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Neuropathological role of alpha-synuclein: major contribution of inflammation in the evolution of both motor and non-motor symptoms of Parkinson's disease.

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Abstract

Neuroinflammation is nowadays considered a cardinal pathological feature of Parkinson's disease (PD), in which glial cells lose their homeostatic function in favour of a pro-inflammatory profile. Such sustained glial response within the brain parenchyma is characterized by a chronic release of a number of pro-inflammatory mediators, likely driven by pathological interactions with toxic forms of α-Synuclein (αSyn). Moreover, the contribution of the peripheral immune system to PD neuropathology has been demonstrated, promoting the view of PD as a systemic condition. While the contribution of inflammation to the neuropathology of motor symptoms has been ascertained, its role in non-motor symptoms is still under-investigated, particularly in relation to cognitive disturbances.

Here, we targeted inflammation in PD by testing the immunomodulatory imide drug (IMiD) Pomalidomide (Pom) for its disease-modifying properties against motor deficits, in a translational rat model of PD based on the intranigral infusion of toxic oligomers of human α-synuclein (H-αSynOs) (study I). Moreover, we investigated the contribution of neuroinflammation in PD cognitive symptoms, in the same PD preclinical model (study II).

Study I: The neuroprotective effect of Pom (20 mg/kg; i.p. three times/week for two months) was tested in the early stage of the disease. We found that the infusion of H-αSynOs induced an impairment in motor performance that was fully rescued by Pom, as assessed via a battery of motor tests. Moreover, H-αSynOs-infused rats displayed a 40–45% cell loss within the substantia nigra (SN), that was largely abolished by Pom. The inflammatory response to H-αSynOs infusion and the Pom treatment was evaluated both in CNS and peripherally. After H-αSynOs infusion, microglia displayed a proinflammatory profile, producing a large amount of the cytokine tumour necrosis factor (TNF)-α. In contrast, Pom inhibited the TNF-α overproduction and elevated the anti-inflammatory cytokine interleukin (IL)-10. Moreover, the H-αSynOs infusion induced a systemic inflammation with a dysregulated production of serum cytokines and chemokines, that was largely restored by Pom.

Study II: We asked whether the H-αSynOs-based model of PD is an effective tool to study PD-related cognitive disturbances, and investigated the contribution of neuroinflammation. We show that H-αSynOs-infused rats displayed memory deficits three months after the infusion. These were underpinned by an altered electrophysiological neuronal activity and altered expression of the neuron-specific immediate early gene (IEG) Npas4 (Neuronal PAS domain protein 4) in cognitive

regions, such as the anterior cingulate cortex (ACC). Moreover, the brain of cognitively impaired rats showed a neuroinflammatory response in the ACC and discrete subareas of the hippocampus, in the absence of any evident neuronal loss, supporting a role of neuroinflammation in cognitive decline. Such neuroinflammatory response was epitomized by the acquisition of a pro-inflammatory phenotype by microglia cells, as indicated by the increased levels of TNF-α.

Taken together, results of the present study indicate that neuroinflammation is a common feature of both motor and non-motor aspects of PD, and suggest that targeting inflammation might represent a novel therapeutic strategy to treat the disease as a whole.

Contents

List of abbreviations

6-OHDA	6-hydroxydopamine
ACC	Anterior cingulate cortex
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	Antigen presenting cell
BBB	Blood brain barrier
BG	Basal ganglia
CA	Cornu Ammonis
COMT	Catechol-O-methyltransferase
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DA	Dopamine
DAMPs	Damage-associated molecular patterns
DG	Dentate gyrus
DI	Discrimination index
DLB	Dementia with Lewy Bodies
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCL	Granular cell layer
GFAP	Glial fibrillar acidic protein
GID	Gastrointestinal dysfunctions
GluR1	Glutamate receptor subunit 1
GPe	Globus pallidus external part
GPi	Globus pallidus internal part
ICAM-1	Intercellular adhesion molecule 1
IEG	Immediate-early gene

IFN	Interferon
IL	Interleukin
LBs	Lewy bodies
LC	Locus coeruleus
L-DOPA	Levodopa
LID	L-DOPA-induced dyskinesia
LNs	Lewy neurites
LTP	Long term potentiation
MAO	Monoamine oxidase
MCI	Mild Cognitive Impairment
MMSE	Mini-Mental State Examination
Mol	Molecular
MPTP	1-methyl,-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple System Atrophy
MSNs	Medium Spiny Neurons
NAC	Non-amyloid-β component of AD amyloid plaques
NCI	Not cognitively impaired
NMDA	N-methyl-D-aspartate
NM-MRI	Neuromelanin-sensitive magnetic resonance imaging
NO	Nitric oxide
NOR	Novel object recognition
Npas4	Neuronal PAS domain protein 4
NSAID	Non-steroidal anti-inflammatory drugs
PAMPs	Pathogen-associated molecular patterns
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PET	Positron Emission Tomography

PL	Pyramidal layer
Pom	Pomalidomide
PTMs	Post-translational modifications
Rad	Radiatum
RBD	REM sleep behavior disorder
REM	Rapid eye movement
ROS	Reactive oxygen species
SN	Substantia nigra
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TH	Tyrosine Hydroxylase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VA	Ventral anterior nucleus
Veh	Vehicle
VL	Ventral lateral nucleus
VMAT2	Vesicular transporter of monoamines
αSyn	Alpha-synuclein
αSynOs	Alpha-synuclein oligomers

Background

In 1817, the doctor in London James Parkinson first described "*paralysis agitans*" in his work *An Essay on the shaking Palsy* [1] as:

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

The disease was later renamed as Parkinson's disease (PD). PD represents one of the most common chronic progressive neurodegenerative disorders, second only to Alzheimer's disease (AD). Is it foremost characterized by a selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of proteinaceous aggregates referred to as Lewy bodies (LBs) and Lewi neurites (LNs), which together lead to the appearance of the characteristic motor symptoms, including resting tremor, bradykinesia, rigidity, and postural instability[2]. This cardinal motor symptomatology may initially be subtle, and is commonly preceded by a long prodromal phase characterized by a broad spectrum of non-motor signs and symptoms including constipation, olfactory disturbances, neuropsychiatric symptoms and sleep disorders[3,4]. In many cases, with the progression of neuronal loss and advancing disease, these non-motor symptoms dominate the clinical scenario, appearing as the main determinants of quality of life and institutionalization[3,5].

Despite the huge effort in understanding PD aetiology, the specific mechanisms and underlying causes still remain uncertain. Genetic forms of PD only account for 3-5% of total cases, indicating that the majority of them are sporadic, as the results of a complex interplay between genetic and environmental factors[6]. The complex nature of this pathology, which encompasses diverse epi-phenotypes, opens to several clinical challenges, including the inability of early diagnosis and difficulties in the management of symptoms at later stages[7]. Moreover, at the current state level, only symptomatologic treatments are used in the clinical practice, with Levodopa (L-DOPA) as the gold standard. In this regard, a big effort has been aimed at finding therapeutical approaches suitable for slowing or even stopping the pathology. However, to date, this intense research failed to find potential disease-modifying therapies to be used in the clinical practice, in part for the misleading categorization of idiopathic PD patients as a uniform group, not taking into account the complexity underlying the pathology itself [6]. This opens to the important question whether it would be more appropriate to reconsidered PD as a syndrome rather that a disease.

1. Clinical features

PD symptoms are generally classified into two major categories, depending on whether they are related to body movement abnormalities or not, respectively named as motor and non-motor symptoms. The spectrum of both motor and non-motor symptoms and signs may vary from patient to patient as well as in onset and progression.

1.1. Motor symptoms

Motor symptoms classically define and describe PD. They generally appear in the early/mid stages of the pathology, after about 60% of dopaminergic neurons have died and striatal dopamine (DA) concentration falls below 60-70% [8,9]. The onset of motor manifestations is commonly asymmetric, with the upper limb and the hand affected first[10]. The symptomatology then spreads to the other limb on the same side to affect later the opposite side. Typically, motor abnormalities include bradykinesia, tremor, rigidity and postural instability.

The term bradykinesia refers to slowness of movement, which encompasses difficulties in performing sequential and repeated movements and, in the advanced stages of PD, it is subjected to rapid fluctuations from ease to inability of movement. Initial bradykinetic manifestations are generally characterized by a slowness of reaction times and difficulties in performing simultaneous tasks[11,12]. Manifestations of bradykinesia also include loss of facial expression (named hypomimia), impaired swallowing, monotonic and hypophonic dysarthria, decreased blinking and loss of gesturing.

Tremor might be considered the most easily recognised symptom of PD, which occurs in at least 75% of patients[13]. Usually it starts asymmetrically, affecting only one side of the body to become bilateral as the disease progresses. Tremor in PD tends to occur at rest at a frequency of 4-6 Hz, while diminishes during an intentional movement or during sleep. This cardinal symptom generally occurs in the hands, but sometimes also in the lips and chin, and is often described as “pill-rolling” (to depict the fingers and thumbs that move in opposition, as though a small object was being rolled between them). Such kind of signature represents an important diagnostic value, since it is present only in a limited number of conditions. In fact, apart from PD, it can also be present in some atypical parkinsonism and in drug-induced parkinsonism[14].

Another cardinal motor feature of PD is rigidity, which is experienced as stiffness of limbs, neck and/or trunk. Differently from bradykinesia, which only promotes a slowness of the movement, here the motion is limited to a reduced range, due to muscle stiffness and the lack of relaxation capability[11].

Postural instability usually manifests at late PD stages. It is related to loss of postural reflexes, resulting in impaired balance and coordination. Such impairment drastically changes patients' posture, with their shoulders hunched forward, which leads to an unbalanced posture and contributes to falls. A manifestation of that might result in the so-called festinating gate, characterized by an increase in speed with a shortening of stride. Thus, the patient takes multiple short steps in an attempt to "catch up" with his barycentre which is propelled forward due to stooped posture. Such manifestations are generally transient and worsen as the pathology progresses[15].

1.2. Non-motor symptoms

Even though motor symptoms remain central to the diagnosis of PD, in the last few decades non-motor symptomatology is recognized as being of similar relevance, long enough to consider PD as a complex multiorgan and multisystemic pathology. The non-motor symptomatology falls into broad categories of signs and symptoms (Fig. 1), can precede motor features and progresses in severity and diversity as the disease evolves [5]. Non-motor symptoms can be schematically categorized in central and autonomic/peripheral symptoms.

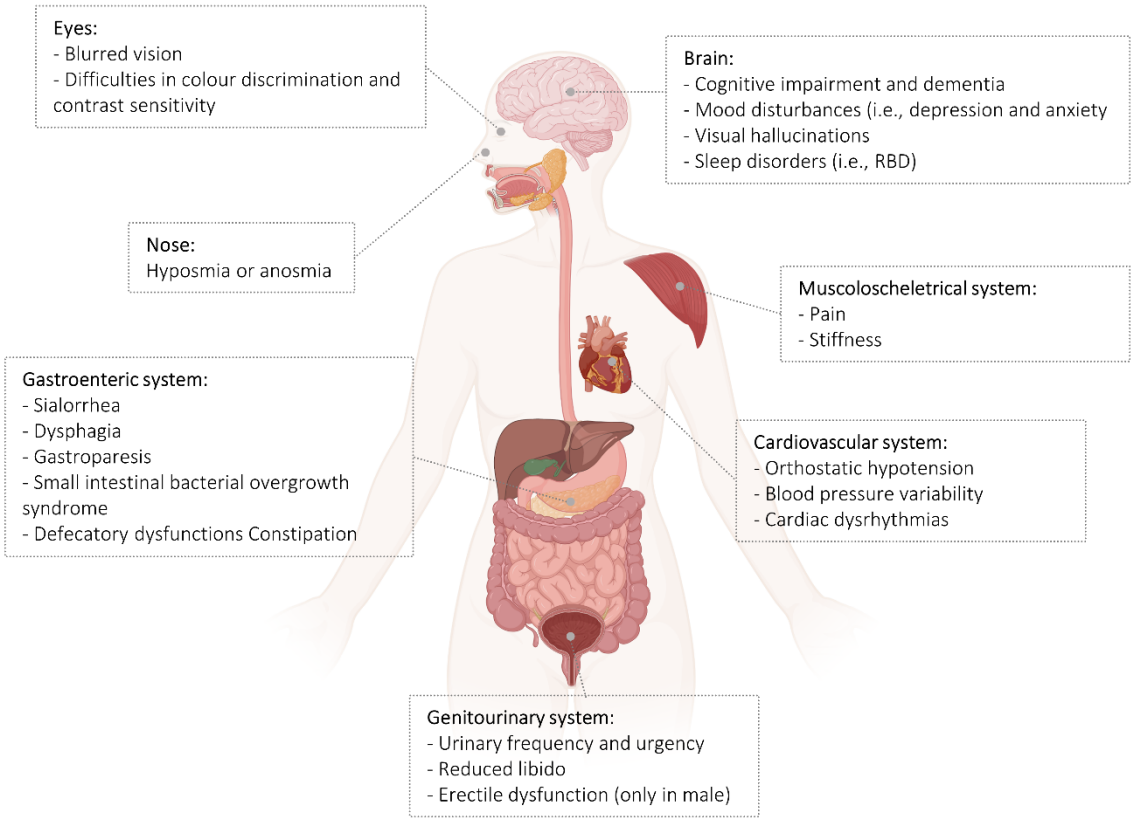


Figure 1. Non-motor features associated with PD.

1.2.1. Sensory impairments

Pain and other sensory abnormalities, including olfactory and visual deficits, are present in a large proportion of PD patients. They generally appear in the prodromal phases of the pathology, then increasing in prevalence and severity as the disease progresses.

Deficits in olfaction, including hyposmia and anosmia, are one of the most frequent non-motor manifestations of PD, which affect more than 90% of PD patients[5]. On a neuropathological level, these alterations are tightly associated with a degeneration of those neuroanatomical areas involved in olfactory perception, including the olfactory bulb and the anterior olfactory nucleus[5,16]. Because the olfactory disturbances may occur many years prior to the development of a clinical motor symptomatology, evaluation of olfactory function has been mentioned as a potential early marker of the disease, particularly in combination with other clinical, imaging and/or biochemical markers, such as reduced noradrenergic denervation of cardiac tissue and cognitive abnormalities[5,17–21].

Other sensory impairments frequently reported in PD patients are visual, including a wide range of symptoms from difficulties in colour discrimination and contrast sensitivity, double and blurred vision to complex visual hallucinations. Interestingly, visual hallucinations have been linked to the presence of LBs at the level of both occipital lobe – centre of visual processing – and retinal neurons, as well as to the loss of dopaminergic amacrine cells[5,22]. Moreover, visual hallucinations seems to be associated with another common non-motor PD symptom, the cognitive impairment, so as to suggest that such hallucinations might be a good predictor of cognitive decline[23].

Pain is highly prevalent in PD patients, further worsening their quality of life. PD-related pain is heterogeneous, enough to be subdivided into many different domains, including neuropathic, musculoskeletal, dystonia-related, akathitic or central pain[24]. Among them, musculoskeletal pain (for example dystonia, muscle cramps or stiffness) is the most frequent in PD-population, being reported in almost 50% of individuals experiencing pain[25]. Besides pain, PD patients may report other somatosensory disturbances, including peripheral paraesthesia, burning sensation and the so-called burning mouth syndrome, a medical term used to indicate the chronic or recurrent burning in the mouth, unrelated to any obvious cause[5].

1.2.2. Autonomic dysfunctions

Autonomic dysfunctions represent an important non-motor phenotype of PD, of increasing interest in the last years as predictive and early diagnosis factors of PD[26,27]. Among them gastrointestinal malfunctions, cardiovascular dysregulations and genitourinary disturbances are listed.

Gastrointestinal dysfunctions (GID) appear with high frequency among PD population, especially in the prodromal phase of the disease. Accordingly, it has been reported how 88.9% of PD patients will develop GID prior to the onset of any motor symptom[28]. GID include sialorrhea, dysphagia, gastroparesis, small intestinal bacterial overgrowth syndrome, defecatory dysfunctions and constipation.

Among cardiovascular dysregulations, orthostatic hypotension appears to be the most frequent, occurring in over half the patients[29], although only a minority of them manifest classical PD symptoms[30,31]. Orthostatic hypotension is generally experienced by patients with light-headedness upon standing, accompanied by disturbances in vision, clouded thinking and shortness of breath, all signs that increase the risk of recurrent fallings.

1.2.3. Sleep disturbances

Sleep disturbances include a broad spectrum of signs and symptoms such as insomnia, restless leg syndrome, excessive daytime sleepiness, obstructive sleep apnea, and Rapid eye movement (REM) sleep behavior disorder (RBD)[5]. Among them, RBD is found to best correlate with PD progression[11]. RBD is a kind of parasomnia particularly frequent in several proteinopathies including PD, Dementia with Lewy Bodies (DLB) or Multiple System Atrophy (MSA)[32]. It is characterized by vivid, often frightening dreams associated with simple or complex motor behavior during REM sleep. Many studies indicate RBD as a good predictor of severity for PD and one of its most important manifestations since its onset precedes any evident motor symptom by several years or even decades. As a matter of fact, this kind of manifestation has a prevalence of 0.5-1% in the general population, but up to 50% in PD patients[33–35].

1.2.4. Mood disturbances

Mood disturbances such as depression and anxiety occur in comorbidity with PD, from the prodromal to the late stage of the disease.

Anxiety affects up to 60% of PD patients, with a prevalence of approximately 30%[36]. Anxiety disorders in PD encompasses generalized anxiety disorder, panic disorder, agoraphobia, obsessive-compulsive disorder, social or specific phobia, and anxiety not otherwise specified and may be present in comorbidity with depression. Anxiety in PD represents one of the main determinants of quality of life among patients[37], although underrecognized. In this regards, clinical ascertainment of anxiety in PD is complicated by some atypical presentations[38]. A first limiting factor is the mentioned comorbidity between depression and anxiety, which is relatively frequent making very difficult to dissociate

between these two syndromes[39]. Moreover, autonomic dysfunctions typically present in PD patients might be experienced as anxiety by some (e.g., heart palpitations, increased tremor), further complicating an accurate diagnosis[40].

Similarly to anxiety, the diagnosis of depression in PD is particularly difficult, mainly due to the overlapping with other motor and non-motor PD symptoms, including sleep disturbances, anhedonia, anxiety itself, loss of energy and psychomotor retardation[41,42]. Accordingly, PD depression is often unrecognized and untreated. In line with this assumption, some studies have reported a significant proportion of PD patients not being treated for depressive disorders[43,44], or even not screened for depression[45,46]. In this regard, implementing the screening for both depression and anxiety in PD patients appears, at the current state level, of fundamental importance. In fact, awareness that these conditions might be related to PD is pivotal to the quest for treatment and causative factors which are the cornerstone for delivering a comprehensive modern treatment for these two disorders.

1.2.5. Cognitive decline and dementia

Cognitive dysfunctions are nowadays considered as an integral part of non-motor features in PD, often emerging early in the disease's course. Accordingly, such disturbances are up to six times more common in PD patients than in the healthy population[47]. In PD, such manifestations fully cover the entire spectrum of cognitive impairment, from subjective cognitive decline and mild cognitive impairment (MCI), to full-blown dementia (PDD)[48].

MCI is a common PD manifestation that can be present prior or at the time of the diagnosis – some studies report a frequency of PD-MCI cases of 15-20%[49] – which is often underrecognized. In fact, these kinds of alterations are generally subtle and only detectable with proper neuropsychological tests[50]. On a clinical level, the main features of PD-MCI are represented by impairment in executive functions and visuospatial domains, making PD-MCI different from those present in other neurodegenerative disorder, such as AD, which are characterized by a more severe memory impairment[51]. There are robust evidence that indicate PD-MCI as an important risk factor for developing dementia[52–54]. Interestingly, according to the "dual syndrome hypothesis", only those cognitive impairments linked with recognition memory and visuospatial domains are associated with subsequent dementia, while those with attentional and executive deficits seems to be more stable[55,56].

Differentially from PD-MCI, PDD is associated with a more severe impairment in almost all cognitive domains, including visual spatial constructional deficits and recognition, as well as semantic and

episodic memory loss[5]. Furthermore, the clinical scenario of PDD is complicated by the co-presence of other disabling non-motor symptoms typical of the late stages of the disease, including psychotic symptoms, apathy, excessive daytime sleeping and others[57]. Such comorbidities further shrink the quality of life of both patient and caregivers.

2. Pathophysiology of PD

2.1. Anatomy and pathophysiology of basal ganglia

Parkinson-related motor behaviours are notoriously the result of abnormalities that occur within the basal ganglia (BG) circuit, a group of subcortical nuclei primarily involved in motor control and motor learning. On a neuroanatomical level, BG are composed of a telencephalic core, represented by the globus pallidus and the striatum, and associative nuclei located in the diencephalon, mesencephalon and pons, respectively the subthalamic nucleus (STN), the substantia nigra (SN) and pedunculopontine nucleus.

On a functional level, these subcortical structures can be categorized as input, output and intrinsic nuclei[58]. Input nuclei, including the dorsal striatum and other structures, such as the nucleus accumbens and olfactory tubercle, receive afferents from different sources, preferentially from the cortex, the thalamus and the SN. On the other hand, output nuclei exert a direct control over the thalamus which in turn projects directly to the cortex, and consist of the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNpr). The intrinsic nuclei include the STN, the externa segment of the globus pallidus (GPe) and the SNpc; they are located between the input and output relays.

Over the years, different circuit models have been formulated to explain the functional interconnection between these structures, mostly to address the pathophysiological changes in their activity following DA depletion, as occur in PD. Among them, the so-called classical model will be briefly discussed, which offers a simplified vision of BG function. The classical model of PD has been postulated at the end of the last century, with the purpose of explaining the direct contribution of DA in the execution of involuntary movements, and how its progressive depletion would trigger those abnormalities typically seen in PD (see Fig. 2). Under this perspective, the striatum represents the central core of the entire circuit, in which several excitatory signals (glutamatergic) converge from different cortical areas, thus representing the major input of the BG. Two distinct neuronal populations are present in the striatum: GABAergic Medium Spiny Neurons (MSNs), representing the

majority of the entire striatal population, and a modest percentage of cholinergic interneurons. From the GABAergic MSNs two different pathways take origin that project to the remaining BG nuclei, indicated as direct and indirect pathways. Both pathways relay information from the cortex, through the basal ganglia into the thalamus, and back to the cortex, working in opposition to one another, in order to allow for the weighing and choosing of motor movements.

The direct pathway is a monosynaptic route projecting from the nucleus striatum to the GPi and the SNpr. The activation of the inhibitory MSNs results in the inhibition of the aforementioned nuclei. Both the GPi and the SNpr are GABAergic nuclei, which in turn projects to two different thalamic nuclei, respectively the ventral anterior (VA) and ventrolateral (VL); that directly send glutamatergic (excitatory) projections to the cortex. Briefly, the striatal activation results in a decreased inhibitory tone of the GPi and SNpr over the thalamus, thereby leading to cortical activation (Fig. 2). Therefore, the activation of the direct pathway, through GABAergic MSNs, promotes movement meant as motor control.

Conversely, the indirect pathway is a polysynaptic route which starts likewise from the MSNs. These inhibitory neurons send their projections to the GPe, another GABAergic nucleus, which in turn send the message to the STN. Glutamatergic neurons from the STN project to the GPi and SNpr. The activation of this pathway results in a total sum of inhibition on thalamocortical loops, thus suppressing movement.

As previously mentioned, the activity of these two pathways is tightly regulated by DAergic neurons originating from the SNpc and which innervate the striatum. At this level, MSNs express two different types of DAergic receptor: D1-like and D2-like. In the direct pathway MSNs express the D1-like excitatory receptor, while in the indirect route MSNs express the inhibitory D2-like receptor. Therefore, DA released from the SNpc would promote the activity of the direct pathway, while reducing the activation of the indirect. Taking into account this concept is fundamental to understand the pathophysiology of PD. In fact, striatal DA depletion consequent to the progressive degeneration of nigral neurons, results in a switched effect on DA receptors and MSNs activity. As a final effect on the BG circuit, the reduced DAergic tone results in the suppression of the thalamocortical loop, thus deteriorating the efficiency of motor control functions.

