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Understanding the role of perivascular macrophages in Parkinson's disease pathophysiology

Jaime Fuentealba

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Ecole doctorale

*Institut du Cerveau et de la Moelle Épineuse / Thérapeutique expérimentale de la maladie
de Parkinson*

Titre de la thèse

“Understanding the role of perivascular macrophages in Parkinson’s disease pathophysiology”

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Dirigée par le Dr. Stéphane HUNOT

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CONTENTS

ACKNOWLEDGMENTS	2
CONTENTS	2
ABBREVIATIONS	6
INTRODUCTION	9
1. PARKINSON'S DISEASE GENERALITIES	9
1.1 A-SYNUCLEIN	10
1.2 THE "PRION HYPOTHESIS" IN PD	14
1.3 A-SYNUCLEIN TOXICITY	16
1.4 CONTRIBUTING FACTORS TO A-SYN AGGREGATION	17
1.5 ANIMAL MODELS OF PD	20
1.5.1 Neurotoxic models	20
1.5.2 Genetic models	21
1.6 CELL-AUTONOMOUS MECHANISMS OF NEURODEGENERATION IN PD	23
1.6.1 Autophagy-lysosomal pathway (ALP) alterations	23
1.6.2 Endoplasmic reticulum (ER) stress	24
1.6.3 Ubiquitin-proteasome system (UPS)	25
1.6.4 Mitochondria dysfunction	25
1.7 NON-CELL AUTONOMOUS MECHANISMS OF NEURODEGENERATION IN PD	27
1.7.1 Evidences for neuroinflammatory mechanisms in PD	27
1.7.2 Microglia in PD	28
1.7.3 Astrocytes in PD	30
1.7.4 Engraftment of lymphocytes in PD	30
1.7.5 Engraftment of blood-derived monocytes in PD	31
1.8 EVIDENCES OF BLOOD-BRAIN BARRIER REMODELLING IN PD	34
2. PERIVASCULAR MACROPHAGES	37
2.1 PHYSIOLOGY OF PERIVASCULAR MACROPHAGES	37
2.2 METHODOLOGICAL PRINCIPLE OF PVMs DEPLETION	39
2.3 PVMs IN PATHOLOGY	40
2.4 PVMs AND THE "GLYMPHATIC" SYSTEM	41
3. THE CNS LYMPHATIC SYSTEM	42

HYPOTHESIS	45
SPECIFIC AIMS AND EXPERIMENTAL APPROACH	46
METHODS	47
1.1 HUMAN SUBJECTS	47
1.2 ANIMALS	47
1.3 ANESTHESIA	47
1.4 TAMOXIFEN	47
1.5 PRODUCTION OF NON-REPLICATIVE CANINE ADENOVIRUS TYPE 2 AND A-SYN FIBRILS ..	48
1.6 STEREOTAXIC INJECTIONS	48
1.7 INTRACISTERNAL INJECTIONS	49
1.8 PARABIOSIS	50
1.9 FLOW CYTOMETRY	51
1.10 <i>IN VIVO</i> PROLIFERATION ASSAYS	51
1.11 IMAGE ACQUISITION	51
1.12 TISSUE PROCESSING	51
1.13 BRAIN TISSUE IMMUNOSTAINING	52
1.14 IMMUNOHISTOCHEMICAL STAINING OF HUMAN BRAIN SECTIONS	53
1.15 TRANSMISSION ELECTRON MICROSCOPY	53
1.16 IMAGE ANALYSIS AND CELL COUNTING	54
1.17 IMMUNOFLUORESCENCE ANALYSIS	54
1.18 STATISTICAL ANALYSIS	55
1.19 MICROGLIA SORTING	55
1.20 RNA-SEQUENCING	55
RESULTS	57
1.1 HISTOLOGICAL AND FUNCTIONAL IDENTIFICATION OF PERIVASCULAR MACROPHAGES 57	
1.2 PERIVASCULAR MACROPHAGES DYNAMICALLY RESPOND TO NEURODEGENERATION IN A MOUSE MODEL OF DEGENERATIVE SYNUCLEINOPATHY	59
1.3 LOCAL PROLIFERATION DRIVES DISEASE-ASSOCIATED PVMS RECRUITMENT	64
1.4 PVMS ABLATION AGGRAVATES NEURODEGENERATION	68
1.5 SYNUCLEINOPATHY SPREADING IS AGGRAVATED IN PVMS-DEPLETED MICE	77
1.6 PVMS-DEPLETED MICE SHOW IMPAIRED CSF INFLUX AND REDUCED LYMPHATIC CLEARANCE	82
1.7 PVMS ACCUMULATE IN THE SNPC OF PD PATIENTS	84
GENERAL DISCUSSION	86
1.1 PVMS DYNAMICALLY RESPOND TO NEURODEGENERATION	86

1.2 PVMS PROLIFERATE IN RESPONSE TO NEURODEGENERATION (FIGURE 27)	88
1.3 PVMS POSSESS NEUROPROTECTIVE FUNCTIONS	91
1.4 MECHANISMS OF PVM-ASSOCIATED NEUROPROTECTION	93
1.4.1 Role of PVMs as immune regulators	93
1.4.2 Role of PVMs as phagocytes	96
1.4.3 Role of PVMs as potential regulators the CSF flow.....	98
1.5 PERSPECTIVES	101
1.6 GENERAL CONCLUSION	102
REFERENCES	103

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
α -Syn	α -synuclein
A β	Amyloid beta
AD	Alzheimer's disease
ALP	Autophagy-lysosome pathway
APP	Amyloid precursor protein
AQP4	Aquaporin-4
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BM	Bone marrow
BMT	Bone marrow transplantation
CAA	Cerebral amyloid angiopathy
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAT	Dopamine transporter
DBS	Deep brain stimulation
dcLNs	Deep cervical lymph nodes
DLB	Dementia with Lewy bodies DLB
DNs	Dopaminergic neurons
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Gliofibrillary acidic protein
GFP	Green fluorescent protein
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
HSC70	Heat shock cognate protein 70
ICAM-1	Intercellular adhesion molecule-1
IFN γ	Interferon γ
IL-1 β	Interleukin-1 β
IL-2	Interleukin-2

IL-6	Interleukin-6
iNOS	Nitric oxide synthase
ISF	Interstitial fluid
LAMP2A	Lysosome associated membrane protein type 2A
LB	Lewy bodies
LFA-1	Leukocyte function- associated antigen-1
LN	Lewy neurites
LPS	Lipopolysaccharide
LRRK2	Leucine rich repeat kinase 2
Lyve-1	Lymphatic vessel endothelial hyaluronan receptor 1
MAO-B	Monoamine oxidase-B
MHC	Major histocompatibility complex
MMs	Meningeal macrophages
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple system atrophy
mtDNA	Mitochondrial DNA
MyD88	Myeloid differentiation primary response gene 88
NAC	Non-amyloid-beta component
NF- κ B	Nuclear translocation of nuclear factor κ B
Nox	NADPH oxidase
NSAIDs	Nonsteroidal anti-inflammatory drugs
NVU	Neurovascular unit
PD	Parkinson's disease
PET	Positron emission tomography
PFFs	Preformed α -Syn fibrils
PINK1	PTEN-induced novel kinase 1
PVMs	Perivascular macrophages
ROS	Reactive oxygen species
SNpc	Substantia nigra pars compacta
SR-BI	Scavenger receptor class B type I
sVEGFR-2	Soluble vascular endothelial growth factor receptor-2
TGF α	Transforming growth factor α
TH	Tyrosine hydroxylase
Th	T helper

thioS	Thioflavin S
TLR	Toll-like receptor
TNF α	Tumor necrosis factor α
TOM20	Translocase of the outer membrane 20
TSPO	Mitochondrial translocator protein
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
VEGF	Vascular endothelial growth factor
YFP	Yellow fluorescent protein

INTRODUCTION

1. Parkinson's disease generalities

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by both motor and non-motor system manifestations that affects approximately 1% of individuals older than 60 years old. The disease results from an intricate interaction of genetic and environmental factors affecting several cellular processes. The first detailed clinical description of the disease was written by James Parkinson in 1817, later expanded and refined by Jean-Martin Charcot in the mid-1800 (Goetz, 2011). The majority of PD cases are sporadic, and only about 10% of patients report a positive family history. The classical motor features of PD are resting tremor, slowness of movements (bradykinesia), rigidity and postural instability, which become progressively worse as the disease advances. On the other hand, non-motor features include olfactory dysfunction, cognitive impairment, psychiatric symptoms, sleep disorders, autonomic dysfunction, pain, and fatigue. Some of these features are frequently present before the onset of the classical motor symptoms (Kalia and Lang, 2015).

The cardinal neuropathological feature of PD is the loss of dopaminergic neurons (DNs) within the *Substantia Nigra pars compacta* (SNpc), which is the cause of motor features. When these features are clinically recognized, about 60% of DNs are already lost (FEARNLEY and LEES, 1991), resulting in 80% reduction of striatal dopamine. Besides nigrostriatal degeneration, neuronal loss of various degrees also occurs in many other brain structures including the *locus coeruleus*, the nucleus basalis of Meynert, the pedunculo-pontine nucleus, the raphe nucleus, the dorsal motor nucleus of the vagus, the amygdala, and the hypothalamus (Dickson, 2012).

Nowadays, the available therapies only treat the symptoms of the disease. Indeed, drugs that enhance intracerebral dopamine contents, or stimulate dopamine receptors, remain the mainstay of treatment for dealing with motor symptoms. These drugs include levodopa, dopamine receptor agonists, monoamine oxidase type B (MAO-B) inhibitors and, less commonly, amantadine; none of which has proven to be neuroprotective nor disease-modifying (Kalia and Lang, 2015). When medication no longer provides adequate relief of motor symptoms in advanced stages of the disease, deep brain stimulation (DBS) offers a powerful therapeutic alternative. DBS is a surgical therapy consisting in the implantation of electrodes into specific brain regions, commonly the subthalamic nucleus

and the internal segment of the globus pallidus. Electrodes deliver electrical stimuli to modulate or disrupt abnormal patterns of neural signalling within the targeted region. Although the efficacy of DBS has been clearly demonstrated, it is used in less than 2% of patients with PD for reasons that include a lack of familiarity with the procedure and concerns for potential surgical risks in elderly individuals, among others (Hickey and Stacy, 2016).

Another key neuropathological hallmark of the disease is the presence of neuronal inclusions mainly composed of aggregated α -synuclein (α -Syn) and known as Lewy pathology. While the inclusions located in neuronal perikarya are referred to as Lewy bodies (LB), similar inclusions within neuronal cell processes are called Lewy neurites (LN). Besides PD, there are other degenerative disorders characterized by inclusions composed of α -Syn: Multiple system atrophy (MSA), which is defined by abnormal deposition of α -Syn in the cytoplasm and nucleus of oligodendrocytes and some nerve cells; and dementia with Lewy bodies (DLB), characterized like PD by the presence of LB and LN. Whereas LB and LN are found in particularly high numbers within the brainstem in PD cases, their widespread distribution through almost all brain areas, but particularly numerous in the cortex, is a characteristic of DLB. Since α -Syn aggregation is a common feature of the three diseases, they are frequently referred to as α -synucleinopathies (Spillantini et al., 2000).

1.1 α -Synuclein

α -Syn is a 140-amino acid cytosolic protein with three domains (i) the N-terminal lipid-binding α -helix, which contains seven imperfect repeats of 11 amino acids each, (ii) a central domain called the non-amyloid-beta component (NAC), which is highly hydrophobic and is both necessary and sufficient for the aggregation and toxicity of α -Syn, and (iii) a C-terminal acidic tail that provides structural flexibility to the protein (Rodriguez et al., 2015) (Villar-Piqué et al., 2016). The expression of α -Syn is relatively specific to the nervous system, however some non-neural tissues, like red blood cells also express it (Barbour et al., 2008). Within the brain, α -Syn is preferentially localized in presynaptic terminals, interacting mostly with membranes that possess high curvature, like synaptic vesicles (Middleton and Rhoades, 2010).

Although the function of α -Syn is far from being fully established, the enrichment at the presynaptic compartment and the interaction with synaptic vesicles strongly suggest a role in modulating neurotransmitter release. When over-expressed, α -Syn reduces neurotransmitter release both *in vivo* and *in vitro* (Nemani et al., 2010). However, targeted gene deletion of α -Syn in mouse has shown more contradictory effects on synaptic transmission (Bendor et al., 2013). Alternative roles for α -Syn have been described, nevertheless so far none is fully consensual: e.g. it is believed to modulate vesicle trafficking (Scott and Roy, 2012), act as a molecular chaperone (Witt, 2012) and suppress apoptosis by reducing protein kinase C activity (Jin et al., 2011).

α -Syn reached prime time in the late 90s when two important studies were published revealing the pathological association between α -Syn and PD. On one hand, the identification of the first mutation responsible for PD indisputably showed that the disease may be hereditary: the A53T mutation described was located in the SNCA gene (coding for α -Syn), causing autosomal-dominant PD (Polymeropoulos et al., 1997). Ever since, other mutations as well as duplications and triplications have been identified in the SNCA locus, however they are all very rare events. On the other hand, and thanks to the identification of the A53T mutation, α -Syn was recognized as the major component of LB and LN, immediately suggesting its implication in sporadic PD pathogenesis, given that Lewy pathology is a signature and a criterion for the definite diagnosis of sporadic PD (Spillantini et al., 1997).

Based on the distribution of Lewy pathology, a staging scheme for PD was proposed (Braak et al., 2003a) known as the Braak model. The model reflects the progression of Lewy pathology in a stereotyped pattern over the course of PD, with both dopaminergic and non-dopaminergic areas being affected. The model proposes six stages with α -Syn depositions starting from the peripheral nervous system and progressing in a caudal-to-rostral direction towards the neocortex (Figure 1). In stage 3, Lewy pathology reaches the basal portions of the midbrain and forebrain, with the SNpc being affected and, consequently, it is believed that it would occur when motor symptoms manifest, due to a significant dopamine deficiency. The predictable topographic sequence of brain regions affected by Lewy pathology suggests that the process is actually not random. In support of this, it was suggested that the neurons susceptible to develop Lewy pathology share some common features: they have long, thin and unmyelinated or poorly myelinated axons (Braak et al., 2003b). Braak and colleagues also hypothesised that PD might originate due

to an environmental insult (e.g. a neurotropic pathogen) that enters the body through the olfactory and the enteric epithelium, where it spreads trans-synaptically from one vulnerable region to the next (Hawkes et al., 2009).

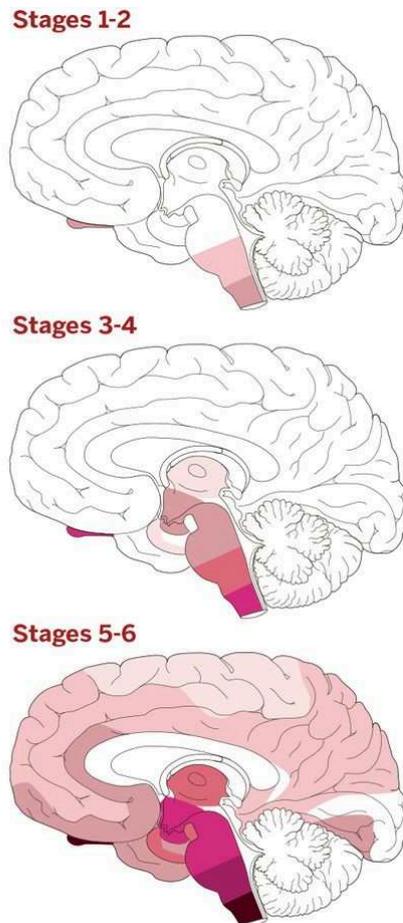


Figure 1. Staging of Lewy pathology according to the Braak model. The model states that Lewy pathology progresses in a stereotyped and temporal pattern that ascends caudo-rostrally. Within the brain, the first inclusions occur in the olfactory bulb, the anterior olfactory nucleus and the dorsal motor nuclei of the vagal and glossopharyngeal nerves in the brainstem (stages 1 and 2). Then the pathology spreads into the pons, midbrain and basal forebrain (stages 3 and 4) to finally reach the neocortex (stages 5 and 6). Adapted from (Goedert, 2015).

However, in the recent years many studies have convincingly shown that, instead of an exogenous pathogen, disease progression could be mediated by a prion-like transmission of α -Syn between neurons. As it will be discussed in the next section, the prion-like hypothesis postulates that in pathology, misfolded α -Syn form high molecular weight structures like oligomers and fibrils, leading to the formation of inclusions (Lewy pathology) that can propagate to neighbouring neurons (Figure 2).

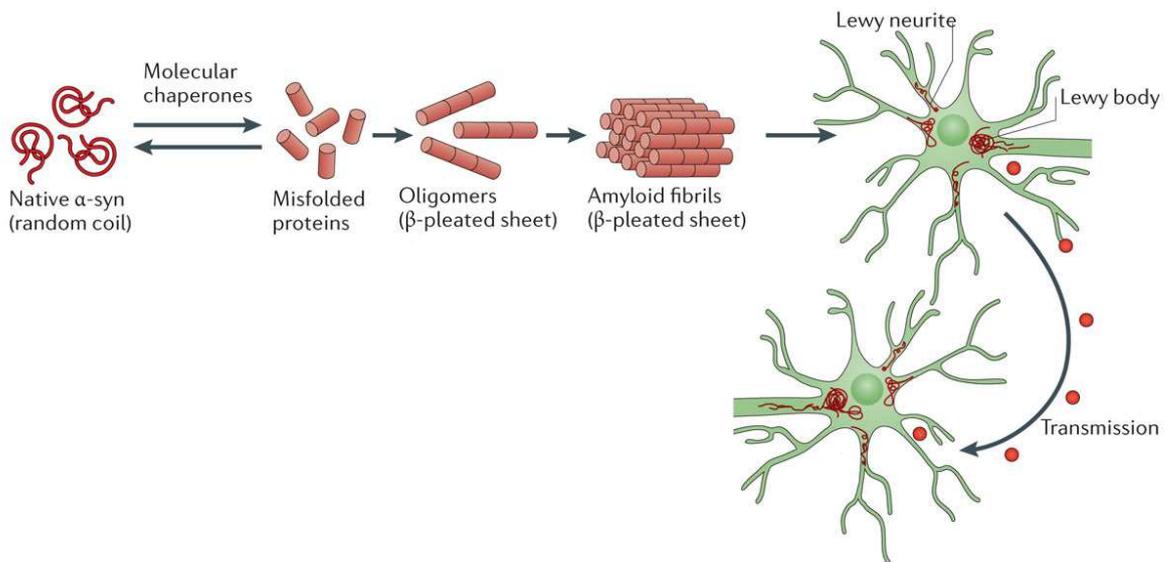


Figure 2. Hypothetical model of α -Syn aggregation and propagation. In normal conditions, α -Syn exists in a soluble random coil state. Nevertheless, the native protein in pathology undergoes misfolding into toxic species: from oligomers to higher-order structures like amyloid fibrils. The accumulation of these amyloid fibrils leads to the formation of intracellular inclusions (Lewy pathology: LB and LN). α -Syn “seeds” (red dots) from diseased neurons can be transferred into neighbouring neurons to induce further aggregation of monomeric α -Syn. Adapted from (Kingwell, 2017).

1.2 The “prion hypothesis” in PD

One of the first reports that led to the hypothesis that PD may be a prion-like disorder, was the identification of Lewy-like pathology within embryonic mesencephalic neurons grafted into the putamen of PD patients more than a decade before the autopsies, suggesting that the pathology spread from the host tissue to the graft (Kordower et al., 2008). Besides this observation, many studies are in favour of the prion hypothesis of synucleinopathy spreading. First, monomeric α -Syn in a test tube spontaneously form fibrils, reminiscent of those found in LB in PD (Conway et al., 1998). Moreover, it was shown that the aggregation of α -Syn is a nucleation-type process, meaning that it can be accelerated by the presence of α -Syn pre-formed fibrils (PFFs) acting as seeds (Wood et al., 1999). Second, it was demonstrated that the introduction of PFFs into cells engineered to overexpress α -Syn, but not monomers nor oligomers, induces the aggregation of endogenous α -Syn (Luk et al., 2009a). Third, by transplanting green fluorescent protein (GFP)-expressing mouse cortical neuronal stem cells into the hippocampus of transgenic mice expressing human α -Syn under the control of the Thy-1 promoter, the authors observed human α -Syn immunoreactivity in the grafted cells, showing a host-to-graft transmission of synucleinopathy *in vivo*. Yet, by examining the ultrastructural features of the inclusions formed in the grafted cells, only amorphous electron-dense material was found, with no signs of truly aggregated α -Syn (Desplats et al., 2009). Despite the evidences showing that α -Syn can be transferred from cell-to-cell and induce the formation of inclusions similar to LB, the first study to clearly show a link between PD-like Lewy pathology and neurodegeneration was recently published (Luk et al., 2012a). The authors demonstrated that a single intrastriatal injection of α -Syn PFFs into wild-type mice was sufficient to induce the spreading of α -synucleinopathy in anatomically interconnected brain regions, causing a selective loss of DNs in the SNpc. Later, the injection of LB-containing homogenates from PD brains into the SN and striatum of non-human primates and mice gave similar results regarding α -Syn pathology and neurodegeneration (Recasens et al., 2014). These recent evidences strongly suggest that aggregation of α -Syn *per se* can direct the degenerative process.

Even if α -Syn has been considered exclusively cytoplasmic, the prion hypothesis implies that in order to spread, α -Syn has to be secreted into the extracellular milieu, where it could induce the aggregation of α -Syn in neighbouring neurons once internalized. This is supported by the presence of α -Syn in the cerebrospinal fluid (CSF) and plasma of humans

(El-Agnaf, 2003) suggesting that cells can release α -Syn, even though it does not contain a secretory peptide sequence. However, how could α -Syn be released from the cell? α -Syn can be found in the culture media from cells expressing human α -Syn, and it was shown that the secretion is very likely mediated by exocytosis, since the release can be inhibited by low temperature, a condition usually used to block vesicular exocytosis (Lee, 2005). Another mechanism of secretion described for α -Syn is through exosomes. Indeed, cells can excrete α -Syn inside exosomes, in a pathway that is negatively influenced by autophagic activity (Danzer et al., 2012) (Lee et al., 2013). After being secreted, α -Syn must be internalized by surrounding cells in order to induce further aggregation. It has been shown in cultured neurons that monomeric α -Syn can diffuse across the plasma membrane in a process that is not inhibited by low temperature or other endocytosis blockers, suggesting an endocytosis-independent internalization route. However, larger assemblies (fibrils and oligomers) are able to gain access into the intracellular milieu in an endocytosis-dependent manner (Lee et al., 2008).

As mentioned previously, it is nowadays well accepted that the aggregation of α -Syn is a nucleation-dependent process. From its monomeric form, α -Syn can organize into oligomers enriched in β -sheet structures and then aggregate into more complex amyloid fibrils, in a process accelerated by α -Syn seeds (Figure 2). This process, that is considered to be an essential characteristic for α -Syn self-propagation, in combination with all the evidences presented in this section, argue in favour of the prion hypothesis. Nonetheless, the existence of strains, an additional key feature of prion diseases was missing, until Bousset and collaborators identified two strains (or polymorphs) of fibrillar α -Syn (named ribbons and fibrils) with different structures, levels of toxicity, and both *in vitro* and *in vivo* seeding and propagation properties (Bousset et al., 2013a). The importance of the study is that one might consider that such strain differences could explain the different clinicopathological characteristics within the synucleinopathy spectrum. Indeed, the injection of ribbons and fibrils into rat brains demonstrated that the two strains hold different seeding capacities and, more intriguingly, induce different pathological phenotypes. In rats that overexpress human α -Syn, the inoculation of fibrils induces intraneuronal α -Syn accumulation and dopaminergic cell death, a hallmark of PD. Remarkably, the inoculation of ribbons promotes α -Syn inclusions in oligodendrocytes, the histopathological characteristic of MSA (Peelaerts et al., 2015a).

The evidences pointing towards a prion-like propagation of α -Syn pathology are robust, however what are the consequences of such propagation in terms of cell death?

1.3 α -Synuclein toxicity

Over the past decades, great efforts have been undertaken to identify how α -Syn can cause cellular dysfunction. Most of the studies show that α -Syn can disrupt biological processes at different levels by targeting several organelles including synaptic vesicles, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, autophagosomes and the nucleus (Wong and Krainc, 2017a). Although the evidences showing the toxicity of α -Syn are convincing, a fundamental question about which α -Syn conformers hold such toxicity is still open and currently a matter of intensive research. By analysing fresh homogenates of minimally processed human brain extracts through biochemical and biophysical techniques, it was observed that α -Syn exists physiologically as both stable monomers and metastable conformers distinct from oligomeric intermediates, which are sodium dodecyl sulfate (SDS) and heat stable (Gould et al., 2014). In addition, an independent study reported that endogenous α -Syn exists as folded tetramers of 58 kDa when isolated under non-denaturing conditions from different cell lines, human cells and brain tissue (Bartels et al., 2011). Therefore, both studies suggest that multiple forms of α -Syn may occur physiologically, but not higher molecular weight species like oligomers or fibrils. Since monomers and tetramers seem to be the physiologically occurring conformers, they are unlikely the culprits involved in toxicity. This assumption has oriented research towards the study of the toxic capacities of oligomers and fibrils that are present in animal models of the disease and in the brain of PD patients. Yet, the question of which α -Syn assemblies are more toxic still remains controversial. Some reports suggest that oligomers are more toxic: for instance, the expression of α -Syn variants that promote oligomers formation induce severe dopaminergic loss, whereas the variants that promote fibrils formation were less toxic *in vivo* (Winner et al., 2011). By contrast, recent work has suggested that α -Syn fibrils, rather than their oligomeric precursors, are highly toxic to cultured cells and induce apoptosis. Additionally, human α -Syn fibrils were more toxic than oligomers when co-injected with a recombinant adeno-associated viral vector to express human α -Syn into rat brains (Peelaerts et al., 2015a).

Nowadays, the aggregation/accumulation of α -Syn is considered an unquestionable process and a hallmark of PD. Therefore, questions like what upstream factors could influence α -Syn aggregation, have become the focus of intense investigation.

1.4 Contributing factors to α -Syn aggregation

Since mutations in the SNCA locus are responsible for familial cases of PD, one can imagine that such mutations might contribute to the aggregation of α -Syn. In line with this, *in vitro* studies have revealed that the PD-related A53T, A30P, E46K and H50Q point mutations enhance α -Syn oligomerization and fibril formation (Conway et al., 1998), (Narhi et al., 1999), (Pandey et al., 2006), (Khalaf et al., 2014). On the other hand, the level of α -Syn in the brain is tightly controlled by a balance of synthesis and degradation. An imbalance between these processes should result in abnormal levels of α -Syn that might favour the formation of aggregated species. Indeed, in some familial forms of PD, duplications or triplications of the SNCA locus result in an increased accumulation of α -Syn, probably because of an increased protein expression (Singleton et al., 2003) (Chartier-Harlin et al., 2004). Furthermore, the overexpression of α -Syn induces aggregation in some animal models of PD (Outeiro, 2003), although it does not always result in an aggregative response and it heavily depends on the promoters used to drive transgene expression, as well as the model organism (Dawson et al., 2010).

On the degradation side, there are some reports implicating the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP) in the clearance of α -Syn (Webb et al., 2003). Interestingly, both processes seem to be implicated in PD pathophysiology, as it will be reviewed in the section “cell-autonomous mechanisms of neuronal death” (Michel et al., 2016).

By contrast, in sporadic forms of PD, in which protein accumulation cannot be explained by point mutations or gene duplications and triplications, some alternative contributing factors must intervene. Among these contributing factors, a special interest has been devoted to study the influence of posttranslational modifications of α -Syn. It is well known that α -Syn undergoes numerous of these modifications including phosphorylation, ubiquitination, nitration and truncations, among others. These modifications can influence the aggregation and toxicity of α -Syn in experimental settings. However, how a specific modification or a combination of several modifications would contribute to disease mechanisms is still unclear.

Phosphorylations are among the most studied posttranslational modifications of α -Syn and, over the past decade, a growing interest has been focused on the phosphorylation at serine 129 (Ser129) (Fujiwara et al., 2002). This modification is particularly interesting because around 90% of α -Syn deposited in LB is phosphorylated at this residue, whereas only a small fraction (<4%) of total α -Syn is phosphorylated in a normal brain, suggesting that it may play an important role in regulating α -Syn aggregation and LB formation (Popova et al., 2015). Despite the extensive research, the exact implication of Ser129 phosphorylation on α -Syn aggregation and toxicity *in vivo* still remains under debate, because the results in different model systems are controversial (Tenreiro et al., 2014). It has been reported that in a test tube, using phosphorylation mimicking α -Syn mutants (Ser129E/D), Ser129 phosphorylation negatively modulates fibrillogenesis (Paleologou et al., 2008). However, this observation has not been validated *in vivo* and it seems that Ser129 phosphorylation is not required for the formation of intracellular LB-like inclusions, since the addition of truncated α -Syn¹⁻¹²⁰ PFFs (lacking amino acid 129) into cells stably expressing phosphorylation-incompetent α -Syn^{S129A} (S129 replaced by alanine: S129A), can still induce the formation of inclusions (Luk et al., 2009b).

Another rather common modification of α -Syn is nitration at tyrosine residues (Y39, Y125, Y133 and Y136). Nitration of α -Syn is enhanced under conditions of elevated oxidative stress. On the other hand, increasing evidences have suggested that oxidative stress plays a major role in PD pathophysiology. Therefore, this could partly explain the high levels of nitrated α -Syn found in Lewy bodies (Giasson et al., 2000). Interestingly, the exposition of α -Syn to nitrating agents results in the formation of nitrated α -Syn dimers, trimers and oligomers, stabilized through the formation of dityrosine bonds (Souza et al., 2000). Additionally, although purified nitrated α -Syn monomers are not able to form fibrils, nitrated dimers accelerate the fibrillation of unmodified α -Syn (Hodara et al., 2004).

