco et al., 2017). We therefore infused such α-syn oligomers in the rat SNpc, in order to evaluate whether they may model neuropathological traits of PD *in vivo* first, and then investigate the interaction with microglia through *in vivo* and *in vitro* experiments. Our results demonstrated that particularly structured α-syn oligomers can reproduce features of PD *in vivo*, included motor impairment, cell loss, neuroinflammatory response with microgliosis and p-α-syn deposits in the SNpc. Moreover, we found that α-syn-oligomers impaired the phagocytic function of microglia *in vitro*.

The dose-finding experiment indicated that 0.5 mg/ml was the optimal dose for the aim of our experiment. In fact, this dose did not induce any mechanic damage to the tissue, did not cause the immediate deposition of the α-syn oligomers or their diffusion along the injector-trace.

Once established the optimal dose, we injected the α-syn oligomers in the rat SNpc and we performed behavioral and immunohistochemical analysis at progressive time points. The behavioral assessment revealed that α-syn oligomer-induced motor deficits were still absent at one month post-infusion, while were detected three months and worsened five months post-infusion. According to the behavioral results, stereological analysis of TH in SNpc showed that one month post-infusion no detectable dopaminergic loss was present in the oligomer-injected SNpc, while three and five months-post infusion a consistent and progressive decrease in the

density of TH+ neurons was measured. The reduction in the density of dopaminergic neurons was associated with a decrease in the striatal dopamine content.

Previous studies have tested the toxicity of different α-syn forms when overexpressed or directly injected into the brain. Overexpressed WT or mutant α-syn caused progressive dopaminergic neurodegeneration accompanied by α-syn inclusions and aggregates within neurons and motor symptoms, which well reproduced the progressive disease stages observed in PD patients (Decressac et al 2012, Kirik et al 2002). However, a massive overexpression of α-syn, about four- to five fold above the normal and much higher than in human disease, was necessary to obtain substantial degeneration in the AAV-based models (Decressac et al 2012, Thakur et al 2017). Interestingly, AAV-mediated expression of human α-syn closer to that seen in PD patients, combined with a low no toxic dose α-syn PFF injected 4 weeks apart from AAV, induced a progressive dopaminergic degeneration (Thakur et al 2017). The striatal infusion of PFFs induced a progressive α-syn neuropathology with nigrostriatal degeneration and mild motor deficits which became consistent six months post-infusion (Luk et al 2012a, Paumier et al 2015, Peelaerts et al 2015, Thakur et al 2017). Another study showed that the intracerebroventricular infusion of α-syn oligomers induced non-motor symptoms and late motor symptoms accompanied by dopaminergic loss in mice (Fortuna et al 2017). In our study the infusion of α-syn oligomers induced a neurodegenerative process which was detectable as early as three months after the infusion. Notably, the dopaminergic loss was homogeneous and similar among the animals, suggesting an elevated degree of reproducibility. The α-syn oligomers used in the present study have been well-characterized for their ability to induce neuronal toxicity based on their structural properties, where an exposed N-terminal and a rigid core enriched in β-sheet structures confer the ability to insert and damage the lipidic bilayers (Fusco et al 2017). Such structure related-toxicity has been further confirmed by studies showing that blocking the binding of α-syn oligomers to lipidic membranes by aminosterol molecules (Perni et al 2018, Perni et al 2017) or by an antibody against the N-terminal region (Cascella et al 2019), reduced the α-syn oligomers-induced toxicity in primary cortical neurons, neuroblastoma cells and C. Elegans. All together, these evidences confirm that α-syn toxicity is strictly dependent upon its form, with oligomers being the most toxic species, but also on the secondary structure of the oligomer itself, in which confer toxic properties to the protein.

Interestingly, the analysis of inflammatory markers showed that the α-syn oligomers infusion triggered a pro-inflammatory process mediated by microglia which preceded frank neurodegeneration. Hence, one month post-infusion increased levels of Iba-1 and TNF-α were detected in the oligomer-injected SNpc in the absence of dopaminergic loss. Three months post-infusion, Iba-1 levels further increased in the SNpc while TNF-α levels remained stable. These results are in line with the early and persistent inflammatory response detected in other PD models, where the inflammatory response preceded and then accompanied the dopaminergic degeneration (Maia et al 2012, Su et al 2008, Walsh et al 2011, Watson et al 2012). Moreover, the infusion of α-syn oligomers in our study induced a severe and persistent inflammatory response, reproducing the chronic microgliosis observed in human PD (Gerhard et al 2006, Ouchi et al 2009). Previous studies have reported a mild and transient inflammatory response after AAV-based α-syn overexpression, further suggesting that structural properties mediate the toxic effects of α-syn species and may differently affect their interaction with microglia (Chung et al 2009, Sanchez-Guajardo et al 2010, Thakur et al 2017, Theodore et al 2008).