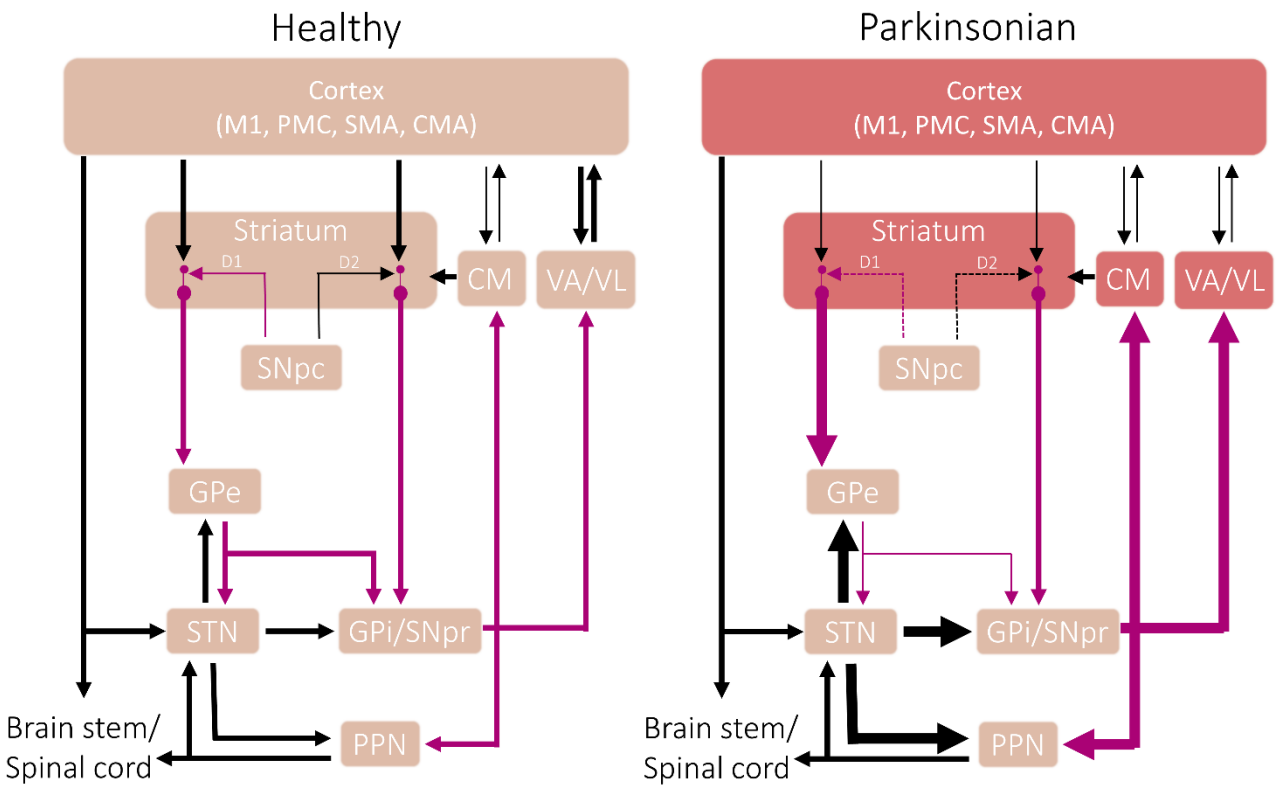


Figure 2. Schematic diagram of the direct and indirect pathways of the BG motor circuits in normal and parkinsonian states. The left panel indicates the circuit in the "healthy" state, while the right shows the overall changes in activity that have been associated with parkinsonism. Purple and black arrows indicate inhibitory and excitatory connections, respectively. Note that the thickness of the arrows directly correlates with firing-rate activity of that specific connection (larger arrow: increase firing-rate; thinner arrow: decrease firing rate). Abbreviations: CM, centromedian nucleus; CMA, cingulate motor area; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; M1, primary motor cortex; PMC, pre-motor cortex; PPN, pedunculopontine nucleus; SMA, supplementary motor area; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; STN, subthalamic nucleus; VA/VL, ventral anterior/ventral lateral nucleus. (Modified from Smith et al., 2012)

2.2. Beyond the basal ganglia: a focus on cognition pathophysiology

Albeit cognitive decline is a recognized predominant trait in PD, the pathophysiological bases underneath is debated. Most of our knowledges on the pathophysiological bases of cognitive symptoms in PD come from studies in PD patients, while preclinical investigations in this regard are sporadic due to the lack of reliable preclinical models. Imaging studies have reported volume changes across different cognition-related brain regions. Among these, the atrophy of the hippocampus has been associated with impaired memory in PD[59,60], in line with the pivotal role of hippocampal synaptic plasticity in memory in PD patients[61]. Interestingly, volume decline in specific hippocampal subfields were suggested as predictive of the conversion from PD-NCI (not cognitively impaired) to PD-MCI[59].

According to clinical evidences, PD-MCI and its eventual progression in PDD might be the result of a progressive hypometabolic state that from the anterior cingulate cortex (ACC) spreads to other cortical regions, including temporal and parietal cortices[62,63]. In the context of memory and learning, ACC plays an important role in certain higher-level functions, including decision-making, impulse control, and error detection, all of which resulted impaired in PDD[64]. Specifically, the ACC, together with other neuroanatomical areas such as the anterior insula, takes part to the so-called salience network, involved in attention and orientation to stimuli[65]. In this regard it has been demonstrated a disconnection between these two neuroanatomical areas in association with PD-related cognitive impairment[66].

Several hypotheses have been investigated over the last years, including the neurochemical changes occurring across multiple cognition-related brain regions consequent to degenerative processes[48].

The dopaminergic system is the foremost neurochemical domain implicated in PD. In this context, a widespread dopaminergic degeneration that goes beyond the BG circuit has been considered as a possible contributor to the appearance of cognitive decline. In line with this theory and according to imaging studies, dopaminergic degeneration follows a gradient of severity when considering PD-MCI versus PDD. While in PD-MCI the dopaminergic degeneration occurs preferentially in subcortical areas (i.e., the caudate nucleus), in PDD it progresses to limbic and neocortical brain region[67]. Moreover, it has been demonstrated how cognitive symptoms are associated with a reduction in D2-receptor density in the insular cortex, ACC and parahippocampal gyrus[68].

Similar to DA, deficits in noradrenergic (NAergic) transmission occur in PD-NCI patients. However, in cognitively impaired PD patients, a more widespread NAergic degeneration has been associated with cognitive disturbances. Several studies have in fact reported a correlation between the clinical severity of PDD and the magnitude of NAergic neuronal loss in the Locus Coeruleus (LC), one of the main NAergic nuclei of the central nervous system (CNS)[69–72]. NAergic dysfunctions have been also reported in PD-MCI cases. In this regard, a recent study conducted on PD-MCI cases using neuromelanin-sensitive magnetic resonance imaging (NM-MRI) has revealed a reduced intensity in NM signal in the LC of PD-MCI patients compared to control subjects. Thus, authors argued how LC and in general NAergic dysfunctions might act as a contributing factors to the development of PD-MCI[73]. The involvement of LC degeneration in the pathophysiology of cognitive dysfunctions is also corroborated by the neuroanatomical connections between this NAergic nucleus and areas

implicated in learning and memory, such as the hippocampus and the frontal cortex (including the ACC)[74].

It is generally assumed that the disruption of the ascending cholinergic pathway is of great importance to the progression of cognitive decline in PD[75]. In the brain there are three major sources of cholinergic innervation: 1) basal forebrain which projects to the cortex through the nucleus basalis of Meynert; 2) pedunculopontine nucleus; 3) cholinergic interneurons widespread throughout the brain, but preferentially found in the striatum. It has been shown that loss of structural integrity and connectivity of these region with their respective projective areas is associated with impairments in several cognitive domains. Cholinergic dysfunctions assume in the context of cognitive disturbances a predictive trait. In this regard, it has been demonstrated how in newly diagnosed patients, a reduction in both volume and density of the basal forebrain and its projections to neocortex, hippocampus and amygdala is associated with cognitive decline over a 2-year period[76–78], being also predictive of cognitive impairment in those with PD-NC over 5 years[48,79]. . Importantly, progressive DAergic degeneration within the striatum strongly contributes to the cholinergic hypo-functionality, thus having a cumulative effect in the appearance of cognitive decline[77,80]. In terms of memory disfunction, cholinergic hypofunctionality and especially cholinergic projections to the hippocampus strongly correlated with memory deficits and conversion to PDD[78,81].

Most of our knowledges about the pathophysiology of cognitive decline resulted from clinical studies. However, in the last years basic research has also attempted to study this peculiar trait in preclinical model, though with several limitations. For example, toxin-based models of PD, including the 6-hydroxydopamine (6-OHDA) and 1-methyl,-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-based models, display traits of impairment in cognition, however these deficits are thought to reflect the severe mesencephalic Dopaminergic lesion rather that the progressive pathology toward cognition-related areas, being poorly useful to investigate the pathophysiology of cognitive disturbances observed in human patients.

3. Neuropathology

As mentioned above, the main neuropathological hallmark of PD is the progressive loss of dopaminergic neurons in the SNpc, accompanied by the presence of typical intraneuronal LBs. The pathological events leading to neurodegeneration are complex, involving oxidative stress as the final result of different mechanisms, such as mitochondrial dysfunctions, abnormal protein aggregation,

modification on DA metabolism and neuroinflammation. The present work will focus on the latest as a main recognized contributor to PD pathogenesis.

3.1. Contribution of central and peripheral inflammation to PD neuropathology

Inflammation is nowadays considered as a pivotal factor in the pathogenesis and progression of different neurodegenerative diseases, including PD. Several studies have in fact reported the presence of numerous innate and adaptive inflammatory responses in PD patients, both at the CNS level and peripherally[82,83], though the precise mechanisms and their role in disease progression are still poorly understood. These evidences have been further corroborated by an extensive pre-clinical literature[84–89]. The presence of such inflammatory responses both at the CNS level and peripherally is motivating the researchers to abandon the classic view as a CNS-centric' pathology in favour of a more systemic one[90].

3.1.1. Evidence for neuroinflammation in PD

In their seminal study McGeer et al., reported for the very first time the presence of HDL-DR+ microglial cells within the SN of *post-mortem* PD brain samples, thus suggesting the potential involvement of neuroinflammatory responses in PD neuropathology[91]. Thereafter, these evidences have been further confirmed by other groups demonstrating the presence of microgliosis in different PD-affected areas, characterized by reactive morphology of microglial cells and increased levels of inflammatory markers such as Major Histocompatibility Complex-Class II (MHC-II), CD68, and Toll-like receptors (TLRs)[92–98]. Additionally, reactive astrocytes were also reported, based on increased immunostaining for glial fibrillar acidic protein (GFAP)[91]. Furthermore, subsequent *post-mortem* studies have described the presence of glial cells expressing apoptotic markers in the SN in parallel with the occurrence of αSyn inclusions in astrocytes in different regions (SN, striatum, amygdala, thalamus, septum, claustrum, and cerebral cortex), whose abundance appeared to correlate with disease progression[99–101].

The presence of such neuroinflammatory environment was further confirmed on a molecular level, as suggested by studies in autoptic tissue from PD patients showing an increase of inflammatory mediators in the brain parenchyma as well as in cerebrospinal fluid (CSF). Accordingly, increased levels of pro-inflammatory cytokines Tumour Necrosis Factor (TNF)-α, Interleukin (IL)-1β, IL- 2, IL-6, and IL-4, Interferon (IFN)-γ, but also of the anti-inflammatory cytokine IL-10 and the chemokine CXCL12, have been described to correlate with the clinical course of the disease[102–107].

In parallel, several *in vivo* imaging studies have been carried out in PD patients at progressive disease staging, thus offering the advantage over *post-mortem* analysis of investigating markers of neuroinflammation along with disease evolution. In this regard, most of Positron Emission Tomography (PET) neuroimaging studies have focused primarily on microglial cells, representing main source of cytokines in the brain. These studies, by using the microglial ligand [^{11}C]-PK11195 have revealed the presence of activated microglia in several brain regions of PD patients compared to healthy-matched controls, including pons, basal ganglia, as well as frontal and temporal cortex[108–111]. A similar pattern of microglial activation in PD brains[112–114] have been pointed out by means of other PET ligands – including [^{11}C]-DPA713, [^{18}F]-FEPPA, and [^{18}F]-DPA714 – therefore supporting a role of neuroinflammation in the clinical stages of the disease.

3.1.2. Microglial cells in the healthy brain

Microglia, the innate immune cells resident in the brain, represent the principal actors in the CNS's immune surveillance system, capable of orchestrating inflammatory responses and ensuring brain repair and protection[115]. Unlike neurons and other CNS-resident glial cells which share a neuroectodermal origin, microglia arise from myeloid precursors born in the yolk sac, which then migrate into the CNS during development. Depending on the specific neuroanatomical region, microglia account for 0.5–16.6% of the total cell population that inhabit the human brain[116,117]. In this regard, regional differences have been described in microglia distribution, that might account for different regional vulnerability in diseases[118–121]. Remarkably, the SN appears as one of the brain regions with highest microglia density[122,123]. This aspect, together with the high concentration of iron and neuromelanin may contribute to the high vulnerability of the SN in PD[124]. Additionally, not only the density, but also the morphology of microglia seems to be brain-region dependent.

As a component of the innate immune system, microglial cells constantly patrol and sense the microenvironment, readily responding to external insults such as pathogen invasion or internal signals released by damaged tissue[125–127]. When some of these insults occurs, resting microglia become activated, leading to a series of morphological and functional changes addressed at solving the damage and promoting tissue repair[128]. Morphological alterations are generally described as a transition from a highly ramified morphology, characterized by a small cell body and long ramified processes, to an ameboid shape, with an enlargement of the cell body and retraction of processes. As mentioned, these morphological states' transitions occur together with alterations in gene expression pattern, conferring to microglia specific phenotypes that can be beneficial or detrimental

according to the trigger stimulus[129,130]. As a result, microglial cells may exhibit a dynamic repertoire of surface receptors, perform phagocytic activity, increase the release of pro- or anti-inflammatory mediators[126,131]. In fact, depending on the type of the stimulus and the resulting response, microglial activation has been traditionally distinguished into two main types, referred to as M1 and M2. The M1 activation phenotype is generally associated with the secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, chemokines, nitric oxide (NO) and reactive oxygen species (ROS)[132,133], as well as the upregulation of cell surface markers such as the MHC-II and CD68[134,135]. On the other hand, M2-like microglia has been further classified in the phenotypes M2a (associated with the suppression of inflammation), M2b (with prevalent phagocytic activity), and M2c (involved in tissue remodelling)[136–139]. However, it is widely recognized how the activation process produces a heterogeneous population of microglial phenotypes which coexist within the same microenvironment and consist of a continuum of intermediate activation states with various pro- and anti-inflammatory functions[140]. In this regard, the categorization between M1 and M2 phenotype represents an oversimplification. Given the recent advances in the scenario of microglia research, according to Ransohoff[141] such dichotomous categorization should be abandoned in favour of a more comprehensive perspective, which takes into account several aspects of microglial heterogeneity (differential transcriptomic and proteomic profiles, regional heterogeneity, sexual dimorphism, diverse functions in both healthy and diseased brain).

Besides their pure immunological role, microglial cells are involved in many processes throughout the organism' life, from its development to mature and elderly phase. During development microglia are essentially involved in the refinement and remodelling of synaptic networks[121,142–145], actively participating in synaptic pruning[121,146], promotion of developmental apoptosis[147], secretion of neurotrophic factors[148] and positioning of neurons within developing barrel cortex[149]. In the mature brain, microglia constantly interact with neuronal and nonneuronal elements in order to maintain brain homeostasis and function. Interactions include phagocytosis of synaptic structures during postnatal development, phagocytosis of new neurons during adult neurogenesis, active remodelling of the perisynaptic environment and release of soluble factors in the mature and aging brain[121]. In this regard, reciprocal interactions between microglia and neurons occur in order to guarantee a fine-tuned regulation of the CNS-microenvironment. On one hand, the release of several pro- and anti-inflammatory mediators such as IL-1β, IL-10 and TNF-α by microglial cells influences neuronal activity by the regulation of synaptic plasticity, learning and memory[150–153]. Moreover, microglia-released cytokines could act in turn in an autocrine manner, thus promoting self-regulation of these cells[151,154].

On the other hand, microglial cells express on their surface a dynamic and extended repertoire of receptors for neurotransmitters and neuromodulators[155], thus regulating their responses according to changes in neuronal activity[156].

3.1.3. Microglial cells in the parkinsonian brain

Compelling evidence for a role of neuroinflammation in PD neuropathology comes from studies conducted on animal models of the disease[157–159]. In pathological conditions, as occur in PD, microglial cells lose progressively their capacity to self-regulate, leading to an imbalance between pro- and anti-inflammatory phenotypes and promoting a chronic inflammatory state that feeds neurodegeneration[160–162]. Preclinical evidences have demonstrated how microglial cells are able to dynamically shift their functional phenotype in relation to the disease-state, which may account for the coexistence of pro- and anti-inflammatory molecules described in PD[102,103,160]. Increased levels of antigen-presenting molecules such as MHC-I, MHC-II, as well as intercellular adhesion molecule 1 (ICAM-1) have been linked to early-disease stages[163–165], while in late stages unremitting pro-inflammatory microglia seem to prevail[160,162,165–167]. In line with that, as the disease progresses microglial cells lose their antigen presenting cell (APC) capacity, while maintaining a pro-inflammatory phenotype[168] to the detriment of the anti-inflammatory phenotype[160].

3.1.4. Evidence for peripheral inflammation in PD

Increasing evidence from both human studies and experimental animal models are suggesting that immune response in PD is not restricted to the CNS. Accordingly, elevated levels of several inflammatory mediators, including the pro-inflammatory cytokines TNF, IFNγ, IL-1β, IL-6, IL-2, and the chemokines CXC-chemokine ligand 8 (CXCL8) and CCL2 have been found in the serum of PD patients. Interestingly, such increment in pro-inflammatory mediators correlates with the disease severity and disability[169,170].

Recently, different studies have proposed a contribution of peripheral inflammation in the progression of PD. In this regard, La Vitola et al[171] tested the influence of peripheral inflammation induced by intraperitoneal LPS injection, on αSynOs' effects *in vivo*. Specifically, systemic LPS injection trigger a long-lasting neuroinflammatory response, that ultimately worsen the toxic effects of αSynOs given centrally at subliminal doses. The same authors observed a detrimental effect of systemic LPS injection on the cognitive performance in the A53T mouse model of PD. Accordingly, in a study examining a patient cohort with incident parkinsonism, the unbalanced ratio of increased serum proinflammatory cytokines versus decreased anti-inflammatory cytokines correlated positively with

a faster disease progression and cognitive deterioration[172]. However, the direct contribution of peripheral inflammation to the progression of PD has been recently questioned. A recent study showed lack of correlation between CSF versus plasma cytokines in PD patients, suggesting that central and peripheral cytokine levels may at least partially behave independently, and may be driven by different factors[173].

3.1.5. Role of neuroinflammation in PD-related cognitive disturbances

Despite the clear contribution of neuroinflammation in the neurodegenerative process within the BG, little is known about its role in the neuropathology of PD-related cognitive symptoms[162]. In recent years, several studies have attempted to fill this gap, yet with uncomplete results. At the current state, a better understanding of the biological factors underlying cognitive decline in PD appears crucial, both to improve the understanding of disease neuropathology and for designing therapeutic approaches suitable for these non-motor disturbances, possibly aimed at slowing their progression towards a full-blown dementia.

Most of the evidence suggesting a contribution of neuroinflammation in the development of cognitive disturbances in PD is the result of studies conducted on PD patients. By measuring inflammatory markers in the CSF of PD patients, Lindqvist et al[174]. observed increased levels of C-reactive protein (CRP) in PDD patients compared with non-demented. In another study, higher levels of pro-inflammatory cytokines such as TNF-α and IFN-γ as well as CRP have been associated with a lower Mini-Mental State Examination (MMSE) score in newly diagnosed PD patients, whileIL-1β and IL-2 have been related to a faster rate of cognitive decline[172]. Importantly, the upregulation of pro-inflammatory cytokines has been also observed in limbic regions of PDD patients compared to age-matched controls, with a concomitant upregulation in TLR-4 in these regions[175].

By using the microglial ligand [^{11}C]PK11195 Edison et al.[110] have shown the presence of reactive microglia in several cortical regions of PD-NCI cohort as well as in PDD patients. In this latter group, however, spatial extent of microglial activation was greater, being additionally involved the frontal and cingulate regions. Moreover, microglial response appeared to be negatively correlated with the cognitive function in PDD cohort. More recently [^{11}C]PK11195 PET scanning was performed in PDD and AD and age-matched controls. Results demonstrated an inverse correlation between hippocampal volume and microglial activation in the same area in both AD and PDD compared to the control group[176].

Although not investigated in relation to cognitive disturbances, an increased expression of some microglial markers (specifically CD68 and MHC-II) has been described in limbic and cortical regions of PD patients, including the hippocampus and cingulate cortex[93,177]. Interestingly, HLA-DR$^+$ microglia described in some limbic regions of PDD patients did not consistently overlap the LB pathology[175]. Moreover, markers of reactive microglia are not a feature of dementia with LB[178,179]. Recently, a dysregulation of innate immune response has been observed in a cohort of PD patients at high risk of developing dementia. Specifically, increased levels of classical monocytes and TLR$^+$ monocytes have been observed in PD patients, though in a large extent in those at increased early dementia risk; moreover, monocyte TREM2 expression was also elevated in the higher dementia risk group[173].

Taken together, these data provide evidence that adaptive and innate immune responses may be involved in the development of PD-related cognitive disturbances. In this context, the role of microglial cells appears to be fundamental, being involved not only in orchestrating neuroinflammatory response within the CNS level but also as a main actor in remodelling synapses, as well as influencing membrane properties, thus actively participating in cognitive processes[152,180,181]. As previously stated, in pathological conditions microglia lose their capacity to self-regulate. Specifically, in PD chronically dysfunctional microglia have been suggested to affect synaptic connectivity in motor areas through the excessive release of inflammatory cytokines[156,182]. Among them, TNF-α represents a key regulator of neuronal excitability, synaptic strength and plasticity[183,184]. All these functions are in part mediated by the direct regulation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor expression on the cell-surface, that, in large part, oversee glutamatergic mechanism underlying synaptic plasticity[153,185].

3.2. Alpha synuclein: from physiology to pathology

Post-mortem analyses of brains from patients diagnosed with PD display atrophy, cell death and several inclusions of aggregated proteins. Such aggregates, known as LBs and LNs mainly consist of aggregated α-synuclein (αSyn)[186–188], but also contain other proteins involved in a broad range of cellular processes. Since its discovery as the main component of LBs and LNs, a large body of evidence has been accumulated, suggesting a pivotal role of αSyn in the neuropathology of PD and more in general of synucleopathies. Missense mutations in the *SNCA* gene encoding for αSyn have been linked to familiar forms of PD and DLB, as well as duplication or triplication of the entire *SNCA locus*[189–191]. Moreover, genome-wide association studies (GWAS) have revealed that genetic variants in the αSyn encoding gene represent an important risk factor for sporadic forms of PD[192,193]. Finally, animal

models of PD based on the overexpression of αSyn[194–198] as well as exogenous αSyn-based models[168,199–201] show, albeit in a variable way[202], histopathological and symptomatologic features typical of PD.

3.2.1. Alpha synuclein: conformational properties and physiological functions

α-Syn is a protein belonging to the synuclein protein family. Specifically, human αSyn is a protein of ∼14 kDa (containing 140 amino acid residues) encoded by the gene *SNCA*. It is widely distributed throughout the entire body but is particularly abundant in neurons[203–205], mostly at the pre-synaptic terminal. On a structural level, this protein is characterized by an amphipathic lysine-rich amino (N)-terminus, which plays a fundamental role in modulating its interactions with the cellular membranes and is predisposed to fold into α-helices, and an acidic carboxy (C)-terminal tail, intrinsically disordered, responsible for the nuclear localization of the protein as well as for its interactions with small molecules, metals and other proteins[206,207]; at this level are present several phosphorylation sites on Tyr-125, 133, 136 and Ser-129. In between the N-terminal and C-terminal domains is the central core of the protein, known as non-amyloid-β component of AD amyloid plaques (NAC). It contains an hydrophobic domain essential for α-Syn aggregation when it acquires a β-sheet structure[208]. Accordingly, deletion of a large segment of this central core decreased considerably the oligomerization and the fibrillization of the protein[201,209,210]. Moreover, certain mutations linked with synucleinopathies have been found in this region[191,211,212].

In physiological conditions αSyn exists in an equilibrium between a soluble unfolded status, and a membrane-bound state[213]. Soluble αSyn is natively monomeric and structurally disordered, which allows to adopt different conformations upon the interactions with cell membranes or other proteins[214,215]. These different conformations are strictly dependent on intramolecular interactions between amino acid residues[216]. Both under physiological and pathological conditions αSyn may undergo several post-translational modifications (PTMs), including ubiquitination, nitration[217], acetylation[218], glycosylation[219], SUMOylation[220,221] and phosphorylation, which affect the activity of the protein and may be relevant for its pathological function[222–224]. Among them, phosphorylation is the most studied PTM. In physiological condition, about the 4% of total αSyn is phosphorylated, while this percentage increases up to 90% under pathological conditions[224–226]. Most of the phosphorylated residues are located at the C-terminus domain of the protein, which is believed to be involved in αSyn pathology. Of these, the most frequent phosphorylation occurs at Ser-129, thus nowadays considered a specific marker for synucleopathies[225,226].