C-terminal truncation is an additional common posttranslational modification of α -Syn found in PD brains. Indeed, it has been reported that in patients, it constitutes up to 15% of the α -Syn present in LB (Baba et al., 1998). Several recombinant C-terminal truncated forms of α -Syn have been shown to be more prone to aggregate *in vitro* (Serpell et al., 2000), (Murray et al., 2003). Also, a transgenic mouse line with an α -Syn null background and expressing human α -Syn¹⁻¹²⁰ under the control of the rat tyrosine hydroxylase (TH) promoter, presents pathological inclusions in the SNpc and the olfactory bulb (Tofaris et al., 2006). Moreover, the co-expression of human α -Syn and human α -

Syn¹⁻¹¹⁰ in rat midbrain using recombinant adeno-associated virus vectors exacerbates the accumulation of human α -Syn and leads to dopaminergic cell death. (Ulusoy et al., 2010).

While major efforts have been spent to understand the role of α -Syn posttranslational modifications, a deeper understanding of the interplay between these modifications and its aggregation/toxicity is still missing. This will be essential to contribute to the development of future therapeutics for PD and other synucleinopathies aiming at targeting the aggregation and toxicity of α -Syn. However, in this respect some precautions must be taken since it is currently unclear whether aggregation of α -Syn into high molecular weight species to ultimately form LB or LN is harmful or neuroprotective. Although Lewy pathology has been traditionally considered as detrimental to neuronal survival, in the recent years the hypothesis that the formation of LB and LN is an attempt of neurons to sequester toxic α -Syn assemblies, has gained considerable acceptance (Olanow et al., 2004).

Although the current view is that Lewy pathology appears early in the disease and spreads in synaptically coupled brain networks driving neuronal dysfunction and death, some reports are inconsistent with this simple model. Indeed, post-mortem analysis of human brains and connectome-mapping studies in rodents show that the pattern of Lewy pathology in PD is not entirely driven by trans-synaptic transmission of pathological α -Syn. Moreover, the correlation between Lewy pathology and neuronal death is rather weak. Thus, a central question arises from this set of paradox observations: if the patterns of Lewy pathology and neuronal death in PD are not simply a consequence of the spreading of misfolded α -Syn through neuronal networks, then what governs them? One current concept proposes that cell-autonomous (neuronal) determinants such as bioenergetic control and proteostatic capacities are likely important. Nonetheless, given the ability of many neurons to tolerate Lewy pathology for decades, it is apparent that the emergence of α -Syn pathology alone does not necessarily cause cell death. Therefore, cellular context must be important and, in that respect, non-cell autonomous mechanisms may also play a critical role by creating a tipping point beyond which neurodegeneration occurs. In the past years, a great deal of effort has been dedicated to investigate cell-autonomous and non-cell autonomous mechanisms involved in neuronal death. Most of the studies have used animal models of the disease, therefore before describing these mechanisms, it is essential to quickly review some of the most used models in the field of PD.

1.5 Animal models of PD

A large array of models is available in the field of PD: from invertebrate organisms like *Drosophila*, to species closer to humans, like macaque and squirrel monkey. The experimental models can be classified into two categories: neurotoxic or genetic, and sometimes a combination of both. In this section, a special emphasis on mammalian models will be placed. It should be stated that none of the PD models established to date can precisely phenocopy the disease, and since they are just models of an extremely complex pathology, the success of a particular research will depend on the appropriate question being asked to the optimal model for addressing that question.

1.5.1 Neurotoxic models

One of the most important and frequently used neurotoxins to induce parkinsonism is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), administered to primates and mice (rats being resistant to this toxin when administered peripherally). This molecule was identified in the late 80's as the agent causing a clinical picture almost indistinguishable from PD, manifested by drug addicts that self-administered a synthetic opioid that was contaminated with MPTP. Both in primates and mice, MPTP causes nigrostriatal degeneration and neuroinflammation, but no obvious Lewy pathology. MPTP is highly lipophilic and after systemic administration rapidly crosses the blood-brain barrier (BBB). Once into the brain, it is metabolized by MAO-B expressed by glial cells to produce MPP⁺, the active metabolite taken up later by neurons through dopamine transporter (DAT). This explains the selective toxicity against DNs. Inside the neuron, MPP⁺ inhibits the mitochondrial complex I leading to the release of reactive oxygen species (ROS), as well as decreased ATP production (Blesa et al., 2012).

Before MPTP, a different neurotoxin was used to induce degeneration of the nigrostriatal pathway in rats: 6-hydroxydopamine (6-OHDA). It happened 50 years ago and it was the first neurotoxin ever used for this purpose. The chemical structure of 6-OHDA has similarities to that of dopamine, which explains the high affinity of 6-OHDA for catecholaminergic transporters such as DAT. The only structural difference with dopamine is the presence of an additional hydroxyl group that makes it toxic to DNs through a combination of ROS and quinone production (Blesa et al., 2012). Since 6-OHDA cannot cross the blood-brain barrier, it has to be injected directly into the brain at

different levels of the nigrostriatal pathway (SNpc, striatum or medial forebrain bundle) (Blandini et al., 2008).

Although 6-OHDA and MPTP are the most used neurotoxins to induce PD-like neurodegeneration, some other neurotoxic agents have been employed as well, including paraquat and rotenone, which are commonly used as pesticides. All the toxin-induced models possess the ability to stimulate the production of oxidative stress and to cause degeneration of DNs. However, only paraquat and rotenone have been reported to additionally induce the aggregation of α -Syn in rodents (Manning-Bog et al., 2002; Sherer et al., 2003), even though these results have not been widely reproduced.

1.5.2 Genetic models

In the past decade, many genetic mouse models have been developed based on genetic mutations identified in monogenic forms of PD. To date, various transgenic models have been established to induce the expression of human α -Syn and its mutated variants (A53T, A30P), as well as α -Syn knockouts and intracerebral injection of α -Syn toxic species. Despite the fact that some of these models cause a variety of abnormalities in the brain and spinal cord (e.g. mitochondrial dysfunction, gliosis, loss of motor neurons, α -Syn aggregation), they do not necessarily recapitulate DNs degeneration (Blesa et al., 2016). Besides transgenic models, viral vector-based α -Syn models have been developed in rats, mice and primates. In contrast to most of the α -Syn transgenic mice, viral-based models display more convincing α -Syn pathology and clearer dopaminergic neurodegeneration (Blesa et al., 2016).

Apart from α -Syn mutations, other genes responsible for PD have been identified: while LRRK2 (leucine rich repeat kinase 2) mutations cause autosomal dominant PD, PINK1 (PTEN-induced novel kinase 1), Parkin and DJ-1 mutations cause autosomal recessive PD (Klein and Westenberger, 2012). LRRK2 transgenic mice do not show overt gross neuropathological abnormalities, except when combined with a model that expresses A53T α -Syn (Lin et al., 2009). On the other hand, the models based on knocking out the genes for Parkin, PINK1 or DJ-1 display some mitochondrial dysfunction, however they all failed to replicate dopaminergic degeneration (Duty and Jenner, 2011).

Overall, it seems difficult to fully recapitulate the complexity of the human disease in a single model. Obviously, each model has advantages and disadvantages and while

toxic models offer some of the hallmarks of the disease, particularly a clear nigrostriatal degeneration, some genetic models offer alternative pathological features such as Lewy-like pathology.

Together with clinical and post-mortem studies in humans, preclinical models have been essential to understand the mechanisms underpinning PD. Obviously, understanding these mechanisms is key to find new neuroprotective therapeutic strategies. In the next section, some of the cell-autonomous mechanisms will be described (Figure 3), followed by the non-cell autonomous mechanisms, which are the main focus of this thesis work.

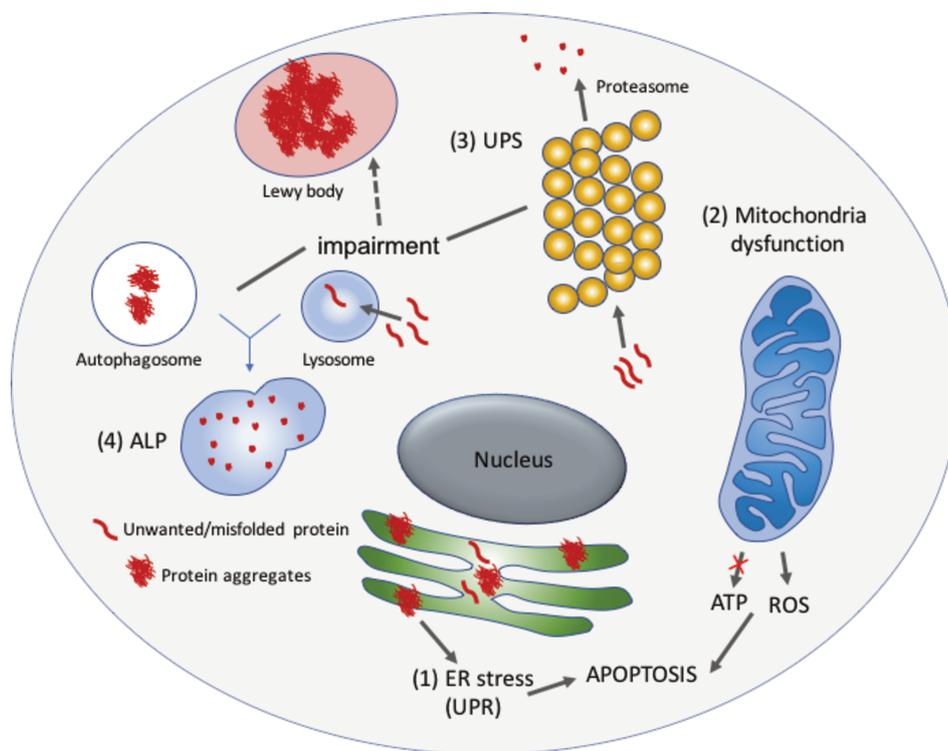


Figure 3. Autonomous mechanisms of neuronal death in PD. Neurodegeneration in PD seems to occur as the result of several cellular systems dysfunction, including endoplasmic reticulum (ER), Ubiquitin-Proteasome System (UPS), mitochondria and the autophagy-lysosome pathway (ALP).

Scheme:

(1) ER stress: The accumulation of misfolded/unfolded proteins in the endoplasmic reticulum results in the activation of an adaptive stress pathway, known as the unfolded protein response (UPR). Under chronic stress conditions, the UPR can promote apoptosis.

(2) Mitochondria dysfunction: Defective mitochondrial respiration has long been associated with the pathogenesis of PD. The consequences of mitochondria deficiency are a reduction in ATP synthesis and an increased production of reactive oxygen species (ROS), ultimately leading to apoptosis.

(3 and 4) UPS and ALP impairment: UPS and ALP are the most important machineries responsible for protein degradation. Aberrant protein homeostasis leads to the toxic accumulation of intracellular proteins, detrimental to neuronal survival.

1.6 Cell-autonomous mechanisms of neurodegeneration in PD

1.6.1 Autophagy-lysosomal pathway (ALP) alterations

Autophagy is a dynamic process involved in the degradation of damaged organelles and misfolded/aggregated proteins. It can be classified as macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), depending on the way the cellular components are being delivered to lysosomes for degradation (Klionsky et al., 2016).

Macroautophagy is the process by which double membrane structures called autophagosomes engulf cytosolic cargo like organelles or protein aggregates and then fuses with lysosomes to allow the cargo to be degraded by proteolytic enzymes. It has been reported that in post-mortem tissue from PD patients, markers of autophagosomes and lysosomes are altered within DNs, indicating that macroautophagy is deeply altered. Also, the overexpression of α -Syn impairs macroautophagy both *in vitro* but also in transgenic mice expressing wildtype human α -Syn under the control of the prion protein promoter (Winslow et al., 2010). Additionally, α -Syn inclusions induced by PFFs of α -Syn cannot be efficiently degraded and cause a partial impairment of autophagosome clearance (Tanik et al., 2013).

On the other hand, CMA is a selective type of autophagy that requires chaperone heat shock cognate protein 70 (HSC70) to recognize proteins that have a KFERQ consensus sequence, and to translocate them into the lysosomes for degradation, via lysosome associated membrane protein type 2A (LAMP2A). The expression of both LAMP2A and HSC70 is significantly decreased in the SNpc and amygdala of PD brains (Alvarez-Erviti et al., 2010). Interestingly, while LRRK2 can be degraded by CMA,

pathogenic mutants as well as high concentrations of wild-type LRRK2 can inhibit it by a mechanism that blocks the formation of the CMA translocation complex at the lysosomal membrane (Orenstein et al., 2013). One of the consequences of inhibiting CMA is that the degradation of α -Syn, also a substrate of CMA (Cuervo et al., 2004), is compromised. This increases the burden in α -Syn and as a consequence, aggravates Lewy pathology. Furthermore, A53T and A30P α -Syn bind LAMP2A more tightly than wild-type α -Syn, preventing degradation and cargo loading of other CMA substrates into lysosomes (Cuervo et al., 2004), hence massively disturbing protein CMA machinery.

Interestingly, genome-wide association studies (GWAS) have identified many genes involved in several functions of the ALP associated with PD. Some of these genes encode for lysosomal enzymes like beta-glucocerebrosidase (*GBA gene*) (Gan-Or et al., 2015). Additional genes include ATP13A2 (Ramirez et al., 2006), which encodes for the transmembrane lysosomal protein P5-type, and whose deficiency impairs degradation of lysosomal substrates leading to α -Syn accumulations (Usenovic et al., 2012). Mutations in the gene encoding for the protein VPS35 are also associated with PD (Vilariño-Güell et al., 2011). VPS35 is a protein required for endosome-to-Golgi recycling of membrane proteins, including LAMP2A (a member of the CMA) and M6PR protein, responsible for transporting many of the lysosomal hydrolases to the lysosome (Bonifacino and Rojas, 2006).

1.6.2 Endoplasmic reticulum (ER) stress

The endoplasmic reticulum is essential for protein folding, trafficking to the Golgi, calcium buffering and the unfolded protein response (UPR). UPR is a complex physiological protective response that has evolved to maintain a productive ER protein-folding environment. Perturbed protein folding leading to the accumulation of misfolded proteins causes ER stress and results in UPR activation. Under chronic ER stress conditions, sustained UPR activity can trigger the activation of pro-apoptotic pathways (Hetz, 2012). The immunoreactivity for UPR activation markers (pPERK and peIF2 α) has been detected in DN containing LB within the SNpc of PD brains, but not in the brains of control individuals, suggesting that the UPR may be involved in PD (Hoozemans et al., 2007). This is not surprising considering that misfolded proteins and aggregates are the main activator of the UPR. Indeed, it has been reported that α -Syn accumulates within

the ER of PD brains (Colla et al., 2012). Furthermore, UPR activation has also been seen in mammalian and invertebrate models of synucleinopathy (Matus et al., 2011).

1.6.3 Ubiquitin-proteasome system (UPS)

The UPS is the main cellular pathway to degrade and remove damaged and unwanted proteins. It consists of two separate and consecutive steps: ubiquitination and proteasome degradation. The ubiquitination of proteins, which is the transfer of a 76 amino acids polypeptide, is an ATP-dependent process that requires the concerted action of three general families of ubiquitin enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3). Multiple forms of ubiquitination exist, with different functions, however it is polyubiquitination that acts as a signal for degradation by the proteasome, the proteolytic arm of the system (Dantuma and Bott, 2014). Dysregulation of the UPS has been implicated in the pathogenesis of PD. Indeed, post-mortem data obtained from sporadic PD cases suggest that the expression of some proteasome subunits as well as proteasomal enzymatic activity are reduced in the SN (Bukhatwa et al., 2010; McNaught and Jenner, 2001). Interestingly, rotenone and MPTP have been reported to negatively influence the function of the UPS (Betarbet et al., 2006; Zeng et al., 2006). Also, mutated or aggregated forms of α -Syn can bind to and impair the activity of the proteasome *in vitro* (Emmanouilidou et al., 2010; Stefanis et al., 2001). Furthermore, *parkin*, another gene responsible for familial PD, codes for an E3 enzyme, strongly suggesting that pathological mutations would very likely affect the UPS (Giasson and Lee, 2001). Therefore, the main consequence of a dysfunctional UPS would be the over-accumulation of misfolded α -Syn, in turn leading to enhanced aggregation.

1.6.4 Mitochondria dysfunction

Mitochondria is an essential organelle for ATP synthesis, calcium storage, lipid metabolism and neuronal survival. Therefore, damage to this organelle has disastrous consequences such as bioenergetics failure, ultimately leading to apoptosis. The first evidence suggesting that mitochondria plays a role in PD pathogenesis was indeed the accidental intoxication with MPTP that suffered drug addicts in the 80's (see section: animal models of PD). Intoxicated people developed an irreversible neurodegenerative condition with clinical and histological features very similar to sporadic PD. The scientific community came to the conclusion that it was due to the inhibition of mitochondrial complex I caused by MPP⁺, the product of MPTP metabolism by MAO-B in glial cells.

MPTP and some other complex I inhibitors like rotenone can induce neurodegeneration of DNs in flies, rodents and humans, implicating mitochondrial dysfunction in PD pathogenesis (Betarbet et al., 2000; Gash et al., 2008). A reduced activity of mitochondrial complex-I has been reported in the SNpc of PD patients, but also in platelets and skeletal muscle (Beal, 2005). Similarly, reduced complex-I activity and an increased vulnerability to MPP⁺ were observed in cybrid cell lines that contain mitochondrial DNA (mtDNA) from PD patients (Swerdlow et al., 1996). This observation suggests that mtDNA might encode or accumulate mutations linked with PD. Indeed, genetic studies have suggested that mutations in mtDNA also play a role in the pathogenesis of PD. A point mutation in a subunit of mitochondrial complex-I was identified in a family with atypical PD (Simon et al., 1999). Moreover, a mutation in mitochondrial DNA polymerase gamma has been identified in five families with idiopathic sporadic PD (Luoma et al., 2007). Finally, loss-of-function mutations in the genes encoding PINK1 and Parkin mediate autosomal recessive PD, as mentioned. Interestingly, both proteins play important roles in mitochondrial quality control, a pathway required to identify and discard dysfunctional mitochondria (Narendra et al., 2012). On the other hand, toxic α -Syn might disrupt mitochondrial functions, since mice expressing A53T α -Syn manifest mtDNA damage and increased mitochondrial autophagy (mitophagy) (Choubey et al., 2011; Martin et al., 2006). Moreover, using phosphomimetic α -Syn mutants, it was shown that posttranslational modifications, but also oligomers, may alter mitochondrial function by binding with high affinity to the receptor TOM20 (translocase of the outer membrane 20), required for mitochondrial protein import (Maio et al., 2016).

In summary, although neuronal cell death mechanisms in PD are still incompletely defined, defects in several cellular pathways have been implicated, including altered protein degradation via ALP and UPS, mitochondrial dysfunction and ER stress. The sustained failure of proper protein homeostasis leads to the accumulation of α -Syn. Toxic α -Syn can in turn induce damage of ALP and UPS and consequently facilitate further α -Syn accumulation and aggregation. Additionally, protein aggregation causes ER stress and the activation of the UPR, eventually leading to apoptosis, which can also occur as a consequence of a bioenergetic failure resulting from mitochondria dysfunction. Therefore, it is likely that very complex interactions between these biological processes regulate DNs function and death.

However, as it has been mentioned above, apart from dysregulated processes happening inside DNs, neuronal death can be influenced by external factors derived from the interaction of DNs with non-neuronal cells. These pathological processes, often referred as non-cell-autonomous, will be developed in the next section.

1.7 Non-cell autonomous mechanisms of neurodegeneration in PD

The mechanisms of cell death presented in this section originate from the interaction of DNs with glial cells (e.g. brain macrophages and astrocytes) and peripheral immune cells (e.g. lymphocytes and monocytes), which are grouped together under the term of neuroinflammatory mechanisms.

1.7.1 Evidences for neuroinflammatory mechanisms in PD

Several lines of evidence show that neuroinflammatory mechanisms indeed occur in PD. For instance, GWAS have led to the identification of genetic markers linking PD and inflammation. Indeed, an association was evidenced between PD and the human leukocyte antigen (HLA) region, which is a group of genes encoding the proteins of the major histocompatibility complex (MHC) in humans. The association was strong in sporadic and late-onset cases and the variant most strongly associated with PD was rs3129882 in intron 1 of HLA-DRA, believed to impact the expression of HLA-DR and HLA-DQ (Hamza et al., 2010). Moreover, epidemiological studies have suggested that the chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a delay or prevention of PD onset (Chen et al., 2003). However, different studies found that the lower risk of PD was associated only to ibuprofen, but not to aspirin nor other NSAIDs, raising the question of whether the association observed was due to some specific effects of ibuprofen (Chen et al., 2005; Gao et al., 2011). On the other hand, at the molecular level, several inflammatory mediators have been found in the striatum of PD brains, including tumor necrosis factor α (TNF α), epidermal growth factor (EGF), transforming growth factor α (TGF α), TGF β 1, interleukin 1 β (IL-1 β), IL-2 and IL-6. The following cytokines were also identified in the SNpc: TNF α , IL-1 β , and interferon γ (IFN γ). Some of these inflammatory mediators can also be found in the cerebrospinal fluid (CSF) of patients with PD: TNF α , IL-1 β and IL-6 (Hirsch and Hunot, 2009).

1.7.2 Microglia in PD

At the cellular level, there are several reports showing that microglia, the most abundant macrophage of the central nervous system (CNS), might play a role in the pathogenesis of PD. Almost 30 years ago it was first reported that microglia was activated in the SNpc of PD brains. This was assessed by analysing the expression of HLA-DR, a member of the MHC class II cell surface receptor (McGeer et al., 1988). This observation was confirmed by others, using different markers such as CD68 and CD11b (Croisier et al., 2005; Ghosh et al., 2007). Moreover, microglia activation has been reported in living patients through positron emission tomography (PET) studies using radiolabelled PK11195, which is a ligand for the mitochondrial translocator protein (TSPO), highly expressed during microglial activation. In the normal brain, there is a low binding of PK11195, however it increases following neuronal injury due to TSPO expression in microglia. It has been shown that in 18 PD patients there is an increased PK11195 binding in the striatum, thalamus, pons, frontal and cingulate cortex, as compared to 11 controls (Gerhard et al., 2006). This result was recently confirmed in early stages of the disease by a study that reported an increased binding of PK11195 in the SN and the putamen. The study also included patients with DLB, which showed extensive cortical microglial activation in addition to subcortical changes (Iannaccone et al., 2013). The most straightforward evidences showing a link between microglia and PD pathophysiology originate from observations described in animal models and in cell culture experiments. Indeed, microglia activation has been seen in several models of PD, including neurotoxic models like MPTP (Brochard et al., 2008) and 6-OHDA (Akiyama and McGeer, 1989), but also in genetic models of α -Syn toxicity, either transgenic- or viral-based ones (Chung et al., 2009; Lin et al., 2012; Theodore et al., 2008), recapitulating post-mortem observations of PD brains. In these models, microglia activation is often present before detectable neuronal dysfunction or death occurs. Remarkably, the administration of anti-inflammatory compounds reduce microglia activation and neuronal damage in different models of PD (Sanchez-Guajardo et al., 2013). For instance, the administration of minocycline, a tetracycline derivative, inhibits microglial activation and mitigates the demise of nigrostriatal DNs both in the MPTP and 6-OHDA models (He et al., 2001; Wu et al., 2002). Yet, these results have not been widely replicated and there are some reports showing that minocycline might enhance MPTP-induced degeneration (Sanchez-Guajardo

et al., 2013; Yang et al., 2003). These contradictory effects of minocycline may depend on dose and frequency of administration.

Based on the observations from PD patients and animal models, the implication of microglia in the pathogenesis of the disease seems likely. However, how can this cell population drive cell death? One has to keep in mind that, as part of the innate immune response of the CNS, microglia represents the first line of defence against microorganism invasion and injury. However, in sterile inflammation conditions (in the absence of infection), microglia response is similar to the one induced by microorganisms and, as a consequence, it leads to the release of cytotoxic factors (e.g. TNF α , IL-1 β , ROS). Therefore, those factors aimed at destroying the invading pathogen, can enhance neurodegeneration. For instance, activated microglia through the activity of NADPH oxidase (Nox) and inducible nitric oxide synthase (iNOS) can produce superoxide and nitric oxide free radicals, respectively. Both free radicals can combine to form peroxynitrite, which is highly reactive and triggers nitrative modifications of TH (required for the synthesis of catecholamines like dopamine), affecting its enzymatic activity. Peroxynitrite can also induce the nitration of α -Syn, possibly potentiating its aggregative response, as it was mentioned before (Hirsch and Hunot, 2009). Therefore, microglia response might influence α -Syn toxicity. Interestingly, α -Syn can also have an impact on microglial response. Indeed, it has been recently demonstrated that α -Syn, which can act as a chemoattractant to promote microglia migration (Wang et al., 2015), can also directly activate microglia by acting as an endogenous agonist for toll like receptor 2 (TLR2) (Kim et al., 2013). Also, oligomeric forms of α -Syn can elicit a proinflammatory response *in vitro* through the activation of the heterodimer TLR1/2. This leads to the nuclear translocation of nuclear factor κ B (NF- κ B) and the increased production of TNF α and IL-1 β in a myeloid differentiation primary response gene 88 (MyD88)-dependent manner (Daniele et al., 2015).

Most of the evidences regarding microglia activation indicate that it might negatively influence dopaminergic survival, however since phagocytosis is required for the elimination of dead neurons and the clearance of tissue debris, potentially neuroprotective effector functions should not be ruled-out, especially when designing therapeutic strategies aiming at completely inhibiting microglia response. Ideally, therapeutic strategies should target specific detrimental functions while maintaining or even boosting beneficial ones.

1.7.3 Astrocytes in PD

Some studies show that astrocytes might also play a role in the pathophysiology of PD. A 30% increase of astrocytes has been reported in the SNpc of PD brains at post-mortem using the markers gliofibrillary acidic protein (GFAP) or glutathione peroxidase (Damier et al., 1993). Evidence of astrocytic activation has also been found in PD models. Indeed, reactive astrocytes have been described both in the 6-OHDA and the MPTP model, and characterized by an increased expression level of GFAP and a hypertrophic cell body (Parillaud et al., 2017; Rodrigues et al., 2001). On the other hand, high concentrations of extracellular α -Syn have been shown to induce a TLR4-dependent inflammatory response in primary astrocyte cultures (Fellner et al., 2013). Furthermore, astrocytes can uptake α -Syn released by neurons via endocytosis, leading to increased expression of pro-inflammatory cytokines and chemokines including IL-1, IL-6, and TNF α . (Lee et al., 2010). Although further studies are needed to assess the role played by astrocytes, the current opinion is that they might exert both protective and detrimental functions. Through the production of antioxidant molecules like glutathione, but also neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), astrocytes might protect neurons from dying. However, reactive astrocytes can also release pro-inflammatory cytokines, therefore potentially enhancing dopaminergic cell death (Hirsch and Hunot, 2009).

1.7.4 Engraftment of lymphocytes in PD

Regarding lymphocytic infiltration, the presence of cytotoxic T lymphocytes (CD8+) has been identified in the SNpc from one PD patient at post-mortem (McGeer et al., 1988). This result was later confirmed in additional cases of PD, but more importantly, the presence of CD4+ T cells was additionally reported. No B cells nor natural killer cells were identified (Brochard et al., 2008). As it was mentioned, GWAS have shown the association of PD with alleles of the MHC. The function of MHC is to bind and present antigens to T cell receptors, thereby triggering an adaptive immune response. Interestingly, it has been recently demonstrated that two α -Syn-derived peptides are recognized by and activate T cells from PD patients, but rarely from healthy controls. This opens up the possibility of an autoimmune response underlying PD (Sulzer et al., 2017). The same group showed that DNs from the SNpc express MHC class I, suggesting that they may display antigens and therefore rendering them selective targets for T-cell mediated death (Cebrián et al., 2014). The presence of lymphocytes has been described in different PD models as well. In the MPTP model, both CD4+ and CD8+ T cells have been identified

(Kurkowska-Jastrzebska et al., 1999). The authors demonstrated that infiltrating lymphocytes express leukocyte function-associated antigen-1 (LFA-1) protein, which is an integrin that binds to intercellular adhesion molecule-1 (ICAM-1) expressed by activated endothelial cells to facilitate the diapedesis across blood vessels. Indeed, in the same study ICAM-1 expression by endothelial cells was also described. Interestingly, the infiltration of CD4⁺ and CD8⁺ lymphocytes into the SNpc was confirmed in a different study, also using the MPTP model (Brochard et al., 2008). In this second study, no B lymphocytes were found, suggesting that adaptive immune cell infiltration is selective for T cells. Furthermore, T cells were found within the SNpc as soon as two days and until seven days after MPTP intoxication. In the same study, MPTP induced neurodegeneration of DNs was attenuated in immunodeficient mice that lack mature lymphocytes (Rag1^{-/-} and Tcrb^{-/-} mice), revealing the potentially detrimental role of the adaptive immune system in PD pathogenesis. Moreover, mice lacking CD4⁺ T-cells, but not those lacking CD8⁺ T-cells, are also protected in the MPTP model, suggesting that most of the deleterious outcome associated with infiltrated T cells is mediated by CD4⁺ T-cells. Interestingly, the toxicity displayed by CD4⁺ T cells requires the expression of FasL but not IFN γ , since reconstitution of Rag1^{-/-} mice with FasL-mutant splenocytes mitigates DNs death, while reconstitution with IFN γ ^{-/-} splenocytes did not. On the other hand, MPTP-induced neurodegeneration is aggravated when T cells polarized to Th17 (T helper 17: IL-17 producing) or Th1 (T helper 1: IFN γ -producing) phenotypes obtained from mice immunized with nitrated α -Syn, are adoptively transferred into wild-type mice. In contrast, neurodegeneration was not exacerbated by adoptive transfer of T cells polarized to Th2 (T-helper 2: IL-4 producing) phenotype (Reynolds et al., 2010). Finally, adoptive transfer of *ex vivo* differentiated regulatory T cells into MPTP treated mice attenuated neurodegeneration and microglia activation (Reynolds et al., 2007). The novelty of this study is that it shows that by modulating the adaptive immune response, it is possible to improve the outcome of the neurodegenerative process in a PD model.