Morphological evaluation of microglia indicated that one month post-infusion cells in the SNpc displayed an activated pro-inflammatory morphology with highly ramified processes and high levels of TNF-α. Three months post-infusion most microglia displayed an ameboid or round-shaped phagocytic morphology but still high levels of TNF-α. These observations suggest that microglia with mixed phenotypes coexisted in the SN after α-syn oligomers infusion, with some cells displaying a pro-inflammatory phenotype while others a clear phagocytic morphology. According with our observation, previous studies demonstrated the activation of TLRs by α-syn (Kim et al 2013, Stefanova et al 2011). TLRs are involved in the activation of both the proinflammatory and the phagocytosis-activator pathways in microglia. Notably, confocal analysis revealed the presence of pathologic p-αsyn following to the infusion of the α-syn oligomers. While one month post-infusion few and small deposits of p-αsyn were present in microglia, three and five months post-infusion, increasingly bigger p-αsyn deposits were present inside cells, indicating that the pathologic p-αsyn was up-taken from the extracellular milieu.

Altogether, results showed that motor impairments, dopaminergic loss, neuroinflammatory responses and deposition of pathologic p-αsyn were of increasing severity at progressive time points, reproducing the progressive neuropathology of PD.

Hence, particularly structured toxic α-syn oligomers reproduced the cardinal neuropathological traits of PD after one single unilateral infusion into the SNpc, offering an interesting advantage in respect of complex protocols requiring multiple surgeries or use of viral vectors.

Microglial cells are the main phagocytic cells of the brain and they are in charge of clearing the extracellular α-syn in the brain (Ferreira & Romero-Ramos 2018, Lee et al 2008b). In PD, the contribution of phagocytosis to disease progression and the interaction between α-syn and phagocytic cells remains unclear (Janda et al 2018). In our study, the presence of p-αsyn deposits inside microglia *in vivo* suggest an active phagocytosis of pathologic α-syn from the extracellular space. Moreover, when we measured the effect of α-syn oligomers on cultured microglia by a functional phagocytosis assay, we found that the short-term exposure to α-syn oligomers did not alter the phagocytic activity of microglial cells, while a prolonged exposure to α-syn oligomers inhibited the phagocytic process.

Previous *in vitro* studies aimed to investigate the effect of α-syn variants on microglia-mediated phagocytosis led to contrasting results, but suggest that changes in phagocytic activity depend upon the α-syn species and conformations (Park et al 2008, Rojanathammanee et al 2011, Roodveldt et al 2010). Hence, WT and A53T α-syn promoted phagocytosis in microglial cells, while the A30P and E46K α-syn induced the opposite effect (Roodveldt et al 2010). Furthermore, in one study oligomeric α-syn inhibited both basal and LPS-stimulated phagocytosis, while in another study all forms of α-syn, including fibrils increased the phagocytosis of fluorescent beads (Fellner et al 2013, Park et al 2008). Altogether, our *in vivo* and *in vitro* results suggest that the pathological α-syn may be efficiently up-taken in early disease stages by microglia, which however lose their phagocytic ability after prolonged exposure to α-syn oligomers.

Previous studies suggested that microglia in PD lose the ability to self-regulate leading to a disbalance between pro- and anti-inflammatory phenotypes (Carta et al 2011, Hirsch & Hunot 2009). For instance, the exposure to soluble or fibrillar α-syn induced an increased microglial phagocytic activity together with increased production of ROS and pro-inflammatory cytokines, confirming that mixed microglial phenotypes may coexist in pathological conditions (Fellner et al 2013).

Our findings support an aberrant activation of microglial cells as a mechanism of α-synuclein oligomers-induced-neurotoxicity. In fact, while previous studies demonstrated a direct toxic action of α-synuclein oligomers on neurons by disrupting membrane integrity (Fusco et al 2017, Winner et al 2011), our study also shows that α-syn oligomers activated microglia in their pro-inflammatory phenotype, which lead to the release of pro-inflammatory mediators and contribute to neuronal damage. Damaged and dying neurons in turn may release pathological p-αsyn in the extracellular space, which is cleared by phagocytic microglia until this process fails. We suggest that microglia are not able to efficiently process the large amount of pathological α-syn, which accumulates inside the cells leading to the engulfment of the phagocytic machinery and to phagocytosis impairment. A mixed reactive phenotype, with concurrent pro-inflammatory microglia and poorly-efficient phagocytic microglia may represent a dangerous milieu for nearby neurons which favors neurotoxicity, posing microglia as a key player in α-syn-mediated neurodegeneration in PD.