Although the physiological functions of αSyn are still highly debated, its localization at the pre-synaptic terminal and its association with synaptic vesicles[227,228], suggest a role of the protein in the regulation of neurotransmitter release, synaptic function and plasticity[229]. At the presynaptic space αSyn would act as a chaperone, promoting the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex formation and regulating synaptic vesicle trafficking as well as neurotransmitter release and re-uptake[230–232]. In the presynaptic compartment, αSyn can also interact with the vesicular transporter of monoamines (VMAT2)[233–236], even though its proper function is not well understood yet. Nevertheless, it has been demonstrated how the overexpression of *wild-type* αSyn in SH-SY5Y cell line increase the level of cytosolic DA[234], while *in vivo* depletion of αSyn decreased the reuptake of this neurotransmitter at striatal level[237] and causes functional deficits in the nigrostriatal system[238]. Moreover, it has been shown that αSyn is able to inhibit the DA synthesis with a Tyrosine Hydroxylase (TH)-related mechanism[239–241]. Taken together, evidence supports the hypothesis that αSyn may play an important role in the regulation of DAergic neurotransmission at the presynaptic level.

Several studies have demonstrated the presence of αSyn in other cellular compartments, such as endoplasmic reticulum (ER) and Golgi apparatus[242,243], mitochondria[244–246] and nucleus[204,247–249]. In the mitochondrial compartment, αSyn plays a role in improving ATP-synthase efficiency and, in general, mitochondrial function[250], while within the nucleus αSyn expression levels were shown to increase under oxidative stress conditions and appear to regulate the expression of genes related to DNA repair and mitochondrial biogenesis[251,252].

3.2.2. States of aggregation

As previously stated, primary components of both LBs and LNs are fibrils of αSyn. This has led to the hypothesis that molecular mechanisms at the origin of PD may be attributable to the fibrillization of αSyn into amyloids[200,253–256]. A number of factors, including mutations, modifications of the microenvironment, and PTMs[190,191,211,212,222,226,257–259] contribute to the conformational changes and subsequent aggregation of αSyn in PD. In this regard, the association with lipidic membranes is relevant. The interaction with lipid bilayers has been shown to modulate kinetics and mechanisms for αSyn aggregation, with effects ranging from inhibition to enhancement[260–262].

The aggregation cascade of αSyn occurs through a multistep process which follows a nucleation-dependent model. The cascade starts with misfolding monomeric protein leading to the formation of oligomeric structures (so-called lag phase), followed by an elongation phase where the aggregates

rapidly grow, which are finally deposited as LBs and LNs. Importantly, a dynamic equilibrium exists between all these different conformations. As a result, different species and states of αSyn aggregation have been detected in the parkinsonian brain, including unfolded monomers, soluble oligomers, protofibrils, and insoluble fibrils of elevated molecular weight[263]. As opposed to the insoluble fibrils found in both LBs and LNs, soluble forms of αSyn are nowadays considered the most toxic. In this regard, several studies have in fact indicated a poor correlation between the LB pathology and some clinical features of PD. Moreover, some genetic forms of PD (i.e., mutations in *PARKIN* and *LRRK2 G2019S* genes) showed a neuronal loss within the nigra in the absence of LBs and LNs[264,265]. Finally, *in vitro* studies have demonstrated a poor capacity of amyloid fibrils in inducing neurotoxicity[226,266]. Therefore, according to these and other evidence, it has been suggested even a neuroprotective role of LBs and LNs. According to this hypothesis, these insoluble aggregates may act by sequestering toxic species such as soluble oligomers and prefibrils from the cytoplasm, thus slowing their spreading[267–271].

The interaction with membranes assumes a pivotal role in the pathological scenario of PD. As previously mentioned, all αSyn species (including monomers, oligomers and fibrils) bound the membranes. However, these kinds of interactions assume different features (if pathological or not) according to the diverse conformational states of these species. With respect to oligomeric forms of αSyn, given the high variability in terms of structures taken by these aggregates, in a recent study Fusco and colleagues[272] characterized two different forms of αSyn oligomers (αSynOs), named as type A and type B. Specifically, they differentiate these two forms basing on the presence of a core β-sheet-folded region together with a dynamic and accessible N-terminal region, which is typically present in the type B but not in the type A; the presence of these domains conferred toxicity to the oligomer. When incubated with neuroblastoma cells, type B oligomers, but not type A oligomers or monomers, impaired mitochondrial machinery and induced oxidative stress in these cells. Importantly, this latter effect was mild when neuroblastoma cells were incubated with type A oligomers and even fibrils of αSyn. Furthermore, toxic gain of function of type B oligomers is also related to their different ability to disrupt the membranes. Upon incubation with lipidic vesicles, both types of oligomers bound to membrane, but type B displayed a more stable binding than type A. Moreover, the presence of the rigid fibrillar core allowed type B oligomer to insert into the lipid bilayer, disrupting its integrity (Fig. 3).

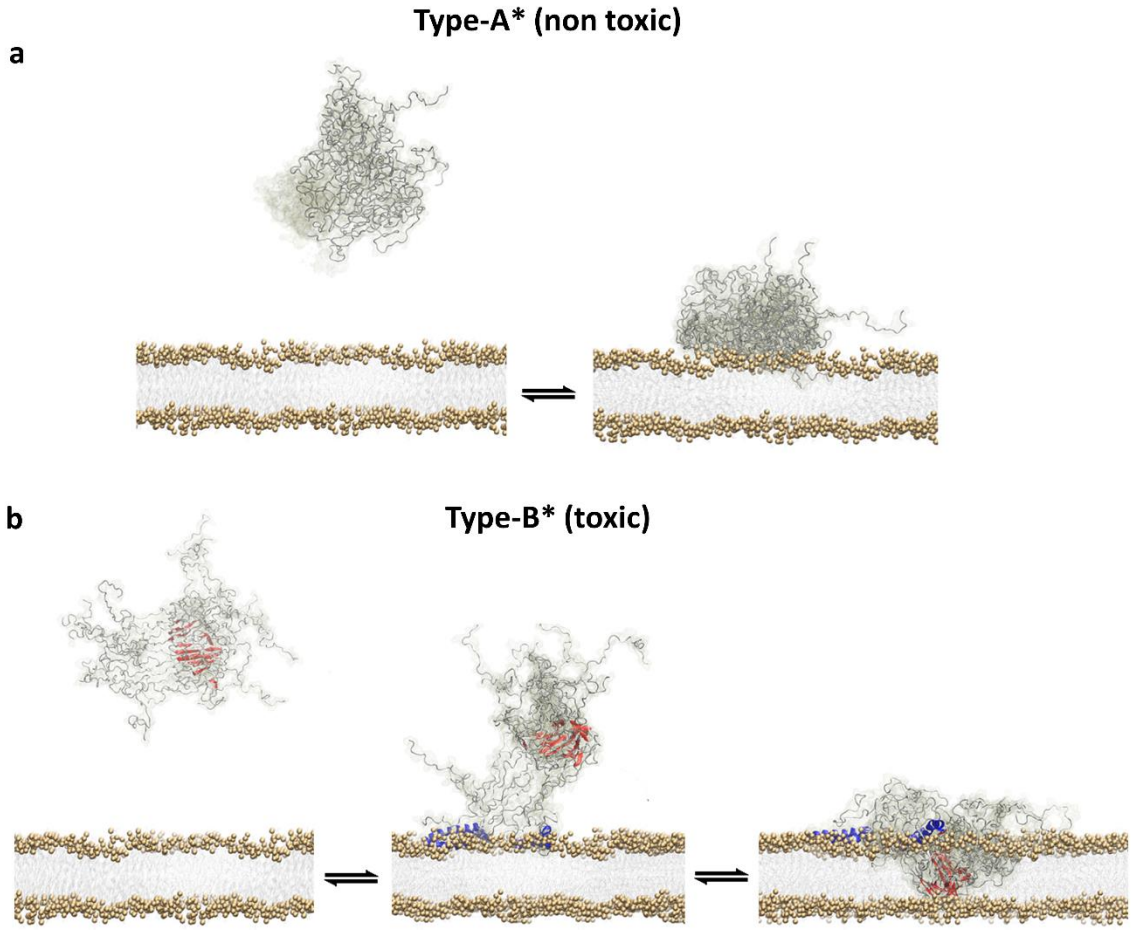


Figure 3. Structural basis of αSynOs' membrane binding. Non-toxic type-A* αSynOs (a) and toxic type-B* αSynOs (b) are represented. In type-B*, the presence of β-sheet-folded region (here presented in red) allows it to insert within the lipid bilayer, thus promoting the disruption of membrane integrity. (Modified from Fusco et al., 2017).

3.2.3. Alpha-synuclein oligomers: mechanisms of neurotoxicity

Within the boundaries of CNS, αSynOs may exert their neurotoxic role by directly targeting neurons (both at the membrane and organelle levels) or interacting with glial cells.

Neurons: As referred to neurons, toxic mechanisms may involve a direct interaction with neuronal membranes as well as with subcellular organelles (Fig. 4). Intracellularly, αSynOs might induce mitochondrial dysfunctions[168,246,273,274], as well as inducing oxidative stress into the ER[275], or disrupting the integrity of cytoskeletal apparatus[276]. As previously mentioned, αSynOs exert their neurotoxic effect by primarily disrupting the integrity of cell membranes. Toxic oligomeric forms have in fact the capacity to bind and then permeabilize the lipid bilayer, causing the influx of several ions within the cytoplasm[277,278], including calcium. As known, increase calcium influx leads to cell

cytotoxicity and consequently cell death. Remarkably, it has also been observed how αSynOs might stabilize pre-existing membrane defects, thus accelerating its disruption[279].

Several evidences have shown that αSynOs negatively impact neuronal signaling and excitability by targeting specific synaptic components, in line with the key role played by αSyn in presynaptic-protein complexes[280–282]. Notably, it has been suggested that αSynOs-induced synaptic deficit should be considered as a starting event for neurodegeneration, being reported before any evident neuronal loss[283,284]. Several mechanisms have been proposed to explain α-SynOs-detrimental effects at the synaptic level, one of which is the disruption of microtubule integrity and dynamic. Indeed, by binding to varying extents a broad range of proteins involved in microtubule assembly and dynamics, oligomeric species might disrupt the integrity and the proper functionality of the axonal network and negatively affect axonal transport machinery[285,286]. Besides their interactions with microtubule machinery, α-SynOs might influence neurotransmitter release by inhibiting synaptic docking. In this regard, the interaction between α-SynOs and the protein sinaptobrevin-2, a component of SNARE multicomplex, plays a critical role. It has been demonstrated how, when bound by α-SynOs, sinaptobrevin-2 becomes unavailable for promoting the t-SNARE complex assembly, thus inhibiting the neurotransmitter release[280]. The neuropathological role of αSynOs at the synaptic level is assuming great relevance in the context of PD related non-motor symptoms, especially for cognitive disturbances. Several groups have observed a reduction in long term potentiation (LTP) currents in hippocampal slices exposed to αSynOs[281,287] which might be relevant to memory impairment. In this respect, it has been proposed that αSynOs might alter LTP currents, by causing an impairment of both AMPA and N-methyl-D-aspartate (NMDA) receptors permeability. Interestingly, αSynOs seem to enhance AMPA receptor transmission in cultured hippocampal cells, leading in turn to increased calcium influx and cell death. These *in vitro* experiments have been further corroborated by *in vivo* evidences, that demonstrated a direct effect of oligomeric species on cognitive function[287]. Albeit these data support an important role of αSynOs in cognitive function, the clear mechanism underneath is still missing. Therefore, additional investigations are needed to acquire new insights on possible processes which may occur.

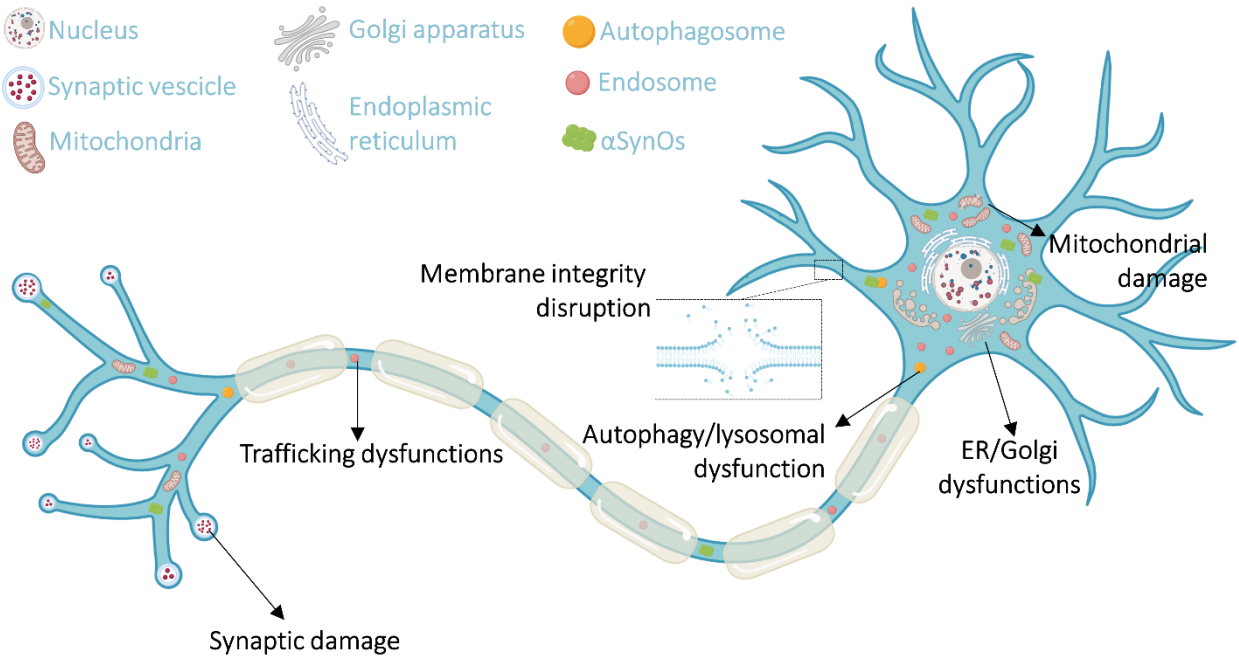


Figure 4. Intracellular target of αSynOs-mediated neurotoxicity. Schematic representation of different intracellular neuronal target of αSynOs.

Microglia:

The presence of detectable αSynOs in extracellular fluids as well as the direct observation of their release by neuronal cells has suggested how these species may target other cell types besides neurons. Importantly, αSynOs may spread within the brain and establish contacts with microglia and astroglia[202,288–290]. Under a pure immune perspective, microglial cells should be considered the principal actors in the innate immune response within the CNS, responsible, amongst others, of phagocytic clearance. In pathological condition, increased levels of aberrant proteins, may lead to microglial phagocytic exhaustion, promoting in turn protein accumulation and the establishment of a neurotoxic environment.

αSyn seems to interact with microglial cells by binding a specific class of receptors widely distributed on microglial surfaces, namely TLRs. This class of protein is primarily involved in microglia-mediate innate immune responses. They act as sentinels able to detect and recognize a number of extracellular molecules, generally referred to as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The latter are found generally on molecules endogenous to the host[291]. Once activated by PAMPs/DAMPs these receptors trigger non-specific immune responses, such as initiation of pro-inflammatory cytokine production and release,

phagocytosis, as well as the activation of adaptive immune system[292]. In this regard, TLRs appear to be the principal mediators in microglia-αSyn interactions[293–295]. It has been in fact demonstrated how αSyn interacts with microglial cells by stimulating two different subclasses of TLRs, respectively TLR2 and TLR4. While αSyn-TLR2 interaction leads to the production and release of inflammatory mediators[293], stimulation of TLR4 seems to be preferentially involved in αSyn phagocytosis[294]. Interestingly, it has been suggested that interaction between αSyn and TLR2 occurs in a conformation-dependent manner with the oligomers, but not monomers or dimers, acting as a TLR2 agonist, triggering the inflammatory cascade via NF-kB activation[293,296].

As previously stated, TLR4 are preferentially involved in microglial phagocytosis of extracellular αSyn[294] and this process seems to depend upon levels and conformational state of αSyn itself[202]. In this regard, it has been observed an opposite effect exerted by αSyn monomers or oligomers on microglia phagocytosis, with the first behaving as stimulating factor while the latter as inhibitor[297]. Moreover, when incubated with A53T mutant αSyn, microglial cells acquired a pro-inflammatory profile associated with impaired phagocytic function[298]. Conversely, other evidences have depicted a promoting effect on microglia phagocytosis triggered by A53Tα Syn[299]. Finally, recent evidences have shown a time-dependent impairment of microglia phagocytic activity after the exposure to αSynOs[168]. Despite a lack of consensus, all these data clearly highlight that the microglia phenotype and phagocytic function are differently affected by αSyn structural variants.

Astrocytes: As well as other glial cells, in physiological conditions astrocytes do not express αSyn, or at least in a very low extent[300,301]. However, several studies have largely demonstrated the interactions between these cells with aberrant forms of αSyn in pathological conditions, both *in vitro* and *in vivo*. Changes in astrocytic gene expression profile have been observed following the exposure to αSyn aggregates, promoting in turn the production of pro-inflammatory cytokines and chemokines, and triggering the recruitment of other glial cells, such as microglia[302]. Such neuroinflammatory environment is also exacerbated by the production and release of ROS, which will affect in turn neuronal survival and neuronal functions[302,303]. When co-cultured with neurons and αSynOs, astrocytic cells can rapidly internalize and then degrade oligomers via the lysosomal pathway. However, it has been shown a partially degradation of these toxic species, suggesting not only an important role played by astrocytes in their clearance, but also how αSynOs might overburden the astrocyte clearing machinery[274]. In the same study, the capacity of these oligomeric species to induce mitochondrial damage in charge of astrocytic cells has also been described, resulting in

alteration in their morphology and in a reduced functionality and efficiency. Moreover, treatment with an antibody selective for αSynOs, fully prevented the accumulation of the toxic species within astrocytes and thereby rescued them from mitochondrial impairment[304].

It is interesting to note how the entity of cellular responses triggered by the interaction between astrocytes and αSyn aggregates seems to be dependent on their aggregation status. Both fibrillar and soluble oligomeric forms induced, in a similar extent, both morphological and phenotypical alterations in astrocytic cells (i.e., increased levels of pro-inflammatory cytokines). However, only oligomers can target astrocyte mitochondria, resulting in a number of dysfunctions and a significant increase in the extracellular production of hydrogen peroxide by these cells[303,305].

Finally, αSynOs seem to modulate glutamatergic neurotransmission and synapse formation via their interactions with astrocytic cells. Oligomers may exert their toxicity on neurons by indirectly inducing glutamate release from astrocytic cells, thus contributing to synaptic loss[306]. Moreover recent *in vivo* and cell culture data from MSNs suggests that αSynOs applied to neurons alone lead to neuronal spine loss, but when taken up by neighbouring astrocytes, lead to neuronal spine gain posing a potential neuroprotective role exerted by these cells[307].

3.2.4. Seeding and spreading properties of αSynOs

As with many other prion-like diseases (such as AD, MSA and others), PD is marked by a stereotypical distribution of αSyn pathology. For many years, this peculiar pattern has been uniquely imputed to differences in vulnerability of neuronal subtypes among the different brain regions[308,309]. However, in the last years several studies have demonstrated cell-to-cell transmission for αSyn, thus reviewing the classical hypothesis[310–313]. The presence of LBs in foetal DAergic grafts implanted in a PD brain 15 years earlier provided the very first evidence in favour of a prion-like spread of αSyn [314,315]. Since then, a growing body of research has demonstrated that αSyn seeds made from recombinant proteins or aggregate-containing lysates from diseased brains may spread in a prion-like fashion in neuronal cells, organotypic slice cultures, and mouse models of PD[201,253,316–320]. Several mechanisms have been proposed to explain the cell-to-cell propagation. These include passive diffusion within the plasma membrane[321–323], passage through membrane pores[324], exosomal transport[325], transport through carrier proteins[326,327] and tunneling nanotubes[313,328–332].

The propensity of αSyn to spread throughout the connectome is extremely relevant in the context of PD non-motor symptoms. In this regard, the progressive nature of cognitive disturbances typically present in parkinsonian patients, might be seen as a reflection of the spreading pathology that

underlies PD. Good evidence from *post-mortem* studies have shown a correlation between limbic and cortical spread of LBs pathology in PDD. In these patients, αSyn-pathology in cortical and limbic regions, such as hippocampus, amygdala, as well as the entorhinal cortex and ACC, strongly correlated with cognitive decline, suggesting how the presence of LBs in these areas may predict the development of PDD[175,177,333,334]. However, such link is not straightforward regarding PD-MCI. As previously mentioned, MCI is particularly common in the very early phases or even in newly diagnosed PD patients, when limbic and cortical proteinaceous depositions are not detectable[48,335,336]. Therefore, this evidence leaves uncertain the role of αSyn in MCI, suggesting that the neuropathology beneath cognitive disturbances initiates before any clearly detectable protein deposition.

4. Therapeutic strategies

At the present state, no disease-modifying strategies have been approved for PD treatment, leaving the pharmacological scenario dominated by the so-called symptomatic therapies. In this regard, DA replacement therapy (DRT), with the metabolic precursor of DA levodopa (L-DOPA) as the gold standard, remains the mainstay of treatment for motor symptoms[7]. Therapeutic effect of L-DOPA strictly depends on its capacity to cross the blood-brain-barrier (BBB), unlike DA. Once in the brain, L-DOPA is converted into DA by the enzyme L-amino-acid decarboxylase, thus restoring DA levels. Generally, it is administered in formulation with carbidopa or benserazide, aromatic acid decarboxylase inhibitors that prevent L-DOPA peripheral metabolism, thus increasing its bioavailability at CNS level. Though effective, the treatment with L-DOPA is associated with severe side effects that constitute an important part of the disabilities experienced by the patient, particularly in advanced stages. Chronic L-DOPA treatment might in fact result in significant motor complications including the so-called L-DOPA-induced dyskinesia (LID) and on-off motor fluctuations[337]. The firsts are described as monophasic, dystonic, choreiform, and non-rhythmic abnormal movements, which affect the facial muscles, neck, upper and lower limbs, and body axis[156]. The second mainly refers to those unpredictable shift from an *ON period,* in which the patients experience a good response to medication, to an *OFF period*, in which characteristic motor symptoms reemerge[338]. These motor complications appear to be less severe when L-DOPA is administered in co-therapy with both catechol-O-methyltransferase (COMT; entacapone, tolcapone, opicapone) and monoamine oxidase-B (MAO-B, selergiline, rasagiline; safinamide) inhibitors[339,340]. Finally, DA

agonists such as pramipexole and ropinirole appear to be most effective during the early stages of the pathology, and are sometimes associated with L-DOPA in late-stages of the disease to increase [341].

4.1. Disease-modifying therapies: state of art to 2022

To date, despite numerous clinical trials, no intervention has been demonstrated to modify the progression of PD. Of note, the annual clinical trials pipeline for 2022 has been recently published, reporting a total of 147 clinical trials registered on the ClinicalTrials.gov website. Of these, 93 investigate symptomatic therapies (STs) while 54 focus on disease-modifying therapies (DMTs)[342]. Among DMTs, the pipeline reports 24 clinical trials in phase 1, 28 in phase 2 and only 3 trials in phase 3. In Fig. 5 pie charts are reported, relative to the therapeutic categories for each clinical phase. As shown, several targets have been addressed for potential disease-modifying properties in testing therapies including antioxidants, anti-inflammatories, immunotherapies, neurotrophic factors. Interestingly, pie chart relative to phase 1 shows an increasing interest towards innovative approaches targeting the immune system, including classical anti-inflammatory compounds and last generation immunotherapies. In this regard, in the last years different epidemiological studies have been carried out to ascertain the potential neuroprotective role of non-steroidal anti-inflammatory drugs (NSAIDs), however reporting contrasting evidences[159,343–345]. In the last decade, pre-clinical evidence has successfully reported the neuroprotective activity of immunomodulatory compounds, thus prompting for their repositioning in PD. In this regard, the immunomodulatory agent Sargramostim (Leukine)[346] and antidiabetic compounds acting on glucagon-like peptide (GLP)-1 pathway, such as exenatide and liraglutide are currently in clinical investigation for PD. Exenatide testing for repositioning in PD has produced positive outcomes in motor and cognitive measures, and possibly delayed disease progression, and has now reached the clinical trial phase 3[347,348].