1.7.5 Engraftment of blood-derived monocytes in PD

Apart from lymphocytes, some studies have attempted to assess whether circulating monocytes infiltrate the degenerating SN. The main complication of studying monocytic infiltration is the technical challenge to differentiate them from brain-resident myeloid cells. Indeed, there are no specific antibodies working adequately in tissue-sections that specifically identify infiltrating monocytes. Yet, by using different paradigms of MPTP

intoxication, several groups have demonstrated that blood-derived monocytes can infiltrate the degenerating SN (Depboylu et al., 2012; Kokovay and Cunningham, 2005; Rodriguez et al., 2007). To overcome the lack of specific markers, all these studies used GFP-expressing bone marrow chimeric mice. However, as we shall see further in the following sections, it has now become clear that the use of bone marrow chimeras leads to the artefactual extravasation of peripheral myeloid cells. This is essentially due to whole body irradiation protocols followed by bone marrow transplantation usually used to establish such chimeras (Ajami et al., 2007a; Mildner et al., 2007). Therefore, it should be confirmed using alternative approaches whether or not monocytes can gain access to the brain parenchyma, and, if so, determine what role they might play in PD pathogenesis. Very recently, a reporter mouse for CCR2 has been developed and characterized (Serbina et al., 2009). CCR2 is a chemokine receptor expressed by inflammatory monocytes, which are CCR2⁺Ly6C^{hi} and different from “patrolling” monocytes, which are CX3CR1⁺Ly6C^{lo} (Shi and Pamer, 2011). This new tool was useful to determine that microglial cells do not express CCR2 and therefore it can be used to identify infiltrating monocytes. Using this reporter mouse, it has been shown that early, yet limited nigral monocyte infiltration occurs after MPTP intoxication (Parillaud et al., 2017). However, blocking monocyte infiltration through CCR2 deletion had no influence on DNs survival, suggesting that either infiltrated monocytes are not deleterious or that the number of infiltrated cells is too low to reveal any pathological potential in this disease model. Remarkably, transgenic over-expression of CCL2 (a ligand for CCR2) by astrocytes not only increases CCR2⁺ monocyte infiltration after MPTP intoxication, but also exacerbates neurodegeneration, revealing the potential detrimental role of infiltrating monocytes under nigrostriatal pathway injury (Parillaud et al., 2017). To date, whether monocytes are present in affected brain regions in the human condition remains unknown. Interestingly, higher CCL2 levels as well as increased blood-derived inflammatory monocytes have been described in PD patients (Grozdanov et al., 2014). This suggests that infiltrating monocytes could be relevant for the human disease, although the final confirmation is still missing.

In summary, inflammation caused by brain damage can aggravate a pathological situation. Indeed, activated glial cells can produce inflammatory mediators that trigger BBB alteration. This in turn may facilitate the influx of not only immune cells from the periphery, but also toxic blood-derived factors that could worsen neuronal injury and enhance or perpetuate inflammation, engaging a positive-feedback loop of

neurodegeneration. This model has been considered as a reasonable explanation for the progressive nature of PD (Figure 4).

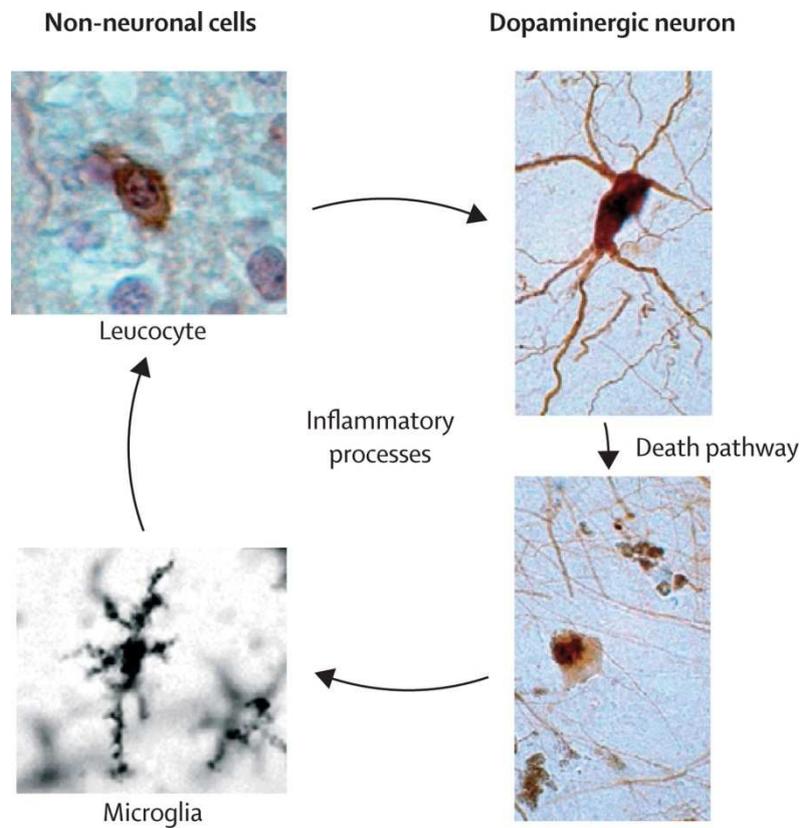


Figure 4. A vicious circle of neurodegeneration in PD. Neuronal distress and death leads to an inflammatory response mediated by activated glial cells and infiltrated lymphocytes. This response is accompanied by the subsequent production of proinflammatory cytokines and neurotoxic molecules that will influence the course of the disease, entering in a vicious circle of degeneration. Scheme from (Hirsch and Hunot, 2009)

The presence of peripheral immune cells inside the brain parenchyma in a region-specific manner is the result of a concerted action of molecular events involving the production of cytokines that can induce BBB alterations, including the expression of adhesion molecules by endothelial cells. Adhesion molecules are required for the diapedesis of circulating leukocytes that follow a gradient of chemokines released by suffering neurons and activated glia. However, apart from the observation of blood-derived cells within the degenerating SN, there are several reports suggesting that BBB remodelling occurs in PD.

1.8 Evidences of blood-brain barrier remodelling in PD

The BBB is a diffusion barrier important for the normal function of the CNS. It is comprised of specialized endothelial cells joined continuously by tight junction complexes (Reese and Karnovsky, 1967). It limits the entrance of hydrophilic as well as high molecular weight molecules, potentially toxic plasma components, circulating cells and pathogens. In contrast, small lipophilic molecules like O₂ and CO₂ can freely cross plasma membranes following concentration gradients. Vascular endothelial cells also express several ATP dependent transport systems to dynamically regulate the influx of nutrients and efflux of metabolic waste and toxins between the blood and the brain parenchyma. The formation, function and maintenance of the BBB require the coordinated activity of multiple cell types other than endothelial cells, including mural cells (pericytes and smooth muscle cells), glial cells (astrocytes and microglia), neurons and **perivascular macrophages**. BBB endothelial cells together with all other cells that regulate them, are collectively referred to as the neurovascular unit (NVU) (Zhao et al., 2015) (Figure 5).

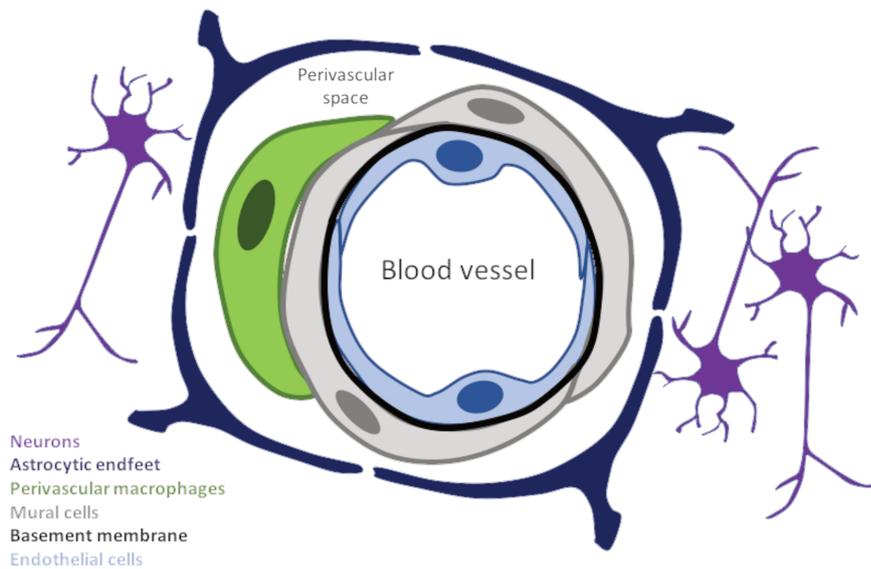


Figure 5. The neurovascular unit (NVU). Endothelial cells connected by junctional complexes (tight, adherens and gap junctions) are in close proximity with mural cells (essential to control the blood flow) and perivascular macrophages. Endothelial cells basement membrane and the *glia limitans* (formed by astrocytic endfeet) delineates the perivascular space. In close contact with astrocytic endfeet there are neuronal cells that also integrate and regulate the NVU.

In PD patients, there are some evidences indicating that the BBB is partially impaired. For instance, the function of the P-glycoprotein, an efflux pump that removes multiple drugs and toxins from the brain, has been shown to be compromised in the midbrain of 5 PD patients as compared to controls, through PET measurements of ^{11}C -verapamil uptake, a ligand of P-glycoprotein (Kortekaas et al., 2005). However, this finding could not be replicated in a similar study that included 10 PD patients enrolled at early disease stage (Bartels et al., 2008a). In another study analysing 10 *de novo* PD patients (diagnosed in the past year and without PD medication) and 10 patients with advanced PD, it was reported that there is a reduced activity of the P-glycoprotein in frontal regions during later disease stage. Interestingly, an increased rather than decreased P-glycoprotein activity was also observed in the midbrain within the group of *de novo* patients, suggesting region-specific early compensatory mechanisms (Bartels et al., 2008b).

Moreover, the analysis of protein content on lumbar CSF showed that there is an increased transfer of albumin from the blood into the CSF (increased CSF/plasma albumin ratio), indicative of a possible permeability of the BBB and/or the blood-CSF barrier (BCSFB) (Pisani et al., 2012). The BCSFB is found at the choroid plexus (the structure that produces two-thirds of the CSF volume) and it is made of epithelial cells linked by tight junctions. Unlike the vessels that form the BBB, choroid plexus vessels are fenestrated and have no tight junctions (Engelhardt and Sorokin, 2009).

Besides increased BBB permeability, it was recently demonstrated that in three independent cohorts, PD patients displayed higher CSF levels of vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and sVEGFR-2 (soluble VEGF receptor-2) compared to controls, suggesting that angiogenesis might occur in PD. It is important to state that newly created vessels are immature and lack the restrictive properties of the BBB, meaning that angiogenesis could also be a “hallmark” of BBB dysfunction. Interestingly, the authors showed that higher levels of VEGF and PlGF were associated with increased CSF levels of neurofilament light subunit and monocyte chemoattractant protein-1 (MCP-1), a neurodegeneration and a glial activation marker, respectively (Janelidze et al., 2015). They also reported an increased CSF/plasma albumin ratio, validating the observations from Pisani and colleagues three years before.

Several post-mortem studies have also suggested that the BBB might play a role in PD pathophysiology. For instance, an increased number of endothelial cell nuclei in the SNpc was reported in PD patients compared to control individuals, suggesting disease-associated angiogenic processes (Faucheux et al., 1999). In line with this, the presence of integrin $\alpha\beta3$ reactive vessels, which is a sign of angiogenesis, was found in the SNpc, putamen and *locus coeruleus* (Bradaric et al., 2012). However, and contrasting with the above data, vascular degeneration at the level of capillaries has also been suggested to occur in the SNpc of PD patients by assessing the number, length, thickness and complexity of vessels (Guan et al., 2013). It is possible to speculate that an interplay between angiogenesis and vascular degeneration might occur, meaning that BBB properties are affected in either case. Moreover, electron microscopy studies revealed a thickening of the cerebral capillary basement membrane as well as an accumulation of collagen in PD patients as compared to age-matched controls (Farkas et al., 2000). Finally, a recent study shows a loss of integrity of the BBB in the striatum of patients with PD, by

detecting extravasation of erythrocytes, presence of perivascular hemosiderin deposits and leakage of the serum proteins haemoglobin and fibrin (Gray and Woulfe, 2015).

Altogether, these data suggest that the BBB is very likely altered in PD. Yet, besides these evidence, little is actually known about the implications of the BBB cellular residents and in particular, one of its immune components: perivascular macrophages (PVMs). Being the focus of the present thesis work, in the next section we will learn the current knowledge of PVMs in health and disease.

2. Perivascular macrophages

2.1 Physiology of perivascular macrophages

PVMs are a cell population strategically positioned within the perivascular space (also known as the Virchow-Robin space), enwrapped between endothelial cells and the *glia limitans* (Figure 5). More than two decades ago, the injection of particulate matter (Indian ink) into the striatum of rats allowed the identification of PVMs that readily engulfed the injected material (Zhang et al., 1992) and revealing their strong phagocytic capacities. It has been shown that in normal human brains, PVMs constitutively express MHC class II, suggesting that they represent one of the most potent antigen-presenting cell population in the brain (Graeber et al., 1992). In mouse, PVMs can be readily identified by the expression of the scavenger receptor CD206, a type I transmembrane C-type lectin that recognizes branched mannose-containing carbohydrate structures on both microbial and host proteins. Unlike CD163 which is expressed by PVMs but also by activated microglia, CD206 is exclusively expressed by PVMs in both physiological and in several inflammatory conditions (Galea et al., 2005), making it a very useful tool to specifically study this cell population. PVMs localize in the perivascular space deep inside the brain, but also in the meninges, where they are often referred to as meningeal macrophages (MMs) (Figure 6).

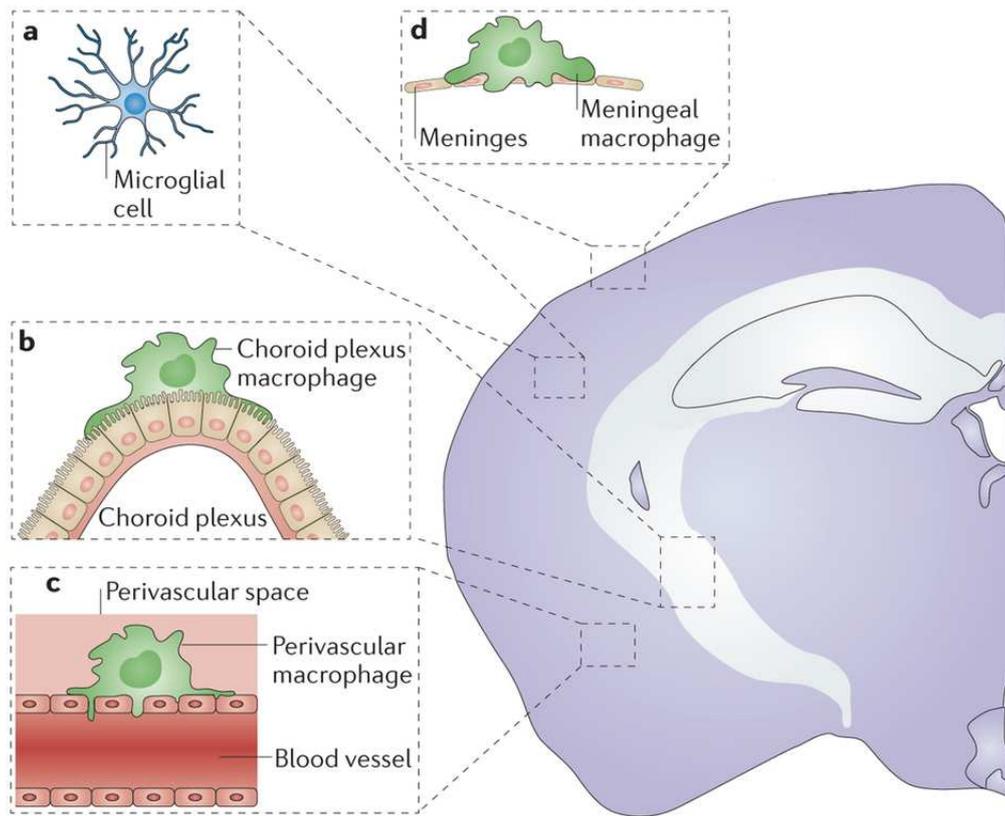


Figure 6. Brain macrophages of the central nervous system. Under physiological conditions, the CNS hosts a heterogeneous population of myeloid cells that can be distinguished according to their anatomical location, morphology and surface marker expression. These are (a) ramified parenchymal microglia and non-parenchymal (b) choroid plexus, (c) perivascular and (d) meningeal macrophages. Modified from (Prinz and Priller, 2014)

For several decades, PVMs were believed to originate from short-living blood monocytes after birth, which are quickly renewed by bone marrow (BM)-derived cells. The demonstration for making that assumption originally came from studies using BM chimeras (Bechmann et al., 2001; Prinz and Priller, 2014). However, all of these studies used irradiation as conditioning regimens and, as a consequence, induced an artificial engraftment of injected BM-derived cells due to damages of the BBB and the local induction of chemoattractant factors in the host brain (Kierdorf et al., 2013). More recently, by using a combination of fate mapping and parabiosis experiments, it was

demonstrated that in contrast to choroid plexus macrophages, PVMs and MMs just like microglia persist throughout the entire life of the organism without any significant contribution from peripherally derived cells, due to their capacity to self-renew (Goldmann et al., 2016). This was clearly demonstrated by the induction of peripheral blood chimerism through parabiosis, which consists in subcutaneously conjoining two syngeneic mice, one of which ubiquitously expresses GFP. Notably, after 5 months of parabiosis there was a complete absence of GFP expressing PVMs, MMs or microglia, meaning that the extent of circulatory exchange was negligible. Only choroid plexus macrophages pools received a contribution from donor-derived cells. In conclusion, parabiotic studies in mice provided, for the first time, unequivocal evidence that the PVMs population during the steady state is able to maintain itself throughout adult life by local renewal, independent of circulating precursors. However, whether peripheral precursors may give rise to PVMs in pathological conditions still remains an open question.

In order to study the function of PVMs in pathology, only one depletion method has been described and extensively used. Therefore, and since it is the method used in this thesis work, it is essential to explain in details its principle before going through the literature illustrating the role of PVMs in pathology.

2.2 Methodological principle of PVMs depletion

The technique used to deplete PVMs was originally developed to deplete all kind of phagocytic macrophages. It is based on the administration of liposomes (artificial lipid vesicles made of concentric phospholipid bilayers) loaded with clodronate (a bisphosphonate class of drug targeting cellular metabolism and inducing apoptosis at high concentration). Indeed, once macrophages ingest liposomes by phagocytosis, the phospholipid bilayers of the liposomes are disrupted under the influence of lysosomal phospholipases, causing the release of clodronate inside the cell, which induces a pro-apoptotic response (van Rooijen et al., 1996). Free clodronate has an extremely short half-life, explaining why non-phagocytic cells are unaffected. Macrophages are present in almost every tissue, meaning that a systemic administration would target macrophages from multiple organs. Therefore, tissue-specific macrophage depletion is possible, but depending on the administration route of the liposomes. It was demonstrated that the intraventricular injection of clodronate liposomes can reach a complete depletion of PVMs and MMs throughout the CNS. The selectivity of the depletion method was investigated

using fluorescently labelled liposomes that can be engulfed by PVMs and MMs, but not microglia (Polfliet et al., 2001a).

2.3 PVMs in pathology

In the context of in Alzheimer's disease (AD), the first data implicating PVMs used the TgCRND8 mouse model. It encodes a double mutant form of human amyloid precursor protein (APP, KM670/671NL+V717F) under the control of the prion protein promoter. In this model, vascular amyloid deposition in leptomeningeal and small cortical blood vessels develops over time, making the model useful to study cerebral amyloid angiopathy (CAA), which affects the majority of patients with Alzheimer's disease. The role of PVMs was analysed in this model through the intraventricular injection of clodronate liposomes, by assessing the severity of CAA. The authors demonstrate that depleting PVMs results in a 5-fold increase in the percentage of total cortical area covered by thioflavin S (thioS)-positive blood vessels. ThioS binds amyloid fibrils but not monomeric amyloid beta (A β), so it is very frequently used to stain pathologically relevant amyloid plaques. In contrast, the stimulation of PVMs turnover through the administration of chitin (a naturally occurring biopolymer of N-acetyl- β -D-glucosamine, believed to be taken up via binding to CD206 receptor) causes the opposite phenotype, i.e. a reduction in the number of thioS-positive vascular amyloid deposits. Overall, these data are consistent with a role of PVMs in the clearance of A β from the cerebral vasculature (Hawkes and McLaurin, 2009).

Additionally, in the J20 mouse model that also expresses a double mutant form of human APP (KM670/671NL+V717F), but under the regulation of platelet-derived growth factor beta (PDGF- β) promoter, it was shown that the expression of CD206 was increased. This observation was suggested to occur due to the recruitment of PVMs at the level of A β deposits. In this model, the authors also demonstrated that PVMs express the scavenger receptor class B type I (SR-BI) (Thanopoulou et al., 2010). SR-BI, which is expressed by brain macrophages and microglia in AD brains, has been suggested to have a role in AD pathogenesis (Husemann et al., 2002). The authors evaluated the role of SR-BI in AD by inactivating one SR-BI allele in the J20 transgenic mice (J20/SR-BI^{+/-}). SR-BI reduction in J20/SR-BI^{+/-} mice enhanced vascular amyloid deposition and fibrillary amyloid plaques numbers in the hippocampus, and also exacerbated learning and memory deficits. This observation was attributed to the depletion of SR-BI in PVMs only, because the phagocytic capacities of peripheral macrophages and microglia from SR-BI^{+/-} *in vitro*, were not

affected. Interestingly, the analysis of SR-BI mutant mice alone revealed a significant increase in the number of CD206 positive PVMs, which was hypothesized as an effort to compensate for the loss of SR-BI (Thanopoulou et al., 2010). The study shows a clear association between SR-BI function and PVMs, as well as the fact that SR-BI depletion modifies amyloid pathology. However, the invalidation of SR-BI specifically in PVMs would be needed to show that SR-BI is required by PVMs to clear vascular amyloid deposits in a cell-autonomous fashion.

These above studies have revealed that PVMs could hold neuroprotective potential through their capacities to clear toxic amyloid plaques from vessels. However, it has been shown that in alternative AD models, PVMs could also mediate neurovascular dysfunction. When administered directly into the cerebral cortex, intravascularly or when over-produced in the brain of Tg2576 mice (transgenic line that expresses a mutant form of APP with the Swedish mutation KM670/671NL), A β induces vascular dysfunction through oxidative stress. The authors demonstrate that the depletion of PVMs through the clodronate liposome technique abrogates the production of ROS and vascular dysfunction induced by A β . They identified PVMs as the major source of ROS. Furthermore, the authors showed that to exert their harmful activity, PVMs require the activation of the scavenger receptor CD36 and NADPH oxidase-2 (Nox2). Indeed, the replacement of PVMs with PVMs derived from CD36^{-/-} and Nox2^{-/-} mice through the establishment of BM chimeras, abolished the cerebrovascular dysfunction and vascular oxidative stress induced by exogenous A β delivery (Park et al., 2017). However, the replacement of PVMs occurs in an artefactual way due to the lethal irradiation applied to mice before BM-transfer. This means that replaced PVMs (from CD36^{-/-} and Nox2^{-/-} mice) possess a different ontogeny and possibly some functional differences compared to PVMs established during development that self-perpetuate postnatally, as it was already mentioned (Goldmann et al., 2016).

2.4 PVMs and the “glymphatic” system

As it was earlier stated, PVMs seem to be important for clearance of vascular A β , very likely due to their phagocytic activity (Hawkes and McLaurin, 2009). However, as its name indicates, PVMs are structural components of the perivascular space. It has been known for a long time that perfusion of tracers into the CSF compartment can diffuse far from the injection site, suggesting the presence of a CSF flow, instead of basic diffusion

movement (Abbott, 2004). Additionally, it was observed that ink injected into the CSF compartment gets access inside the perivascular space of vessels, suggesting that the perivascular space can act as a pathway for CSF circulation (Zhang et al., 1992). Very recently, using *in vivo* two-photon microscopy imaging, it has been possible to live-track the movement of fluorescent tracers injected into the cisterna magna (Iliff et al., 2012a). In particular, it was shown that the CSF enters the brain interstitium through the perivascular space of arteries and penetrating arterioles, clearing the interstitial fluid (ISF) and exiting the brain along the perivascular space of venules. The authors also showed that the flow is facilitated by water channel aquaporin-4 (AQP4) localized to astrocytic end-feet. Indeed, the lack of AQP4 causes a reduction of both the CSF influx into the brain and the clearance of interstitial solutes. Due to the fact that the CSF flow clears ISF from the brain resembling lymphatic functions, but depending on glial water flow, the system was named the “glymphatic” pathway (for **g**lial “**l**ymphatic”) (Iliff et al., 2012a) (Figure 7). Since PVMs stand precisely at the place where the CSF flows, the functional and structural implication of this cell population in building up the glymphatic pathway are expected, but still completely unknown.

3. The CNS lymphatic system

The glymphatic system has been described as the mechanism to clear the ISF of the brain, which lacks a classic lymphatic circulation. Downstream of the glymphatic system, most of the CSF is considered to drain into the bloodstream via arachnoid granulations of the dural sinuses (Raper et al., 2016). On the other hand, a connection between the subarachnoid space and the cervical lymphatic system has been established long ago. Through the injection of tracers into the CSF, it has been observed that the tracers drain into lymphatic vessels external to the cranium, reaching cervical lymph nodes. The presence of tracers has also been observed in the nasal lymphatic vessels underneath the cribriform plate, suggesting that drainage occurs in part via olfactory nerve sheaths through the cribriform plate before reaching cervical lymph nodes (Kida et al., 1993a; Koh et al., 2005). Although the connectivity between the CSF and the extracranial lymphatic compartment was shown, the physical structure connecting both was missing. Yet, very recently, two independent studies demonstrated that a functional lymphatic vascular system actually exists in the meninges (Aspelund et al., 2015; Louveau et al., 2015). This lymphatic system is composed of a simple network of thin vessels aligned to the superior sagittal and transverse sinuses. The lymphatic vessels described express several

characteristic markers of lymphatic endothelial cells like lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1) and VEGFR-3. Also, they hold functional aspects of the peripheral lymphatic vessels like the permissiveness to fluid and drain into lymph nodes. Indeed, the intracerebroventricular injection of a dye leads to its accumulation inside deep cervical lymph nodes (dcLNs), and the ligation of lymphatic vessels just above dcLNs, completely abolished the drainage of the dye into these lymph nodes. Furthermore, the ligation also causes a dilation of the meningeal lymphatic vessels. These observations strongly demonstrate the physical connection between the meningeal lymphatic vessels and the dcLNs. Another hallmark of the classic lymphatic vessels, is their ability to allow cells to travel from tissues to draining lymph nodes. Interestingly, it has been observed through immunohistochemical analysis that leukocytes can be found inside meningeal lymphatic vessels. Moreover, the removal of the dcLN results in an accumulation of T cells in the meninges, indicating that the meningeal lymphatic vessels could represent a path for immune cells to egress the CNS (Louveau et al., 2015). A scheme of the glymphatic clearance and the lymphatic drainage of the CSF is depicted in figure 7.