In conclusion, the α-syn oligomers administration reproduced neuropathological features of PD, including motor deficits, neurodegeneration, α-syn pathology and microgliosis, and might represent a valid and useful model of PD to investigate mechanisms of α-syn-induced toxicity and to test protective strategies.

Discussion II

To further investigate the role of neuroinflammation in PD, we tested the possibility to target neuroinflammatory responses in order to alleviate L-DOPA-induced dyskinetic movements. To this aim, we assessed the efficacy of the immunomodulatory drug TLD and its analogue DTT in attenuating the L-DOPA-induced neuroinflammatory response and LID in the 6-OHDA rat model of PD. Previous studies supported an active role of L-DOPA induced striatal inflammation in the development of LID (Barnum et al 2008, Bortolanza et al 2015a, Mulas et al 2016). Our study showed for the first time that the immunomodulatory drugs TLD and its analogue DTT significantly reduced the severity of LIDs in the 6-OHDA model of PD. Moreover, both IMiDs dramatically diminished the neuroinflammatory response induced by L-DOPA in the Str and the SNpr. As DTT possesses a more potent TNF-α inhibitory profile than TLD (Zhu et al 2003), we broadened our investigation to characterize altered GLUR1 protein levels in the underlying antidyskinetic mechanism of DTT treatment and found that DTT reduced L-DOPA-induced increases in GLUR1 protein. We also found that DTT strongly attenuated L-DOPA-induced angiogenesis. These findings are of clinical relevance for PD, because TLD and several close analogues are FDA-approved drugs, currently in clinical trials for several central nervous system disorders (ClinicalTrials.gov Identifier: NCT00140452, NCT00231140, NCT01094340, NCT02415153 and NCT01553149). Both TLD and DTT reduced the AIM score during the whole treatment. This effect resulted from the induction of less intense AIMs when L-DOPA was administered in association with IMiDs, whereas the duration of the pharmacological effect was not affected. This effect was further highlighted by the linear regression analysis showing that both IMiDs reduced the development of AIMs response upon repeated L-DOPA injections and suggesting a dampening of striatal mechanisms underlying sensitization (Carta et al 2008). Of note, treatment with IMiDs did not affect the severity of contralateral turning behavior in L-DOPA-treated animals, suggesting that these drugs did not interfere with L-DOPA therapeutic efficacy. TLD induced a slightly more intense turning behavior than did L-DOPA, likely a consequence of the dramatic suppression of axial AIMs, which mostly impede motor responses. Interestingly, DTT effects were similar to those of TLD, albeit that DTT was used at a lower dose. This is in line with the 10- to 30-fold higher potency of DTT against TNF-α synthesis compared with TLD (Tweedie et al 2009). We also found that DTT inhibited the striatal overexpression of GLUR1 induced by repeated L-DOPA treatments. The GLUR1 subunit

confers Ca++-permeability to the AMPA channel and is highly expressed in the striatum. Several reports suggest that the AMPA receptor plays a role in dyskinesia (Calon et al 2003, Konitsiotis et al 2000, Ouattara et al 2010). An increase of PKA dependent phosphorylation of GLUR1 and alterations in alternative splicing of AMPA receptor subunits were associated with LIDs in rats (Ba et al 2006, Kobylecki et al 2013), whereas the inhibition of the Ca++-permeable AMPA receptor was associated with antidyskinetic effects (Kobylecki et al 2010, Konitsiotis et al 2000). Of note, TNF-α has been shown to increase the cell surface expression of GLUR1 protein (Kobylecki et al 2013). Therefore, results show that increased TNF-α levels induced postsynaptic neuronal changes in the striatum, suggesting a functional link between neuroinflammation and altered neuronal activity. As previously reported, L-DOPA induced an intense microgliosis and increased TNF-α levels in microglia in the dopamine-depleted striatum, whereas in the intact striatum the drug only induced a mild inflammatory response. This suggests that the preexisting inflammatory milieu, related to the neurodegenerative process dramatically amplified an otherwise mild inflammatory response to L-DOPA. Moreover, a similar inflammatory reaction extended to the SNpr, the output basal ganglia structure that also mediates motor effects and LIDs (De Deurwaerdere et al 2017, Navailles & De Deurwaerdere 2012). The microglial overproduction of TNF-α was associated with decreased levels of the anti-inflammatory cytokine IL-10 in the same cell type. When the cytokine profile was evaluated by multiplex ELISA, we found an increase of inflammatory cytokines IL-6 and IL-1β, and of CXCL1 and IL-10, whereas several other cytokines were unaltered by L-DOPA, including TNF-α. Moreover, TLD reversed IL-6 and IL-1β levels. Therefore, ELISA results suggest that the L-DOPA and TLD effect extended to additional inflammatory cytokines. Interestingly, the ELISA assay also produced some mismatching results when compared with IHC data. This was not surprising because ELISA could not discriminate different cell types, and it was performed in the whole substantia nigra, including the pars compacta, where the neuroinflammatory environment was profoundly affected by the 6-OHDA lesion, in contrast to the focused analysis in single cell types performed by IHC. Moreover, in the ELISA assay the TNF-α levels were in the very low range of detection, whereas IL-6 and IL1-β levels were easily measured. Altogether the results suggest that repeated L-DOPA treatment induced a chronic imbalance in the microglial phenotype, with a prevalence of pro-inflammatory over anti-inflammatory microglial phenotypes. This phenomenon thus provides a solid scientific rationale for using IMiDs in the treatment of LIDs. Accordingly, TLD