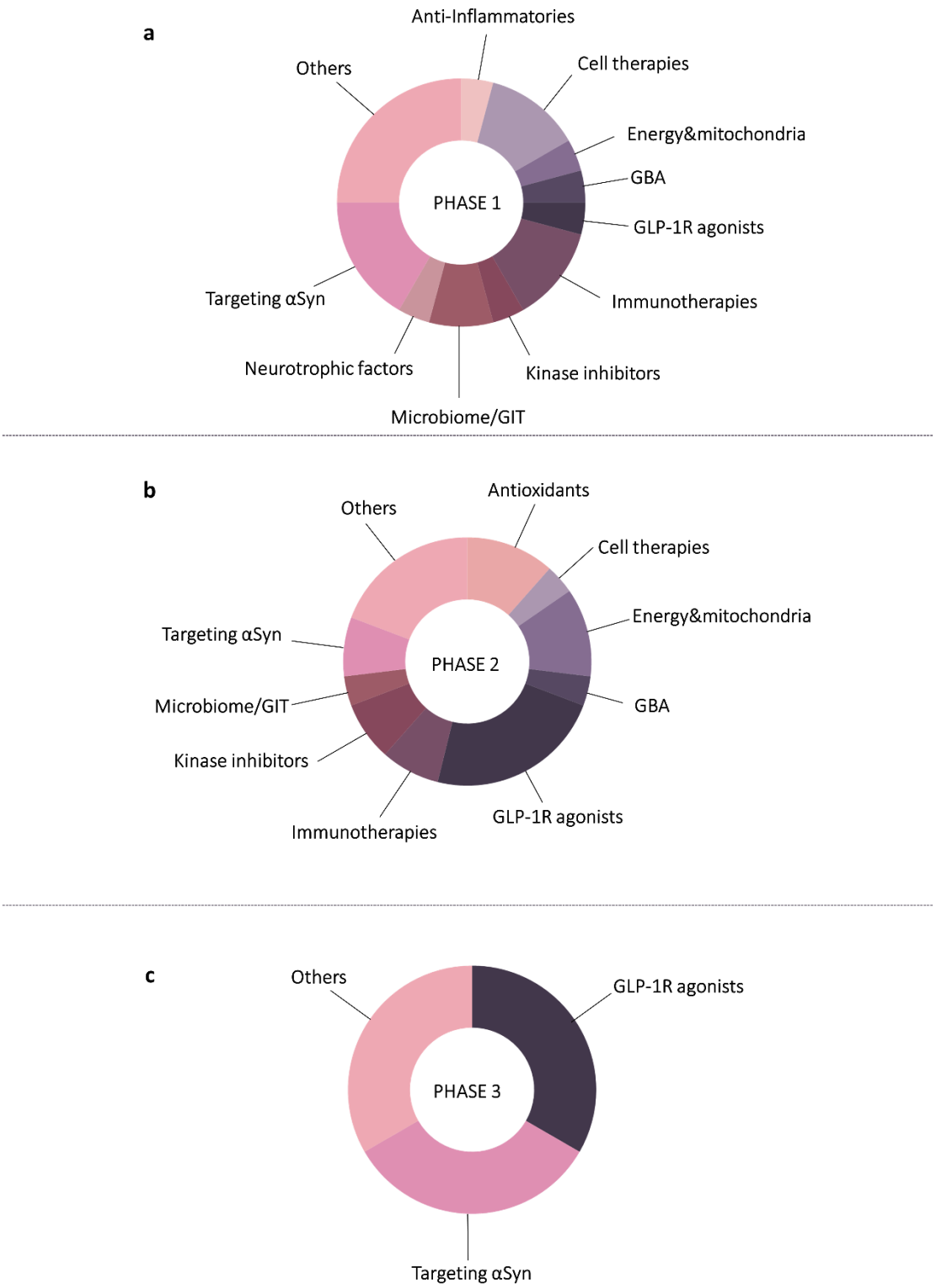


Figure 5. Pie chart of the disease-modifying agents in active (a) phase 1, (b) phase 2 and (c) phase 3 trials for PD, registered on clinicaltrials.gov as of January 31st, 2022. Figure was adapted from McFarthing et al., 2022.

4.1.1. Immunomodulatory Imide Drugs (IMiDs)

Immunomodulatory Imide Drugs (IMiDs) are a class of thalidomide-derivatives used in clinical practice to treat certain forms of tumours (such as multiple myeloma) and in some autoimmune diseases. These therapeutic uses are related to the pleiotropic anti-myeloma actions of this class of compounds that comprise anti-angiogenic, anti-proliferative, immunomodulatory and anti-inflammatory. The history of IMiDs, and more specifically thalidomide, is infamous for causing teratogenic effects when used as an antiemetic in pregnancy. Therefore, for a long period of time thalidomide was withdrawn from the market, until it was serendipitously found to be effective in the treatment of erythema nodosum leprosum, an inflammatory complication of leprosy characterized by high levels of serum TNF-α[349]. Thereafter, thalidomide capacity to inhibit TNF-α synthesis was definitively discovered [350]. As a consequence, novel analogues have been synthesized and evaluated in the hope of enhancing drug potency and decreasing side effects in respect to the parent compound[351].

One of the most important mechanisms of action shared by IMiDs relates to their TNF-α lowering activity through post-translational mechanisms that comprise the destabilization of TNF-α mRNA and consequent inhibition of protein production[352,353]. This anti-inflammatory effect is also evident in the dampening of the production of other pro-inflammatory mediators such as IL-1, IL-6, IL-8, IL-12, GM-CSF and boosting of the production of anti-inflammatory cytokines such as IL-10[354–356]. This aspect is of major interest for all those pathologies characterized by a chronic and sustained cytokine release, such as PD. As mentioned above, whether inflammation is an essential healing response, chronic and dysfunctional inflammatory reactions, with an unbalanced production of pro- and anti-inflammatory cytokines, lose their physiological and adaptive significance, in favour of a pathological role that, in turns, exacerbate the neuropathological scenario.

Apart from its anti-inflammatory property, thalidomide and its derivatives also show strong anti-angiogenic and immunomodulatory properties, including T-cell co-stimulation and activation of Natural Killer (NK) cells.

Compared to classical immunosuppressant and TNF-α-targeting drugs, IMiDs present an optimal pharmacokinetic profile making them suitable for treating chronic neurological disorders[107]. This class of compounds display, in fact, high CNS MPO (multiparameter optimization) score, which reflects the BBB permeability, and so their delivery within the brain at clinically relevant doses[351,357]. Moreover, they follow the Lipinski rule of five, predicting their successful delivery to their drug target

under physiological conditions[358]. In this regard, Pomalidomide (Pom), a III-generation IMiD, is of particular interest for its potent TNF-α lowering activity. In fact, Pom has demonstrated a TNF- α inhibitory action of up to 50,000-fold greater than that of thalidomide[359,360] and has a favourable BBB permeability in mice, achieving a brain/plasma concentration ratio of 0.71[361]. Notably, it has been also shown that neurotoxic and teratogenic side-effects are less prominent than those of the other IMiDs[359,362], making it potentially suitable for repositioning in neurological diseases.

First evidence of a beneficial effect of IMiDs on PD neurodegeneration, came from studies conducted on rodent models. In the MPTP mouse model, thalidomide has been shown to counteract in a dose-dependent manner DA depletion in the striatum[363], though it did not prevent from DAergic degeneration within the SNpc[364]. These evidences have been further corroborated in a recent study conducted by Palencia et al[365], in which the administration of thalidomide before or after MPTP exposure was able to increase the DA levels in the striatum while those of MAO-B in the SNpc were decreased. Moreover, they also observed an inhibition of the lipoperoxidation process in thalidomide-treated mice, suggesting a potential role of this compound in counteracting oxidative stress in the nigrostriatal pathway[365].

Thalidomide and its analogue Lenalidomide were tested in a transgenic mouse model of PD overexpressing αSyn. Specifically, the II-generation compound Lenalidomide has proved higher efficacy than Thalidomide in counteracting the DAergic degeneration and the associated motor impairment, as well as in attenuating cytokine production and microgliosis in the striatum and in the hippocampus[366]. In this regard, it has been shown that Lenalidomide not only reduced the expression of pro-inflammatory mediators such as TNF-α, IL-6, IL-1β but also increased the expression of the anti-inflammatory cytokines IL-10 and IL-13[366].

Recently, the efficacy of Pom in counteracting the motor impairment and the neuronal loss in the transgenic LRRK2 WD40 Drosophila was demonstrated[367]. Interestingly, Pom and its derivative 3,6'-dithioPom (DP) mitigated α-Syn-induced loss of DAergic neuron as well as microglial cell activation in primary cultures[368].

IMiDs have also been recently tested for their effectiveness against LIDs in the 6-OHDA rat model of PD. Both Thalidomide and its analogue 3,6'-dithioThalidomide (3,6'-DTT) have shown a beneficial effect in attenuating the severity of LIDs, as well as an important immunomodulatory effect, showed by the restoration of pro-inflammatory/anti-inflammatory cytokines ratio[182]. Furthermore, these compounds were also able to inhibit the striatal and nigral angiogenesis associated with LIDs, in

accord with the potent antiangiogenic activity of this drugs[182], and with the antidyskinetic properties of anti-angiogenic compounds[107,162,369].

Despite their promising therapeutic potential, more preclinical evidence is needed to support the translatability of these compounds to the clinic for repositioning in PD. Therefore, currently no clinical trial has been launched to test their disease-modifying properties in parkinsonian patients.

STUDY 1

Pomalidomide as a potential disease-modifying strategy for PD treatment

The development of therapeutic approaches to slow or stop disease progression remains the greatest unmet therapeutic need in PD management. At present, only symptomatic therapies are used in the clinical practice, thus making the quest of disease-modifying strategies a priority. Given the high cost and low success rate in new drug development, a complementary strategy based on the repositioning of drugs that are approved for other indications is taken into heavy consideration. Here, based on the pivotal role of central and peripheral inflammation in the neuropathology of PD, we addressed the disease-modifying potential of the IMiD Pom in the progressive model of PD obtained by the intranigral infusion of preformed H-αSynOs.

Materials and methods

This study was conducted in collaboration with Prof. Alfonso de Simone, at the University of Naples Federico II, who produced and provided the oligomers of αSyn to generate the PD model used in the present study. The preparation of such oligomers is briefly summarized below.

Production of recombinant H-αSyn

Recombinants human αSyn (H-αSyn) were expressed and purifies in *E. coli* using plasmid pT7-7 encoding for the protein, as previously described[272]. After several centrifugation-suspension steps, the αSyn fraction containing the monomeric protein was removed by Vivaspin filter devices (Sartorius Stedim Biotech, Gottingen, Germany). The purity of the sample was analyzed by SDS-PAGE and protein concentration was determined from the absorbance at 275 nm[272].

Purification of H-αSynOs

The H-αSynOs were prepared from the purified recombinant H-αSyn as previously described[168,272]. Briefly, lyophilized protein was resuspended in PBS buffer at a pH of 7.4 and a concentration of 12 $mg \cdot mL^{-1}$, and the solution passed through a 0.22 µm cut-off filter before incubation at 37 °C for 24 h. [370]. The sporadic fibrillar species formed during this incubation period were removed by ultracentrifugation for 1 h at 288,000 g, while the excess of monomers was removed using several filtration steps with 100 kDa cut-off membranes, resulting in the enrichment of the oligomeric species. The H-αSynOs are stable for many days at room temperature, but in the present study were used within 2 days of their production.

At the end of the purification procedure, and prior to intracerebral inoculation, oligomers were tested for endotoxin contamination via the LAL (Limulus Amebocyte Lysate) assay (Kairosafe, Italy).

Animals and stereotaxic surgery

Male Sprague–Dawley rats (275–300 g, Envigo) were housed in groups of three to four in standard conditions of temperature (21 ± 1 °C) and humidity (60%) under a 12 h light/dark cycle (lights on 7:00 A.M) with standard diet and water available *ad libitum*. All procedures were performed in accordance with the ARRIVE guidelines and with the guidelines and protocols approved by the European Community (2010/63UE L276 20/10/2010). Experimental protocols were approved by the Italian Ministry of Health (authorization N. 766/2020-PR).

At 3 months of age, rats were deeply anesthetized with Fentanyl (0.3 mg/kg) and medetomidine hydrochloride (0.35 mg/kg) and stereotaxically injected according to previous studies[168]. 5 µL of H-αSynOs were infused bilaterally into the SNpc (n=40) (coordinates relative to bregma; −5.4 mm anteroposterior; ± 1.9 mm from the midline; −7.2 mm beneath the dura, according to Paxinos' and Watson's atlas[371],) at the rate of 1 µL/min via a silica microinjector as previously described[168]. The injector was left in place for additional 5 min after infusion, and then slowly withdrawn. Control animals (Veh, n=22) received an equal volume of PBS (Fig. 6).

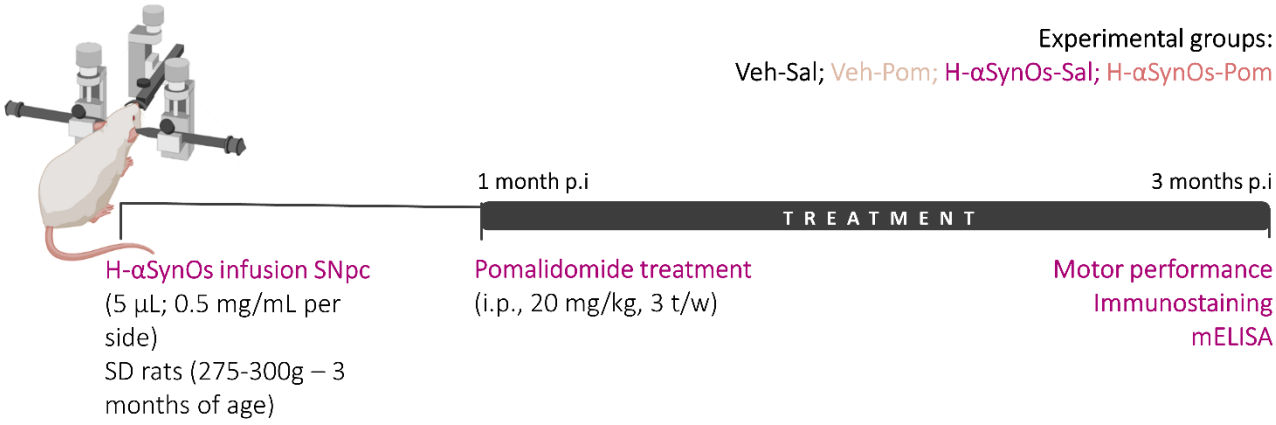


Figure 6. Experimental timeline.

Drugs

In order to increase its bioavailability, Pom was nanosuspended (Pom-NS) in an aqueous solution of Tween 80 (0.75%) using the Wet media milling technique as previously described[372]. One-month post-infusion Veh- and H-αSynOs-infused rats were chronically treated with Pom-NS (20 mg/kg; i.p.) or saline on alternate days 3 days/week for 2 months and then sacrificed 24 h after the last injection.

The experimental groups were as follows: (i) vehicle + saline (Veh-Sal); (ii) H-αSynOs + saline (H-αSynOs-Sal); (iii) vehicle + Pom-NS (Veh-Pom); (iv) H-αSynOs + Pom-NS (H-αSynOs-Pom).

Motor tests

A battery of motor tests was conducted over a 10-day interval, beginning 12 weeks after surgery. In order to avoid any alteration in behavioural parameters induced by the novel environment, animals were allowed to acclimate to the testing room for 30 min prior to each test. All tests and outcome measures were performed and analyzed in a blinded fashion.

Challenging beam walk test

Three-months post-surgery rats were tested for coordination and balance by using the challenging beam walk test as previously described[168], using a protocol adapted from Drucker-Colín and García-Hernández[373] and Korecka et al.[374]. The testing apparatus consisted of a 2 m wooden beam placed between a starting platform, elevated 40 cm from the floor, and the home cage, with a slope of 15°. Three different beam widths were used: 15, 10, and 5 mm. All rats were trained for three days to walk along the three different beams, while on the test day they were videotaped. Briefly, each rat was placed at the lower end of the beam and the number of stepping errors was counted while traversing the beam to reach the home cage. The same procedure was repeated for the three different beam widths[168]. When the animal was not able to complete the task in the established time-frame (120s) or it fell off the beam, the error score resulting from the stepping errors was increased by adding a numerical increment based on the following criteria: (i) 0.25 increment, when the animal completed 75% of the beam; (ii) 0.5 increment, when the animal completed 50% of the beam; (iii) 0.75 when it only completed 25% of the beam.

Vermicelli handling test

The vermicelli handling test was used on order to measure the dexterous forepaw function[375,376]. In order to avoid any neophobic responses to the new food, all rats were exposed to the pasta pieces several times one week prior to testing. On the test day, each rat was placed in a squared arena with dark walls, with a mirror set below it in order to make visible rats' forepaws, and exposed to three 7cm uncooked vermicelli strands. Each trial was videotaped for later analysis. The primary outcome measures for this test were as follows: (a) number of forepaw adjustments per trial, defined as any distinct removal and replacement of the paw on the pasta piece; (b) frequency of atypical handling patterns. These atypical behaviours included: (1) paws together when long—paws placed symmetrically when the piece of pasta was 3.5 cm or greater in length; (2) guide and grasp switch—

the roles of the guide limb and grasp limb are switched during eating; (3) failure to contact—the paw does not contact the pasta piece during eating; (4) drop—the pasta piece is dropped after eating is initiated; (5) paws apart when short—paws placed asymmetrically when the pasta piece is short[375]. These measures were scored 1 if exhibited and 0 when not, and their frequency was then summed. Trials were declared invalid when the rat break the pasta piece during eating or if less than 90% of the recorded eating session showed a clear view of the paws/digits[376,377].

Gait test

A gait test was run on an apparatus consisting of an arena placed on a base with a transparent floor and a mirror mounted inside. The arena was 15 cm wide, 25 cm tall, and 148 cm long. The base was a wooden frame (24 × 24 × 160 cm) provided with a plexiglass top, which served as a floor for the overlying arena. A mirror was housed inside and set 45° below the arena. Once placed in the arena, each rat was allowed to explore voluntarily the arena at self-selected velocities. Three runs across the arena were videotaped at 120 fps. For subsequent analysis the time spent and the number of steps made to cross the arena were measured for each rat. Moreover, strides were analyzed by measuring the distance between paw placements for every step, on a stack of calibrated frames on IMAGE J (https:// imagej. nih. gov/ ij/). For each, subject data are expressed as averaged velocity (cm/s), steps per time unit (n/s), and averaged stride length (cm).

Immunohistochemistry and immunofluorescence

After the motor behaviour assessment and within 24 h of their last injection, rats were deeply anesthetized and transcardially perfused with ice-cold 0.1 M PBS (pH 7.4) followed by 4% buffered paraformaldehyde. After perfusion, the brain was carefully removed from the skull, post-fixed overnight in 4% paraformaldehyde-PBS and then stored in 0.1% NaN3-PBS at 4 °C. 40-µm-thick serial coronal sections of midbrain were then cut using a vibratome as previously described[378].

For stereological quantification of TH^+ cells, midbrain sections were pre-incubated in normal donkey serum (NDS) and then immunoreacted with polyclonal rabbit anti-TH (1:1000, Millipore, Burlington, MA, USA) primary antibody. The reaction was amplified using a biotinylated secondary antibody and visualized by the classic avidin-peroxidase complex (ABC, Vector, UK) protocol, using 3,30-diaminobenzidine (Sigma- Aldrich, St. Louis, MO, USA) as a chromogen. Sections were then counterstained with Cresyl violet. For immunofluorescence, midbrain sections were preincubated with a blocking solution with normal serum/BSA and then immunoreacted with the following unconjugated primary antibodies for double immunolabeling: goat polyclonal anti IBA-1 (1:1000;

Novus Biologicals, Littleton, CO, USA); rabbit polyclonal anti TNF-α (1:500, Novus Biologicals, Littleton, CO, USA); rabbit polyclonal anti IL-10 (1:200, Abbiotec, Escondido, CA, USA); mouse monoclonal anti CD3 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA). For fluorescence visualization of IBA-1 and CD3 a two-step indirect labelling protocol was used, while a three-step detection was performed to increase the signal of TNF-α and IL-10 as previously described[168]. Images were acquired using a spinning disk confocal microscope (Crisel Instruments, Rome, Italy) with a × 63 magnification.

Stereological counting of TH immunoreactivity

TH-immunoreactive (IR) neurons or Nissl-stained cells were counted bilaterally in the SNpc, as previously described[379]. Counting was made by using a dedicated software (Stereologer, System Planning and Analysis, Inc., Alexandria, VA, USA), linked to a motorized stage on a BX-60 Olympus light microscope (Olympus, Segrate, Italy). The total number of TH-stained cells was estimated by means of the optical fractionator method, which combines the optical dissector with the fractionator sampling scheme, giving a direct estimation of the number of 3-D objects unbiased by shape, size, and orientation[380]. A systematic random sampling of cells within the area of interest was achieved by "Stereologer" software. Equidistant counting frames (frame area = 50 μm2) 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.

Microscopy analysis

Qualitative and quantitative analyses for inflammatory markers (IBA-1, TNF-α, IL-10, and CD3) were performed using a spinning disk confocal microscope (Crisel Instruments, Rome, Italy) with a ×63 magnification. 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 7.3).

To determine the total volume occupied by IBA-1$^+$ cells, a stack was obtained from each dataset (40 images). In the resulting stacks, 10 regions of interest for the SNpc (x = 700 μm; y = 700 μm; z = 40 μm) in each acquired section and for each animal were randomly chosen, and the volume of the elements calculated. For colocalization analysis, a colocalization channel was automatically generated by Imaris 7.3. In the resulting stacks each IBA-1+ cell was identified and selected, and the volume of the colocalized cytokine (TNF-α and IL-10) was calculated. Mean colocalization values

obtained from cells analyzed in each animal from each experimental group (n = 6) were plotted as a frequency distribution displaying the percentage of colocalization between the selected cytokine signal and the selected IBA-1$^+$ cell. Histogram inspection indicated a different cytokine expression across experimental groups in a cell subpopulation, whereas a large population of cells maintained colocalization values similar to the vehicle (Fig. n). For this reason, a deconvolution analysis was applied to the histograms in order to unmask subpopulations of cells affected by H-αSynOs infusion and/or Pom treatment. Based on the deconvolution results, an appropriate cut-off value was set in order to categorize the identified cell populations into high and low expressing cells; mean values within each class were calculated for each experimental group and then statistically compared.

Serum cytokine and chemokines analysis by Multiplex ELISA

Serum samples were assayed using the Cytokine & Chemokine 22-Plex Rat ProcartaPlexTM Panel (EPX220-30,122-901, Thermo Fisher Scientific, Vienna, Austria), according to the manufacturer's instructions. The concentrations of cytokines and chemokines were detected with the Luminex MAGPIX instrument (Luminex Corporation, Austin, TX) and data were analyzed with xPONENT® software (Luminex Corporation, Austin, TX). Any analyte with a concentration outside the linear range was excluded from the analysis.

Statistical analysis

Outcome measures were evaluated by observers blinded to experimental conditions. Results are presented as mean ± SEM, using Statistica 8 (Stat Soft Inc., Tulsa, OK, USA). Behavioral data were analyzed by two-way analysis of variance (ANOVA) with intranigral infusion and pharmacological treatment as factors, followed by Tukey's post hoc test, or by t-test where appropriate. The results from the stereological analysis were statistically analyzed with a two-way analysis of variance (ANOVA) followed by Tukey's post hoc test, whereas the dataset of IBA-1 IR in the SNpc was analyzed by a Kruskall-Wallis non-parametric test followed by Dunn's post hoc test. For the colocalization analysis, the effect of drug treatments on the identified subpopulation was determined by one-way ANOVA followed by Fisher's post hoc test for comparison between individual groups. Levels of serum cytokines and chemokines were statistically compared among the experimental groups by one-way ANOVA followed by Fisher's post hoc test. For all the analyses, the level of significance was set at $p < 0.05$.

Results

Pomalidomide rescued from the sensorimotor impairment induced by H-αSynOs infusion

A battery of motor test was performed 3 months after H-αSynOs infusion, in order to test the efficacy of Pom against sensorimotor impairment, and to verify the functional outcome of Pom-induced neuroprotection. In line with our previous results[168], three months after H-αSynOs infusion rats developed a significant motor impairment in the challenging beam walk test, expressed as the number of errors per step on the 10mm and 5mm beams (Fig. 7a) compared with control rats (Veh-Sal group). Moreover, H-αSynOs-infused animals displayed abnormalities in the gait test in terms of decrease speed, steps/second and stride length (Fig. 7b-d). Strikingly, chronic treatment with Pom, initiated one-month post-infusion of H-αSynOs, fully mitigated these sensorimotor abnormalities, as shown in Fig. 7a-d. Specifically, in the challenging beam walk test Pom-treated rats committed a similar number of stepping errors as the control group, which was significantly lower than errors recorded for H-αSynOs-infused rats for the 10 and 5 mm width beams (Fig. 7a). Likewise, in the gait test, Pom treatment significantly restored speed, number of steps/second and stride length to control values, as compared to H-αSynOs-infused rats (Fig. 7b-d). As shown in Fig. 7e, f, when tested in the vermicelli handling test H-αSynOs-infused rats showed fewer normal adjustments and an increased number of abnormal eating behaviors, as compared with the control PBS-infused group. Again, Pom treatment significantly increased the number of total adjustments per trial (H-αSynOs-Pom group) while decreased the frequency of abnormal eating behaviour as compared to H-αSynOs-infused group, restoring the control values (Fig. 7e, f). All together motor tests indicated that the treatment with Pom restored a physiological sensorimotor function.