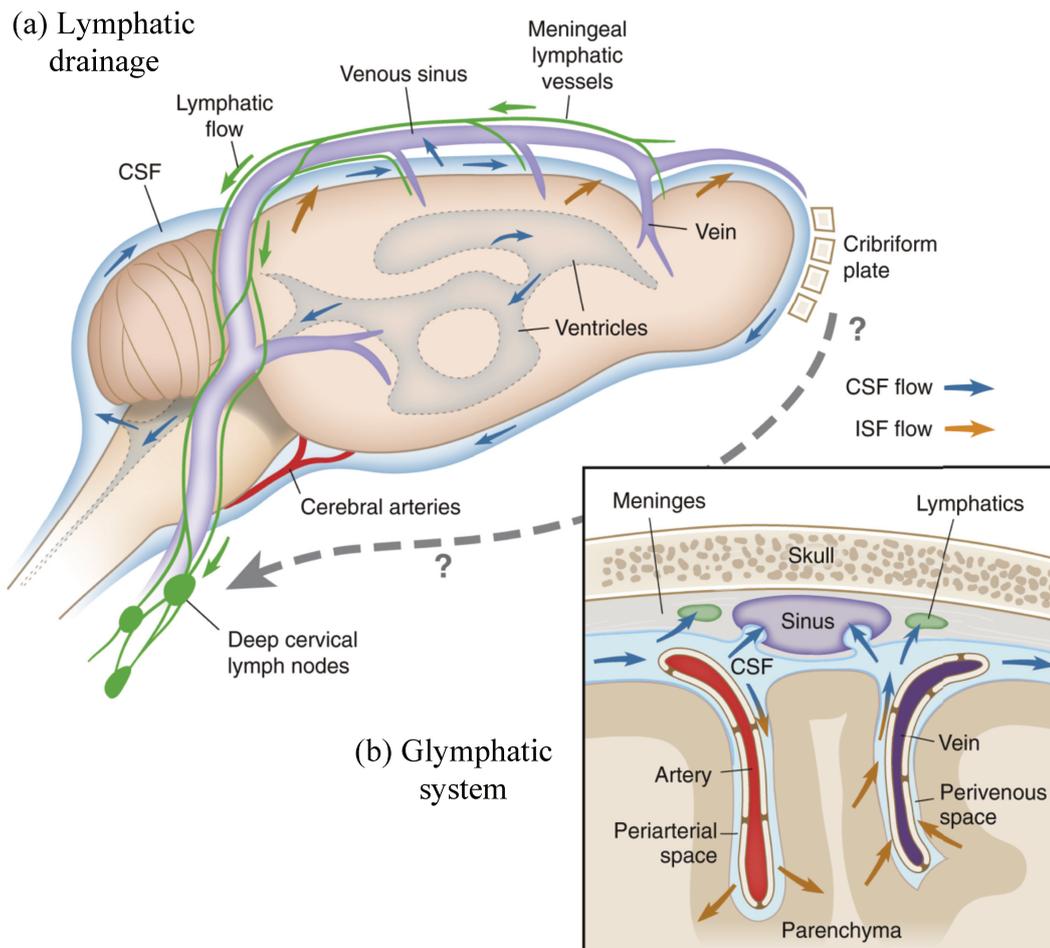


Figure 7. Circulation of cerebrospinal fluid within the central nervous system and drainage into the deep cervical lymph nodes. (a) CSF is produced by the choroid plexus of the lateral and fourth ventricles, and flows to the subarachnoid meningeal space between the pia and the arachnoid mater. The two major routes of drainage of the CSF are the arachnoid granulations, which drain aqueous content directly into the venous sinus and the meningeal lymphatic vessels, which drain macromolecules and immune cells from the meningeal spaces and the brain parenchyma into the dcLN (green arrows). Whether the lymphatic vessels pass through the cribriform plate and the nasal mucosa to finally drain into the dcLN is currently not clear (grey dotted arrow). (b) The glymphatic system describes the flow of CSF across the brain parenchyma, entering through the perivascular space of arteries (periarterial space) and exiting through the perivascular space of veins (perivenous space). The system is responsible for the removal of macromolecules and hydrophilic compounds from the brain parenchyma into the CSF. Modified from (Louveau et al., 2016)

HYPOTHESIS

In summary, PD is a complex disorder driven by an interplay between genetic and environmental factors. The cause of PD is unknown in most of the cases, however all the efforts devoted in the last decades by the scientific community have allowed the identification of several mechanisms of cell death holding invaluable therapeutic potential. Among these mechanisms, the non-cell autonomous ones may contribute to the progressive nature of PD, through the potential engagement of a vicious circle of degeneration (Figure 4). In line with this, the BBB has been implicated in PD pathophysiology. Yet, the BBB is built by multiple cellular partners and their respective pathological contributions have not been well defined so far. Among these partners, PVMs are particularly interesting in the context of non-cell autonomous mechanisms in PD as these cells fulfil numerous functions whose alteration may be directly linked to neuroinflammation (innate immune function), but also to protein aggregate clearance, thanks to both their constitutive phagocytic activity and their strategic location within the perivascular space, so important for the circulation of both ISF and CSF through the glymphatic system. Taking into consideration all the above evidences, we postulate the following hypothesis:

“Perivascular macrophages alterations, in response to neurodegeneration, could modulate Parkinson’s disease pathophysiology”

SPECIFIC AIMS AND EXPERIMENTAL APPROACH

In order to test and validate the hypothesis, two specific aims were formulated:

- 1- How PVMs dynamically respond to nigrostriatal degeneration?**
- 2- What is the role of PVMs in the development of inflammatory processes, neurodegeneration and the clearance of toxic α -Syn aggregates?**

To address the first aim, a viral-based model of synucleinopathy was developed and used to induce nigrostriatal degeneration, without mechanically disrupting the BBB around the SNpc due to intrastriatal viral delivery. In order to assess the dynamic response of PVMs, the specific marker CD206 was used in immunohistochemistry assays. Post-mortem tissue from PD patients was used to confront the results observed in the experimental model with the real pathology. Finally, the origin of PVMs in the context of PD-like neurodegeneration was determined using a combination of parabiosis and cell proliferation experiments *in vivo*.

For the second aim that deals with the functional aspects of PVMs in PD, cell ablation assays were conducted using clodronate-containing liposomes. The readouts of PVMs depletion in the viral-induced pathological context were DNs degeneration, microglial activation and lymphocytic infiltration: *In vivo* experiments in mice, immunohistochemistry techniques and different methods for image quantification and analysis were used for this purpose.

In order to address the role of PVMs in the clearance of toxic α -Syn aggregates, fibrils of α -Syn were injected into the striatum of mice devoid of PVMs. The readouts were neurodegeneration and α -synucleinopathy propagation. Finally, the consequences of PVMs depletion on the glymphatic pathways and the lymphatic drainage of CSF into dcLN was determined by assessing the fluorescence of tracers that follow CSF movements.

METHODS

1.1 Human Subjects

The study was performed on autopsy brain tissue (Hôpital de la Pitié-Salpêtrière) from 14 PD patients and 9 control subjects. All cases were well-characterized clinically and neuropathologically. PD patients and control subjects did not differ significantly with respect to mean age at death (PD patients: 75.85 ± 2.29 years of age; controls: 75.67 ± 3.89 years of age; mean \pm SEM, p-value = 0.96, Student's t test) nor mean post-mortem delay (PD patients, 21.98 ± 3.80 hours; controls, 19.30 ± 2.98 hours; mean \pm SEM; p-value = 0.62, Student's t test). Experiments using human postmortem material were approved by the "Comité de Protection des Personnes" review board (Ile de France 1, Paris, France). Within 2 hours after autopsy, the brains were dissected, frozen in dry ice and stored at -80°C . Serial $25\mu\text{m}$ -thick tissue sections (at the level of the striatum and the ventral mesencephalon) were cut from frozen blocks at -12°C using a cryostat, thaw-mounted onto gelatin /chromalun-coated glass slides, desiccated and stored at -80°C until further use.

1.2 Animals

8- to 12-week-old male C57BL/6J mice were purchased from Janvier Labs (<https://www.janvier-labs.com>). The transgenic lines: Actin-GFP, Rosa26-mT/mG and PDGFR β -CreERT2 have a C57BL/6 background. Mice were bred in-house under pathogen-free conditions. PDGFR β -CreERT2 were crossed to Rosa26-mT/mG in order to identify arteries/arterioles and veins/venules according to the differential morphology of mural cells (Hill et al., 2015a). All experiments were performed in accordance with the European Community regulations and the Inserm ethical committee and were approved by the local Darwin ethical committee.

1.3 Anesthesia

In all experiments, animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture.

1.4 Tamoxifen

For induction of the Cre recombinase in adult animals, PDGFR β CreER^{T2}:Rosa26-mT/mG mice received two intraperitoneal injections of tamoxifen (100mg/kg, Sigma) solved in corn oil (Sigma), 24 hours apart.

1.5 Production of non-replicative canine adenovirus type 2 (CAV) and α -syn fibrils

Vector production and purification of CAV (CAVTR110 and CAVGFP) was performed as previously described (Szelechowski et al., 2013a). Briefly, the gene coding for α -syn^{TR110} was cloned into a shuttle plasmid (pShuttle) between NotI and NheI restriction sites. The resulting pShuttle-CMV- α -syn^{TR110} plasmid was purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). Competent *E. coli* BJ5183 bacteria were transformed for homologous recombination with pShuttle-CMV- α -syn^{TR110} and pCAV2- Δ E1-CMV-EGFP, a vector carrying the CAV2 genome, but in which a CMV-EGFP cassette was inserted instead of E1 gene, required for replication. The resulting vector pCAV2- Δ E1-CMV- α -syn^{TR110} and pCAV2- Δ E1-CMV-EGFP were used to transfect canine kidney cells expressing the complementing CAV2-E1 gene (DK-E1), in order to produce CAVTR110 and CAVGFP viruses, respectively. Viral particles were then purified by ultracentrifugation through cesium chloride gradients and desalted by dialysis using PD-10 desalting columns (Amersham). Real-time PCR analysis was used for genomic copy number determination. Infectious titer was determined as median tissue culture infectious doses (TCID₅₀), using the Reed and Muench statistical method (Reed and Muench, 1938).

Atto647 labelled mouse α -Syn fibrils were generated as previously described (Bousset et al., 2013a). The nature of all α -Syn fibrils was verified using a Jeol 1400 (Jeol Ltd, Peabody, MA) transmission electron microscope after adsorption of the samples onto carbon-coated 200-mesh grids and negative staining with 1% uranyl acetate. The images were acquired with a Gatan Orius CCD camera (Gatan).

1.6 Stereotaxic injections

Following anesthesia, mice were placed in a Kopf stereotaxic frame. Body temperature was maintained at 37±0.5°C with a temperature-regulated heating pad. Injections were performed with a 30-gauge needle and a 10- μ l Hamilton syringe. 2 μ l of CAV2 vectors (CAVTR110 and CAVGFP, 2 x 10⁶ TCID₅₀/ μ l) or 2 μ l of α -Syn fibrils (10 μ g) in Ca²⁺-Mg²⁺ free PBS were injected into the dorsal striatum (coordinates: AP +0,85mm, ML 2mm, and DV -3,4mm below the skull) at a rate of 0.2 μ l/min. The needle was left for an additional 10 minutes before it was retracted.

1.7 Intracisternal injections

To deplete PVMs, clodronate- or PBS- containing liposomes were purchased from <http://clodronateliposomes.org>. Anesthetized mice were fixed in a stereotaxic frame and 10 μ l of clodronate or PBS liposomes were infused into the cisterna magna using a 30GA needle at a rate of 1 μ l/min during 10 minutes. Body temperature was maintained at 37 \pm 0.5 $^{\circ}$ C with a temperature-regulated heating pad (Figure 8).

The same protocol was used to infuse Alexa 647 conjugated ovalbumin (1% in Ca²⁺, Mg²⁺ free PBS, ThermoFisher). The visualization and posterior analysis of the infusion of Alexa 647 conjugated ovalbumin into the brain parenchyma was performed as previously described (Iliff et al., 2012b). Briefly, 30 minutes after tracer injections mice were transcardially perfused with 2% PFA in PB buffer and both brain and dcLN were collected and post-fixed in 2% PFA overnight. Brains were cryoprotected in 30% sucrose and cut in 100 μ m sections using a freezing microtome. 6-7 coronal brain sections around the striatum and whole dcLN were imaged by epifluorescence microscopy and fluorescence intensity was assessed.

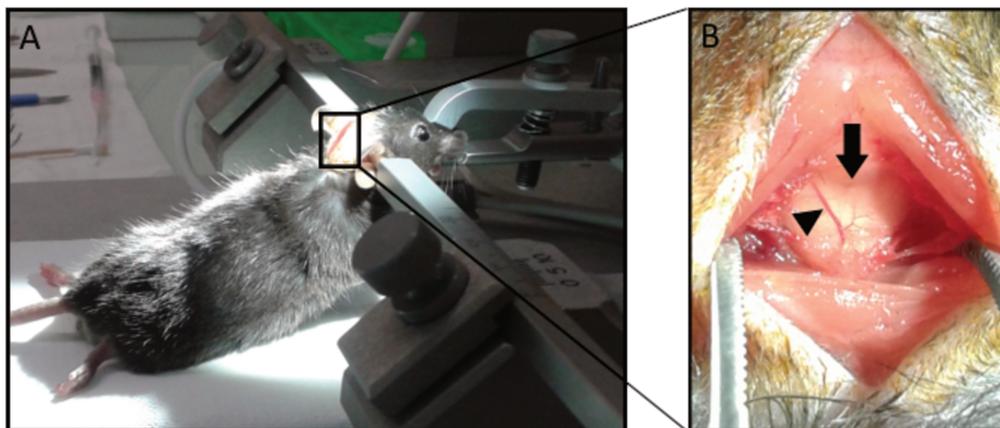


Figure 8. Intrathecal delivery via cisterna magna. (A) Mice were properly positioned on the stereotaxic frame so that the dura mater around the cisterna magna was sufficiently exposed. (B) Higher magnification photo to show the cisterna magna (arrow) and the spinal artery (arrowhead).

1.8 Parabiosis

Parabionts of wildtype and Actin-GFP^{+/-} mice were surgically conjoined for three to six weeks as previously described (Figure 9) (Ajami et al., 2007b). Briefly, two 8-weeks old male mice similar in body weight were anesthetized. Then, the fur was thoroughly removed from the right side of the mouse placed on the left and the left side of the mouse placed on the right, starting at approximately 1 cm above the elbow, to 1 cm below the knee. Body temperature was maintained at 37±0.5°C with a temperature-regulated heating pad during the entire duration of the surgery. Using sterile scissors, a longitudinal skin incision was performed from 0.5 cm above the elbow to 0.5 cm below the knee. The skin was detached from the subcutaneous fascia through blunt dissection. Then the right oleocranon of one mouse was attached to the left oleocranon of the other by a double surgical knot. The same procedure was performed to connect the knee joints. After suturing the skin of the two mice using absorbable 5-0 Vicryl suture, the continuity of the suture was verified to confirm the lack of openings. To prevent skin rupture and separation, surgical staples were used for tight skin closure in the area around the elbows and knees. Finally, to prevent dehydration, both animals received subcutaneous injections of 1 ml of 0.9% NaCl. The most important step to avoid discomfort and mortality is the postoperative care. Immediately after the surgery, mice were kept on heated pad until they recovered completely. Mice were then placed in clean cages filled with wet pellets at the level of the bedding. Buprenorphine (vetergesic) was administered subcutaneously for pain relief every 12 hours. When specified, seven, five and three days prior to the surgery, recipient mice received intraperitoneal injections of the alkylating agent busulfan (Sigma, 25mg/kg in 50% DMSO).

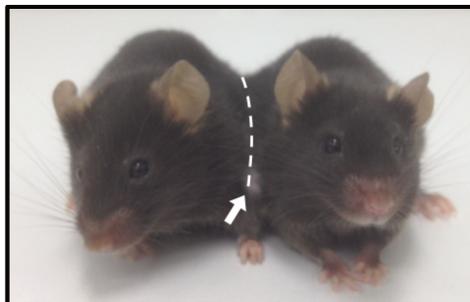


Figure 9. A pair of parabionts. Three weeks after parabiosis, the fur between mice grows back, making hard to visualize the suture line (dotted white line and arrow).

1.9 Flow Cytometry

Whole-blood chimerism after parabiosis was verified through flow cytometry. To do so, blood samples were collected from the facial vein into plastic tubes containing the anticoagulant K3 EDTA (BD Vacutainer K3 EDTA tubes). Red blood cells were lysed with FACS lysing solution (BD). For flow cytometry data acquisition, BD FACSVerser system was used. Data were analysed using the FlowJo v9.0 software. Mice that did not reach a 35% chimerism, or a 70% chimerism when pre-conditioned with busulfan, were excluded from the study.

1.10 *In vivo* proliferation assays

To assess proliferative events *in vivo*, mice received intraperitoneal injections of 5-ethynyl-2'-deoxyuridine (EdU; 50mg/kg, Baseclick EdU *In Vivo* Kit 555). Three injections at 3 days interval, were carried out to determine PVMs proliferation following clodronate- or PBS-loaded liposome injections. Also, CAVTR110- and CAVGFP-injected mice received 10 EdU injections every 2 days during 3 weeks. *In situ* EdU staining was conducted according to the manufacturer's protocol. For the co-localisation of EdU with CD206 or CD3 markers, confocal microscopy images were acquired.

1.11 Image acquisition

When specified, confocal images were acquired on an Olympus FV-1000 microscope with 20X and 40X oil objectives. Maximum projection images are presented throughout the manuscript. Epifluorescence images were acquired on a Leica DM4000 microscope equipped with a digital camera Leica DFC500.

1.12 Tissue processing

For brain tissue immunostaining, mice were deeply anesthetized with sodium pentobarbital (100mg/kg) and transcardially perfused with 0.9% NaCl supplemented with heparin (5 U/ml), followed by 20ml of 2% paraformaldehyde (PFA) prepared in 0.1M PB buffer. After extraction from the skull, brains were postfixed overnight in 2% PFA and cryoprotected in 30% sucrose solution until sinking to the bottom of the glass vial. Brains were then frozen in -30°C isopentane for 1 min and stored at -80°C. Free-floating brain sections (20µm thick) were prepared using a freezing microtome (Leica).

1.13 Brain tissue immunostaining

For immunohistochemistry, free floating coronal sections were permeabilized and saturated for 30 min with 0.5% (v/v) triton X-100 in blocking buffer: 5% (v/v) normal donkey serum and 3% (v/w) BSA diluted in 0.1M PBS. Sections were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. The primary antibodies used in our studies were the following: rabbit anti-tyrosine hydroxylase (TH) (1:500, United States Biological) and rabbit anti-phosphorylated(S129)- α Syn (pS129- α Syn, 1:2000, clone: EP1536Y, Abcam). After 3 washes with 0.1M PBS, tissue sections were incubated with biotin-conjugated secondary antibodies (goat anti-rabbit; 1:300, Vector Laboratories) for 1 hour at room temperature. Sections were washed again with 0.1M PBS and then staining was revealed using the ABC method (Vector Laboratories) with 3,3'-diaminobenzidine (DAB, Sigma) as the peroxidase substrate. Sections were counterstained with thionin solution (Nissl stain) and mounted on gelatin-coated slides with Quick-hardening mounting medium (Eukitt, Sigma)

For immunofluorescence, brain sections were permeabilized for 30min with 0,5% (v/v) triton X-100 in TE buffer (50mM Tris Base, 1mM EDTA, pH 8.0). Sections were incubated with 10 μ g/mL of proteinase K in TE buffer plus 0,5% (v/v) triton X-100 at 37°C during 2 min for antigen retrieval. After 3 washes with 0.1M PBS, tissue sections were incubated in blocking buffer to reduce non-specific hydrophobic binding. Primary antibodies included rat anti-CD206 (1:500, clone MR5D3, Bio-Rad), rat anti-CD68 (1:300 Bio-Rad), rabbit anti-Iba1 (1:500, Wako), mouse anti-TH (1:500, ImmunoStar), rabbit anti-TH (1:1000, United States Biological), rabbit anti-pS129- α Syn (1:2000, clone: EP1536Y, Abcam), chicken anti-GFP (1:1000, Abcam), rabbit anti-collagenIV (1:500, Abcam), rabbit anti-Ki67 (1:200, Abcam), rat anti-CD4 (1:300, clone RM4-5, ThermoFisher), rat anti-CD3 (1:300, clone KT3, Bio-Rad), rat anti-CD8 (1:300, clone KT15, Bio-Rad), rat anti-CD106 (1:400, clone: MVCAM A (429), Bio-Rad) and rabbit anti-GFAP (1:2000, Dako). After incubating with primary antibodies overnight, sections were washed with 0.1M PBS for 3 times. Then sections were incubated with secondary antibodies for 1.5 hours in 0.1M PBS. Secondary antibodies included Alexa488-, Alexa555- and Alexa647-conjugated donkey antibodies (1:1000, ThermoFisher). To label brain vasculature, biotin-conjugated Lycopersicon esculentum (tomato) lectin (1:200, Vector Laboratories) was added with primary antibodies solution. Biotin was revealed with DyLight649-conjugated streptavidin (1:200, Vector Laboratories) diluted in the secondary

antibody solution. Sections were mounted on gelatine-coated slides with Fluoromount aqueous mounting medium (Sigma).

1.14 Immunohistochemical staining of human brain sections

Fresh frozen 25 μ m-thick human sections (at the level of the striatum and the ventral mesencephalon) were permeabilized and fixed in ice-cold acetone, then treated with Sudan black solution (1% in 70% ethanol) for 30 minutes to reduce auto-fluorescence, and rinsed in distilled water for 2 hours. Antigen retrieval was performed by incubating the sections in 10 μ g/mL proteinase K diluted in TE buffer supplemented with 0.5% (v/v) Triton X-100 for 4 min. After washing 3 times with PBS 0.1M, sections were incubated in blocking buffer to reduce non-specific hydrophobic binding. Mouse anti-human CD206 antibody (1:100, clone 15-2, Abcam) and biotin-conjugated Ulex europaeus agglutinin I (1:200, Vector Laboratories) diluted in blocking buffer were used to identify PVMs and the brain microvasculature, respectively. Sections were incubated in primary antibodies overnight. After 3 washes in 0.1M PBS, primary antibody and biotin were revealed with Alexa488-conjugated donkey anti mouse antibody (1:1000, ThermoFisher) and DyLight649-conjugated streptavidin (1:200, Vector Laboratories) diluted in blocking buffer for 2 hours. After 3x 0.1M PBS washes, sections were mounted on gelatine-coated slides with Fluoromount aqueous mounting medium (Sigma)

1.15 Transmission electron microscopy

Mice were deeply anesthetized and transcardially perfused with 0.9% NaCl supplemented with 5 U/ml of heparin followed by 4% PFA + 0.1% glutaraldehyde in 0.1M PB at 4°C. Brains were collected and post-fixed in 4% PFA for additional 12 hours. Then, 50 μ m-thick coronal sections were cut using a vibratome (HM 650V; Microm). Immunostaining against human α -Syn was performed (rabbit anti α -Syn, Clone EP1646Y, Abcam). Staining was revealed using the ABC method with DAB as the peroxidase substrate. After dissecting out the SNpc from immunostained sections, small tissue samples were post-fixed with 0.1% osmium tetroxide in 0.1M PB, dehydrated in alcohol and embedded in epoxy resin. Resin blocks were then cut into ultra-thin sections (50nm-thick) using an ultramicrotome. Uranyl acetate was used as contrast agent and sections were observed in a JEOL JEM 1200 EX2 electron microscope (Peabody, MA, USA) at 80 kV. Images were acquired with a MegaView 3 video camera and analysis software (Soft Imaging System, Munich, Germany)

1.16 Image analysis and cell counting

DAB immunostaining analysis and mapping of α -syn pathology

TH-positive neurons were stereologically quantified on regularly spaced coronal sections covering the whole SNpc (7 sections evenly spaced at 200 μ m intervals), which was delineated according to published boundaries (German et al., 1996). The number of TH-positive neurons was determined in both ipsilateral and contralateral SNpc. Fine mapping of Lewy-like pathology was performed by tracing all visible pS129- α Syn immunoreactive elements including Lewy-like bodies and Lewy-like neurites at 40x magnification. Mapping was performed at multiple rostro-caudal levels around the striatum (3 sections) and the SN (4-5 sections). TH-positive cell bodies within the SNpc, as well as pS129- α Syn positive elements were traced and counted by bright-field microscopy, using a Leitz microscope equipped with the “Mercator ExploraNova” image analysis software, La Rochelle, France.

TH optic densitometry was determined within the SN *pars reticulata* (SNr) using the MCID software (MCID analysis 7.0). Four coronal sections within the mesencephalon, spaced at 200 μ m intervals around the rostral aspect of the SN were used per animal. Sections were scanned using Nanozoomer (Hamamatsu). Background staining measured in the cerebral cortex was subtracted from SNr for optical density measurements.

The investigators performing image acquisition and quantifications were blinded to the treatment groups during the whole analysis process.

1.17 Immunofluorescence analysis

NIH ImageJ software was used to analyze fluorescence microscopy images. Immunoreactivity was defined as the area occupied by a given immunostaining (% of the total area) and it was determined using the threshold method. For assessing CD4, CD8, ColIV and Iba1 immunoreactivity, epifluorescence images were acquired. Immunoreactivity was assessed within the anterior SN, in 4 coronal sections spaced at 200 μ m intervals and ipsilateral to the injected site. The SN was delineated according to TH staining. In the case of Iba1 and ColIV staining, the contralateral immunoreactivity was determined as an internal negative control (for basal immunoreactivity). For vascular VCAM-1 immunoreactivity within the degenerating SN, images were acquired by confocal microscopy, randomly in 4 anterior coronal sections ipsilateral to the injected side, spaced

at 200 μ m intervals around the anterior SN. The investigators performing image acquisition and quantifications were blinded to the treatment groups during the whole analysis process.

1.18 Statistical analysis

Statistics were analyzed by GraphPad Prism 7.0 software. Results are expressed as means \pm standard error of the mean (SEM). Normality of data distribution was assessed with the Shapiro-Wilk test. Statistical significance of normally distributed data was assessed using Student's t-test for comparison between two groups and ANOVA followed by Tukey's post-hoc test for multiple comparisons. When data were not normally distributed, significance was determined with Mann-Whitney U test for comparison of two groups and Kruskal-Wallis test followed by Dunn test for multiple comparisons. Statistical significance level was set as follows: * if $P < 0.05$, ** if $P < 0.01$, *** if $P < 0.001$.

1.19 Microglia sorting

Mice bilaterally injected with CAVGFP or CAVTR110 and treated with liposomes (clodronate or PBS) 4 days and 10 days after the viral injection were perfused with 0.9% NaCl supplemented with 5 U/ml of heparin 2 weeks after the viral injection. The ventral mesencephalon from three mice per condition was dissected out, pooled and mechanically dissociated into single cells, filtered through a 70-micron strainer (BD Biosciences), and centrifuged on a 30%/37%/70% isotonic percoll gradient (Amersham Biosciences) for 40 minutes. Isolated immune cells were subjected to FACS using the following combination of antibodies: CD3-Pecy-7, (1:200, clone 17.A2, BD bioscience), CD206-Alexa647 (1:20, clone MR5D3, Bio-Rad), CD11b-PE (1:20, clone M1/70, R&D Systems). Optimal compensation settings were determined for each experiment based on single stained samples. Propidium iodide was used to exclude dead cells (Propidium Iodide Staining Solution, eBioscience). Cells were sorted using a BD FACSAria II cell sorter (BD Biosciences) and collected directed into lysing buffer RA1 (Macherey Nagel). Data were analysed using the FlowJo v9.0 software.

1.20 RNA-sequencing

RNA extraction was performed using NucleoSpin® RNA XS kit (Macherey Nagel). RNA quality was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies). All RNA samples had RNA integrity numbers (RINs) greater than 7. For library preparations, TruSeq Stranded mRNA kit (Illumina, San Diego, CA) was used.

Paired-end sequencing was performed on the Illumina NextSeq 500 platform. All samples were assessed separately for overall read quality using FASTQC. Reads were trimmed using the Trimmomatic tool (Lohse et al., 2012) then aligned to the mouse genome using Tophat (Trapnell et al., 2009) and fragments were assigned to genes using FeatureCounts (Liao et al., 2014). Differential gene expression analysis was performed with Bioconductor package edgeR (Robinson et al., 2010). Three replicates per condition were used in the study.

RESULTS

1.1 Histological and functional identification of perivascular macrophages

To study PVMs, we took advantage of their known specific expression of mannose receptor (CD206) which is nevertheless shared with other non-parenchymal macrophages, including subdural meninges (MMs) and choroid plexus macrophages (Goldman et al., 2016). In the central nervous system, CD206 is exclusively expressed by non-parenchymal macrophages both in physiological conditions and in the context of acute and chronic neurodegeneration (Galea et al., 2005). We confirmed that in the ventral mesencephalon, PVMs are found deep inside the brain tissue, particularly along large and medium-size blood vessels (Figure 10A). CD206 staining also identifies MMs lining the cerebral peduncle (Figure 10A). As expected, PVMs were found exclusively around blood vessels (Figure 10B) and isolated from the brain parenchyma due to the presence of astrocytic end-foot processes forming the *glia limitans* (Figure 10C). They are known to possess constitutive phagocytic activity and therefore express the phagocytic marker CD68 (Figure 10D). PVMs were extremely efficient at engulfing CSF molecules. Indeed, 30 minutes after intrathecal infusion of fluorescent tracer Alexa647-ovalbumin (Ova) via the cisterna magna, PVMs readily take up the tracer that was not found in other surrounding perivascular or parenchymal cells (Figure 10E). Next, we sought to determine what type of blood vessels PVMs are associated to. It was previously shown that PVMs populate mostly pial and parenchymal vessels larger than 10 μ m in diameter (Faraco et al., 2016). Yet, among these small to medium-size vessels, no clear identification has been provided regarding the precise vessel identity i.e. arterioles vs venules. To differentiate vessel types, we made use of transgenic reporter mice expressing membrane GFP (mGFP) exclusively in mural cells (smooth muscle cells and pericytes), that are positive for the expression of PDGF receptor β (PDGFR β). Indeed, the morphology of mural cells allows the differential identification of arterioles, capillaries and venules (Hill et al., 2015). We found that PVMs are associated with medium and large caliber arterioles and venules, with mural cells showing circumferential band-like morphology around arterioles, or fenestrated patchwork-like morphology around venules (Hill et al., 2015). On the other hand, PVMs were never observed around capillaries, which are covered by pericytes with long and thin parallel processes (Figure 10F).