and DTT were able to reduce the microgliosis and restore the cytokine imbalance induced by L-DOPA to near control levels, indicating that these drugs drove the activity of microglial cells to that of an anti-inflammatory phenotype. TLD and DTT rapidly inhibit TNF-α protein production at the posttranscriptional level via key elements within the 30 untranslated regions (Moreira et al 1993), as well as transcriptionally by downregulating NF-kB and myeloid differentiation factor88 (Majumder et al 2012). TNF-α is a key inflammatory cytokine that is rapidly upregulated and released by microglial cells on receiving inflammatory stimuli. If this response is not restored to normal levels in a timely manner, TNF-α can trigger a cascade of events that generate a chronic pro-inflammatory microenvironment and affect the activity of neurons driving neuronal dysfunction. Moreover, TNF-α is a master regulator of the inflammatory response inducing the expression of other cytokines, chemokines, and growth factors (John et al 2003). IL-10, in turn, is constitutively present in the brain microenvironment, holding a pivotal role in maintaining homeostasis in microglial cells; moreover, it is an important contributor to remission of inflammation, downregulating the expression and secretion of TNF-α and other pro-inflammatory cytokines and growth factors (Frei et al 1994, John et al 2003). Whereas L-DOPA disrupted the complex cross talk between cytokines promoting the proliferation of inflammatory microglia, IMiDs restored the balance to dampen neuroinflammation, breaking the self-fueling circle instigated by L-DOPA. Again, the lower dose of DTT used here reduced TNF-α levels to an extent similar to TLD, in accordance with DTT's higher potency as a TNF-α inhibitor. In this regard, DTT displays a greater potency (10- to 30-fold increased activity at lowering TNF-α protein compared with TLD, reaffirming that TNF-α inhibition is the pharmacological target of these IMiDs for LID attenuation (Baratz et al 2011, Tweedie et al 2009, Zhu et al 2003). The doses used in our present study compare favorably with prior preclinical research (Baratz et al 2011, Russo et al 2012, Tweedie et al 2012), and are in line with translational doses used in humans in which up to 1200 mg singly and 1000 mg daily of TLD have been administered (Rehman et al 2011). More routine in humans is a daily TLD dose on the order of 200 to 400 mg, and this dose proved to be poorly tolerated and inefficacious in a recent clinical trial in Alzheimer's disease (Decourt et al 2017). Common TLD-related adverse events reported in oncologic patients are hematologic such as mild neutropenia, peripheral sensory neuropathy, constipation, and infection. These adverse effects may represent a concern for a possible drug repurposing of TLD, and safety/tolerability of the drug should be scrutinized in the parkinsonian