Pomalidomide induced a neuroprotective effect against the nigral dopaminergic degeneration induced by H-αSynOs infusion

Three months post-surgery, H-αSynOs-infused rats displayed a 40–45% reduction in both density and total number of TH^+ cells in the SNpc in comparison with control rats (Fig. 8a-c). Remarkably, the treatment with Pom, initiated one-month post-infusion of H-αSynOs, significantly arrested the DAergic cell death induced by the H-αSynOs, as shown by the similar number of TH^+ cells in H-αSynOs-Pom rats as compared to Veh-Sal rats (Fig. 8b, c). Thereafter, we counted stereologically the Nissl counterstained neurons to confirm that the TH content reflected the number of surviving cells rather than a decline/increase in enzyme levels. Both in term of density and cell number, the results were

superimposable with those obtained by the TH analysis, demonstrating that Pom reduced H-αSynOs-induced cell loss in the SNpc (Fig. 8d, e).

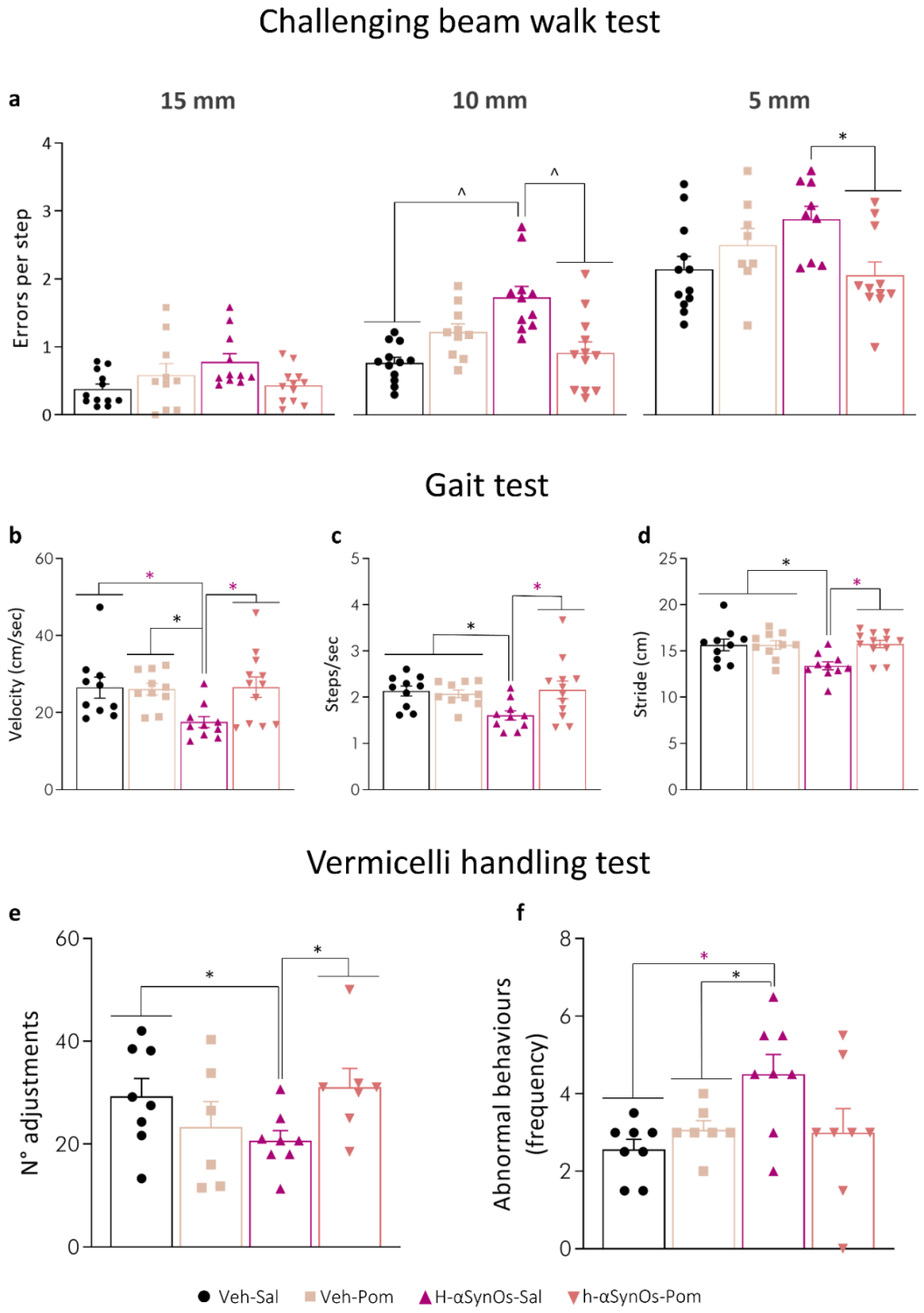


Figure 7. Chronic treatment with Pom mitigates H-αSynOs infusion-induced deficits in sensorimotor function and fine motor movement execution. Challenging beam walk test (**a**) and gait test (**b-d**) were used to asses sensorimotor deficits. Values represent the mean ± SEM (two-way ANOVA and Tukey's post hoc test). ^$p < 0.001$; *$p < 0.01$; *$p < 0.05$. Fine motor movements were evaluated by the vermicelli handling test. Bar charts show the number of normal adjustments (**e**) and the frequency of abnormal behaviors (**f**) made during the eating time. Values represent the mean ± SEM. *$p < 0.05$ vs Veh-Sal and H-αSynOs-Pom (e); *$p < 0.01$ vs Veh-Sal (f) by Unpaired Student's t test.

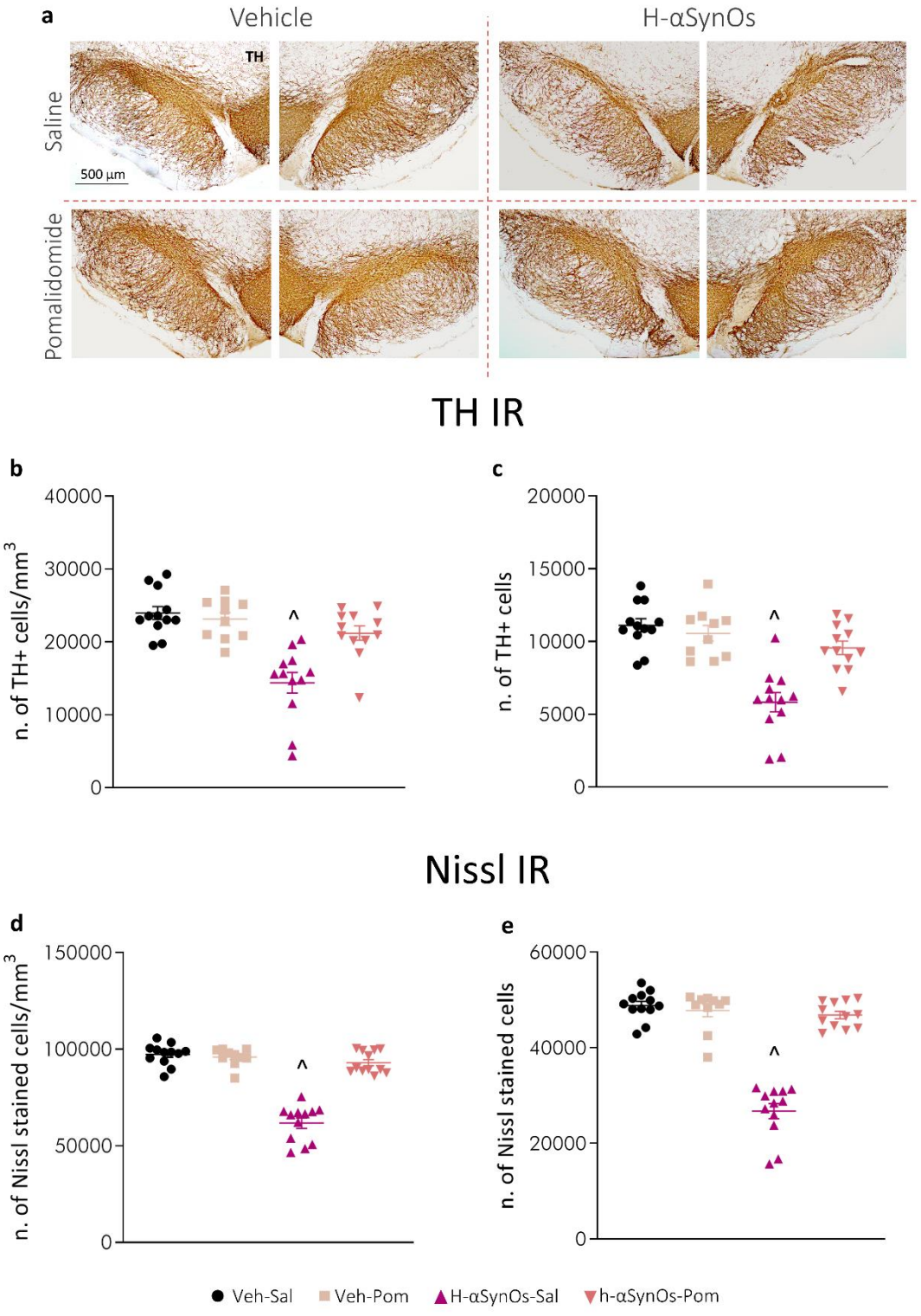


Figure 8. H-αSynOs induced a progressive nigrostriatal degeneration, which is rescued by Pom treatment. (a) Representative images of TH-stained SNpc sections (magnification 5×; scale bar: 500 µm). (b) Density (expressed as number of TH^+cells/mm3) and (c) number of TH^+ cells measured by stereological counting 3 months after the H-αSynOs or vehicle infusion and after 2 months treatment with Pom or Saline. (d-e) Stereological quantification of Nissl-stained cells in the SNpc. Values represent the mean ± SEM. ^$p < 0.001$ vs all other groups, by two-way ANOVA followed by Tukey's post hoc test.

Pomalidomide Effect on H-αSynOs-Induced Microgliosis in the SNpc

Microglial reactivity within the SNpc was evaluated by using IBA-1 as a marker of reactive microglia; results are expressed as the total volume occupied by IBA-1$^+$ cells. While the infusion of H-αSynOs induced a significant microgliosis, as shown in Fig. 9a, b, the treatment with Pom did not abolish such effect, suggesting that this compound did not target this neuroinflammatory aspect.

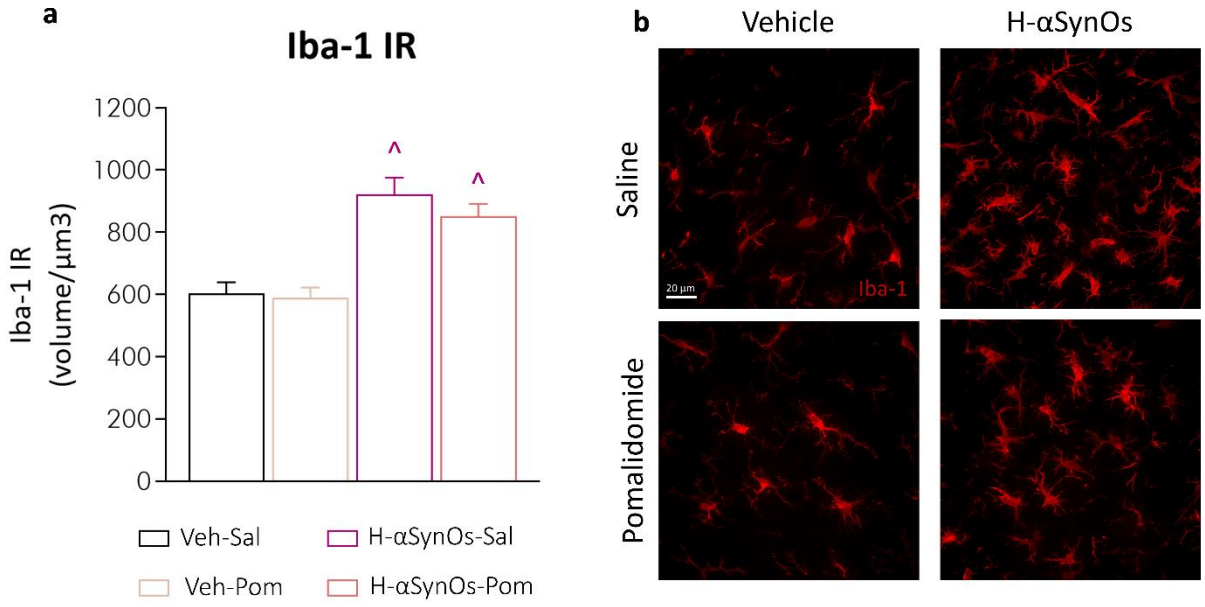


Figure 9. Microgliosis after H-αSynOs infusion. (a) Bar chart showing the total volume occupied by IBA-1$^+$ cells in the SNpc. **(b)** Representative images of IBA-1$^+$ cells (red, magnification 63X; scale bar: 20 µm). Values represent the mean ± SEM. ^$p < 0.0001$ vs. Veh-Sal and Veh-Pom, by Kruskall-Wallis and Dunn's post hoc test.

Functional characterization of IBA-1$^+$ population after Pomalidomide treatment

Functional phenotype of IBA-1$^+$ cells was further evaluated by means of cytokine assessment. Specifically, a colocalization analysis was carried out for the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10 within microglial cells. The dataset followed a non-normal distribution, and the analysis revealed a high variability in the levels of both cytokines in each experimental group, likely reflecting the high functional dynamicity of microglial cells. Therefore, to highlight this aspect and to investigate how the H-αSynOs challenge and Pom treatment affected the heterogeneity of cytokine content in microglia, data were expressed as a frequency distribution of cytokine volume within microglial cells (Fig. 10a, b). While the histogram of the Veh-Sal group was characterized by a single peak/population that expressed low level of TNF-α, the histogram of the H-αSynOs-Sal group showed two peaks, produced by the low TNF-α expressing cells and a new—high TNF-α expressing population. This second population was completely absent in the H-αSynOs-Pom group (Fig. 10a, a_1, a_2, a_3). Microglia were further characterized by assessing the content of the anti-inflammatory cytokine IL-10 across experimental groups (Fig. 10b, b_1, b_2, b_3). As shown in Fig. 10b,

histograms of all experimental groups were characterized by a large peak of cells showing low IL-10 labelling. However, the H-αSynOs-Sal histogram displayed a slight left-shift, which indicates adecrease in cytokine content. Moreover, the H-αSynOs-Pom histogram showed an additional small peak associated with higher IL-10 volumes.

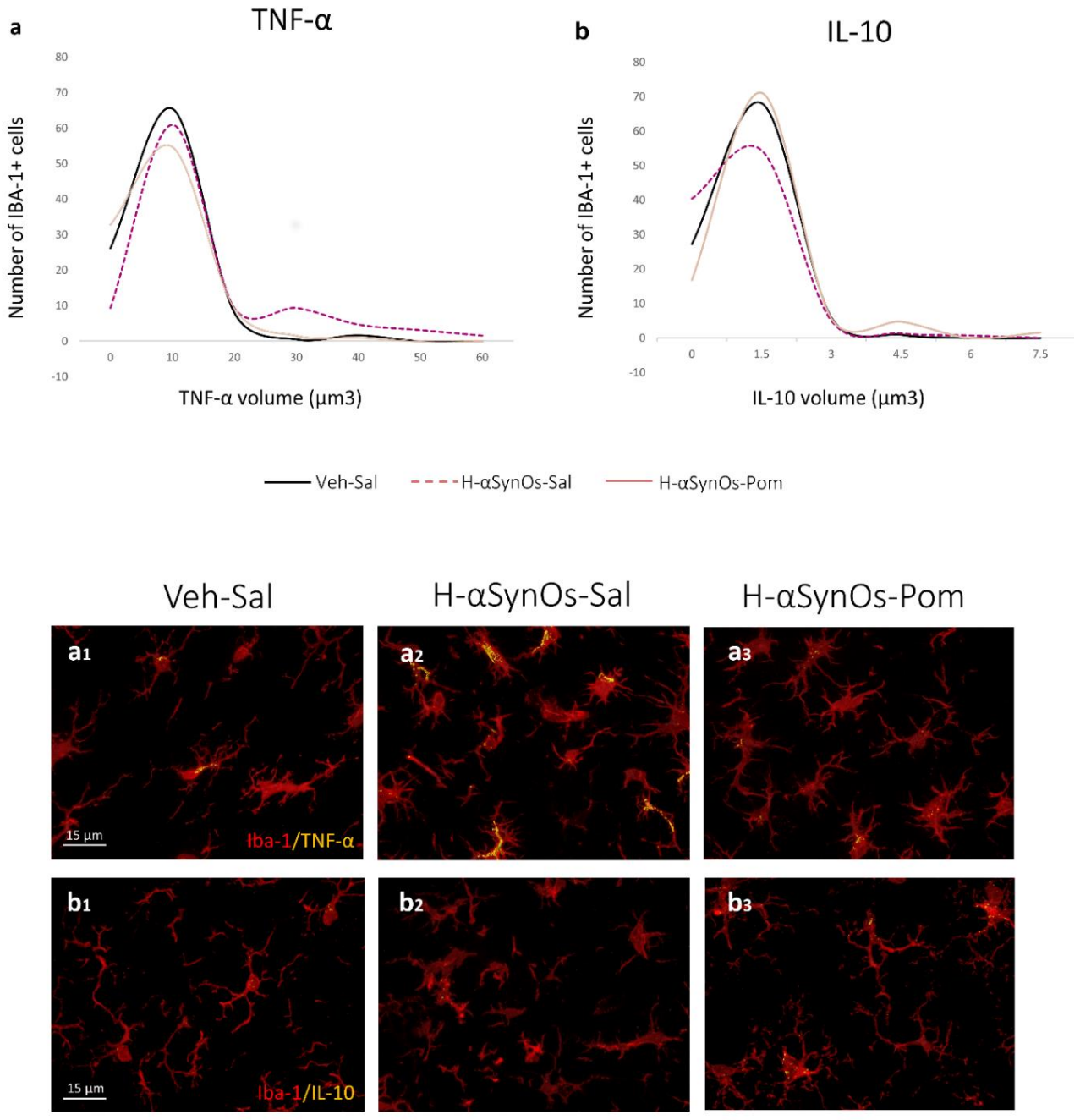


Figure 10. Pom treatment reverses the imbalance in microglial cytokine expression within the SNpc of H-αSynOs-infused rats. Frequency distribution of TNF-α **(a)** and IL-10 **(b)** colocalization within Iba-1 IR cells. Representative images of colocalized TNF-α (yellow) (a1–a3) and IL-10 (b1–b3) (yellow) within Iba-1^{+} cells (red). Magnification 63 ×, scale bar: 15 µm.

Since two different populations of cells for each cytokine – named as low and high TNF-α/IL-10 labelled –emerged from the deconvolution analysis, an appropriate cut off value was set in order to separate and statistically compare them (Fig. 11a, b and corresponding images a1-a2 and b1-b2). As shown in Fig. 11a, a significant difference was found among groups in the TNF-α content, both in the low and in the high labelled cells. Specifically, H-αSynOs-Sal rats showed a significant decrease of low labelled cells and an increase of highly labelled cells, as compared to control rats, suggesting that a subpopulation of microglia produced a supraphysiological amount of TNF-α (Fig. 11a, a_1, a_2). In contrast, H-αSynOs-Pom-treated rats displayed similar labelling to the control group for both cell populations, thereby indicating that Pom treatment fully counteracted the H-αSynOs-induced increase above physiological values of the proinflammatory cytokine (Fig. 11a, a_1, a_2). A similar analysis was conducted for IL-10. While we did not appreciate any difference among highly labelled cells across experimental groups, in the low labelled population Pom significantly increased the IL-10 content in a subpopulation of microglia above control values (Fig. 11b, b_1, b_2). Importantly, the H-SynOs infusion caused a dysregulated ratio toward the pro-inflammatory phenotype by comparing the percentage of pro- versus anti-inflammatory microglia across the total population under study. In contrast, Pom treatment reversed such imbalance, by inhibiting TNF-α and boosting IL-10 production (Fig. 11c). Despite not eliminating microgliosis and microglia reactivity, our data indicate that Pom changed the microglia phenotype in an allostatic way to re-establish an appropriate pro-/anti-inflammatory balance.

Pomalidomide attenuated systemic inflammation triggered by H-αSynOs

The analysis of serum cytokines and chemokines revealed a severe dysregulation in their expression pattern following H-αSynOs infusion (Figs. 12 and 13). Serum levels of several pro-inflammatory markers, including the cytokines IL-1, IL-5, IL-6, IL-17, and GCSF and the chemokines RANTES, eotaxin, MCP3, CXCL 1/2, and MCP1, were increased three months after the intracerebral administration of H-αSynOs (Fig. 12 and 13). In contrast, serum levels of the anti-inflammatory cytokine IL-10 as well as of IL-2 were decreased. Interestingly, serum TNF-α levels were not significantly affected by the infusion of H-αSynOs (data not shown), suggesting a different regulation of this cytokine at the central and peripheral level. Moreover, we observed the presence of $CD3^+$ infiltrating lymphocytes within the nigral parenchyma of H-αSynOs-infused animals (Fig. 14). Notably, Pom reversed most of the αSynOs-induced changes in the serum, by restoring the physiological levels of several inflammatory and anti-inflammatory cytokines and chemokines, in line with the immunomodulatory activity of this drug (Figs. 12 and 13). Accordingly, CD3 immunostaining was very low in Pom-treated rats (Fig. 14).

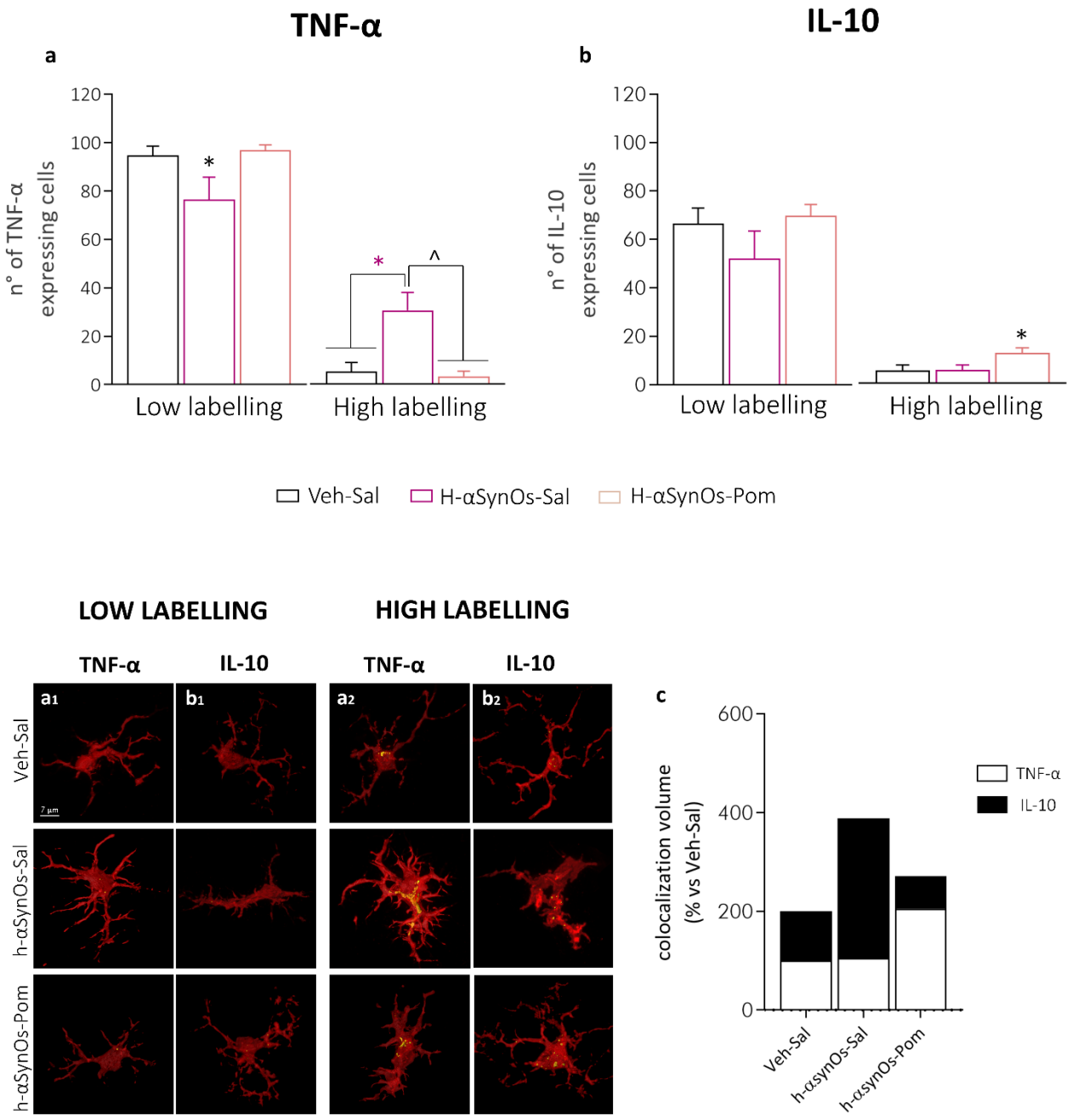


Figure 11. H-αSynOs infusion altered microglial functional phenotype. According to the expression levels of (**a**) TNF-α and (**b**) IL-10, Iba-1$^+$ cells were categorized into two different subpopulations, referred to as low labelled and high labelled. Values are the mean ± SEM (one-way ANOVA followed by Tukey's post hoc test). *$p < 0.05$; *$p < 0.01$; ^$p < 0.001$. Representative images of Iba-1$^+$ cells (red) expressing low or high TNF-α (**a1-a2**, yellow) and IL-10 (**b1-b2**, yellow). Magnification 63 ×, scale bar: 7 µm. (**c**) Bar plot showing the relative percentage of each cytokine among the experimental groups.