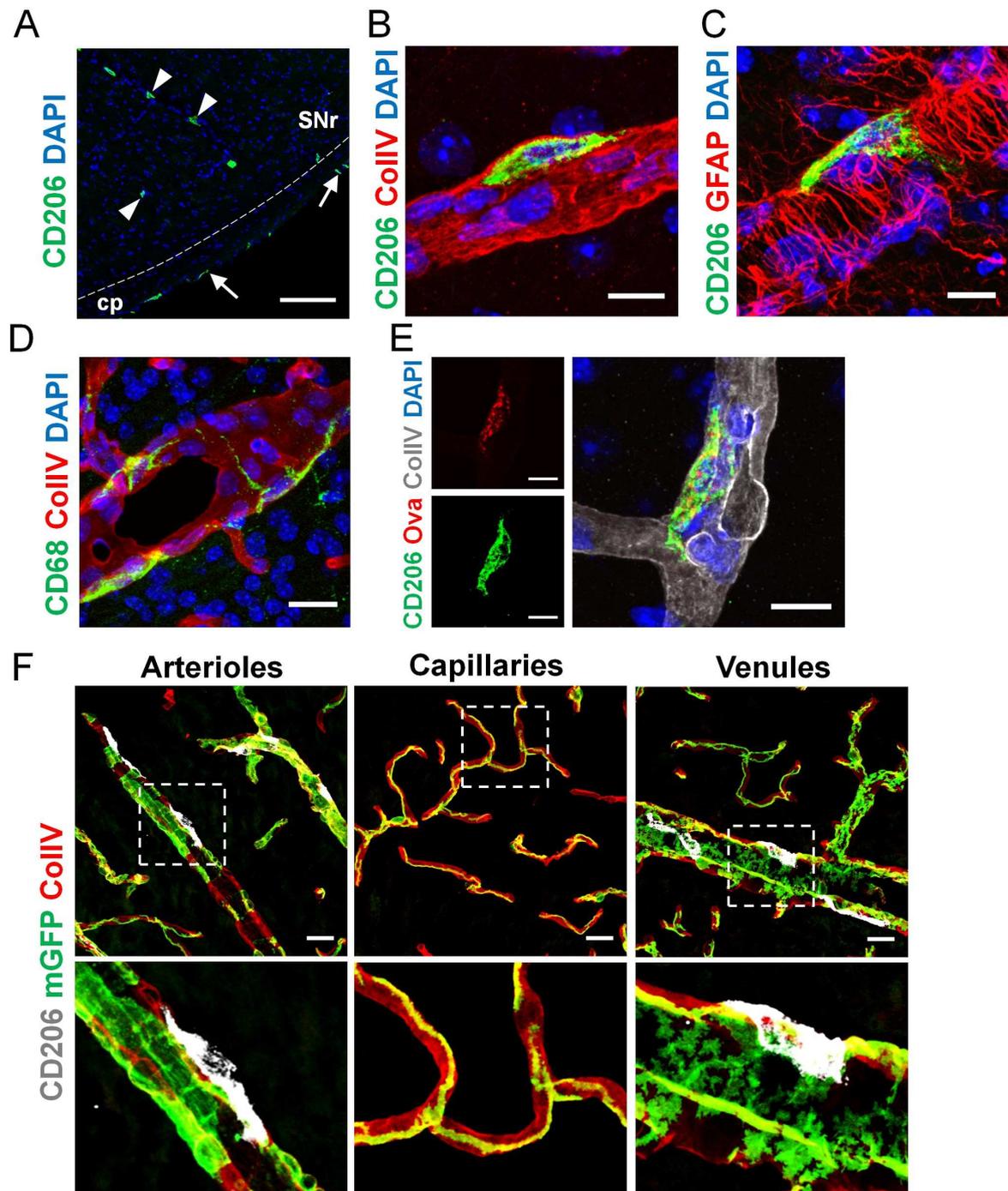


Figure 10. PVMs characterization. Confocal imaging of (A) PVMs (white arrowheads) specifically identified by the expression of CD206. PVMs can also be found in the meninges (white arrows). White dotted line is shown to delimit the substantia nigra pars reticulata (SNr) and the cerebral peduncle (cp), bar=100 μ m. (B) PVMs are embedded inside vessels (ColIV), bar=15 μ m and (C) in close contact with GFAP positive astrocytic end-feet, bar=15 μ m. (D) They express the phagocytic marker CD68, bar=20 μ m, and (E) engulf fluorescent tracers (Ova: Alexa647

conjugated ovalbumin) as fast as 30 minutes after intrathecal delivery into the CSF through the cisterna magna, bar=10 μ m. (F) PVMs are found in arterioles and venules, but never in capillaries. The three vessel types were identified according to the differential morphology of the surrounding mural cells (pericytes and smooth muscle cells) that express membrane GFP, mGFP (for more details see methods), bar=20 μ m. Higher magnification images from white dotted boxes are shown at the bottom of the figure.

1.2 Perivascular macrophages dynamically respond to neurodegeneration in a mouse model of degenerative synucleinopathy

The pathological response of PVMs to nigrostriatal pathway injury was studied in a viral-based model of degenerative synucleinopathy. Since canine adenovirus type 2 (CAV2) are highly neurotropic, with a very efficient retrograde transport in axons (Szelechowski et al., 2013b), we produced non replicative vectors expressing human C-terminal truncated α -Syn (α -Syn^{TR110}) or green fluorescent protein (GFP) as control (CAVTR110 or CAVGFP, respectively). The interest of an efficient retrograde axonal transport was that expression of α -Syn^{TR110} or GFP can be induced in the SN upon stereotaxic striatal injection, therefore avoiding mechanical disruption of the vasculature by direct nigral injections. This aspect was essential to preclude any non-specific vascular alterations and confounding effects on disease-related PVMs response. Unilateral intrastriatal injections of CAVTR110 caused a significant reduction in the number of tyrosine hydroxylase (TH)-positive dopaminergic neurons (DNs) starting at 3 weeks after vector delivery, as compared to CAVGFP injections or sham operated mice (25% cell loss, figure 11, A and B, top panels). DNs degeneration progresses very slowly to reach 33% cell loss at 27 weeks post viral injections. Besides neuronal loss, density of TH-positive neurites was significantly reduced in the ipsilateral SN *pars reticulata* (SNr), also starting at 3 weeks (29% neurites density reduction, figure 11, A and B, middle panels). Within 1 week, microgliosis quantified as the surface area occupied by Iba1 immunostaining was observed in the SNr as compared to CAVGFP or Sham mice. At 3 weeks, microgliosis dramatically increased, affecting both SNpc and SNr (Figure 11, A and B, bottom panels). Of note, microgliosis completely resolved 27 weeks after CAVTR110 injection (Figure 11A bottom panel).

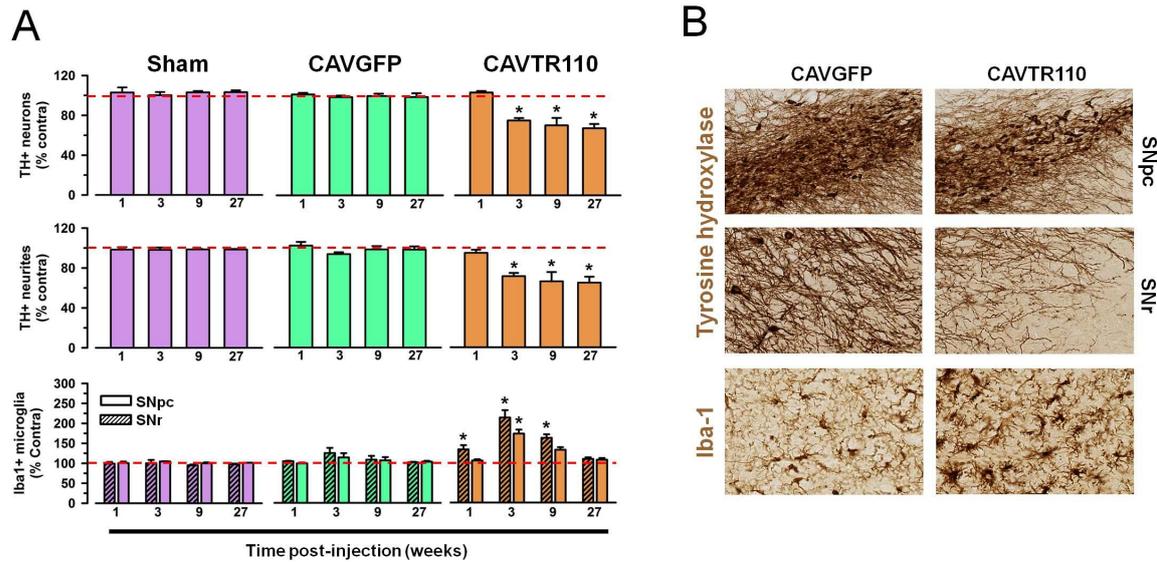
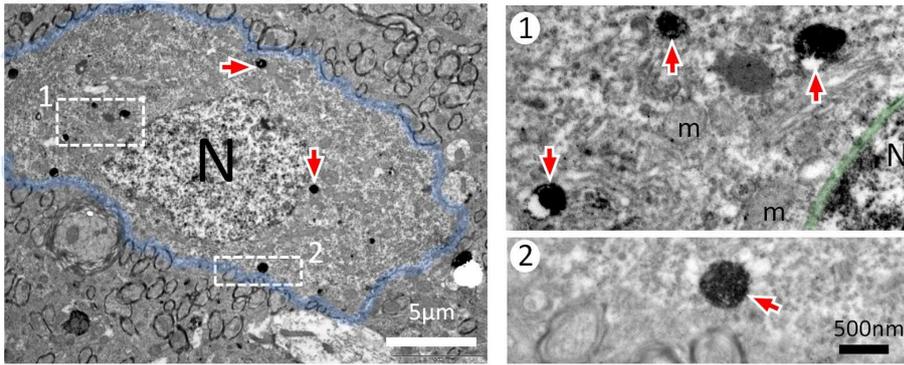


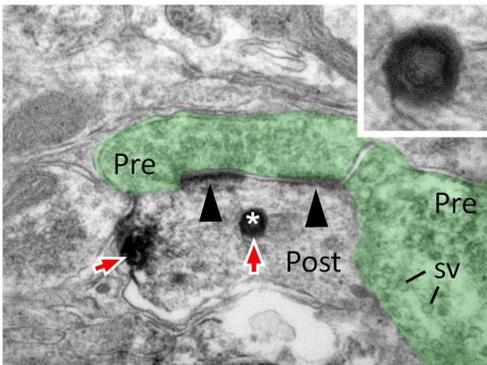
Figure 11. Viral-based model of degenerative synucleinopathy (“CAV model”). (A) Mice were injected with CAVTR110, CAVGFP or Sham operated. Three pathological parameters were quantified at 1, 3, 9 and 27 weeks after viral delivery: quantification of tyrosine hydroxylase (TH)+ dopaminergic neurons within the SNpc (top panels), TH+ neurites density (middle panels) and microgliosis (Iba1 immunoreactivity, bottom panels). Data are expressed as the ratio (%) between ipsilateral and contralateral values. Red dotted lines indicate the 100% threshold. Mean \pm s.e.m., n=5 mice per group. *p<0.05, ANOVA followed by Tukey’s posthoc test. (B) Representative photomicrographs to show TH+ neurons within the SNpc (top panels), TH+ neurites within the SNr (middle panels) and Iba1+ microglia within the SNpc (bottom panels) 3 weeks after CAVGFP or CAVTR110 injections.

Electron microscopy analysis revealed amorphous inclusions of human α -Syn in the cytoplasm of DNs (Figure 12A) and within post-synaptic terminals (Figure 12B). Interestingly, DAB electron-dense precipitates were also found inside PVMs (Figure 12C), within the perivascular space (Figure 12D) and within the vascular endothelium (Figure 12E), suggesting that α -Syn was released into the extracellular space, transported into the perivascular space where it was incorporated by endothelial cells, and engulfed by PVMs.

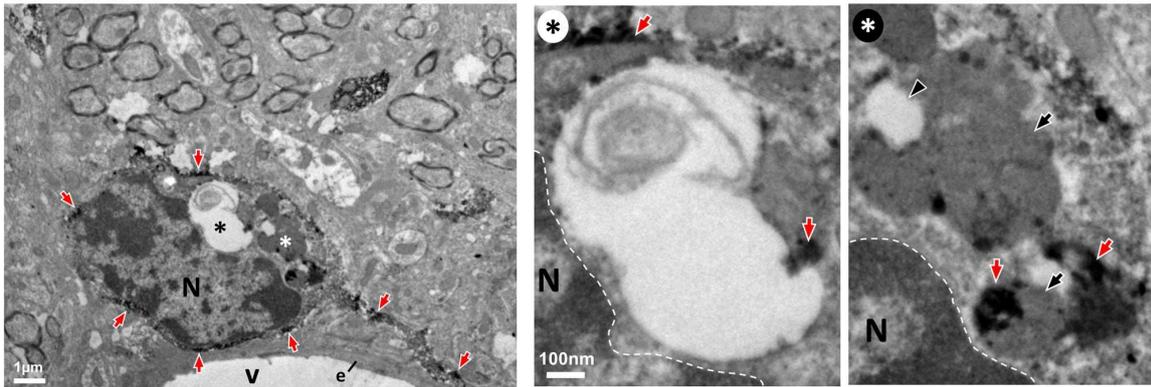
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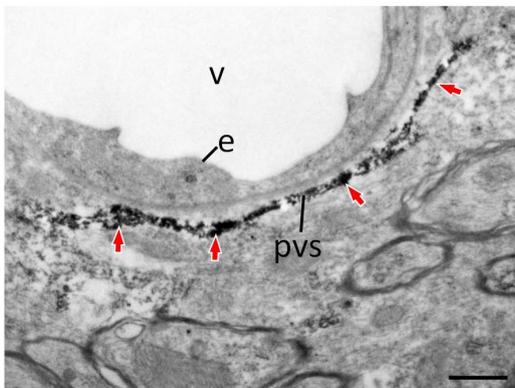
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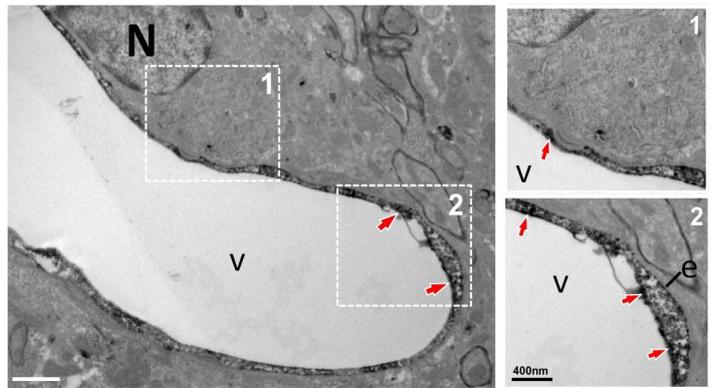


Figure 12. Electron microscopy analysis of α -Syn localization. α -Syn deposits are found in the SN 3 weeks after CAVTR110 injections. (A) Cytoplasmic α -Syn deposits (red arrows) within a DNs. (1) Enlarged view of box 1 showing amorphous α -Syn deposition without limiting membranes. (2) Enlarged view of box 2 showing a round-shaped α -Syn deposit (red arrow). Blue line: neuronal cell membrane. N: nucleus; m: mitochondria. (B) α -Syn deposits (red arrows) at the level of post-synaptic terminals (post) close to electron dense synapses (black arrowheads) within the SNr. (*) Enlarged view of a round-shaped α -Syn deposit. Pre: presynaptic terminals (green surface), sv: synaptic vesicles. (C) α -Syn accumulation (red arrows) inside a PVM, identified by its localization within the perivascular space in the proximities of the vascular lumen (v). Black asterisk: enlarged view of a phagocytic vacuole. White asterisk: enlarged view of amorphous α -Syn deposits (red arrows) without limiting membranes. N: nucleus, e: endothelial cell. (D) α -Syn accumulation (red arrows) within the perivascular space (pvs). v: vessel lumen, e: endothelial cell. (E) The presence of α -Syn deposits is also observed within endothelial cells (e). Boxes 1 and 2: enlarged views of endothelial cells facing the vessel lumen (V). N: nucleus.

The immunofluorescence analysis of CD206-positive PVMs showed that as soon as 1 week after viral injections, greater numbers are found within the SN in CAVTR110 mice as compared to controls. Notably, a peak of PVMs numbers was observed within 3 weeks (Figure 13, A and B), a time point at which no increase of the surface area occupied by ColIV immunostained vasculature was observed (Figure 13, C and D). Thus, these data demonstrate that PVMs respond very early to nigrostriatal injury in a viral-based model of synucleinopathy.

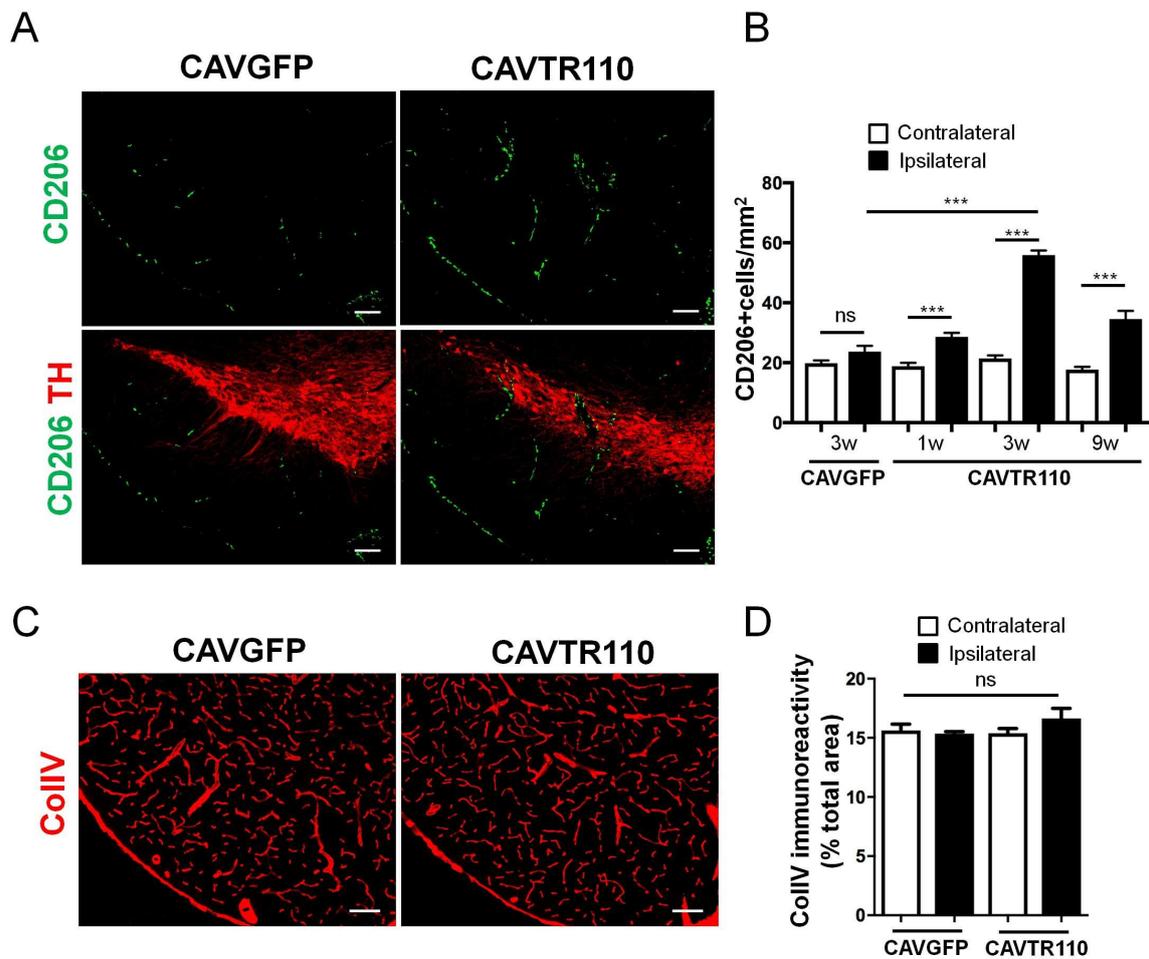


Figure 13. PVMs are recruited to the injured SN. (A) Representative image showing that the number of PVMs (CD206+cells) is increased in the SN (pars compacta and reticulata, delimited by TH immunostaining) three weeks after CAVTR110 injection compared to controls (CAVGFP injected mice), bar=100µm. **(B)** Quantification of CD206+ PVMs cells within the SN 1, 3 and 9 weeks after CAVTR110 injection. The number of PVMs is significantly increased in the injured SN (ipsilateral to the viral injection) as compared to the control SN (contralateral to the viral injection) starting from 1 week after viral injection. At three weeks, PVMs numbers reach its maximum, a time point at which not significant changes are observed in CAVGFP injected animals. Mean ± s.e.m., n=3/CAVGFP, n=5/CAVTR110. ***p<0.001, ANOVA followed by Tukey's posthoc test. w=weeks. **(C)** Representative image of ColIV positive vessels within the SN of mice injected with CAVTR110 or CAVGFP, bar=100µm. **(D)** The surface area occupied by the vasculature identified by ColIV immunostaining (ColIV immunoreactivity) in the SNpc is not significantly modified three weeks after the injection of CAVTR110 as compared to CAVGFP injected mice. Mean ± s.e.m., n=3/CAVGFP, n=5/CAVTR110. n.s., not-significant, ANOVA followed by Tukey's posthoc test.

1.3 Local proliferation drives disease-associated PVMs recruitment

PVMs are believed to renew postnatally from circulating progenitors (Hickey and Kimura, 1988). Classically, the method to track CNS engraftment of peripheral immune cells uses a combination of whole body irradiation followed by bone marrow (BM) transplantation. However, it has been clearly demonstrated that such methodological set up leads to artefactual infiltration of circulating cells into the brain (Ajami et al., 2007a; Mildner et al., 2007). Therefore, to assess a potential contribution of circulating cells to the pool of newly recruited PVMs in the injured SN, we used parabiosis as an alternative method to track blood-derived cells. Our parabiosis approach consisted in surgically providing a shared circulation between two syngeneic mice, one of which, the donor, ubiquitously expressed the reporter fluorescent protein GFP (Actin-GFP mice). Recipient mice were subjected to chemical myeloablation with the alkylating agent busulfan before parabiosis (Figure 14A). This regimen enabled a stable and high whole-blood chimerism of about 90% after surgical separation of the parabionts (Figure 14B). One week after CAVTR110 injections of chimeric mice, we observed very few donor-derived PVMs (CD206/GFP double positive cells) within the ipsilateral lesioned SN. The number of donor-derived PVMs progressively increased with disease progression, reaching more than 50% of all CD206+ cells at 9 weeks. Unexpectedly, a similar proportion of donor-derived PVMs was detected in the non-lesioned contralateral SN, suggesting that physiological rather than pathological recruitment of blood-derived PVMs occurred (Figure 14, C and D). To test whether busulfan treatment combined with parabiosis could have resulted in the artificial and unwanted recruitment of peripheral immune cells, we established parabionts in the absence of busulfan-mediated myeloablation (Figure 14E). Since in the absence of BM reconstitution blood chimerism drops very quickly after parabiont separation (data not shown), CAVTR110 injections were carried out in the recipient mice while still attached to the donor. Under such experimental conditions, whole-blood chimerism of about 45% was achieved (Figure 14F) and not a single donor-derived PVM could be detected in either hemispheres 3 weeks after CAVTR110 injections (Figure 14G). Interestingly, despite the absence of blood-derived PVMs recruitment, we detected a massive parenchymal extravasation of CD4 and CD8 GFP+ lymphocytes and other, yet to be defined, GFP+ cells (Figure 14H). Thus, we concluded that busulfan treatment was necessary to enable the recruitment of blood-derived PVMs observed in BM-reconstituted parabionts (Figure

14C). Additionally, our data indicate that degenerative synucleinopathy induces the recruitment of different blood-borne cells.

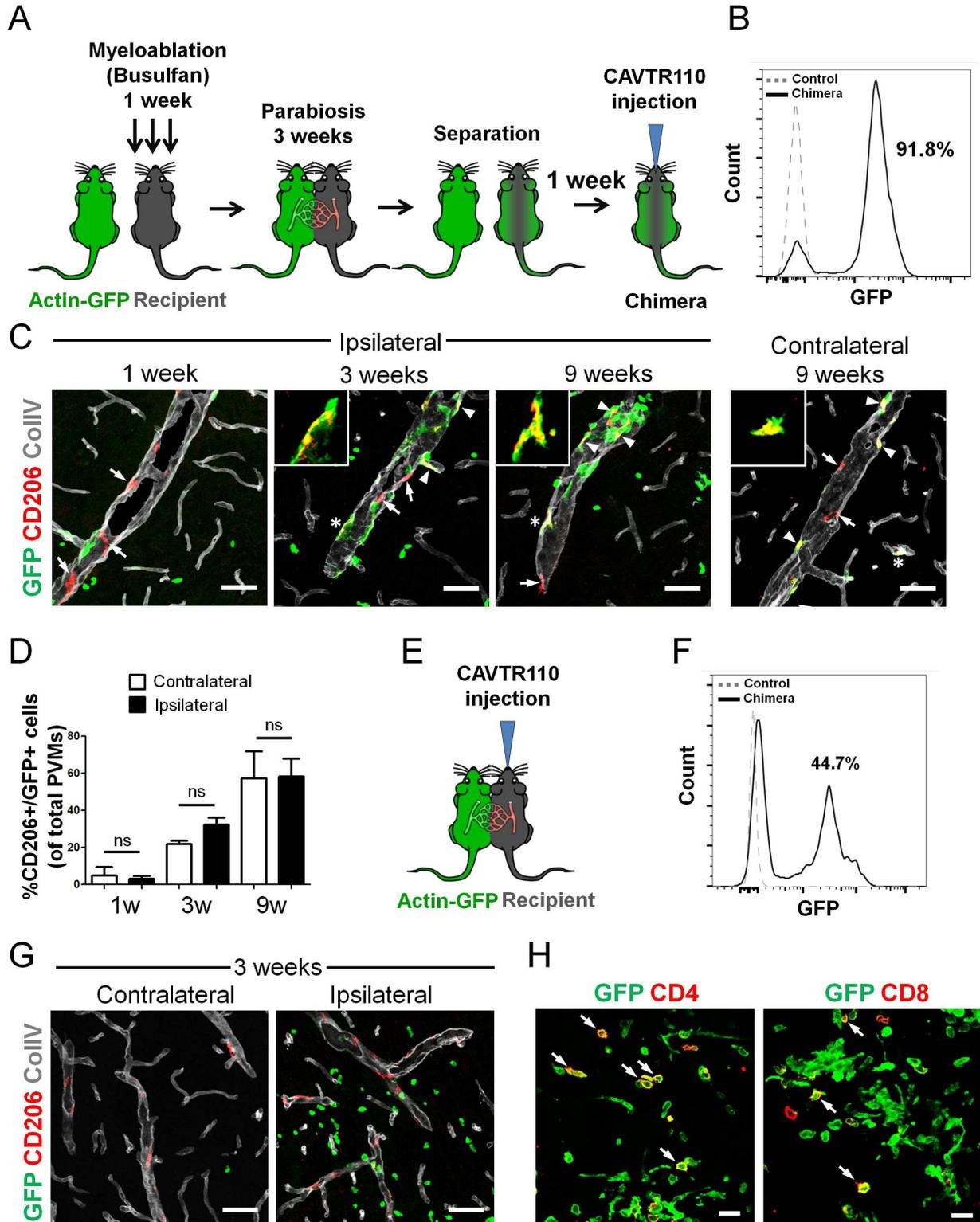


Figure 14. No blood-derived PVMs are found within the injured SN. (A) Schematic diagram of the experimental approach. In order to track blood-borne leukocytes, chimeric mice were established through parabiosis, which consists in subcutaneously conjoining two syngeneic mice, one of which ubiquitously expresses GFP (Actin-GFP) and the recipient is subjected to chemical myeloablation (3 injections of busulfan, i.p.) prior to the surgery, to allow the reconstitution of empty hematopoietic niches by donor-derived GFP positive circulating progenitors (see methods for details). (B) Representative FACS plot showing that whole-blood chimerism of $91.8\% \pm 0.6$ (mean \pm SEM) can be reached at the time of sacrifice. (C, D) Confocal microscopy analysis shows that blood-derived PVMs (CD206⁺/GFP⁺, white arrowheads) can be found in the SN of CAVTR110 injected chimeras 1, 3 and 9 weeks after the viral injection, both in the ipsilateral and contralateral SN. White arrows show CD206⁺/GFP⁻ PVMs. (*) High magnification insets show CD206⁺/GFP⁺ double positive cells, bar=50 μ m. (D) Quantification of blood-derived PVMs shows that equivalent percentage of CD206⁺/GFP⁺ cells (% of total CD206⁺ PVMs) can be identified in the SN of both hemispheres. W: weeks. Mean \pm s.e.m., n=3 per group. ns, non-significant. Kruskal-Wallis test followed by Dunn's posthoc test. (E) Schematic diagram showing the experimental approach: mice are kept conjoined in parabiosis after the injection of CAVTR110 until the day of sacrifice (3 weeks after viral injection). (F) FACS analysis shows that without the use of busulfan, a whole-blood chimerism of $44.7\% \pm 1.2$ (mean \pm SEM) can be reached. (G) Confocal microscopy analysis shows that no blood-borne PVMs infiltrate the ipsilateral nor contralateral SN after CAVTR110 injection when busulfan treatment was avoided, n=3 parabionts, bars=50 μ m. (H) The presence of transmigrated GFP⁺ T cells consisting of both CD8⁺ and CD4⁺ T cell subsets (white arrows) was detected within the injured SN. Bar=20 μ m.