population yet reiterating the need for well-tolerated and more potent analogues. Several studies have provided convincing evidence for a neuroinflammatory component in LID neuropathology (Carta et al 2017). Initial explorative studies showed an intensified inflammatory environment in the striatum of L-DOPA treated dyskinetic rats (Barnum et al 2008, Bortolanza et al 2015a), which was associated with a dramatic increase in the microglial production of TNF-α (Mulas et al 2016). In turn, dyskinesia was exacerbated by an inflammatory stimulus (Mulas et al 2016). Of note, striatal neuroinflammation was linked with the dyskinetic outcome of L-DOPA, because the continuous nondyskinetic treatment was devoid of inflammatory effects (Mulas et al 2016). Interestingly, previous studies have shown that LIDs were more intense in aged than in young rats (Bez et al 2016), which may relate to the physiological presence of senescent reactive-like microglia in the elderly brain (von Bernhardi et al 2010). Moreover, a recent study showed that rats overexpressing brain derived neurotrophic factor, a TNF-α-induced growth factor, were more prone to develop LIDs (Tronci et al 2017). As shown by seminal studies by Picconi et al, LIDs are associated with impaired synaptic plasticity at corticostriatal synapses that become unable to undergo depotentiation (Picconi et al 2003). Of note, TNF-α has a critical neuromodulatory function, and increased TNF-α levels have been related to synaptic deficits and altered synaptic plasticity associated with neurodegeneration (Cavanagh et al 2016, Lewitus et al 2014, Santello & Volterra 2012, Vezzani & Viviani 2015, Wu et al 2015). In line with a TNF-α neuromodulatory role, in the present study excessive production of this soluble cytokine in dyskinetic animals may have contributed to impaired synaptic plasticity at corticostriatal synapses. In addition to immune functions, TNF-α is a potent proangiogenic cytokine acting via the NF-kB transcription factor to stimulate VEGF production and new blood vessel formation (Leibovich et al 1987, Naldini & Carraro 2005, Szade et al 2015). Using vimentin and VEGF as markers of angiogenesis, we report that L-DOPA was highly proangiogenic, and inhibition of TNF-α production by IMiDs completely prevented such an effect. The contribution of angiogenesis in the development of dyskinesia has been elegantly demonstrated in several studies. Elevated angiogenesis was reported in the basal ganglia of dyskinetic PD patients compared with nondyskinetic patients undergoing L-DOPA treatment (Lerner et al 2017, Lindgren et al 2009, Ohlin et al 2011). Moreover, preclinical studies have shown that L-DOPA dose-dependently induced angiogenesis, whereas LIDs were attenuated by VEGF inhibition with vandetanib and candesartan (Hirano et al 2008, Lindgren et al 2009, Munoz et al 2014, Ohlin et

al 2011). Here, we have demonstrated a causal relationship between angiogenesis and L-DOPA induced inflammation and suggest that contrasting the latter by inhibiting TNF-α with IMiDs may be a useful tool to diminish both angiogenesis and dyskinesia. In line with our results, the inhibition of nitric oxide, another proangiogenic product that is potently lowered by IMiDs (Majumder et al 2009), reduced the severity of LID in the 6-OHDA rat model (Bortolanza et al 2015a). Vimentin was analyzed as a marker of mesenchymal cells in newly forming endothelial tissue (Lai et al 2013). Interestingly, vimentin has been described as part of the epithelial-mesenchymal transition process associated with chronic inflammation, a sophisticated pathway enrolled in growing of tissue under inflammatory stresses (Kalluri & Weinberg 2009). Moreover, factors released from degenerating neurons such as lactate dehydrogenase A induce brain angiogenesis through an increase of VEGF in a vimentin dependent manner (Lin et al 2018), confirming the role of vimentin in the pathological central nervous system angiogenesis.

Hence, this study showed that TLD and its analogue DTT attenuated the severity of dyskinesia by breaking the chronic inflammatory cycle induced by L-DOPA and restoring the cytokines to near physiological levels. Mechanistically, the alleviation of dyskinesia by IMiDs may occur at multiple levels, involving inhibition of angiogenesis as well as removal of L-DOPA-induced postsynaptic changes in striatal neurons. TLD and the more recently synthetized analogues are FDA-approved drugs for several chronic inflammatory treatments. In addition, both TLD and derivatives display good BBB permeability and have shown beneficial effects in models of neurological diseases. Taken together, these data suggest that these compounds may be clinically repurposed as an effective therapeutic treatment approach to clinical LIDs in PD.

CONCLUSIONS

In summary, the present work demonstrates the involvement of neuroinflammation in the α-syn-induced neuropathology of PD as well as in the physiopathology of L-DOPA-induced dyskinesia. A detrimental interaction between toxic forms of α-syn and microglia was found as a mechanism of toxicity in the neuropathological process of the disease. Moreover, the exacerbation of such pre-existing inflammatory environment by chronic L-DOPA contribute to the development of LID, which may be efficiently targeted by IMiDs.

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ACKNOWLEDGMENTS

Laura Boi gratefully acknowledges Sardinia Regional Government for the financial support of her PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2014-2020).