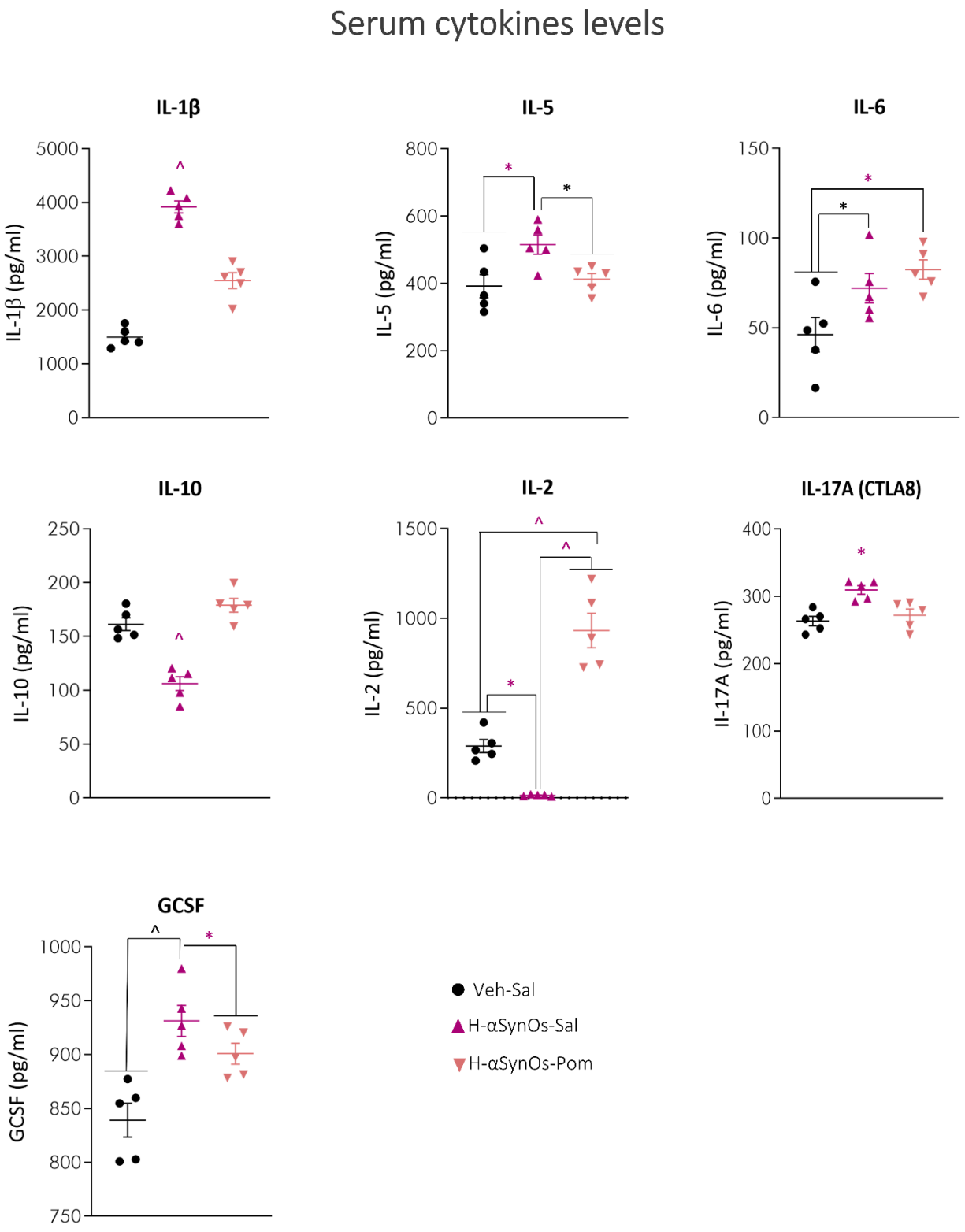


Figure 12. Levels of serum cytokines after H-αSynOs infusion and Pom treatment. Serum cytokines were analyzed by multiplex ELISA. Values represent the mean ± SEM. ^p < 0.0001; ^p < 0.001; *p < 0.01; *p < 0.05, by one-way ANOVA and Fisher's post hoc test.

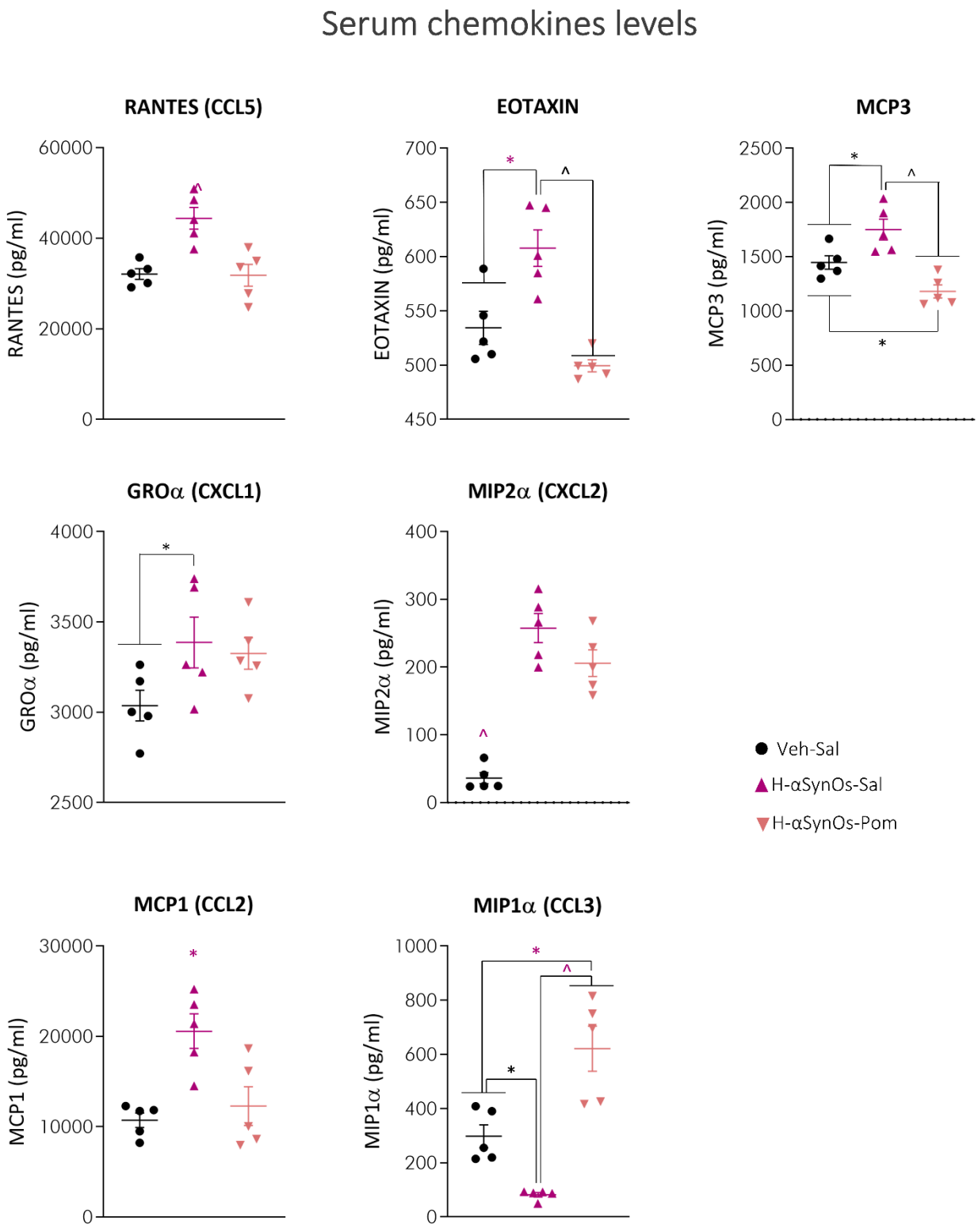


Figure 13. Levels of serum chemokines after H-αSynOs infusion and Pom treatment. Serum chemokines were analyzed by multiplex ELISA. Values are the mean ± SEM. ^p < 0.0001; ^p < 0.001; *p < 0.01; *p < 0.05, by one-way ANOVA followed by Fisher's post hoc test.

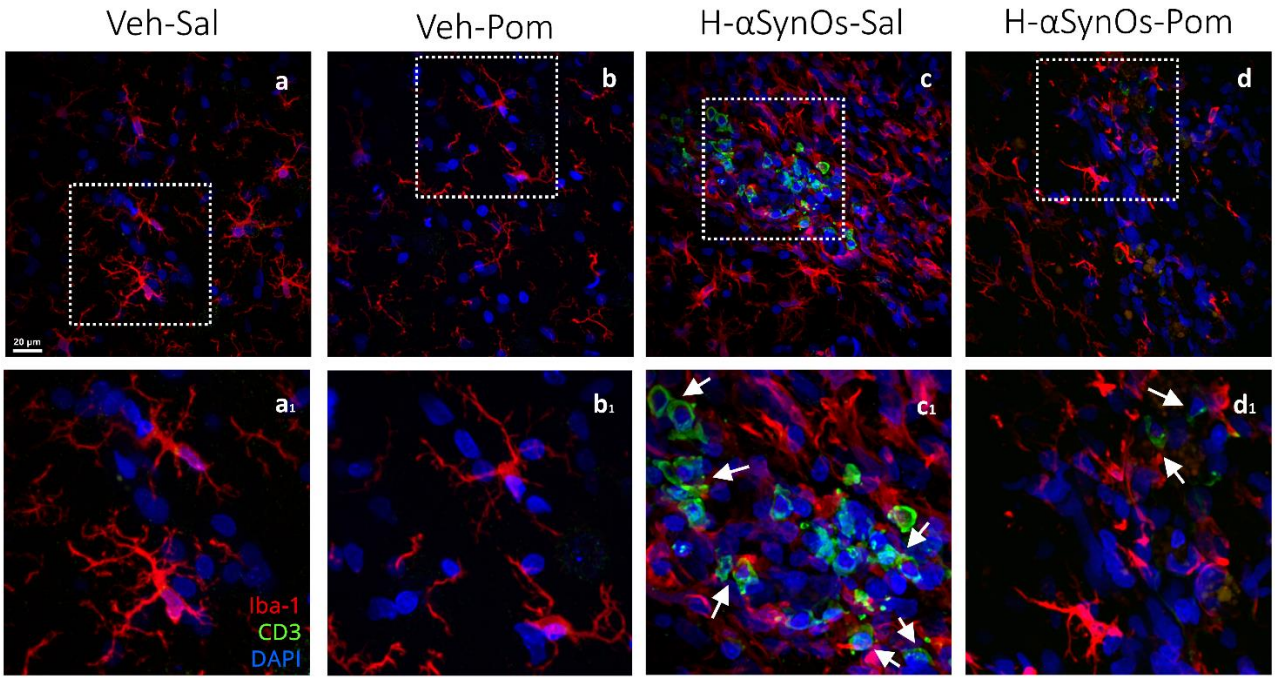


Figure 14. Infiltrated T cells within the nigral parenchyma three months after H-αSynOs infusion. Representative pictures of the SNpc showing CD3+ T cell infiltration after H-αSynOs infusion and after Pom treatment. Double immunostaining was performed using anti-CD3 (green) and anti-IBA-1 (red) antibodies and DAPI staining (blue, nuclei). Magnification 63 ×, scale bar: 20 µm (**a–d**) and 10 µm (**a1–d1**).

STUDY 2

Intranigral infusion of H-αSynOs as a tool for studying cognitive deficits in rodent

Cognitive dysfunctions represent one of the most disabling non-motor symptoms of Parkinson's disease (PD), though, so far, its pathological correlates still remain elusive, mainly for the lack of a valid preclinical neuropathological model that reproduces both motor and non-motor aspects of the disease[4]. Several clinical studies reported the presence of a number of inflammatory markers in parkinsonian patients' brains, pointing at the neuroinflammation as a contributing factor in the development of cognitive decline[175]. Here, we show that the bilateral intracerebral infusion of pre-formed H-αSynOs within the SNpc offers a valid model for studying this aspect of the pathology.

Materials and methods

Production of recombinant H-αSyn and purification of H-αSynOs

For the production and purification of H-αSynOs, the procedures fully described in the material and methods section in Study 1 were used.

Animals and stereotaxic surgery

12-weeks old male Sprague Dawley rats (275-300 g), purchased from Envigo (Envigo, Italy) were housed in groups of four in polypropylene cages, with food and water available *ad libitum* and maintained at 21°C under a 12h light/dark cycle (lights on 7:00 A.M.). All the procedures hereafter described were conducted in compliance with the ARRIVE guidelines and with the European Community-approved protocols and standards (2010/63UE L 276 20/10/2010). Experimental protocols were approved by the Italian Ministry of Health (authorization n° 766/2020-PR). All efforts were made to minimize animal pain and discomfort and to reduce the number of experimental animals used.

Once deeply anesthetized with Fentanyl (0.33mg/kg, i.p.) and medetomidine hydrochloride (0.33 mg/kg, i.p.), 34 rats were stereotaxically injected with 5 µL of H-αSynOs into the substantia SNpc (coordinates relative to bregma; -5,4 mm anteroposterior; ±1.9 mm from the midline; -7,2 mm beneath the dura) bilaterally at the rate of 1 µl/min via a silica microinjector and according to the atlas of Paxinos and Watson[371], as previously described[168,199]. Control animals received an equal volume of sterile phosphate buffer saline (PBS), pH 7.4 at the same in-fusion rate.

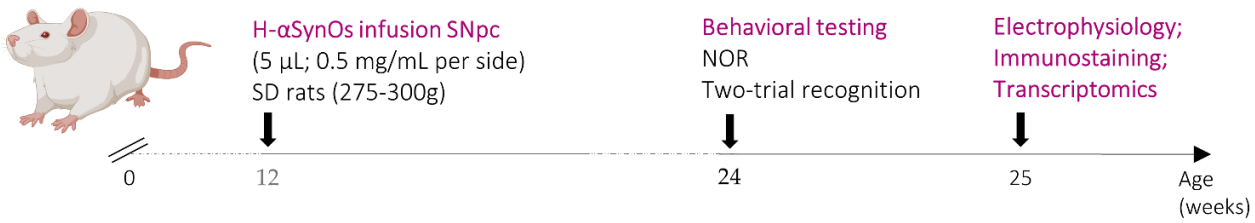


Figure 15. Experimental workflow. SD rats were infused intranigrally with H-αSynOs or PBS at three months of age. After three months post-infusion, one group of rats underwent behavioral testing, followed by immunostaining and transcriptomic analysis. A separate group of rats was used for electrophysiology experiments. BioRender.com was used to compose this figure.

Behavioral testing

Three-months after stereotaxic surgery, animals were tested for short-term memory over a 7-day interval. Prior to each test, rats were acclimated to the testing room for at least 30 minutes, as to avoid alteration of behavioral parameters induced by the novel environment. All tests were carried out during the light phase of the light/dark cycle, thus performed and analyzed by operators blinded to the experimental conditions.

Two-trial recognition task in a Y maze

H-αSynOs-induced alterations of short-term spatial memory were assessed by the two-trial recognition test. Test was carried out in a Y-shaped maze with three identical and symmetrical arms, angled 120° from each other. The three arms of the maze were randomly designated as: “start arm”, in which the rat always started to explore the maze, “novel arm”, and “other arm”. Testing was performed by individually placing each rat in the “start arm” of the maze and involved two trials, separated by a 1h interval[381]. The novel arm was blocked by a guillotine door during trial 1, and left open during trial 2, while the start and the other arms were left open during both trial 1 and 2. To avoid animal discomfort, test was carried out in a quiet room with dim illumination. Moreover, several visual cues were placed outside the walls of the maze, in order to allow maze navigation by rats.

During Trial 1 (10 min), each rat was left free to explore only the “start” and the “other” arm, with access to the “novel” arm being blocked. During Trial 2 (5 min), each rat was left free to explore all the three arms of the maze (“novel”, “start”, “other”). Rats’ performance was videotaped and later analyzed to score the frequency of entrance across the three arms and the amount of time (seconds) spent in each arm. Spatial recognition memory was assessed by evaluating the preference for the “novel” arm vs the combination of “start” and “other” arms, expressed as number of seconds spent and entries performed in the arms during the second trial [382].

Novel object recognition

Novel Object Recognition (NOR) test was performed to assess the short-term object recognition memory. As previously described[383], testing was performed in black boxes (60x60 cm) placed, as described for spatial memory test, in quiet and softly lit room. After the habituation session (10 min, T_0), rats were re-placed individually in the test box containing two identical objects for 10 min, before returning them into their home cage (familiarization phase, T_1). After 1 hour' interval, each rat was placed again for 5 min in the same test chamber, now containing one familiar and one novel object (choice phase, T_2). Training and test sessions were recorded with a camera. Object recognition was expressed by the discrimination index (DI) according to the following formula: (Tn-Tf)/(Tn+Tf) (Tn = time spent exploring the novel object; Tf = time spent exploring the familiar one).

Immunohistochemistry

At the end of the behavioural testing, rats were deeply anesthetized and transcardially perfused as extensively described in the previous section (page 35). After the post-fixation protocol, serial coronal sections of ACC, dorsal hippocampus and midbrain were vibratome-cut (40 µm thickness).

For p129-αSyn visualization, sections from ACC, hippocampus and midbrain were pre-incubated in NDS and then immunoreacted with rabbit monoclonal anti p129-αSyn (1:800, Abcam, Cambridge, UK) primary antibody. The reaction was then amplified using Biotin-SP Donkey Anti-Rabbit (1:500, Jackson Immunoresearch, West Grove, PA, USA) and then visualized by the avidin-peroxidase complex (ABC, Vector, UK) protocol, with 3,30-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) as a chromogen. For immunofluorescence labelling, hippocampal and ACC sections were pre-incubated in a blocking solution and then immunoreacted with the following unconjugated primary antibodies for single or double immunolabeling: goat polyclonal anti IBA-1 (1:1000; Novus Biologicals, Littleton, Colorado, US); rabbit polyclonal anti TNF-α (1:500, Novus Biologicals, Littleton, CO, USA); mouse monoclonal anti GluR1 (1:200, Novus Biologicals, Littleton, Colorado, US). For fluorescence visualization of IBA-1 and GluR1 a two-step indirect labelling protocol was used, while a three-step detection was performed to increase the signal of TNF-α by combining biotin-SP-conjugated IgG (1:500, Jackson Immunoresearch, West Grove, PA, USA) and streptavidin–fluorescein (1:400, Jackson Immunoresearch, West Grove, PA, USA), as previously described[378]. Images were then acquired using a spinning disk confocal microscope (Crisel Instruments, Rome, Italy) with a ×63 magnification.

Cresyl violet staining

In order to obtain a qualitative measure relative to neuronal survival both in the hippocampus and ACC, a cresyl violet (CV) staining was performed. Briefly, once mounted, the brain sections were stained with a solution of 0.1% w/v CV-acetate (ThermoFisher Scientific, Waltham, Massachusetts, US) and then dehydrated in serial ethanol bath.

Microscopy analysis

Qualitative and quantitative analyses for IBA-1, TNF-α and GluR1 were performed using a spinning disk confocal microscope (Crisel Instruments, Rome, Italy) with a ×63 magnification. Surface rendering, colocalization, maximum intensity and simulated fluorescence process algorithms were used (ImageJ and Imaris 7.3). To determine the IBA-1 and GluR1 occupied volume, a stack was obtained from each dataset (40 images). In the resulting stacks, 10 regions of interest for the ACC, granular cell layer (GCL) and hilus of dentate gyrus (DG), CA3 pyramidal layer (x = 700 µm; y = 700 µm; z = 40 µm) and 5 regions of interest for the CA1 subfields (x = 1024 µm; y = 1024 µm; z = 40 µm) in each acquired section and for each animal were randomly chosen, and the volume of the elements calculated. For colocalization analysis, a colocalization channel was automatically generated by Imaris 7.3. Colocalized TNF-α was analyzed in the same sections and ACC/hippocampal regions were IBA-1 was analyzed (see above). A stack was obtained from each dataset (40 images). In the resulting stacks, each IBA-1$^+$ cell was identified and selected, and the volume of the colocalized TNF-α was calculated within each cell (µm^3).

In vivo single unit recordings from the ACC

Three months after the bilateral surgery in the SNpc, rats were deeply anesthetized and then placed in a stereotaxic apparatus. Single-unit activity of putative pyramidal neurons from the ACC (AP: +1.0-1.6 mm from bregma, L: 0.3–0.8 mm from the midline V: 1.5–4.0 mm from the cortical surface, according to Paxinos and Watson….) was recorded with glass micropipettes filled with 2% Pontamine sky blue dissolved in 0.5 M sodium acetate. Each action potential was isolated and then amplified (bandpass filter 10–5,000 Hz) by means of a Neurolog system (Digitimer, Hertfordshire, UK) or a CP511 AC Amplifier (Grass Instruments Co., US). Experiments were sampled with Spike2 software by a computer connected to CED1401 interface (Cambridge Electronic Design, Cambridge, UK). Cells were selected in accordance with electrophysiological characteristics attributed to pyramidal neurons[384–386] which present “regular-spiking” or “intrinsically bursting” activity of biphasic and >2 ms wide action potentials.

RNA isolation, library preparation and sequencing

Total RNA from both ACC and hippocampus was extracted by the PureLink® RNA Mini Kit (Ambion #12183018A) according to the manufacturer's instructions. Yield and purity were then evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA-seq libraries were prepared with an Illumina® Stranded mRNA Prep, Ligation Kit (Illumina #20040532) and IDT® for Illumina® RNA UD Indexes Set A, Ligation (Illumina #20040553). The RNA library concentration was measured using a Qubit® 2.0 fluorometer and success-fully sequenced on an Illumina NovaSeq6000 (Illumina Inc., San Diego, CA, USA).

RNAseq data analysis

RNAseq short reads quality have been evaluated by means of FASTQC (v0.11.9)[387] software. *Rattus norvegicus* reference genome (primary assembly, mRatBN7.2) has been downloaded from ENSEMBL web site (http://ftp.ensembl.org). Short reads have been aligned to the reference genome with STAR software (2.7.9a)[388]. Genes coordinates (Rattus_norvegicus.mRatBN7.2.105.gtf) have been downloaded from ENSEMBL web site (http://ftp.ensembl.org) and gene expression level has been evaluated with HTSeq software[389](0.11.3) with the following command line: htseq-count --stranded=reverse --mode=union --idattr=gene_id --type=exon. Potential latent confounders have been inferred with the svaseq software[390].

Only those genes with at least 5 reads present in minimum the 25% of the samples have been analyzed for differential expression (between treated and untreated) with the DESeq2 software[391] by using defaults settings. Differential expression was performed both incorporating and excluding the svaseq confounders in the DESeq2 model. FDR was computed with the Benjamini-Hochberg method.

Reverse transcription-quantitative PCR

Total RNA was extracted using PureLink® RNA Mini Kit as described above. The expression of specific mRNAs was assayed using fluorescence-based quantitative real-time PCR (RT-qPCR). RT-qPCR reactions were performed using Platinum SYBR® Green Master Mix (Cat # 11744-100 Invitrogen). Quantification reactions were performed in triplicate for each sample using the standard curve method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-Actin were chosen as reference (housekeeping) genes. The following primers were used for the genes indicated:

Gapdh: Fw 5' GGCTGCCTTCTCTTGTGACA 3' - Rev 5' TGAACTTGCCGTGGG-TAGAG 3'

β-Apdh: Fw 5' TCAACACCCCAGCCATGTAC 3' - Rev 5' TCCGGAG-TCCATCACAATGC 3'

Npas4: Fw 5' ATCAGTGACACGGAAGCCTG 3' - Rev 5' AGCTGGGGTTCCTAGGACAT 3'

Npas4: Fw 5' GATCGCCTTTTCCGTTGTCG 3'- Rev 5' CAGGTGGGTGAG-CATGGAAT 3'.

The target gene expression level was normalized to *Gapdh* and *β-actin* mRNA expression levels. At the end of the assay, a melting curve was constructed to evaluate the specificity of the reaction.

Statistical analysis

Outcome measures were evaluated by observers blinded to experimental conditions. Normal distribution was assessed by Kolmogorov-Smirnov test in all data sets and for all experimental designs. Where normality was respected, data were analyzed by two-way ANOVA followed by Tukey's post-hoc test and unpaired Student's t test. Where not, data were analyzed by the non-parametric Mann-Whitney test. Results are presented as mean ± SEM, using Statistica 8 (Stat Soft Inc., Tulsa, OK, USA). In each graph, y-axis error bars represent S.E.M. Level of significance was always set at $p < 0.05$.