Since the pathological recruitment of PVMs could not be explained by the perivascular engraftment of blood circulating cells, we next investigated the possibility that resident PVMs proliferate under the pathological context. To address this issue, we performed EdU injections in CAVTR110 or CAVGFP injected mice. EdU is a thymidine analogue that incorporates into nuclear DNA during the S-phase of the cell cycle and is therefore useful to track dividing cells and their progeny. CAVTR110 or CAVGFP

injected mice were subjected to EdU administration every two days during 3 weeks, starting 2 days after vector delivery. EdU incorporation was assessed 3 weeks after viral injection. Remarkably, many EdU-positive PVMs were found upon CAVTR110 injection, but very rarely in controls (Figure 15, A and B). This indicates that resident PVMs proliferate in response to DN's distress and demise. Alternatively, it is possible that perivascular progenitors rather than committed resident PVMs gave rise to new PVMs, after differentiating into CD206+ cells. While this possibility cannot be totally rule out at this point, we believe that resident PVMs have however the capacity to proliferate, as demonstrated by the expression of the proliferation marker ki67 in CD206-expressing cells (Figure 15C). Importantly, all the newly recruited PVMs were able to take up CSF-delivered Alexa647-ovalbumin, suggesting that they all are functional phagocytes (Figure 15D). Taken together, our data strongly indicate that under degenerative synucleinopathy, functional PVMs are mobilized by local self-expansion rather than peripheral recruitment.

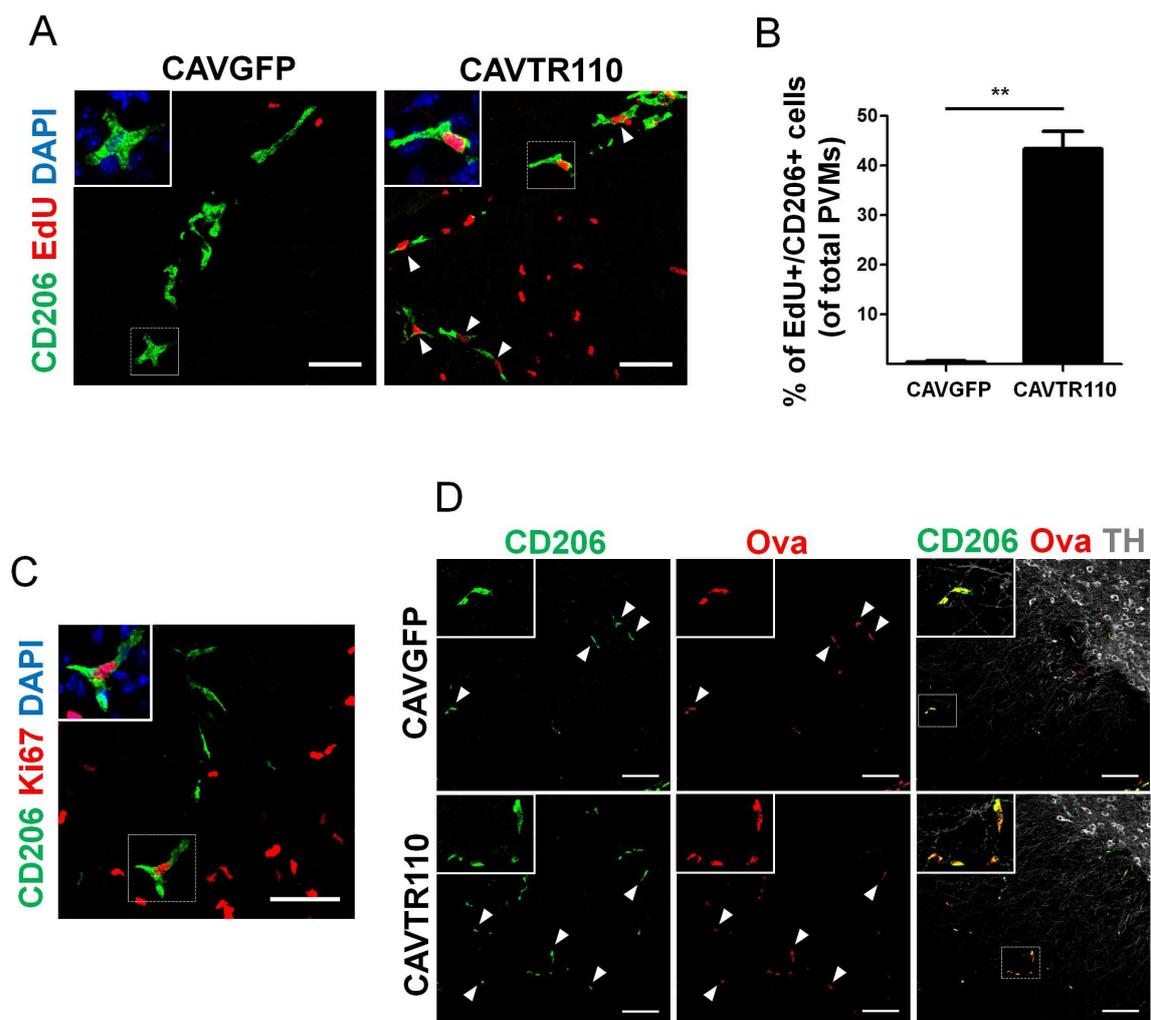


Figure 15. PVMs proliferate as a consequence of nigrostriatal injury. (A) CAVTR110 or CAVGFP injected mice received i.p. injections of EdU every two days during three weeks (10 injections total). Confocal microscopy analysis shows that CD206+ cells with incorporated EdU (white arrowheads) can only be found in the CAVTR110 group and very rarely in CAVGFP injected mice, bar=50µm. Higher magnification insets show co-localization of EdU with DAPI. (B) Quantification of EdU positive PVMs (% of total CD206+ cells) in the SN of mice injected with CAVTR110 or CAVGFP. Mean ± s.e.m., n=5 per group, **p<0.01. Mann-Whitney U test. (C) Confocal microscopy images show that three weeks after CAVTR110 injections, Ki67 expressing PVMs can be identified within the SN. High magnification insets show co-localization of EdU with DAPI within a CD206 positive PVM. Bar=50µm. (D) Confocal microscopy images showing that all PVMs within the injured and healthy SN are able to engulf the fluorescent tracer Ova (alexa647 conjugated ovalbumin) 30 minutes after infusion into the CSF compartment via cisterna magna, suggesting that newborn PVMs are all functional phagocytes. Bar=100µm. Higher magnification insets are depicted inside each image.

1.4 PVMs ablation aggravates neurodegeneration

As shown in figure 13B, PVMs respond very early after CAVTR110 injection, i.e. before significant DNs degeneration occurs, but concomitant to microglial cell activation in the SNr (Figure 11A, bottom panel). The kinetic of this pathological PVMs response may suggest that this macrophage population could contribute either positively or negatively to disease pathogenesis. To test this hypothesis, we selectively depleted PVMs through the delivery of clodronate- or PBS-containing liposomes (ClodroLipo of PBSLipo). ClodroLipo are known to induce apoptosis once engulfed by phagocytes (Polfliet et al., 2001a). Since liposomes were administered directly into the CSF compartment via cisterna magna, a robust decrease of PVMs numbers was achieved in the SN four days after administration (Figure 16, A and B). Importantly, although ClodroLipo administration results in MMs ablation as well (data not shown), they did not deplete microglial cells (Figure 16, C and D), showing the specificity of this approach for brain barrier-associated macrophages.

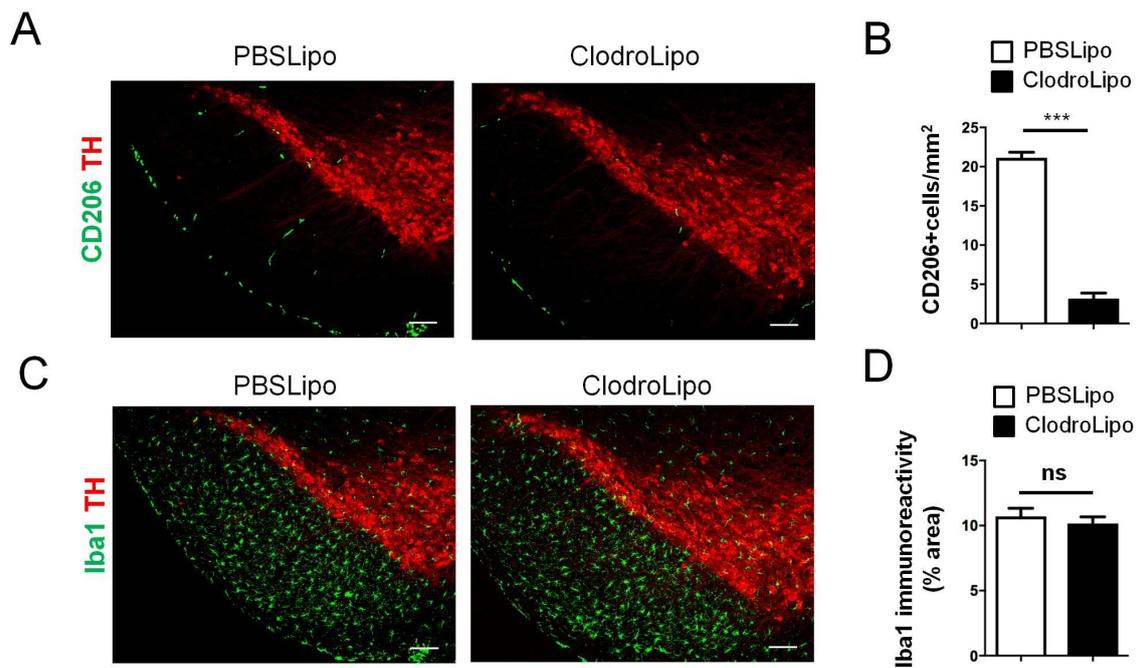


Figure 16. Selective depletion of PVMs. (A, B) The injection of clodronate containing liposomes (ClodroLipo) into the CSF via cisterna magna induces a robust depletion of PVMs after four days, as compared to PBS containing liposomes (PBSLipo) within the SN (delineated according to TH immunostaining). Mean \pm s.e.m., $n = 5$ per group. $*p < 0.001$, Student's t-test, bar = $100\mu\text{m}$. (C, D) Iba1 immunoreactivity (% of total area) within the SN is not modified by the ClodroLipo injections, indicating that microglia is not affected by the treatment. Mean \pm s.e.m., $n = 5$ per group. ns = not-significant, Student's t-test, bar = $100\mu\text{m}$.**

After validating the efficiency and selectivity of ClodroLipo treatment, we assessed the potential contribution of PVMs in synucleinopathy-associated dopaminergic degeneration. Four and ten days after vector injections, mice were subjected to PBSLipo or ClodroLipo administration (Figure 18A). The second round of liposome injections was necessary to guarantee a strong and long-lasting depletion of PVMs during the entire duration of the experiment, since a reconstitution of the PVMs pool was expected to occur very quickly after depletion (Polfliet et al., 2001a). Indeed, PVMs and MMs that incorporated EdU were found already ten days after ClodroLipo, but not PBSLipo administration (Figure 17, A and B).

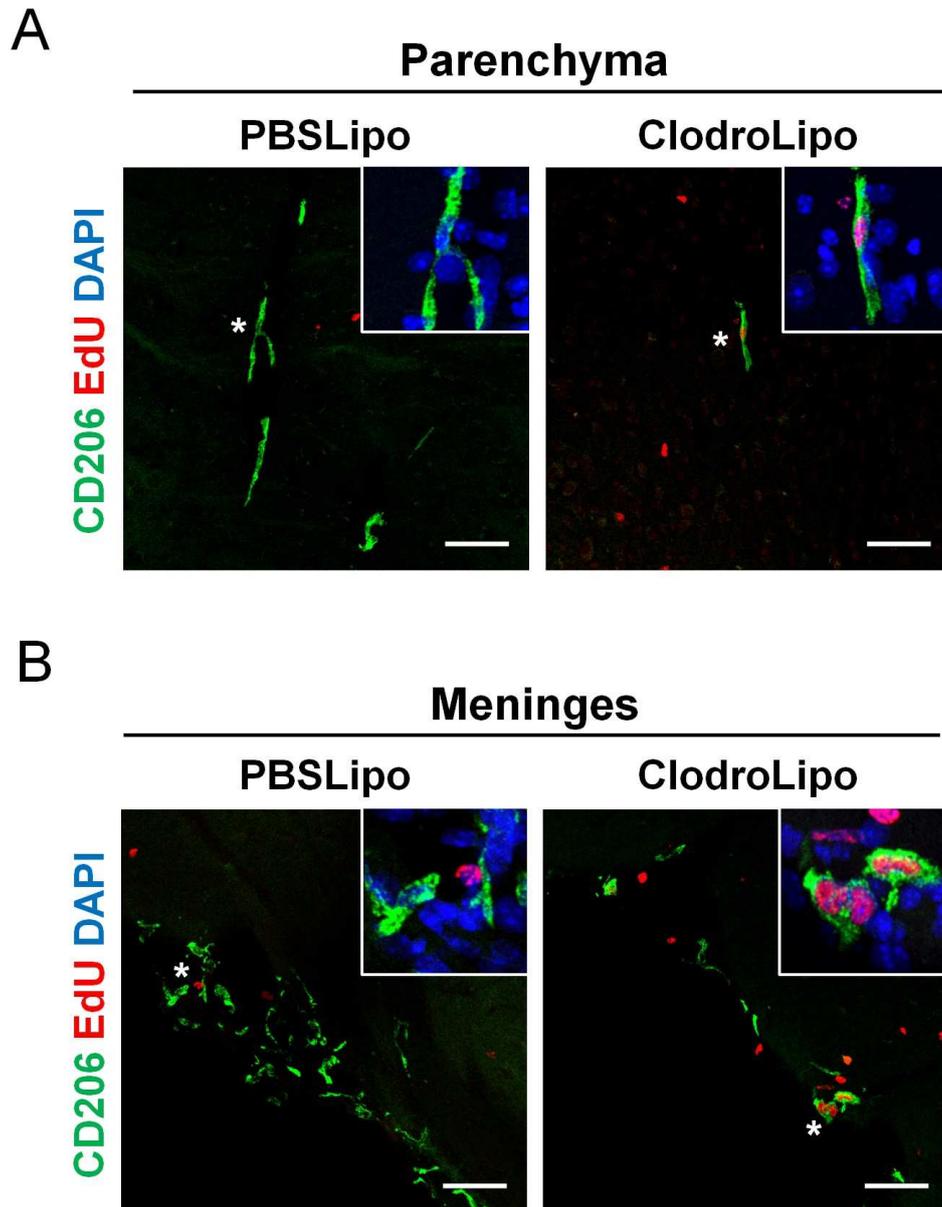


Figure 17. Local proliferation allows the reconstitution of PVMs after depletion. (A, B) Confocal microscopy images demonstrating that ten days after PBS liposomes (PBSLipo) or clodronate liposomes (ClodroLipo) injections, PVMs that incorporated EdU can be found in (A) the SN and (B) the meninges (*pia mater*) only in the ClodroLipo group. (*) High magnification insets show co-localization of EdU with DAPI. Mice received 3 i.p. injections of EdU, 3 days apart. Bars=50 μ m.

Two weeks after CAVTR110 injection, a significant loss of TH+ DN (of 16%) within the SNpc was reached in mice subjected to PVMs ablation, whereas diseased animals with PVMs display normal counts of TH+ DN (Figure 18, B and C). The extent of DN loss was assessed at two weeks, before degeneration reaches the plateau we observed from the third week after disease induction (Figure 11A, top panel). In sum, our data indicate that PVMs hold neuroprotective capacity against synucleinopathy-induced toxicity.

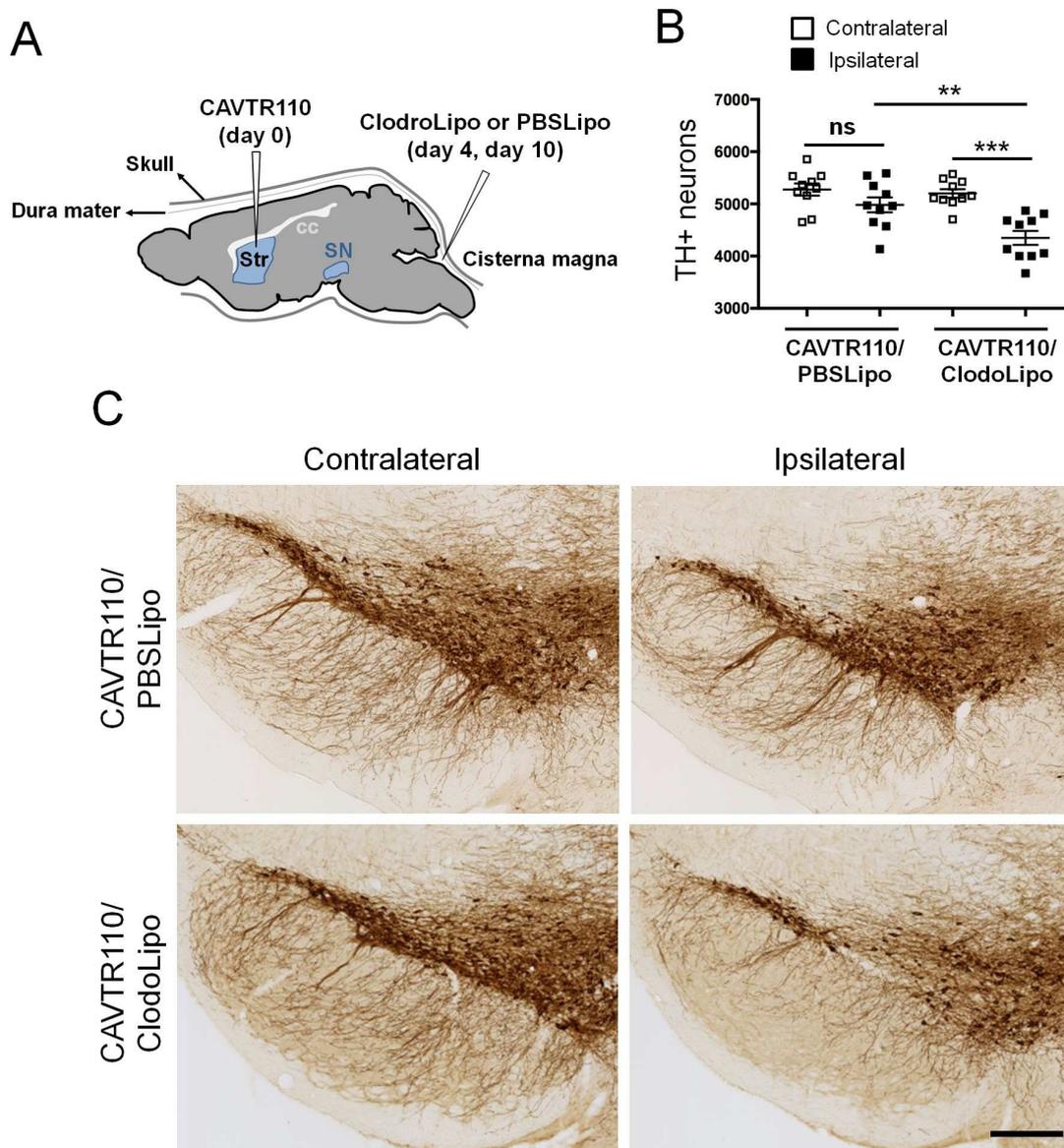


Figure 18. PVMs possess neuroprotective properties. (A) Schematic representation of the experimental approach. CAVTR110 injected mice received two intra cisterna magna injections of either clonate liposomes (ClodroLipo) to deplete PVMs or PBS liposomes (PBSLipo) as control, and were sacrificed two weeks after the viral

injection. Str: striatum, SN: substantia nigra, cc: corpus callosum. (B) Quantification of TH+ DN_s in the SNpc. At two weeks, significant degeneration is observed in CAVTR110 injected mice treated with ClodroLipo (CAVTR110/ClodroLipo) as compared to CAVTR110 injected mice treated with PBSLipo (CAVTR110/PBSLipo). Mean \pm s.e.m., n=10 mice per group. **p<0.01, ***p<0.001, ns not-significant, ANOVA followed by Tukey's posthoc test. (C) Representative photomicrographs of mesencephalic sections immunostained for TH (brown staining) from CAVTR110/ClodroLipo mice and CAVTR110/PBSLipo mice. Bar=100 μ m.

Given the enhanced neurodegeneration observed in the absence of PVMs, we next examined whether microglial cell activation was impacted as well. At the cellular level, microglia activation was quantified by estimating the surface area occupied by Iba1 positive cells (Iba1 immunoreactivity). Surprisingly, we found that ablation of PVMs has no impact on activated microglia cell number and morphology (Figure 19, A and B).

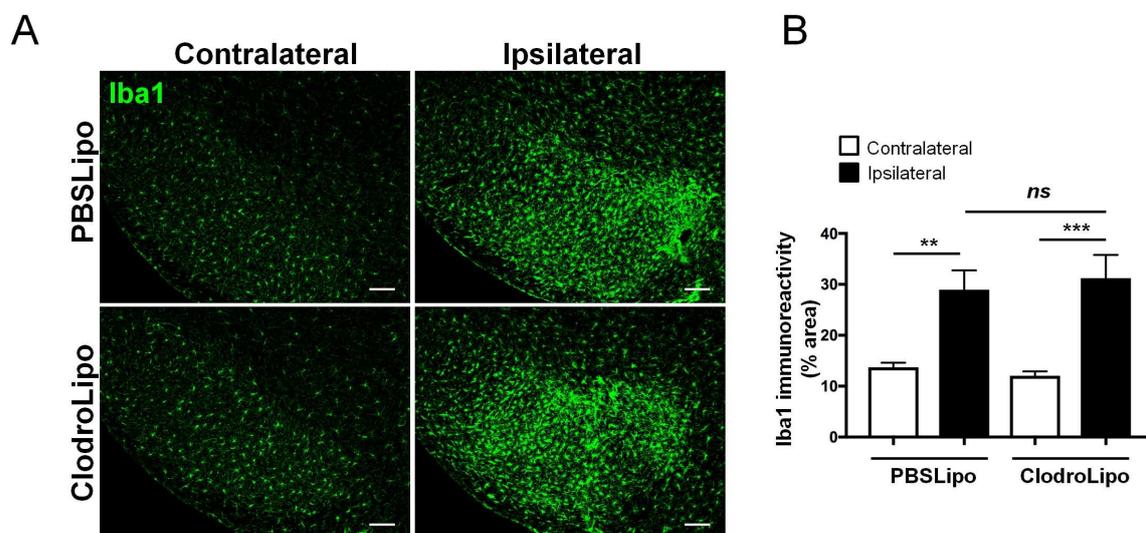


Figure 19. No significant changes of microglia response at the cellular level are observed in the absence of PVMs. (A) Representative images showing microglial cells through Iba1 immunostaining. A robust microglial activation can be observed 2 weeks after CAVTR110 injection, regardless of the depletion of PVMs (PBSLipo vs ClodroLipo, which were injected 4 and 10 days after CAVTR110 injection), bar=100 μ m. (B) Quantification of Iba1 immunoreactivity (% of total area) in the SN (pars compacta + reticulata) shows that at the cellular level, microgliosis is not

modified in the presence (PBSLipo) or absence (ClodroLipo) of PVMs two weeks after the injection of CAVTR110. Mean \pm s.e.m., n=10 mice per group. **p<0.01, *p<0.001, ns not-significant, ANOVA followed by Tukey's posthoc test.**

Since sole histological examination of microglial cells is poorly informative on their activation and polarization state, we conducted in-depth molecular analysis by establishing and comparing the transcriptomic profile of FACS-purified microglial cells (Figure 20A) from the mesencephalon of diseased animals depleted or not for PVMs. We found that gene expression of microglia after CAVTR110 injection was very similar in the presence or absence of PVMs (CAVTR110/PBSLipo: 7 up-regulated genes, CAVTR110/ClodroLipo: 3 up-regulated genes, p-value < 0.05, 2-fold) (Figure 20C). By contrast, microglia sorted from CAVGFP injected mice differed significantly from microglia sorted from CAVTR110 injected mice (CAVGFP: 101 up-regulated genes, CAVTR110: 653 up-regulated genes, p-value < 0.05, 2-fold) (Figure 20B). Together, these results suggest that pathological response of PVMs barely regulates microglial cell response to degenerative synucleinopathy.

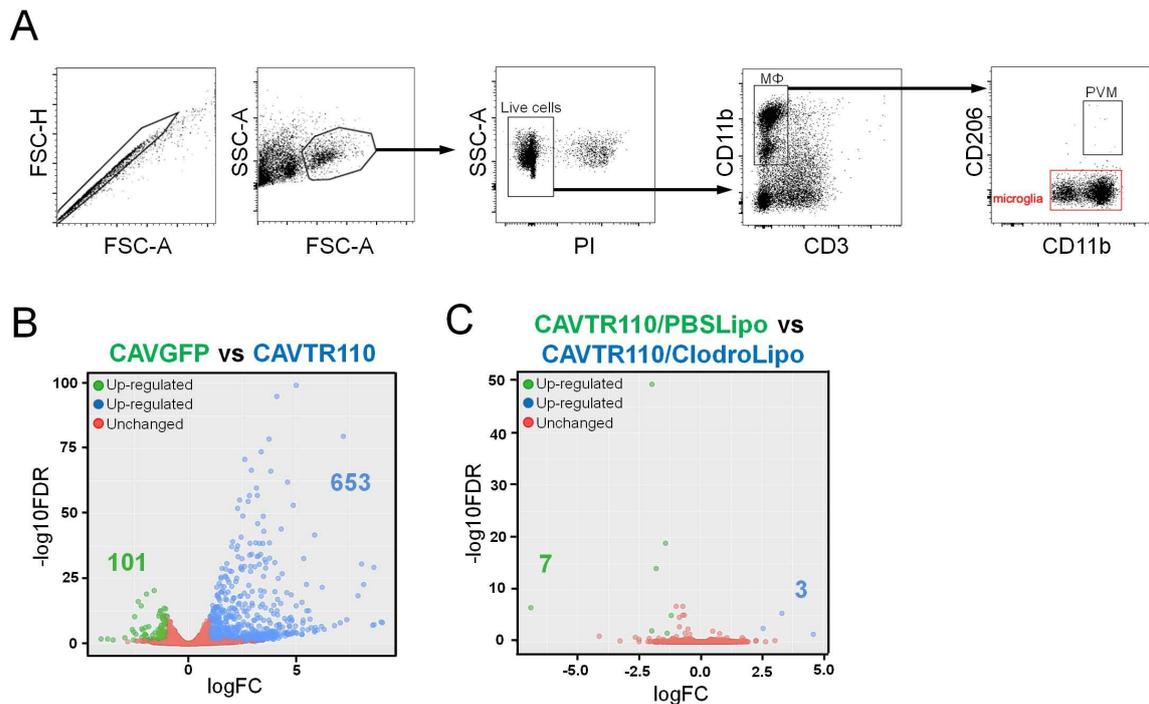


Figure 20. The depletion of PVMs does not seem to greatly impact the molecular signature of microglia. **(A)** Gating strategy for FACS sorting of microglia (CD11b+CD206-). To exclude doublets, cells were gated by plotting forward scatter (FSC)-Area (A) vs. FSC-Height (H). Nucleated cells were then gated by excluding cell debris in forward scatter FSC-A vs. side scatter (SSC)-A. Dead cells were excluded by propidium iodide (PI) staining and lymphocytes were excluded by CD3 staining. **(B)** Volcano plot of microglia RNAseq sorted from CAVTR110 injected mice and from CAVGFP injected mice. Blue and green dots represent up-regulated genes in groups CAVTR110 and CAVTRGFP, respectively. **(C)** Volcano plot of microglia RNAseq sorted from CAVTR110 injected mice that received clodronate liposomes (CAVTR110/ClodroLipo) and from CAVTR110 injected mice that received PBS liposomes (CAVTR110/PBSLipo). Blue and green dots represent up-regulated genes in groups CAVTR110/ClodroLipo and CAVTR110/PBSLipo, respectively **(B, C)** Number of regulated genes per group are shown in each figure. The x-axis shows the \log_2 fold change (FC), and the y-axis shows $-\log_{10}$ False Discovery Rate (FDR) or adjusted p-value (fold change>2, FDR<0.05).

PVMs have been demonstrated to constitutively express MHC class II (Hickey and Kimura, 1988; Kida et al., 1993b). Moreover, PVMs are strategically positioned within the perivascular space, a structure that lymphocytes must cross in order to reach the brain parenchyma. For these reasons, we sought to investigate whether in the pathological context, the extravasation of lymphocytes was modified in the absence of PVMs. To this end, we quantified helper and cytotoxic T cells in the injured SN of mice treated twice with ClodroLipo or PBSLipo, by measuring the surface area occupied by CD4 and CD8 immunostainings, respectively. We found that both CD4 and CD8 immunoreactivity was strongly increased in ClodroLipo treated mice as compared to controls (Figure 21, A-D). In order to facilitate the extravasation of lymphocytes into the brain parenchyma, cerebrovascular endothelium must undergo permissive modifications and express trafficking determinants that include adhesion molecules. Therefore, to further explore the enhanced T-cell infiltration in the absence of PVMs, we analysed the expression of vascular cell adhesion protein 1 (VCAM-1), an adhesion molecule implicated in several steps required for cell trafficking across the BBB (Takeshita and Ransohoff, 2012). We found that vascular expression of VCAM-1 (calculated as the surface area occupied by VCAM-1 immunostaining inside ColIV positive vessels) was exacerbated in CAVTR110 injected mice when PVMs were ablated (Figure 21, E and F). Importantly, ColIV immunoreactivity was not affected by ClodroLipo treatment as compared to controls (Figure 21G). Remarkably, the sole depletion of PVMs leads to the accumulation of proliferating CD3 positive lymphocytes (that incorporated EdU) within meningeal tissue in the vicinities of the SNpc (Figure 22), but also in other regions (data not shown). Together, these observations suggest that PVMs might act as gatekeepers to control the trafficking of lymphocytes into the central nervous system, in physiological and pathological conditions.