Results

Three months post H-αSynOs infusion rats developed a mild cognitive impairment

The results from the two-trial recognition task in a Y maze revealed that while control PBS-infused rats displayed a clear preference for the novel as compared to the other arm, the H-αSynOs rats displayed a similar preference for the two arms. Two-way ANOVA for seconds spent in arms revealed a significant effect of arm ($F_{1,36}$=10.35; p=0.027), but neither an effect of infusion ($F_{1,36}$=1.75; p=0.19) nor an inter-action arm × infusion (F1,36=2.67; p=0.11) (Fig. 16a). Tukey's post-hoc test showed that rats infused with vehicle (n=10) spent significantly more time in the novel arm of the maze than in the remaining two arms (here expressed as the mean value between the time spent in the 'entry' arm and in the 'other' arm) (p<0.01). Conversely, rats infused with H-αSynOs (n=10) spent a similar amount of time in the arms, as revealed by lack of significant effect of arm ($F_{1,36}$=0.58; p=0.45) and infusion ($F_{1,36}$=0.09; p=0.76) and no significant interaction arm × infusion ($F_{1,36}$=0.24; p=0.63) (16b).

Results from the NOR test showed a significant lower discrimination index in H-αSynOs treated rats (n=10) compared to the Veh-infused rats (n=10) ($t_{(18)}$= 3.19; p=0.005 by Unpaired Student's t-test) in the test phase (16c). No differences were seen in the preference for location among the two groups during the training session (data not shown).

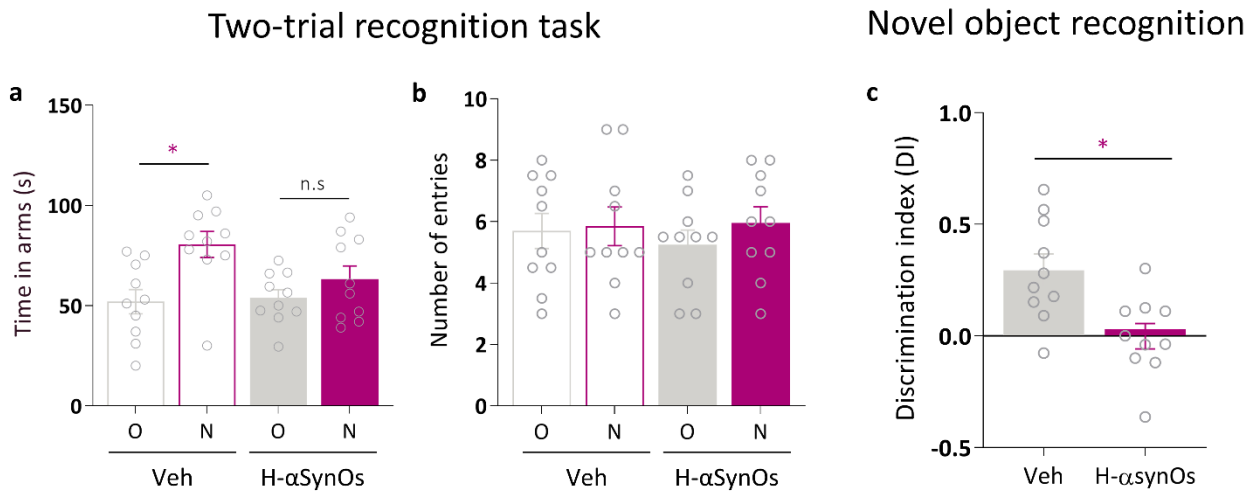


Figure 16. Intranigral H-αSynOs infusion impairs short term spatial reference memory and recognition memory. (a) Time spent (s) in the novel arm (N) vs the other arm (O) in the two-trial recognition test by Veh-infused vs H-αSynOs-infused rats (n=10; data are presented as the mean ± S.E.M; *p<0.01 by two-way ANOVA followed by Tuckey's post hoc test). (**b**) Number of entries in the N and O arms respectively (n=10; p>0.05 by two-way ANOVA and Tuckey's post hoc test). (**c**) Discrimination index measured by the novel object recognition test for Veh-infused and H-αSynOs-infused rats (n=10; *p<0.01 by Unpaired Student's t test).

Altered neuronal activity in the ACC of H-αSynOs-infused rats

Single-unit recording of putative pyramidal neurons within the ACC was performed in order to assess the effect of H-αsynOs infusion on neuronal function. As shown in Fig. 17b, the number of spontaneously active pyramidal neurons was significantly reduced in H-αSynOs-infused rats (n=6) as compared with Veh-infused animals (n=5) (p= 0.026 by Unpaired Student's t test). Additionally, we observed a reduction in the mean firing rate (17c; p= 0.024; Unpaired Student's t test) in H-αSynOs-infused rats ($n_{(treated)}$= 33) as compared with the control group ($n_{(sham)}$=39). Mean coefficient of variation among interspike intervals recorded from pyramidal neurons did not differ between experimental group (n=33-39; p>0.05 by Unpaired Student's t test) (data not shown).

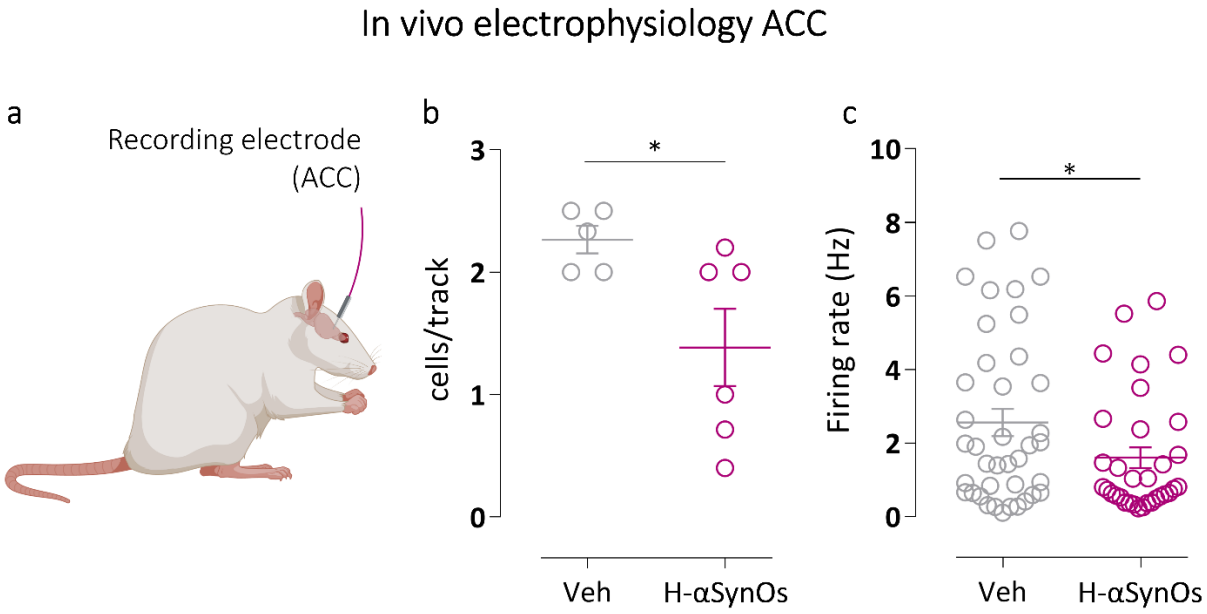


Figure 17. Electrophysiological recordings in the ACC of H-αsynOs and Veh-infused rats. (a) Schematic representation of *in vivo* recordings. (**b**) Mean number of spontaneously active cells recorded per track in the ACC. Single dots represent rat's mean (n=5-6; *p<0.05 by Unpaired Student's t test). (**c**) Mean firing frequency of pyramidal neurons. Dots represent single cells (n=33-39; *p<0.05 by Unpaired Student's t test).

Down-regulation of *Npas4* expression in limbic areas of H-αSynOs-infused rats

Bulk RNA sequencing was performed in dissected ACC and hippocampus to compare the transcriptional profile of H-αSynOs-infused rats (n=6) with those of Veh-infused animals (n=6). After correcting for potential latent confounders, 484 (ACC) and 321 (hippocampus) genes were identified as differentially expressed among the two experimental groups, with nominal p value<0.05[392]. Among differently expressed genes, we focused on the neuron-specific immediate-early gene (IEG) Npas4 (Neuronal PAS domain protein 4), whose expression was downregulated in both the ACC

(log2FoldChange=-7.52E-06; p<0.005) and hippocampus (log2FoldChange=-0.7692225; p<0.001) of H-αSynOs-infused rats as compared to the Veh group (data not shown). RT-qPCR analysis in the same tissues confirmed the H-αSynOs-induced inhibition of Npas4 mRNA expression in the ACC (^p=0.0005; Unpaired Student's t test) (Fig. 18b) and in the hippocampus (^p<0.0001; Unpaired Student's t test) (Fig. 18c), indicating that the memory impairment was sustained by an altered neuronal activity in cognition-related areas.

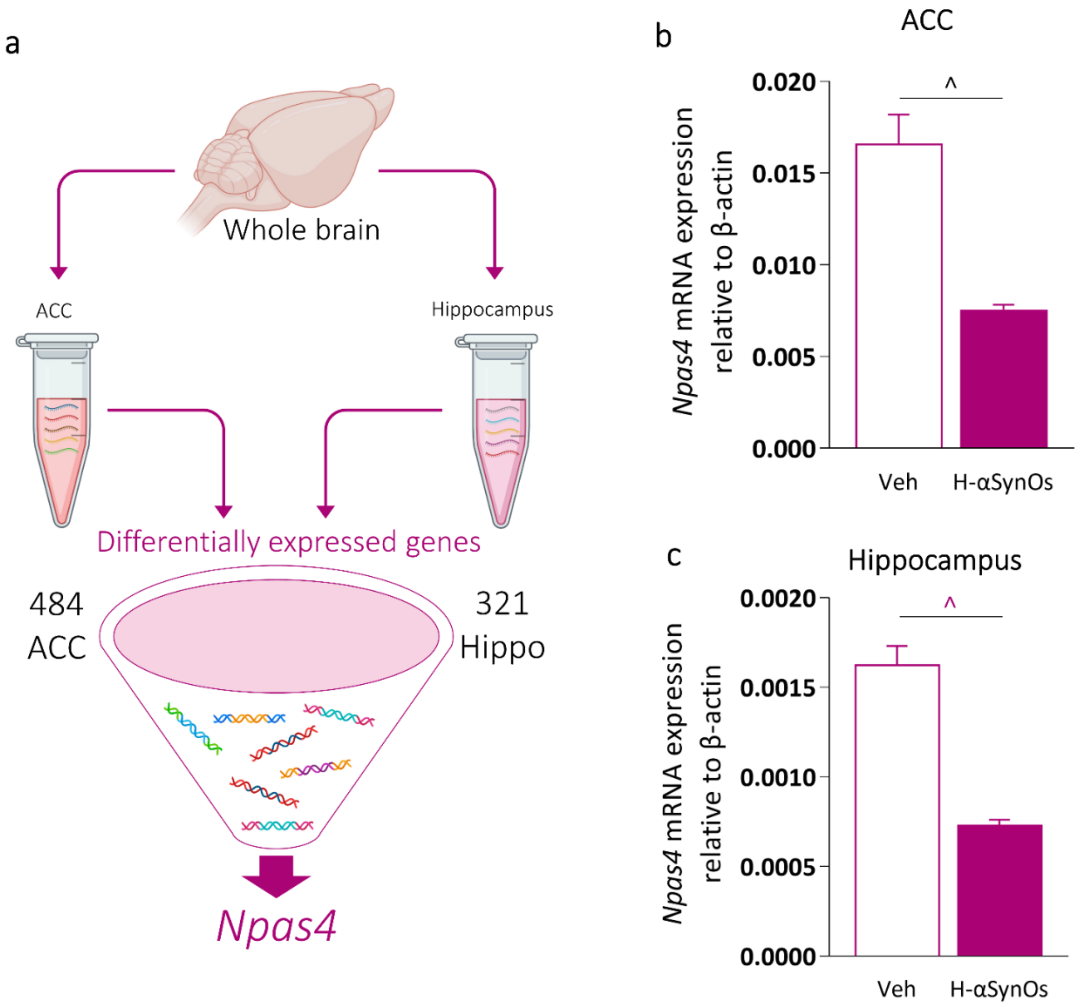


Figure 18. Altered expression of the IEG Npas4 in both ACC and hippocampus of H-αSynOs-infused rats. (a) Schematical workflow of bulk RNA sequencing. (**b**) *Npas4* mRNA expression levels measured by RT-qPCR, normalized to the housekeeping gene *β-actin* in the ACC (n=5; ^p<0.001 by Unpaired Student's t test) and (**c**) hippocampus (n=5; ^p<0.0001 by Unpaired Student's t test).

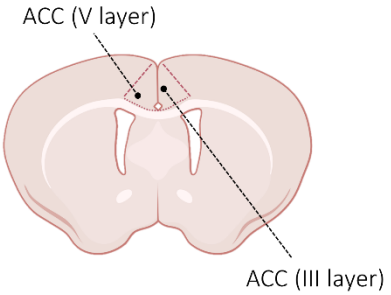
Immunofluorescence and immunohistochemical analyses

IBA-1 immunofluorescence

As a general indicator of microgliosis, IBA-1 IR – expressed as the total volume occupied by IBA-1$^+$ cells – was calculated in both the ACC and hippocampus. Due to the high morphological heterogeneity of microglial cells within the same neuroanatomical area, single subfields were analyzed. As shown in Table 1, no differences were observed in the ACC (both in the III and V layer) between H-αSynOs-infused rats and the Veh counterpart. However, in the dorsal hippocampus, the analysis revealed an increase in IBA-1$^+$ cells in the pyramidal layer of CA1 subfield (p=0.0023) and in the hilus of the DG (p= 0.0335) of H-αSynOs-infused rats (Table 1).

ACC (Iba-1 IR; volume/mm^3)		
	Veh	h-αSynOs
III layer	318.4 ± 23.02	357.1 ± 25.25
V layer	348.6 ± 24.80	380.2 ± 26.42

Dorsal hippocampus (Iba-1 IR; volume/mm^3)		
	Veh	h-αSynOs
DG (GCL)	335.2 ± 16.68	348.6 ± 22.24
DG (hilus)	320.8 ± 16.53	387.2 ± 20.30*
CA3 (stratum pyramidale)	280.8 ± 18.75	249.7 ± 16.52
CA1 (stratum pyramidale)	463.1 ± 28.13	600.5 ± 30.42*
CA1 (stratum radiatum)	564.2 ±28.49	644.5 ± 39.35
CA1 (stratum moleculare)	899.7 ± 40.77	843.6 ± 44.55



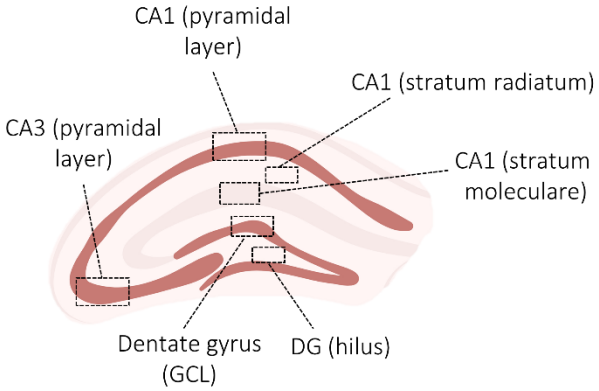


Table 1. Iba-1+ immunoreactivity in ACC and dorsal hippocampus following H-αSynOs intranigral infusion. Each reported value represents the total volume occupied by Iba-1$^+$ cells in the selected sub-regions. Values represent the mean ± SEM. *p<0.05; *p<0.01 by Mann-Whitney test.

TNF-α colocalization analysis

Colocalization analysis was performed for TNF-α within microglial cells in the same areas and subfields analyzed for IBA-1 IF. The analysis revealed a substantial increase in TNF-α levels within microglial cells of H-αSynOs-infused rats in both the ACC and the hippocampus. Specifically, the TNF-α increase was evident in both the cortical layers III and V (Fig. 19; $^{p_{(III\ layer)}}<0.0001$; $^{p_{(V\ 422\ layer)}}=0.005$ by Mann-Whitney non-parametric test) and in the pyramidal layer of both CA1 (*p=0.0238; Mann-Whitney non-parametric test) and CA3 (^p<0.0001; Mann-Whitney non-parametric test) (Fig. 20).

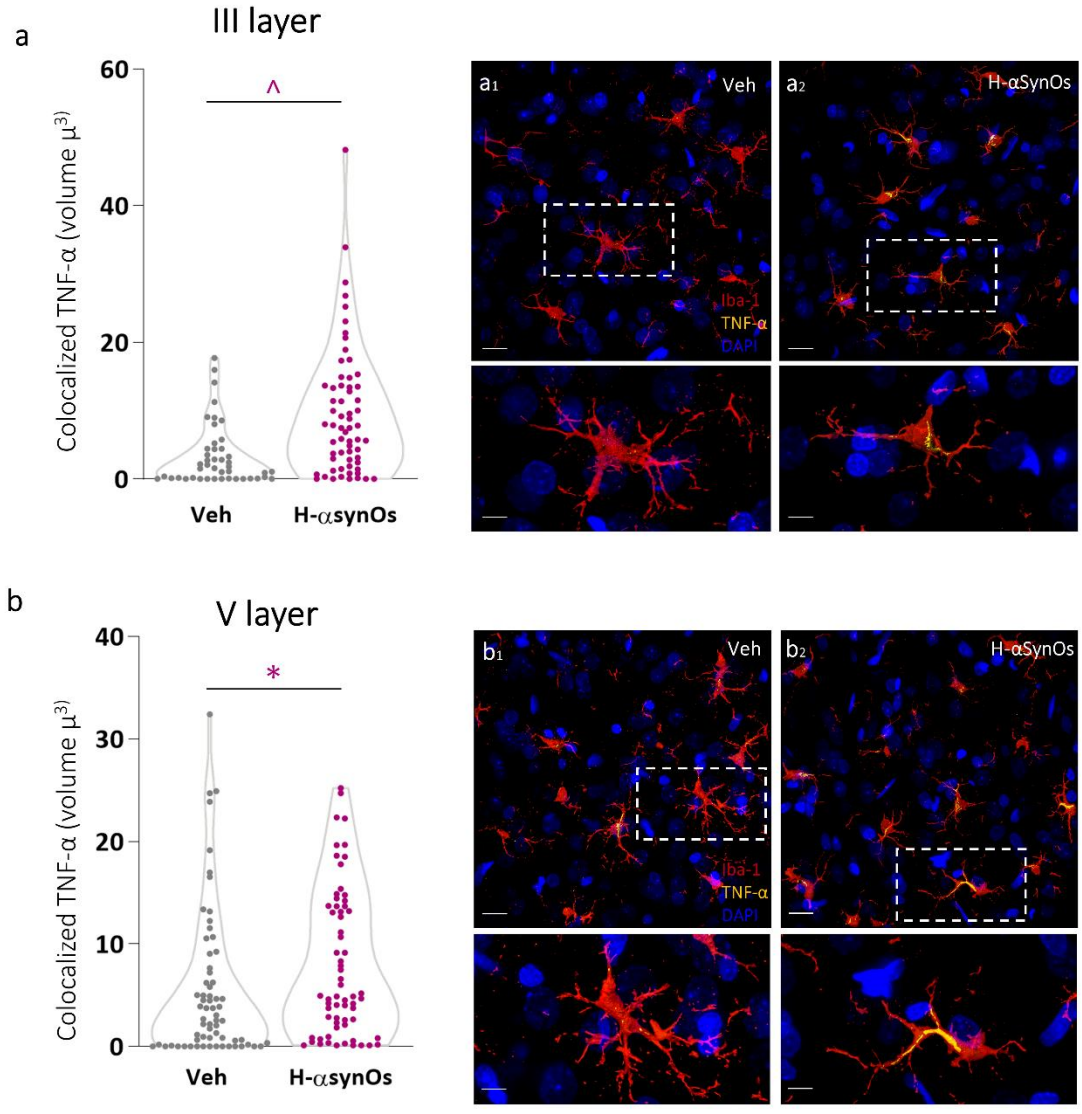


Figure 19. Increased levels of microglial TNF-α in the ACC following H-αSynOs infusion. Volume of TNF-α colocalized within microglial cells in the **(a)** III (n=50-60 cells, n=6 animals per group; ^p<0.0001 by Mann-Whitney test) and **(b)** V cortical layer (n=65 cells, n=6 animals per group; **p<0.01 by Mann-Whitney test). Representative images of TNF-α (yellow) **(a_1-a_2-b_1-b_2)** colocalized with IBA-1^+ cells (red). Magnification 63x; scale bars: 20 μm; 5 μm.

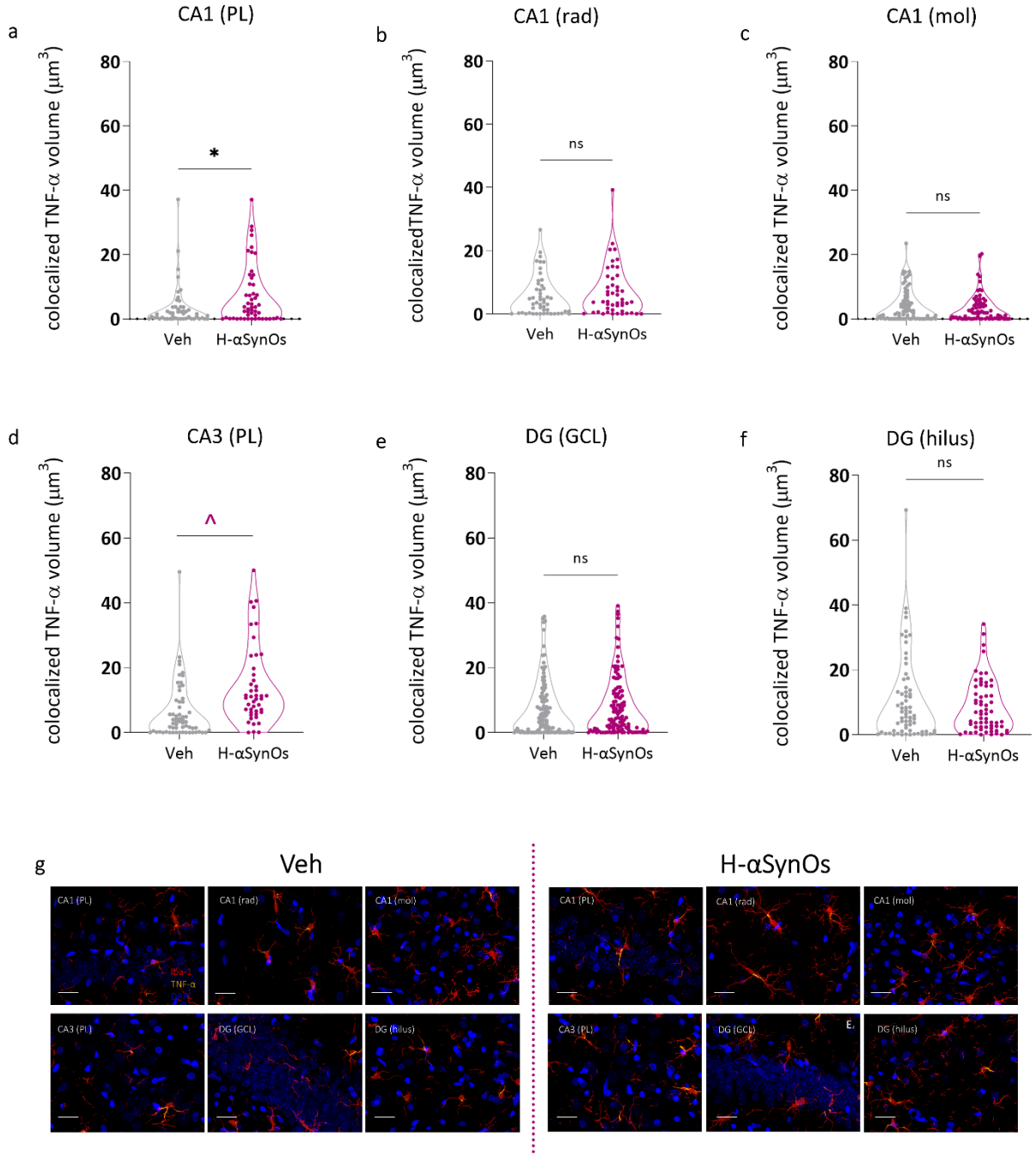


Figure 20. Microglial TNF-α content was increased in the hippocampal CA1 and CA3 subregions following H-αSynOs infusion. (a-f) Volume of colocalized TNF-α within IBA-1$^+$ cells in discrete subfields of the dorsal hippocampus ($n_{(CA1\text{-}GCL)}$=50-55, n=6 animals per group; *p<0.05 by Mann-Whitney test; $n_{(CA3\text{-}GCL)}$=60-45, n=6 animals per group; ^p<0.0001 by Mann-Whitney test). **(g)** Representative images of TNF-α (yellow) colocalized within IBA-1$^+$ cells (red). Magnification 63x; scale bar: 20 µm. GCL (granular cell layer); rad (radiatum); mol (molecular); DG (dentate gyrus), PL (pyramidal layer).

GluR1 IR

Changes in GluR1 protein levels were investigated by IF in the ACC as a neuronal marker of synaptic activity and because it is a recognized target molecule of TNF-α actions. The confocal microscopy analysis revealed a significant reduction in GluR1 levels in the ACC of H-αSynOs-infused rats as compared with vehicle-infused rats (Fig. 21) (*p=0.0057 by Mann-Whitney non-parametric test).

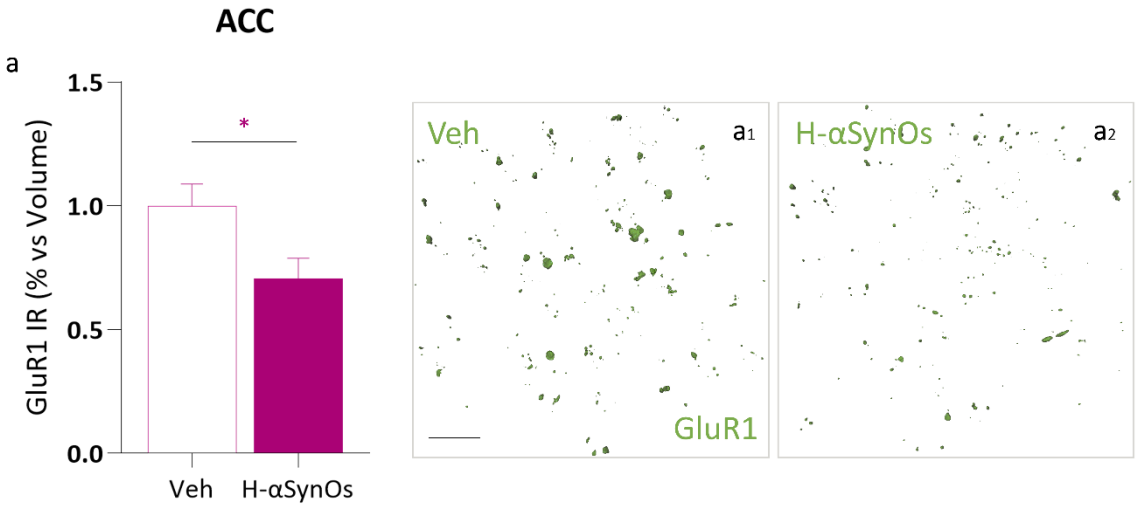


Figure 21. Decreased levels of cortical GluR1 following H-αSynOs infusion. (a) GluR1 expression levels in the ACC (n=50 subfields, n=6 animals per group; *p<0.01 by Mann-Whitney test). Representative images of GluR1 (green) expression in the ACC of **(a_1)** Veh-infused and **(a_2)** H-αSynOs-infused rats, respectively. Magnification 63x; scale bar: 20 µm.

Nissl staining in ACC and hippocampus

Qualitative analysis of Nissl-stained sections of both ACC and hippocampus did not reveal gross differences in the neuronal organisation and density, suggesting that glial activation did not sustain neurodegenerative mechanisms, and the cognitive decline was not underlaid by a neurodegenerative process (Fig. 22)

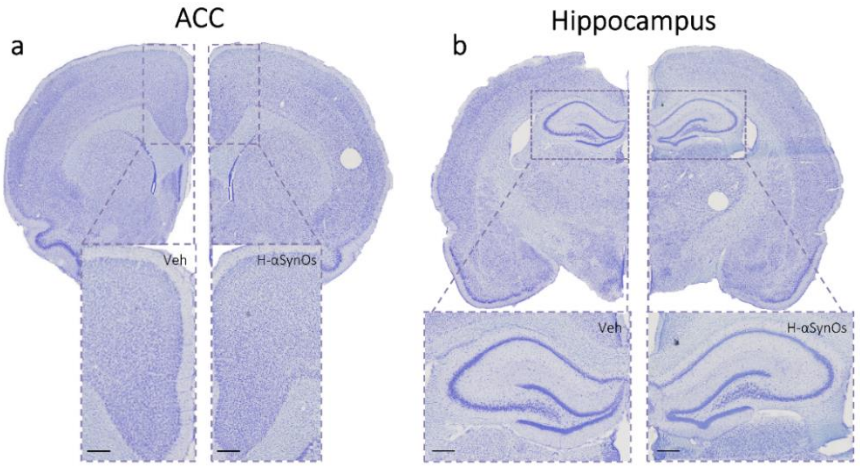


Figure 22. H-αSynOs infusion does not alter neuronal organisation in the ACC and in the hippocampus. Representative images of Nissl-stained coronal sections of (**a**) ACC and (**b**) dorsal hippocampus. Higher magnification pictures are shown (100 µm) below each image.

p129-αSyn staining

Based on previous evidence of αSyn spreading throughout the connectome, we evaluated the presence of aggregated forms of p129-αSyn in the infusion site as well as in the projection area nucleus striatum. Moreover, we sought p129-αSyn in the same cortical regions evaluated for inflammatory responses. Immunohistochemistry showed round-shaped deposits of p129-αSyn in the SNpc and diffused neurite-like deposits in the whole striatum of H-αSynOs-infused-rats (Fig. 23). In the ACC and the hippocampus, besides very small immunopositive dots, we did not detect clear evident aggregates of p129-αSyn (images not shown).

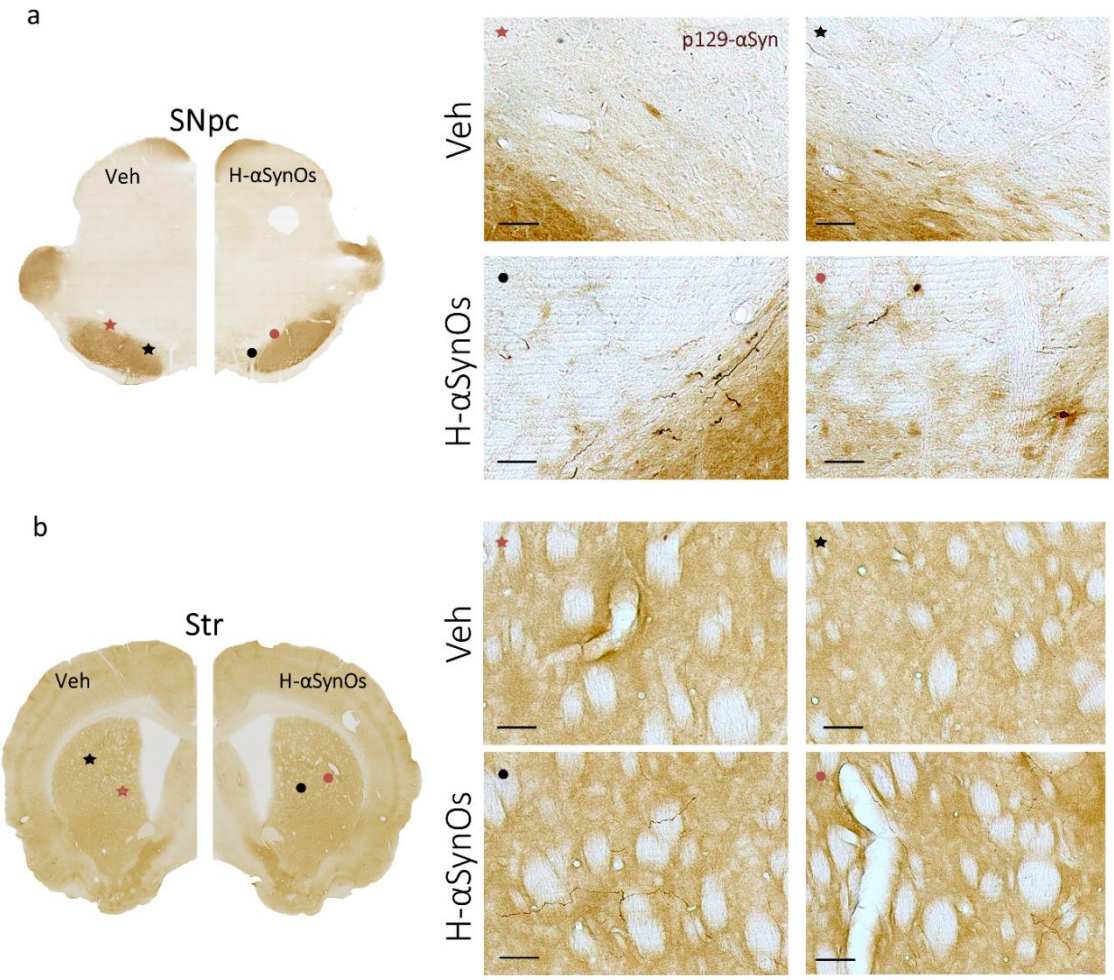


Figure 23. Intranigral infusion of H-αSynOs increases p129-αSyn aggregates in the SNpc (a) and striatum (b). Representative pictures showing aggregates of p129-αSyn in the SNpc and striatum (Str). Higher magnification pictures are shown on right panels (scale bar: 50 µm).

Discussion

Small and soluble αSyn aggregates that include oligomers, prefibrils and protofibrils, are considered as the most toxic species of αSyn in PD[393,394]. Importantly, these species have been widely reported in degenerating brain regions as well as in biological fluids of PD patients[394–398]. Among them, the oligomeric species are considered by several studies the most toxic among αSyn aggregates, capable of exert their toxicity by directly targeting neurons or glial cells, and by triggering a neuroinflammatory environment. In the present study we took advantage of a recently validated rodent model of PD based on the intranigral infusion of preformed H-αSynOs[168] to investigate the contribution of αSyn-induced neuroinflammation in both the neurodegenerative process within the BG circuit and the cognition-related symptoms of PD.

The intranigral infusion of H-αSynOs models both motor and non-motor aspects of PD pathology.

αSyn-based models of PD reproduce critical pathological traits of the disease, epitomized by the αSyn-induced progressive degeneration of DA neurons, spreading of aggregated αSyn and a persistent inflammatory reaction in pathologically affected brain areas. Therefore, these models appear particularly suitable for studying PD neuropathology and to test disease-modifying molecules[202]. However, the symptomatic phenotype of these models has been partially characterized so far, and the coexistence of multiple PD symptoms within the same model remains uncertain. In this study we describe that the intranigral infusion of pre-formed H-αSynOs resulted in motor and cognitive abnormalities, thus demonstrating the face validity of our model.

In line with our previous study[168], three months after H-αSynOs infusion, rats developed motor abnormalities including sensorimotor deficits that were assessed via the challenging beam walk test and the gait test, and coordination impairment in fine movements execution that was evaluated via the vermicelli handling test[375,376]. These motor tests provide an accurate evaluation of common PD deficits, such as stepping falls and bradykinesia, which model gait disturbances in human[399]. Specifically, the challenging beam walk test is a widely validated motor task, highly sensitive for motor deficits associated with partial degeneration of the nigrostriatal pathway, which has been previously used in PD models[160,373,400]. In the present study a sensorimotor impairment, evaluated three-months post H-αSynOs infusion, was indicated by the increased number of errors committed in the challenging beam walk test, as well as by gait abnormalities including a decrease in cadence (steps/s)

and an increase in the time spent to traverse a distance. Moreover, H-αSynOs-infused rats displayed irregular, shorter strides that model the shortening in step amplitude in PD. Finally, the vermicelli handling test revealed an impairment in fine motor movements.

Additionally, MCI was demonstrated by means of the two-trial recognition in a Y-maze and the NOR tests three months after intranigral H-αSynOs infusion. Performance in the two-trial recognition task revealed a mild but significant impairment in the short-term spatial recognition memory, as highlighted by the inability to discriminate between the arms of the maze. Indeed, H-αSynOs-infused rats spent a comparable amount of time in novel and familiar arms[382]. Importantly, the frequency of entrance among arms did not differ across the experimental groups, indicating that spatial memory impairment was not attributable to the presence of motor deficits. Such inability to discriminate the novelty by H-αSynOs-infused rats was further supported by the NOR data. Therefore, the present results extend the characterization of our PD model, indicating that the intranigral H-αSynOs infusion reproduced both motor and non-motor cognitive aspects of the pathology.

Neuropathological and functional alterations in motor and cognitive circuits following H-αSynOs infusion

A previous study demonstrated that motor symptoms were sustained by a neurodegenerative process and neuronal loss born by the nigrostriatal pathway[168]. Accordingly, in the present study we observed a neuronal degeneration within the SN, that paralleled those motor abnormalities previously described. Moreover, we showed that the MCI evaluated by the behavioral tests, although did not induce any frank cell loss in cognitive regions, was functionally associated with an impaired neuronal activity in the ACC and hippocampus. The ACC represents one of the brain regions which highly contribute to cognitive decline in PD[66,401–405]. Specifically, extracellular *in vivo* recordings from the ACC revealed a reduced spontaneous activity of cortical pyramidal neurons following the intranigral infusion of αSyn oligomeric species. Moreover, we found a downregulation in *Npas4* expression both in the ACC and in the hippocampus of H-αSynOs-infused rats. *Npas4* is a neuron-specific IEG, involved in the activity-dependent regulation of the excitatory-inhibitory balance, and is of high relevance for the present study because it represents an important molecular link between neuronal activity and memory[406,407]. Taken together these data provide evidence for a reduced neuronal activity in the ACC and hippocampus, which may be relevant to the impaired cognitive performance observed in behavioral studies.

LB pathology is a hallmark of PD, being LB described both in motor and non-motor areas of the PD brain[186,188], suggesting a pathological contribution of such aggregated forms in the disease. We have previously shown, and confirmed in the present study, the presence of p129-αSyn aggregates within the SN and striatum of H-αSynOs-infused rats[168,408]. Interestingly, a positive correlation between αSyn deposits and nigral degeneration has been previously suggested by preclinical studies[168,409], supporting the pathological role of toxic αSyn species in neurodegeneration and related motor symptoms. Moreover, in our study, despite the presence of p-αSyn deposits and neurite-like aggregates in motor areas, we did not detect the presence of such aggregates in cognitive regions of H-αSynOs-infused rats, such as the ACC and hippocampus. Several studies have pointed to LB pathology in cortical and limbic areas as a neuropathological correlate of PDD[175,177,333,334]. However, in the context of PD-MCI this correlation appears to be less evident. It has been reported that MCI may already develop in the early or even prodromal stages of the disease, when no or few cortical LB are present[336], challenging the assumption of a direct correlation between LB pathology and MCI, and suggesting that other under-explored mechanisms may contribute to the early cognitive decline in PD[410]. Several studies have demonstrated that toxic αSyn species induce the intracellular αSyn aggregation (seeding) and, in turn, promote the intercellular spreading of protein aggregates[202,311,314,315,411,412]. In this regard, the spreading of soluble species of αSyn might be relevant in the neuropathology of PD motor symptoms, but, most importantly, in that of non-motor symptoms. The process of αSyn aggregation generates a variety of intermediate small structures that end up into insoluble fibrillar aggregates within LBs, in a self-fuelling loop that origins and spreads multiple generations of toxic species[277,394,413]. We therefore suggest that soluble oligomeric species resulting from the infusion of HαSynOs, may reach cortical regions and trigger cortical impairment, while the fibrillar aggregates have not yet formed at the analyzed time-point. We suggest that a similar mechanism may apply to mild cognitive decline in the absence of cortical LBs in PD patients with MCI[336].

Another recognized hallmark of PD neuropathology is neuroinflammation, generally characterized by a chronic glial response within the brain parenchyma, likely driven by pathological interactions with toxic α-Syn[414,415]. In such circumstances, glial cells lose their homeostatic functions, thus acquiring a pro-inflammatory phenotype exemplified in the long-lasting and chronic release of a number of pro-inflammatory mediators, such as TNF-α[160,162,165–167,416–418]. Additionally, in the last decades a pivotal contribution of the peripheral immune system has been indicated, robustly enough to reconsider the view of PD not as limited within the boundaries of the CNS, but rather a systemic condition[90].

In line with preclinical and clinical evidence that reported a chronic microgliosis in PD patients' brain[108–111,114,160,168], we observed an enduring microglial proliferation in the SNpc following H-αSynOs infusion. Moreover, activated microglia displayed a pathological gain of toxic functions as indicated by the sustained production of the proinflammatory cytokine TNF-α. Notably, whereas in control rats the majority of microglia cells displayed low physiological levels of TNF-α, in H-αSynOs-infused rats two microglia subpopulations were recognized, displaying low and high TNF-α content, respectively, suggesting a supraphysiological production of the cytokine.

We further observed a long-lasting peripheral inflammatory condition three months post-αSynOs infusion, characterized by the increased serum content of inflammatory cytokines and the concomitant decrease of anti-inflammatory mediators[419,420]. This result is in line with an increasing literature reporting impaired serum cytokines in PD patients, and with the concept of PD as a systemic rather than CNS-specific disease, further validating our model as a translational model of PD[90]. Although the origin of systemic inflammation in PD remains uncertain, our results suggest that it may originate from the presence of toxic αSynOs within the CNS, leaving an open question as to the sequence of mechanistic events. Although there is a convergence of opinion on serum cytokine dysregulation in PD, conflicting findings have been reported with respect to single cytokines[421,422]. Nonetheless, the elevated serum level of IL-17 present in our intracerebrally α-synuclein-infused rats is worthy of note, and is in line with the pivotal role currently attributed to this cytokine in neurodegenerative diseases and in glial cell activation[423]. Moreover, in line with a prevalence of the inflammatory immunophenotype in PD, IL-6 was significantly elevated, whereas IL-10 was significantly decreased in our model[90,419,420]. Finally, chemokines CXCL1/2 and RANTES were increased, in line with the infiltration of immune cells into the inflammatory site, as revealed by CD3 immunostaining in H-αSynOs-infused rats[424–427]. Interestingly, serum TNF-α was unchanged following intracerebral administration of αSynOs. Similarly, in a previous study, we found unchanged levels of serum TNF-α after dopamine depletion and after a chronic L-DOPA treatment, despite an intense inflammatory response within the brain[378].

Despite the clear contribution of neuroinflammation in PD neuropathology, this issue appears to be under-investigated in the context of cognitive decline related to PD. A causal link between these two factors has been often observed in other neurological conditions, thus pushing towards the same direction in relation to PD cognition-related symptoms[428,429]. In this regard, *post mortem* and *in vivo* imaging studies have revealed the presence of inflammatory markers in limbic areas of PDD[110,430] and

DLBs patients[111]. Moreover, a positive correlation between microglial activation and αSyn pathology in cognition-related brain regions have been observed in PDD patients, pointing at αSyn as a potential trigger of such neuroinflammatory response[175]. Specifically, while not observing an evident microgliosis, this study reported increased levels of pro-inflammatory cytokine IL-1β in the frontal cortex of PDD subjects. Albeit a relationship between αSyn toxicity, neuroinflammation and cognitive decline has been suggested by human studies, this issue remains poorly investigated in rodents, likely due to the lack of a proper preclinical neuropathological PD model that recapitulates cognitive symptoms. In the present study we observed that the intranigral H-αSynOs infusion elicited a neuroinflammatory response in cognition-related brain regions, such as the ACC and the hippocampus. Such neuroinflammation was characterized by sporadic microglial proliferation in most of these regions, but a significant increase in pro-inflammatory cytokine production by microglial cells, in line with previous reports[175], and without any frank neuronal loss. Interestingly, a significant increase in Iba-1 IR was specifically found in discrete hippocampal subfields, such as the CA1 pyramidal layer. In this regard, it is important to note that this specific subfield represents one of the most vulnerable regions of the hippocampus in PD-MCI patients [59,431]. Although we didn't observe a diffused microglial proliferation, we found an increased production of the pro-inflammatory cytokine TNF-α by microglial cells, suggesting the acquisition of an altered phenotype in response to the infusion of H-αSynOs. Different studies have reported that αSyn binds the TLR2, thereby stimulating pro-inflammatory responses in microglial cells [432,433]. The entity of such response is strictly dependent on the αSyn conformational state, soluble oligomeric/protofibril forms holding a greater inflammatory potential [432,433] than the native monomeric protein[434,435]. Chronic and long-lasting release of pro-inflammatory mediators, especially TNF-α, might play a crucial role in functional dysfunctions underlying memory deficits in our model. Besides its main role as a master regulator of inflammation, TNF-α is a key player in the regulation of neuronal excitability, synaptic strength and plasticity [153,183,436]. Importantly, increased levels of this cytokine have been observed in several neurological diseases associated with cognitive deficits[437] and plays an important role in age-related cognitive decline [438] and in Alzheimer disease-induced cognitive impairment [439,440]. In this regards, by binding to the neuronal TNF receptor TNFR1, TNF-α inhibits theta-burst-induced long-term potentiation (LTP) in CA1 synapses [441–443] and mediates amyloid-beta-induced inhibition of LTP in the DG [444]. Of note, TNF-α may cause a reduction of synaptic strength via the direct or astrocyte-mediated regulation of AMPA receptor trafficking [152,183]. In the light of this evidence, we evaluated this TNF-α-related effect in the ACC observing a reduced expression of the AMPA receptor subunit GluR1 in rats

infused with H-αSynOs. We therefore suggest that functional changes in the ACC circuit and the related cognitive abnormalities seen three months post-oligomers infusion, might be the result of a long-lasting inflammation. In line with this hypothesis, deficits in spatial learning induced by D-galactosamine administration caused an increase in the expression of proinflammatory cytokines in the hippocampus and a dysregulated glutamate receptor expression, including the downregulation of the GluR1 AMPA subunit [445]. Notably, the differential expression of microglial TNF-α in the hippocampal subfields revealed a highest susceptibility of the Cornu Ammonis region in this animal model. Albeit such a differential inflammatory response has not been reported in PD-MCI patients yet, CA1/CA2/CA3 subfields represent the first hippocampal regions that undergo to atrophy in the conversion from PD-NCI (no cognitive impairment) to PD-MCI [59].

Targeting inflammation as a disease modifying strategy for PD

Given the prominent role displayed by neuroinflammation in human PD neuropathology and confirmed in our PD model, we tested the neuroprotective potential of the clinically approved immunomodulatory agent Pom. One of the main challenges in PD research is the development of proper disease-modifying strategies aimed at slowing or stopping the progression of the disease. In this regard, given the central role of neuroinflammation and more in general of immune system dysregulation and their relation to neurodegeneration, several immunomodulatory compounds have been tested for their disease-modifying potential, showing neuroprotective properties in rodent models of PD, including fingolimod, tacrolimus, cyclosporin and rapamycin[446–450]. Notably, based on these pre-clinical outcomes, rapamycin has been prioritized as a therapeutic candidate to move into clinical trials for repurposing in PD[451]. In contrast, the family of compounds named IMiDs remains relatively poorly investigated in preclinical models of PD. Widely used and effective in cancer treatment, the relative dearth of preclinical research in PD models precludes their current proposition for clinical testing in this disorder. We therefore firstly tested Pom, a III generation IMiDs, for rescuing from the motor impairment induced by H-αSynOs infusion. Notably, motor deficits were fully mitigated by Pom chronic treatment, strongly forecasting a beneficial action of this compound in PD motor symptomatology. The efficacy of Pom was underpinned by the rescue of DAergic cell loss within the SN, as revealed by the stereological counting of TH^+ cells, further confirming the neuroprotective action of Pom.

Given the immunomodulatory nature of this agent, we asked whether Pom was able to dampen both central and peripheral inflammation induced by H-αSynOs infusion. Strikingly, Pom treatment

suppressed the high TNF-α expressing microglia population leaving unaltered the low TNF-α expressing population, thereby restoring physiological levels of the cytokine. Moreover, Pom augmented the microglia production of the anti-inflammatory cytokine IL-10, as expected by the immunomodulatory action of this drug, thus providing an allostatic regulation of microglia phenotype to restore the physiological pro/anti-inflammatory balance. Therefore, chronic treatment with Pom successfully dampened the systemic inflammation, largely restoring normal cytokine levels within the serum. A major point to be considered is that the pharmacological treatment was initiated 1 month after H-αSynOs infusion, a time-point that may be considered to model the prodromal phase of PD, when mitochondrial damage and neuroinflammation are not yet associated with neuronal loss[168]. Therefore, the results of the present study, aside to validate our model in terms of predictive validity, suggest that Pom can mitigate the already initiated and ongoing neurodegenerative processes, as well as successfully dampen central and peripheral inflammation. These aspects are fundamental in view of the translational relevance of this study, since suggest a potential disease-modifying effect of the drug treatment if given to early-diagnosed PD patients.

All together our studies confirm the pivotal role of inflammation in the neuropathology of PD, extending this concept from the neuropathology of motor to that of non-motor symptoms. Results suggest that inflammation not only underpins the neurodegenerative process in motor areas such as the SNpc, but also induces functional changes in the neuronal activity in limbic areas that my account for cognitive deficits. Moreover, the prolonged treatment with the immunomodulatory drug Pom rescued from neurodegeneration and motor symptoms in a PD model, suggesting it for repositioning as a disease-modifying compound in PD. Given the wide involvement of inflammation in multiple aspects of PD, immunomodulatory drugs may provide a beneficial novel tool for clinically target PD as a whole syndrome.

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