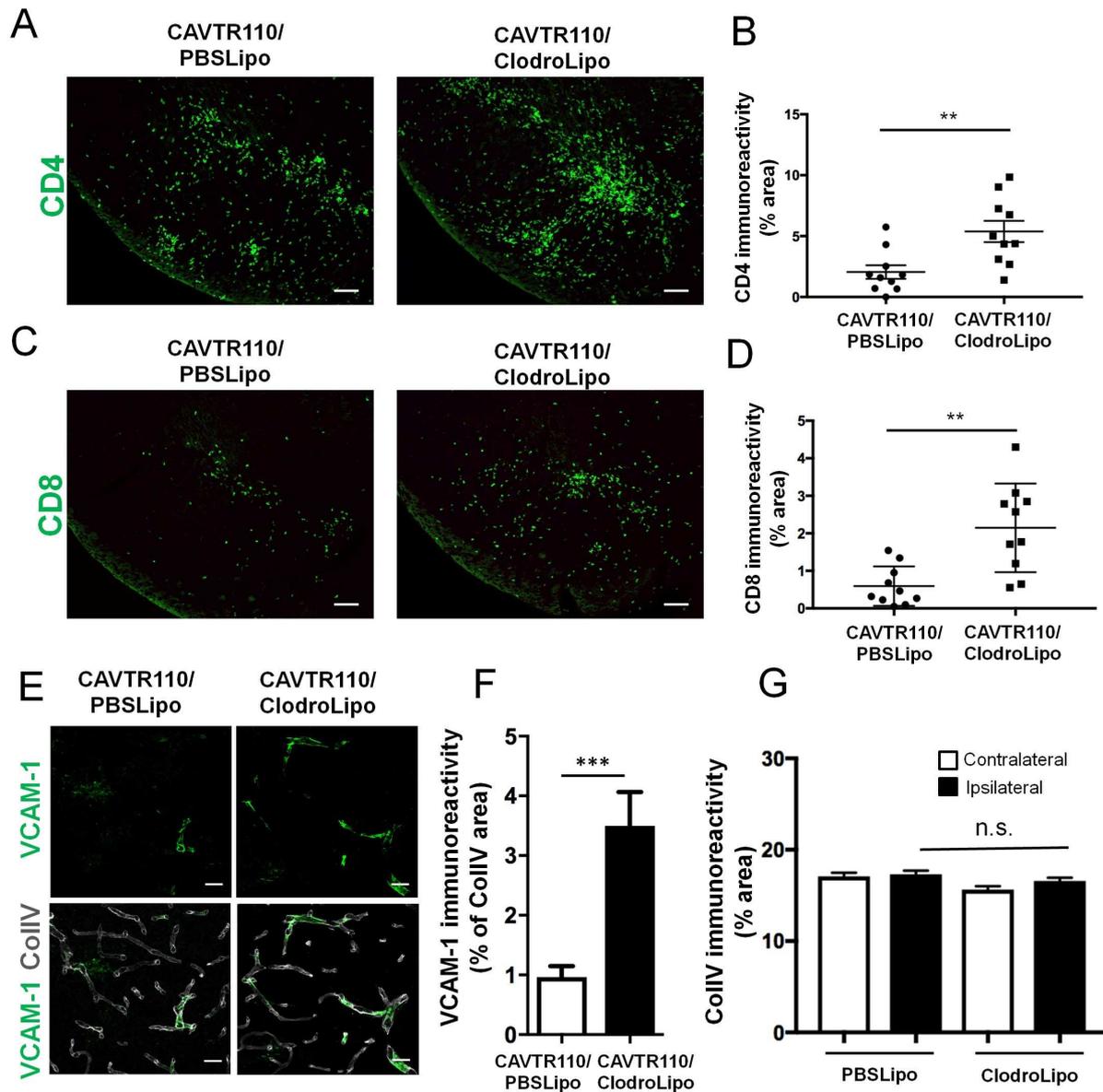


Figure 21. The infiltration of lymphocytes is exacerbated in the injured SN when PVMs are lacking. (A, C) Representative images showing greater numbers of CD4 (A) and CD8 (C) T cells within the SN (pars compacta + reticulata) in CAVTR110 injected mice treated with clodronate liposomes (ClodroLipo) as compared controls (treated with PBS liposomes or PBSLipo), bar=100 μ m. (B, D) Quantification of the surface area occupied by CD4 (B) and CD8 (D) immunoreactive T-cells (immunoreactivity: % of total area) in the injured SN, in the presence (PBSLipo) or absence of PVMs (ClodroLipo). Mean \pm s.e.m., n= 10 per group. **p<0.01, Student's t-test. (E, F) Confocal microscopy analysis shows that in the pathological context, vascular VCAM-1 immunoreactivity (vascular VCAM-1 surface area per total ColiV

surface area) is increased in PVMs deficient mice as compared to controls, bar=20 μ m. Mean \pm s.e.m., n= 10 per group, ***p<0.001 Student's t-test. (G) Quantification of ColIV immunoreactivity (% of total area) demonstrates that vasculature is not significantly modified in CAVTR110 injected mice in the presence or absence of PVMs (PBSLipo vs ClodroLipo). Mean \pm s.e.m., n= 10 per group, ns not-significant, ANOVA followed by Tukey's posthoc test.

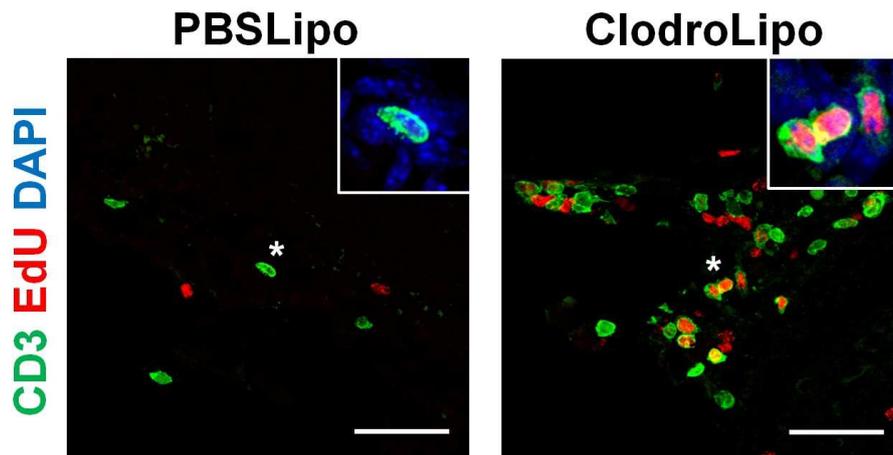


Figure 22. The depletion of PVMs leads to the proliferation of CD3 T-cells in the meninges. Confocal microscopy images showing the proliferation of CD3+ lymphocytes (EdU+) within the meninges (*pia mater*), in the vicinities of the SNpc, ten days after a single injection of clodronate liposomes. High magnification insets show co-localization of EdU with DAPI. Mice received 3 i.p. injections of EdU, 3 days apart. Bars=50 μ m.

1.5 Synucleinopathy spreading is aggravated in PVMs-depleted mice

Converging lines of evidence strongly suggest that misfolded α -Syn propagates between interconnected CNS regions. Importantly, this implicates that toxic α -Syn must be released into the extracellular space and subsequently incorporated into neighbouring neurons in order to seed further aggregation of soluble α -Syn (Wong and Krainc, 2017b). Remarkably, in CAVTR110 injected mice we detected human α -Syn deposits within PVMs (Figure 12C), strongly suggesting that they are capable of taking up extracellular α -

Syn. Therefore, we sought to evaluate whether PVMs may regulate the propagation of α -Syn pathology. To this end, we made use of an alternative model consisting in the intrastriatal injection of mouse α -Syn fibrils with seeding and propagation properties *in vitro* and *in vivo* (Bousset et al., 2013b; Peelaerts et al., 2015b). Interestingly, we observed that 3 and 6 months after a single unilateral injection of mouse α -Syn fibrils into the dorsal striatum resulted in the accumulation of protein inclusions made of phosphorylated α -Syn on serine 129 (pS129- α Syn). Since in PD patients most of α -Syn deposited in LB/LN is phosphorylated on serine 129, pS129- α Syn immunostaining is frequently used as a marker of Lewy pathology (Fujiwara et al., 2002). In our model, these inclusions were observed in neuronal soma and neurites (resembling Lewy pathology) (Figure 23A). Importantly, the amount and distribution of pS129- α Syn-stained elements increased over time, illustrating the propagation properties of this model of synucleinopathy (Figure 23B). Moreover, it is worth noting that from 3 to 6 months post fibril injection, there was a pS129- α Syn pattern distribution switch from neuritic to somatic staining, suggesting that pathological α -Syn deposition starts in neuronal processes to progressively reach and contaminate neuronal cell bodies. After fibril injection, pS129- α Syn was detected in the striatum and cortex of both hemispheres, as well as in the ipsilateral SN (Figure 23B) where α -Syn inclusions were clearly identified within TH+ DN (Figure 23C).

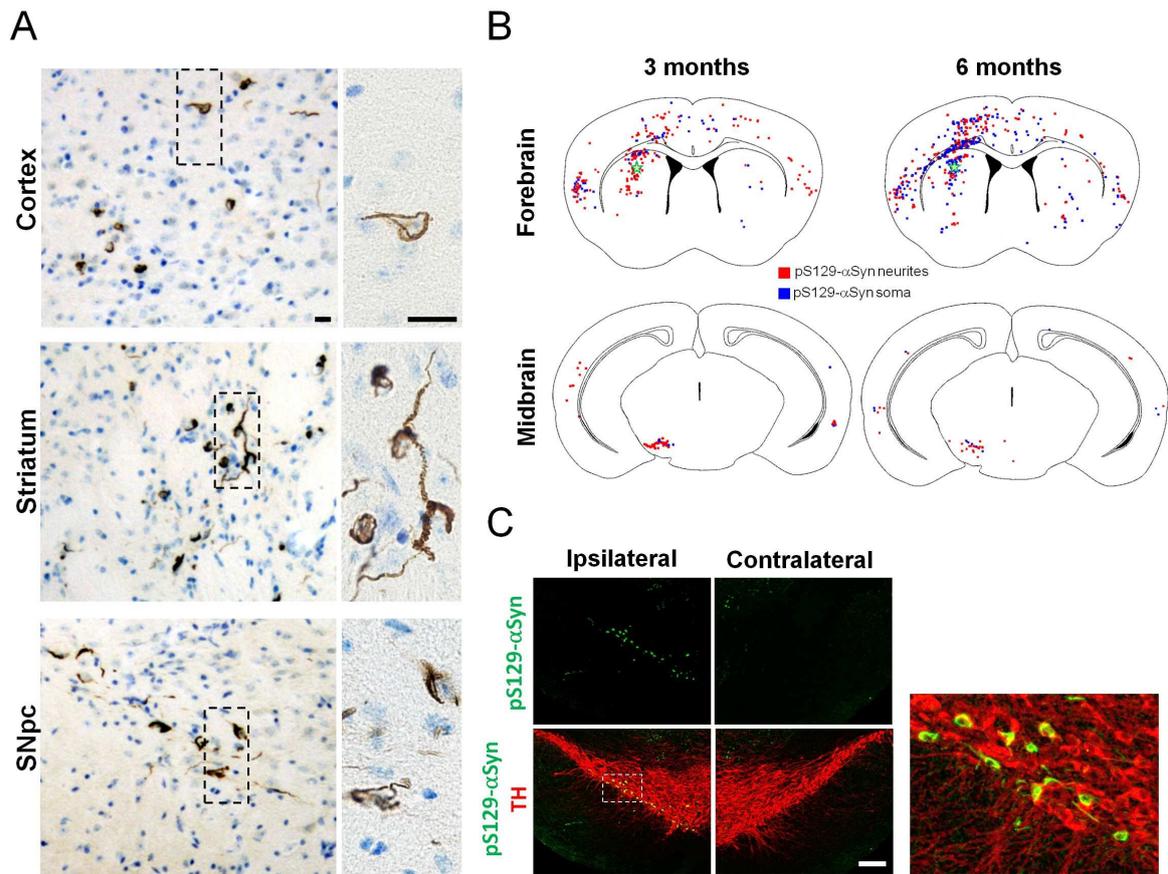


Figure 23. The inoculation of mouse α -Syn fibrils seeds the aggregation of endogenous α -Syn in wildtype mice. (A) The delivery of 10ug of Atto647 tagged mouse α -Syn fibrils into the dorsal striatum can induce Lewy-like pathology 3 months after the injection, as detected by the presence of inclusions of α -Syn phosphorylated on serine 129 residue (pS129- α Syn). pS129- α Syn immunoreactive deposits can be found in neurites (pS129- α Syn neurites) and in cellular bodies (pS129- α Syn soma) within the cortex, the striatum and the SNpc. Bars=20 μ m. (B) The mapping of pS129- α Syn immunostaining shows that pS129- α Syn neurites (red dots) and pS129- α Syn soma (blue dots) can be found in the striatum and the cortex (forebrain), in both hemispheres, but also in the ipsilateral SN (midbrain), 3 and 6 months after the delivery of the fibrils. Green star shows the injected site. (C) pS129- α Syn and TH colocalization within the ipsilateral SN only. High magnification image from white dotted box is shown at the right side of the figure. Bar=100 μ m.

In fibril-injected mice, chronically depleted for PVMs (through the delivery of ClodroLipo every 2 weeks for 3 months), we found that the spreading of Lewy-like pathology was profoundly aggravated. Indeed, both LB/LN-like elements were found in greater numbers within the cortex and striatum of both hemispheres (Figure 24, A and B). Furthermore, although in the absence of PVMs we only detected a trend to increased numbers of LN-like inclusions within the ipsilateral SN, significance was reached in the case of LB-like counts (Figure 24, C and D). No degeneration of DNs was noticed as a result of α -Syn fibrils injection, even in the absence of PVMs, the experimental condition that led to an aggravated Lewy-like pathology (Figure 24E). Overall, our data indicate for the first time that PVMs are important perivascular elements in regulating synucleinopathy spreading *in vivo*.

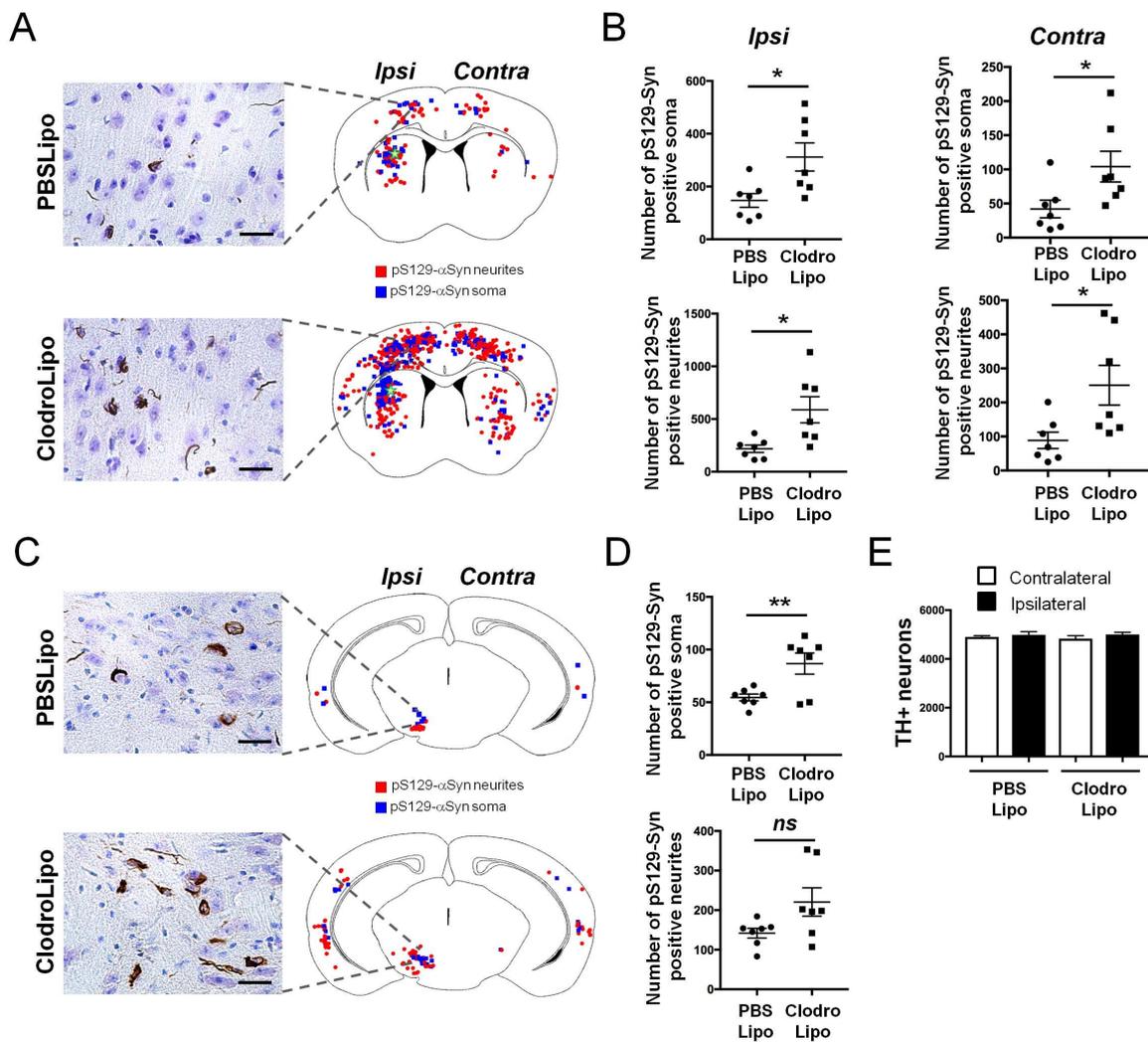


Figure 24. The invalidation of PVMs exacerbates Lewy-like pathology. (A-D) Mice injected with 10ug of Atto647 tagged α -Syn fibrils into the dorsal striatum show more inclusions containing phosphorylated α -Syn on serine 129 (pS129- α Syn) when PVMs are lacking (liposomes were delivered every two weeks during 3 months). (A, C) Representative maps of Lewy-like pathology (pS129- α Syn neurites: red dots and pS129- α Syn soma: blue dots). Green star shows the injected site. Representative high magnification images are depicted to show pS129- α Syn immunostaining in (A) the neocortex and (C) the SNpc. (B, D) Quantification of pS129- α Syn neurites and pS129- α Syn soma throughout (B) the cortex plus the striatum or (D) the ipsilateral SN of mice injected with PBS liposomes (PBSLipo) or clodronate liposomes (ClodroLipo). Mean \pm s.e.m., n=7 per group. *p<0.05, **p<0.01, ns not-significant. Student's t-test, bar=100 μ m. (E) Quantification of TH+ DN in the SNpc of mice injected with α -Syn fibrils treated with PBSLipo or ClodroLipo. Mean \pm s.e.m., n=7 mice per group. ns, not-significant. ANOVA followed by Tukey's posthoc test.

1.6 PVMs-depleted mice show impaired CSF influx and reduced lymphatic clearance

The finding that PVMs ablation leads to increased synucleinopathy spreading and toxicity suggests that PVMs may play an essential role in clearing extracellular α -Syn assemblies. Although direct phagocytosis of α -Syn assemblies by PVMs may concur to limit parenchymal burden of α -Syn, acellular pathways of protein clearance could also be involved. Indeed, it has been recently demonstrated that the perivascular space is required for fluid transport within the brain (Iliff et al., 2012a). While perivascular space of arterioles constitutes the route of CSF influx into the brain, perivascular space of venules carries brain ISF back into the CSF. This pathway for fluid transport has been termed the “glymphatic system” (Iliff et al., 2012a). Considering that PVMs reside within the perivascular space of both arterioles and venules (Figure 10F), we next sought to test whether their depletion would have a functional impact on the glymphatic system. To this end, 4 days after ClodroLipo or PBSLipo delivery, Ova (Alexa647-ovalbumin) was infused into the CSF compartment via the cisterna magna. After 30 minutes, the diffusion of the fluorescent tracer into the brain parenchyma through the glymphatic pathway was assessed *ex vivo*. Remarkably, we found that in the absence of PVMs, diffusion of Ova into the parenchyma was clearly reduced, while staying confined to the contour of brain sections (Figure 25A, bottom panel). On the other hand, Ova properly diffused into the brain parenchyma of PBSLipo treated mice, where PVMs engulfed the tracer (Figure 25A, top panel). Additionally, we noticed that the drainage of Ova into deep cervical lymph nodes (dcLN) was also decreased in ClodroLipo injected mice (Figure 25B), suggesting that a reduced fluid transport within the brain would impact CNS lymphatic drainage as well. In sum, these observations suggest that PVMs are important players in ISF and CSF flows whereby parenchymal solutes and possibly α -Syn assemblies are cleared and drained out.

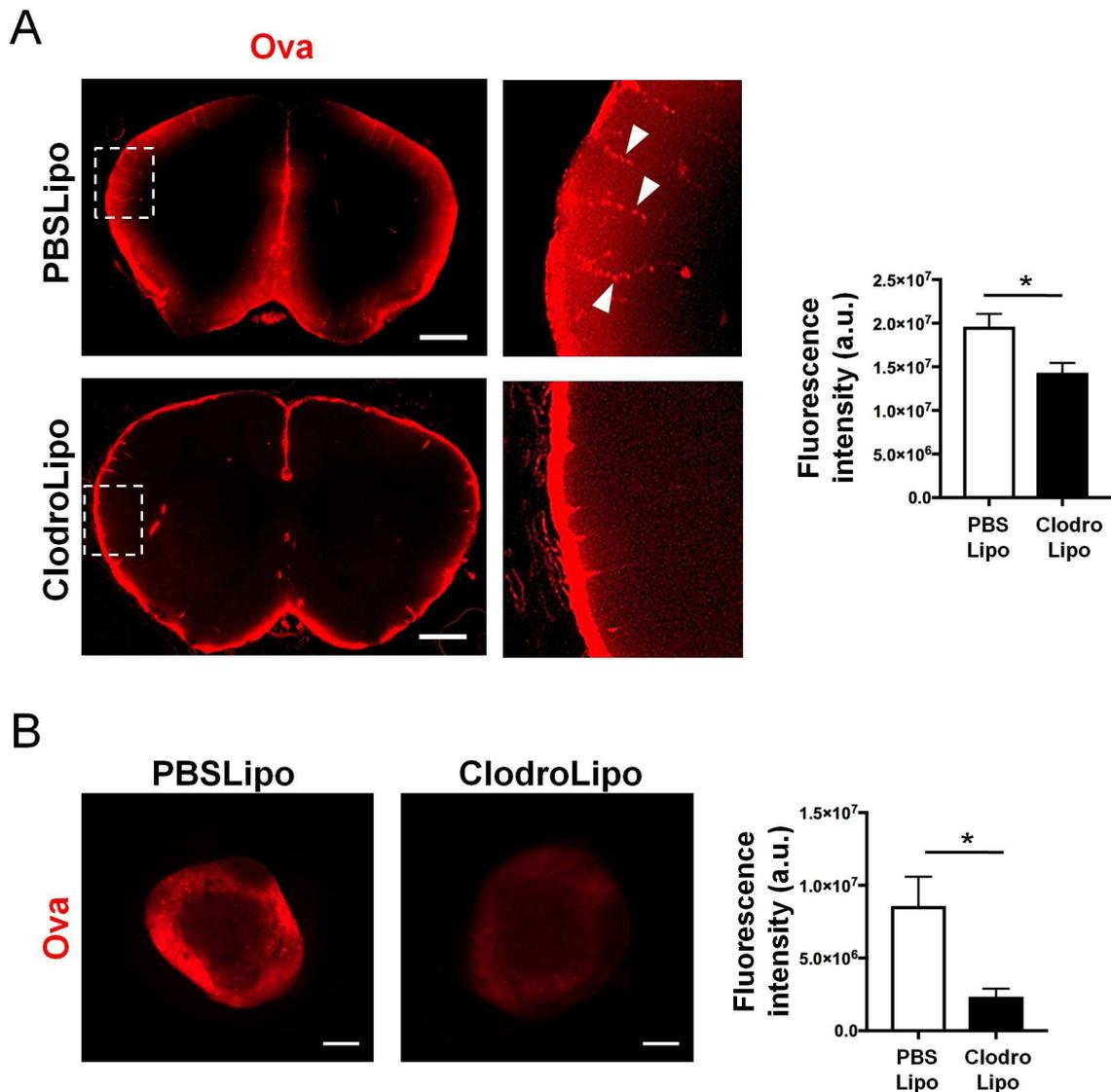


Figure 25. The depletion of PVMs impairs CSF influx into the brain parenchyma and the lymphatic drainage of CSF. (A, B) Four days after a single injection of PBS liposomes (PBSLipo) or clodronate liposomes (ClodroLipo), mice received an intracisternal injection of Alexa647-Ovalbumin (Ova). 30 min after injection, animals were transcardially perfused and fluorescence intensity (integrated density) of (A) whole-slices and (B) deep cervical lymph nodes (dcLN) was determined. CSF/Ova diffusion into the brain parenchyma is significantly reduced in PVMs-deficient mice, as well as the drainage of Ova into the dcLN. Mean \pm s.e.m., $n=5$ per group. * $p<0.05$, Student's t-test. High magnification insets in (A) show the infusion of Ova into the brain parenchyma. PVMs that engulfed Ova can be seen in PBS-injected mice (white arrowheads), but not in the clodronate-treated group, indicating that the depletion of PVMs was efficient. (A) bar=1mm and (B) bar=200 μ m.

1.7 PVMs accumulate in the SNpc of PD patients

Finally, to confront our experimental data to the human condition, we quantified the number of CD206 positive cells within the SNpc, the striatum and the red nucleus of *post-mortem* human brains, from PD patients and neurologically intact age-matched controls. Similar to what we observed in mice, CD206 positive cells were always found associated with lectin-stained blood vessels in the human mesencephalon (Figure 26A). Remarkably, greater numbers of CD206-positive cells with typical elongated morphology of PVMs were found in the degenerating SNpc of PD patients as compared to controls (Figure 26, A and B). In contrast, no accumulation of PVMs was observed in the putamen of PD patients, as well as in the non-lesioned red nucleus (Figure 26B). Together, these observations show that accumulation of PVMs within the SN occurs during PD and suggest that this pathological response may serve protective purposes.

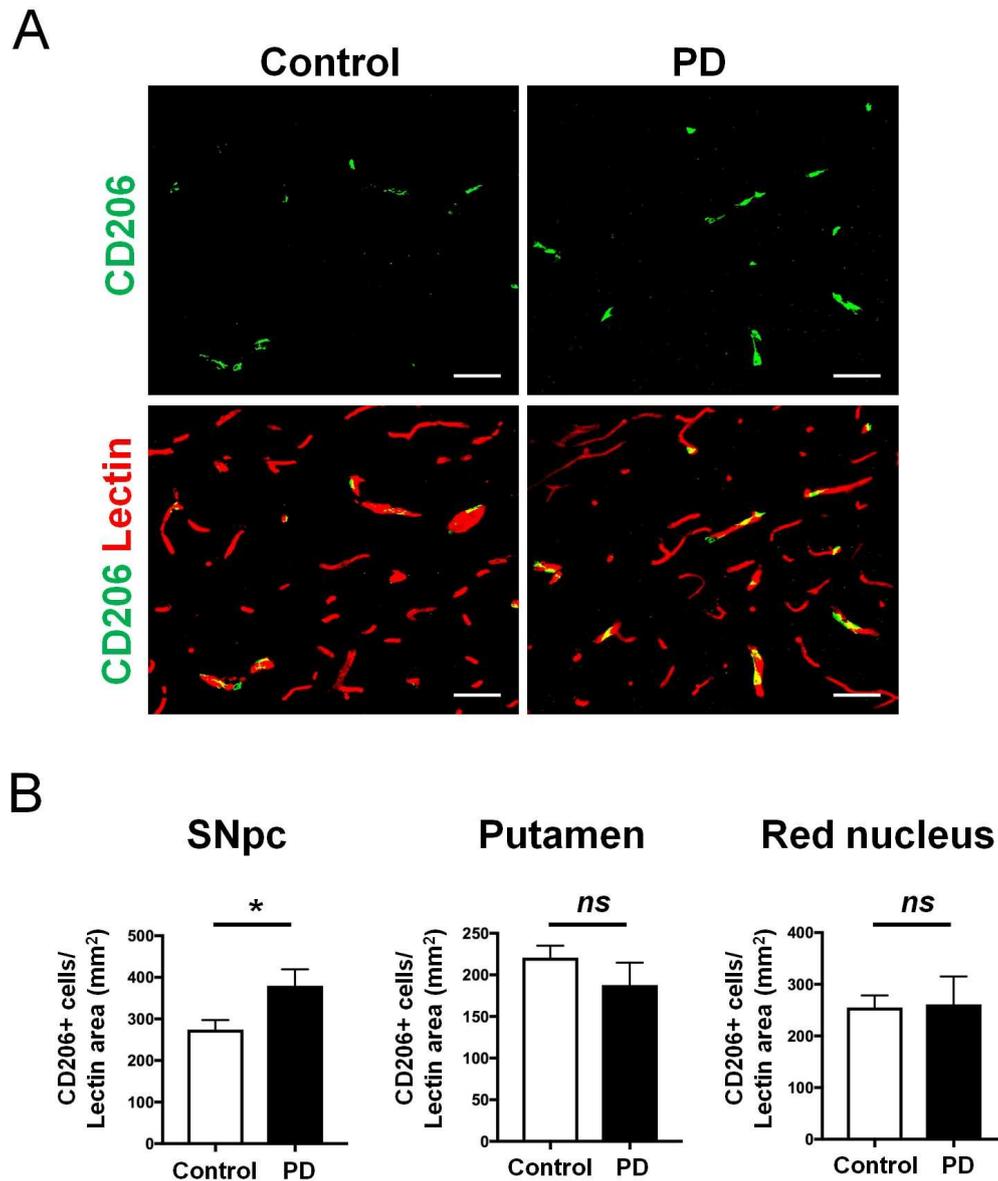


Figure 26. The number of PVMs is increased in post-mortem tissue from PD patients as compared to age-matched controls. (A) Representative image showing greater numbers of CD206 positive PVMs within vasculature stained with lectin in the SNpc from PD patients, compared to neurologically intact subjects, bar=100 μ m. (B) Quantification of the number of CD206 positive PVMs per mm² of vascular surface area occupied by lectin staining in the SNpc (n=9 PD patients and 10 controls), the putamen (n=5 PD patients and 5 controls) and the red nucleus (n=7 PD patients and 9 controls). Mean \pm s.e.m, *p<0.05, ns not-significant. Student's t-test.

GENERAL DISCUSSION

The purpose of this thesis work was to investigate the potential involvement of perivascular macrophages in Parkinson's disease pathophysiology. PVMs are strategically positioned within the perivascular space, between vascular endothelial cells and the *glia limitans* of both arterioles and venules. They were never found, however, along capillaries, which lack a definite perivascular space (Brinker et al., 2014). Although PVMs have been implicated in some neurological conditions (Goldmann et al., 2016; Hawkes and McLaurin, 2009; Park et al., 2017), until very recently nothing was known about their role in PD. Here we provide evidence suggesting that this unique brain macrophage population may hold neuroprotective functions during PD.

1.1 PVMs dynamically respond to neurodegeneration

The possible involvement of a particular cell population in pathology might be evaluated histologically through the examination of several parameters including, among others: morphological changes, strong modifications of protein expression levels and cell numbers. In the present thesis work, we show that CD206-positive PVMs start accumulating very early within the SN in a viral model of degenerative synucleinopathy, i.e. before significant dopaminergic demise occurs. Importantly, this phenotype could not be attributed to an overall increased surface area occupied by the vasculature. These observations suggest that PVMs might be implicated in a PD-like neurodegenerative context. In line with our results, it was observed that PVMs accumulate in the striatum of 6-OHDA injected mice (Tentillier et al., 2016). However, the authors used CD163 immunostaining to identify PVMs, which is not specific. Indeed, it has been observed that activated microglia in post-mortem tissue from PD patients also expresses CD163 (Pey et al., 2014). Therefore, PVMs accumulations in the 6-OHDA model needs to be confirmed using CD206 marker, which is the only specific marker for PVMs identified so far (Galea et al., 2005).

Importantly, greater PVMs numbers were also found in the SNpc of PD patients at post-mortem. This prompted us to speculate that they might not only be implicated in an animal model of PD, but also in the human condition. Despite the fact that the striatum is also affected in PD, PVMs numbers were similar between PD patients and controls. We argue that a differential local inflammatory response mounted in the SNpc versus the striatum might account for such discrepancy. Notably, PVMs numbers were not

significantly changed in the red nucleus either. Since the red nucleus is an area that is not implicated in PD, assessing PVMs numbers was essential to demonstrate that the phenotype observed in the SNpc was not caused by external factors like medication. Indeed, chronic levodopa treatment has been associated with the development of microvascular changes such as angiogenesis (Ko et al., 2015). Finally, although post-mortem studies offer only a snapshot of the very complex and dynamic processes underlying neurodegeneration in PD, our observations revealed that PVMs might be implicated in the human disease. The implication of brain myeloid populations in PD pathophysiology has focus on microglia, yet in most of those studies, pan-macrophage markers including HLA-DR, CD68, CD11b, Iba1 have been used (Gerhard et al., 2006; Hirsch and Hunot, 2009; McGeer et al., 1988; Sanchez-Guajardo et al., 2013). All these markers are also expressed by PVMs, therefore previous reports might have neglected PVMs contributions to mounting the innate immune response.

We have observed that PVMs accumulate in the injured SN, yet which mechanisms could account for such recruitment in our model? PVMs are believed to be slowly replaced by blood-derived cells (Bechmann et al., 2001). This assumption was made on the basis of bone-marrow transplantation experiments, which require whole-body irradiation as a conditioning regimen for allowing the engraftment of donor-derived hematopoietic precursors. However, it has been shown that exposing the brain to gamma irradiation induces disruption of the BBB, leading to a strong production of cytokines and chemokines (Kierdorf et al., 2013). Furthermore, the intravenous injection of whole bone marrow into the recipient's circulation carries progenitor populations that would not be present in the bloodstream under normal physiological conditions. Hence, it has been firmly demonstrated that the combination of both bone marrow transplantation and whole-body irradiation is required for the entry of circulating cells into the CNS (Mildner et al., 2007). Therefore, to avoid any methodological bias, we decided to use parabiosis as an alternative method to track the brain engraftment of blood-derived cells under PD-like pathological conditions. Parabiosis establishes a common blood circulation by surgical pairing; that is, creating a surgical union of twin animals or parabionts (Bunster and Meyer, 1933). Using GFP+ donors to establish parabionts allows the natural (or physiological) introduction of trackable mature immune cells, as well as some of their precursors, into the blood circulation of the recipient mice. Moreover, instead of irradiation, we used busulfan treatment to induce myeloablation of the recipient animals. Busulfan is a clinically

employed chemotherapeutic agent used to perform myelosuppression in patients prior to receiving bone-marrow transplants. Importantly, initial reports indicated that the use of busulfan does not cause overt changes in brain vasculature (Kierdorf et al., 2013). Using parabiosis in combination with busulfan treatment we found that peripherally derived PVMs indeed accumulate within the injured SN with time. Intriguingly however, equivalent proportion of blood-borne PVMs were also observed in the non-lesioned (control) contralateral SN, suggesting that either PVMs were physiologically recruited to the brain or that busulfan treatment was involved. While designing and testing new experimental set up to arbitrate between these two possibilities, Goldmann and collaborators, by using a combination of fate mapping experiments and parabiosis, demonstrated that PVMs are in fact a stable population that do not undergo significant exchange with blood cells (Goldmann et al., 2016). Therefore, suspecting that busulfan was causing the replacement of PVMs by blood-derived cells in our experiments, we established parabionts in the absence of any pre-conditioning busulfan treatment. Remarkably, under such experimental setup, not a single donor derived PVM was found to invade neither the injured SN nor the control one, suggesting that just like brain tissue irradiation, busulfan can induce the artefactual engraftment of bone marrow derived cells. Our data are in line with the observation that bone marrow transplants in busulfan-conditioned mice also led to the brain infiltration of donor-derived cells, very likely due to the long term induction of the chemokine ligand CCL2 (MCP-1), a key regulator of monocyte transmigration (Wilkinson et al., 2013).

Taken together, our observations strongly suggest that in the adult CNS, PVMs are not replaced by blood-derived progenitors, even in pathological conditions. However, the use of busulfan revealed that circulating cells with the potential to enter the CNS and generate PVMs could be mobilized. Since busulfan is already used in clinical protocols, these cells could be therapeutically exploited as vehicles to deliver neuroprotective or immune relevant genes to the diseased CNS.

1.2 PVMs proliferate in response to neurodegeneration (Figure 27)

Instead of a peripheral contribution, we demonstrated using EdU injections that PVM numbers increase due to local proliferation of resident PVMs. However, the presence of perivascular progenitor cells that incorporated EdU within the brain and then gave origin to PVMs, cannot be totally excluded at this point. Indeed, recent developments in

the field of vascular biology have revealed that specific vascular-associated cell populations including mural cells (mainly pericytes) might possess stem cell properties and activities under certain circumstances (Sweeney et al., 2016). Notably, they have been suggested to give origin to parenchymal microglial cells in stroke models (Özen et al., 2014; Sakuma et al., 2016). Yet, given that CD206-positive PVMs expressing the cell cycle marker Ki67 were also found in CAVTR110-injected mice, we believe that either resident PVMs actually proliferate, or that the progenitors they might arise from, also express CD206. Since pericytes do not express CD206, we discard a possible contribution to PVMs pools. In light with these findings, we hypothesized that the expansion potential of PVMs or its progenitors should be sufficient to provide enough progeny for the organism lifetime, both in health and disease.

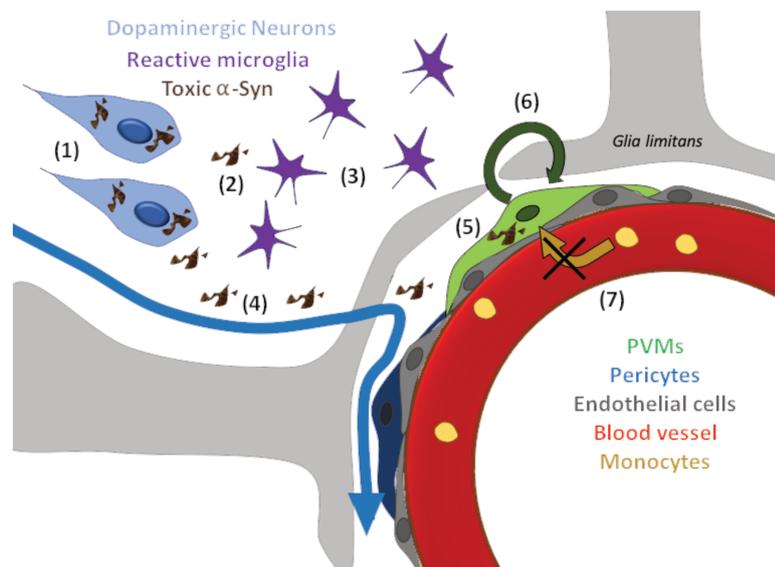


Figure 27. Hypothesis: the expression of a toxic form of α -Syn (1) causes suffering of DNs and degeneration. α -Syn species are released from neurons into the extracellular space (2) probably as a consequence of cell death, through exocytosis or inside exosomes. Extracellular α -Syn and neuronal distress can activate microglia. Also, α -Syn is probably cleared into the CSF following CSF bulk flow (4) (blue arrow) which circulates through the perivascular space of large caliber vessels. Within the perivascular space, α -Syn can be engulfed by PVMs (5), which proliferate (6) as a result of neuronal suffering. It is unclear whether PVMs proliferation is directly triggered by suffering neurons, mediated by reactive microglia or due to the effects of α -Syn. Finally, in this pathological scenario monocytes do not contribute to PVMs pool (7).

Despite the fact that PVMs (or their precursors) proliferate in our disease model, it is far from being a specific feature of degenerative synucleinopathy condition. Indeed, it has been previously shown that PVMs proliferation also occurs in other models of CNS disease, including experimental autoimmune encephalitis (EAE) and simian immunodeficiency virus (SIV) infection (Filipowicz et al., 2016; Goldmann et al., 2016). However, in those studies PVMs were identified through the expression of pan-macrophage markers such as Cx3cr1, CD68 and CD163, none of which being exclusively expressed by PVMs and labelling also several other CNS macrophage populations, as well as circulating monocytes subsets (Filipowicz et al., 2016; Goldmann et al., 2016). Therefore, although it is likely that PVMs proliferate in the context of EAE, SIV-infection and possibly other brain diseases, CD206 marker should be employed to validate these observations.

What could be the factors and/or signals responsible for PVMs proliferation under synucleinopathy-induced neurodegeneration? Here several nonexclusive hypotheses can be proposed. First, it is conceivable that proliferating factors known to be crucially involved in microglial cell expansion during neurodegenerative conditions, may also serve to the expansion of the PVMs population. For instance, in several models of CNS injury including AD, prion disease and facial nerve axotomy, there is an upregulation of colony-stimulating factor 1 (CSF1) (Gómez-Nicola et al., 2013; Guan et al., 2016; Yamamoto et al., 2010). CSF1 promotes microglia proliferation via CSF1 receptor (CSF1R) (Raivich et al., 1998; Yamamoto et al., 2010) and the genetic or pharmacological targeting of CSF1R activity, abolishes microglial expansion (Gómez-Nicola et al., 2013; Guan et al., 2016). Similar secreted factors might account for the proliferation of PVMs. Indeed, despite the fact that PVMs and microglial cells represent distinct specialized population of brain tissue macrophages, they remain however closely related as shown by recent transcriptomic analysis through large-scale single-cell RNA-sequencing (Goldmann et al., 2016). Notably, this study shows that PVMs are much closer to microglial cells than to peripheral monocytes and even to peritoneal macrophages.

Second, as part of an evolutionary ancient system, innate immune macrophages recognize conserved structures in pathogens through the expression of pattern recognition receptors, including Toll-like receptors (TLRs) and scavenger receptors (Kawai and Akira, 2010). The activation of TLR4 in the surface of microglia with lipopolysaccharide (LPS), a bacterial endotoxin, triggers cell proliferation (Rivest, 2009). Remarkably, modified

endogenous proteins such as toxic α -Syn species can activate TLRs as well. For instance, α -Syn oligomers have been recognized as endogenous agonists for Toll-like receptor 2 (TLR2) (Kim et al., 2013). Also, nitrated, misfolded or aggregated forms of α -Syn can activate microglial TLR4, leading to proliferation (Béraud and Maguire-Zeiss, 2012; Reynolds et al., 2008; Su et al., 2008; Zhang et al., 2005). Since the expression of TLRs is a characteristic of macrophages, we speculate that PVMs might proliferate in response to extracellular α -Syn in a TLR-dependent manner. Nonetheless, further investigation would be needed to unravel the exact mechanisms and contributing factors involved in PVM proliferation during the course of PD-like pathology.

1.3 PVMs possess neuroprotective functions

PVMs fulfill a wide variety of homeostatic functions, including immune surveillance, defense from infections, and neutrophil trafficking (Abtin et al., 2014; Polfliet et al., 2001b). Under pathological circumstances, brain PVMs were shown to be beneficial in some conditions (e.g. in models of AD) (Hawkes and McLaurin, 2009; Mildner et al., 2011; Thanopoulou et al., 2010). In contrast, by promoting inflammation they have been also shown to be deleterious in models of myocardial infarction (Yu et al., 2010) and may contribute to functional cerebrovascular alterations associated to hypertension (Faraco et al., 2016), but also mediated by A β peptides (Park et al., 2017). Thus, the pathological function of PVMs strongly depends on the disease context, making difficult to predict their role in PD.

In order to study the role of PVMs in a PD-relevant context, we combined the use of our viral-based PD model with a cell-specific ablation approach. The latter relies on the delivery of clodronate-containing liposomes into the CSF compartment, via the cisterna magna. The selectivity of ventricular-infused clodronate-loaded liposomes to PVMs depletion has been reported previously and was confirmed in our studies by assessing the number of microglial cells, that was similar between cisterna magna-infused clodronate liposomes or PBS liposomes (Hawkes and McLaurin, 2009; Park et al., 2017). Additionally, it has been shown that by injecting DiI (a fluorescent lipophilic cationic indocarbocyanine dye)-containing liposomes, only PVMs appear fluorescently labelled, and not microglia (Polfliet et al., 2001a). Yet, it is essential to mention that CD206-positive cells within the meninges (also referred as subdural meningeal macrophages,

MMs) were also depleted by this treatment. Therefore, this feature has to be taken into consideration when analysing and interpreting the data.

Interestingly, the ablation of PVMs resulted in a significant degeneration of DNs two weeks after CAVTR110 injection, a time at which no degeneration was noticed in control mice. This data strongly suggests that PVMs hold neuroprotective properties in this model of degenerative synucleinopathy. To our knowledge, this is the first report showing that resident PVMs might protect DNs from degenerating during synucleinopathy. Indeed, while a previous report showed that the intravenous administration of liposomes targeting CD163-expressing cells and loaded with the synthetic glucocorticoid dexamethasone (a potent anti-inflammatory drug), could confer neuroprotection in the 6-OHDA model of PD. However, there are uncertainties as to whether the CD163+ targeted cells responsible for the neuroprotection and infiltrating the lesioned areas were PVMs, microglia or infiltrating macrophages (Tentillier et al., 2016). Even though one could consider that most of the neuroprotection might have been possibly conferred by PVMs, the effect of dexamethasone on PVMs would totally mask their response to injury and, therefore, unlike our studies, their inherent protective functions could not be evaluated under such experimental conditions (Tentillier et al., 2016). Nonetheless, this study highlights the principle of macrophage modulation as a potential strategy to achieve neuroprotection and should inspire future therapeutic avenues

Importantly, by showing that PVMs confer neuroprotection, we uncover the dichotomy of functions between two CNS-resident macrophage population in PD pathophysiology: PVMs versus microglia. According to PET and post-mortem studies, microglia is activated in the brain of PD patients (Gerhard et al., 2006; McGeer et al., 1988), and as it was mentioned in the introduction, such activation seems to negatively influence dopaminergic survival. Indeed, in animal models of PD, microglial cells have been suggested to be responsible for the production of toxic molecules like ROSs and reactive nitrogen species (RNSs), as well as pro-inflammatory cytokines (e.g. TNF α and IL-1 β) (Hirsch and Hunot, 2009; Sanchez-Guajardo et al., 2013). Importantly, inhibiting microglial cell activation by anti-inflammatory compounds mitigates dopaminergic cell death in several PD models (Sanchez-Guajardo et al., 2013). The deleterious function attributed to activated microglial cells during PD contrasts with the apparent neuroprotective potential of PVMs, as unravelled in our work. This strongly emphasizes

the importance of carefully and specifically blocking certain immune cell populations for therapeutic purposes in PD.

1.4 Mechanisms of PVM-associated neuroprotection

From a theoretical point of view, several nonexclusive mechanisms may account for the deleterious effects resulting from PVM depletion in our disease model. These mechanisms are likely linked to specific properties that characterize this brain macrophage population and which may be categorized into three main functions: immune regulation, phagocytosis and perivascular functions.

1.4.1 Role of PVMs as immune regulators

PVMs influences on microglial activation state

The brain hosts several myeloid populations other than PVMs, including microglia, meningeal and choroid plexus macrophages. They all act as the first line of defence in the nervous system and react in response to brain injury (Prinz and Priller, 2014). Whether there is a crosstalk between macrophage subsets within the brain in steady state or pathological conditions has not been formally addressed. PVMs are able to produce several inflammatory mediators (Prinz and Priller, 2014), suggesting that they can communicate with other immune cells carrying the specific receptors for these mediators. Moreover, CD206 expression and a high phagocytic activity are two characteristics of macrophages with anti-inflammatory properties, and both are recognised features of PVMs (Cherry et al., 2014). Therefore, we asked whether PVMs might dampen microglial response, which as mentioned before, has been mostly associated with deleterious functions in PD (Hirsch and Hunot, 2009).

To do so, we determined whether pathological microglia response was altered in the absence of PVMs, by analysing the expansion and morphological alterations of the microglial cell population through Iba1 immunoreactivity. Surprisingly, despite greater neurodegeneration in PVM-depleted mice, the extend of microgliosis was similar in the presence or absence of PVMs. Yet, it is increasingly recognized that activated microglial cells are highly plastic and can adopt a great variety of phenotypes and functions that can hardly be defined by morphological parameters and a limited number of cell-surface receptors (Prinz and Priller, 2014). Therefore, a more in-depth molecular characterization was mandatory to fully appreciate the potential impact of PVMs depletion upon microglial

cell polarization and function. To our surprise, the transcriptome profiling of microglial cells was unchanged in the absence of PVMs, indicating that PVMs have little, if any, regulatory functions on microglial cell activation in our disease model. We hypothesize that microglia response in the CAV model is so robust, that the depletion of a cell population that is physically isolated from parenchymal macrophages, has little influence on their functions. Alternatively, we cannot rule-out at this point the possibility that the number of biological replicates in our RNA-seq experiments (n= 3) was too low to reveal any subtle differences.

Role of PVMs in the regulation of T-cell extravasation

Taking into consideration their potential role as antigen presenting cells (APCs) related to their constitutive expression of MHC class II (Hart and Fabry, 1995), we sought to visualize the engraftment of lymphocytes into the injured SN, in the presence or absence of PVMs. Significantly greater numbers of CD4⁺ and CD8⁺ T cells were found in the SN when PVMs were lacking. However, whether the neuroprotective functions of PVMs are associated to the regulation of the infiltration of lymphocytes has to be confirmed. Indeed, we still don't know whether T-cell response in our model of synucleinopathy is protective or detrimental. This question is fundamental since depending on the pathological context, T-cells can execute beneficial or detrimental functions. For instance, in a neurodegenerative model of inherited amyotrophic lateral sclerosis (ALS), the invalidation of CD4⁺ T cells results in an accelerated motoneuron disease, suggesting that they might provide supportive neuroprotection (Beers et al., 2008). In contrast, CD4⁺ T cells have been recognized as a source of neurodestruction in the MPTP mouse model of PD (Brochard et al., 2008). More recently, it has been shown that CD8⁺ T cells infiltrating the hippocampus of the THY-Tau22 mouse model of tauopathy were detrimental in promoting neuroinflammation and cognitive decline (Laurent et al., 2017). Therefore, it would be essential to characterize the nature of the adaptive immune response mounted in the injured SN in our synucleinopathy mouse model, both in the presence and absence of PVMs.

The role of PVMs as APCs has been studied to some extent in the EAE model. PVMs were shown to undergo extensive self-renewal by local proliferation before the onset of clinical symptoms and the infiltration of leukocytes into the CNS. Interestingly, PVMs depletion in the EAE pathological context results in a suppression of the clinical symptoms, indicating that they are instrumental during early stages of EAE (Goldmann et

al., 2016; Polfliet et al., 2002). As mentioned before, PVMs constitutively express MHC class II and it has been observed that interactions between CD4⁺ T cells and MHC class II⁺ PVMs/MMs readily occur within the subarachnoid space, well before the onset of the neurobehavioral signs associated with EAE (Kivisäkk et al., 2009). These data further highlight the importance of PVMs in regulating the access and activation state of adaptive immune cells into the CNS and should be considered in the future as a possible physiopathological mechanism in PD.

In an attempt to understand the mechanisms by which PVMs depletion might influence T-cell extravasation, we analysed the vascular expression of VCAM-1, an adhesion molecule of the immunoglobulin superfamily required for leukocyte transmigration (Cook-Mills et al., 2011). Adhesion molecules including ICAM-1 have been found to be upregulated in several PD models as well as in post-mortem PD specimen, and are likely important for the extravasation of lymphocytes in PD (Armentero et al., 2011; Brochard et al., 2008; Miklossy et al., 2006). We showed that PVMs depletion increases the expression of VCAM-1 within the lesioned SN. Interestingly, similar observations regarding enhanced VCAM-1 expression in the absence of PVMs were made in a model of meningitis (Polfliet et al., 2001b). In this study, PVM depletion caused enhanced clinical symptoms, and was unexpectedly associated with a reduced influx of granulocytes, known to play a major role in bacterial clearance. Whether granulocyte infiltration is a characteristic of our model is not known. Nonetheless, these data illustrate the fact that PVMs might differently control the recruitment of distinctive leukocyte populations which hence could result in different disease outcomes.

Another striking observation from our studies is that PVM depletion in non-diseased mice was sufficient to cause the accumulation of proliferating lymphocytes within the meninges close to the SN. Meningeal structures are now a recognized route for lymphocyte entrance and access to the brain parenchyma (Engelhardt and Ransohoff, 2005). In the context of EAE, it was recently shown that the parenchymal invasion of effector T-cells coming from the meninges require the interaction with PVMs/MMs (Schläger et al., 2016). The authors suggested that in the absence of PVMs/MMs, T-cells would not be licensed to enter the CNS parenchyma, which could explain the suppression of the EAE clinical symptoms in PVM-depleted mice (Polfliet et al., 2002). However, in PVM-depleted synucleinopathic mice we found opposite results, i.e. an increased parenchymal T-cell invasion, suggesting that the pathological context is again an important

variable or that an alternative SN-vasculature transmigration operate independently from the meningeal route.

1.4.2 Role of PVMs as phagocytes

Constitutive phagocytic activity is a recognized key functional feature of PVMs. Interestingly, in CAVTR110-injected mice developing degenerative synucleinopathy, we observed α -Syn deposits within PVMs, suggesting that they are efficient in clearing toxic α -Syn from the extracellular space. Moreover, all the newly recruited PVMs to the injured SN are able to engulf fluorescent tracers that circulate within the CSF, attesting that they are functional phagocytes. Whether their phagocytic activity is essential for their neuroprotective potential in our disease models was a tantalizing hypothesis that we sought to address.

We hypothesized that PVMs may play an important role in scavenging extracellular α -Syn, thereby controlling α -Syn pathological burden in the brain parenchyma and therefore limiting the spreading of Lewy-like pathology. To test the later, we made use of an alternative model to visualize the spreading of α -Syn pathology. The model consisted in the direct intracerebral injection of α -Syn fibrils with seeding and propagation capacities *in vitro* and *in vivo* (Bousset et al., 2013a; Peelaerts et al., 2015b). Three months after the delivery of α -Syn fibrils, we observed the deposition of phosphorylated α -Syn at Ser129 (a marker of Lewy pathology in PD patients) into somatic and neuritic inclusions throughout the brain in distinct areas distant from the injection site. These data confirm the primary observation reported in several recent studies showing that α -Syn pathology can propagate *in vivo* (Luk et al., 2009c, 2012b).

It is currently believed that aggregated α -Syn transfers from one neuron to another, in a process that requires the release and subsequent incorporation of α -Syn seeds. Once taken up by a target (still non-diseased neuron) α -Syn seeds would induce endogenous α -Syn aggregation. We hypothesized that PVMs might take up extracellular α -Syn once released, therefore modifying the propagation potential of α -Syn seeds. Indeed, when PVMs are lacking, α -Syn pathology is significantly aggravated in different brain areas, including the neocortex, the striatum and the SN of both hemispheres. The importance of this result is that to our knowledge, this is a pioneer study demonstrating that the propagation of α -Syn can be influenced by surrounding immune cells. The role of

microglial cells to promote clearance of extracellular α -Syn aggregates has been suggested before. However, the observations were made on the basis of passive immunization experiments (using α -Syn specific antibodies), and direct evidence for microglial cell-dependent clearance mechanisms of α -Syn is lacking. The authors determined that antibodies against α -Syn help the clearance of extracellular α -Syn, preventing cell-to-cell aggregates transmission, in a mechanism that involves microglia through the Fc γ receptor (Bae et al., 2012). Some other passive immunization studies have been successful in blocking propagation of α -Syn and neurodegeneration *in vivo* (Games et al., 2014; Tran et al., 2014). Nonetheless, the cellular machinery by which immunization prevents α -Syn spreading remains largely unknown. Our data strongly suggest that besides microglia, PVMs are important players in such clearance mechanisms.

Our results are consistent with previous reports showing that PVMs also play a crucial role in the clearance of protein aggregates in AD. In a mouse model of AD, the invalidation of PVMs leads to an exacerbated accumulation of vascular A β . In contrast, stimulating the turnover of PVMs causes reduced A β deposition (Hawkes and McLaurin, 2009). In line with this, a different study reported that the invalidation of the expression of scavenger receptor class B type I (SR-BI), impairs the response of PVMs to A β , causing enhanced A β deposition and learning and memory deficits in a different mouse model of AD (Thanopoulou et al., 2010). These data further demonstrate that PVMs might be important for the clearance of toxic protein aggregates. Since the accumulation of A β is generally considered causal for synaptic loss and neurodegeneration in AD, it is possible to speculate that decreasing A β burden by stimulating PVMs might be neuroprotective and a similar reasoning may be applied for PD regarding brain α -Syn burden.

It has been observed that PVMs express the chemokine receptor CCR2 (Gómez-Nicola et al., 2014). In a model of AD (APP^{swe} mice), CCR2 deficiency (APP^{swe}CCR2^{-/-} mice) reduces the clearance of A β and increases mortality (El Khoury et al., 2007; Mildner et al., 2011). Recently, by using the same APP^{swe}CCR2^{-/-} mice, it was demonstrated that CCR2 deletion has no influence on PVMs recruitment, however their capacities to clear A β were perturbed (Mildner et al., 2011). Whether CCR2 is also required for the clearance of α -Syn has not been addressed so far. Yet, in the MPTP model CCR2 deletion did not influence DNs loss (Parillaud et al., 2017). This reveals that the phagocytic activity of PVMs might be neuroprotective, yet only in the context of proteinopathies. In line with

this hypothesis, preliminary experiments have suggested that depletion of PVMs in the MPTP model has no influence on nigrostriatal degeneration (data not shown).

Despite the fact that α -Syn pathology can propagate *in vivo* upon α -Syn fibril injections, we were not able to notice neurodegeneration, even in the absence of PVMs, a condition where α -Syn pathology was significantly exacerbated. Our data are consistent with reports showing that even direct intra-nigral injection of α -Syn fibrils does not necessarily cause nigral degeneration (Masuda-Suzukake et al., 2013; Peelaerts et al., 2015b). Yet, opposite results were also reported (Luk et al., 2012b, 2012c) suggesting that experimental variables could contribute to the differential results obtain by various groups. This may include the time course of *in vivo* studies, the conditions of α -Syn fibrils preparation and purification, the site of injection, the model organism, etc.

1.4.3 Role of PVMs as potential regulators the CSF flow

Since we have detected α -Syn accumulations within PVMs in the CAVTR110 model, we wondered how PVMs could have access to extracellular α -Syn if they are confined to the perivascular space and structurally isolated from the brain parenchyma thanks to the *glia limitans*. It has long been shown that tracers injected within the brain parenchyma can be readily taken up by PVMs (Kida et al., 1993b). The removal of extracellular products within the parenchyma is thought to be facilitated by the constant flow of CSF through the brain interstitium. This flow uses the perivascular space to circulate. In order to function, this “glymphatic system” requires the activity of aquaporin-4 on astrocytic end-feet. Importantly, this pathway has been implicated in the clearance of soluble A β (Iliff et al., 2012a). Using cell-specific ablation approach, we were able to demonstrate that PVMs may play an essential role in the functioning of this clearance pathway. It is tempting to speculate that PVMs might directly participate in the maintenance of the pathway, for instance through the production of extracellular matrix components that are important to maintain the structural properties of the perivascular space. In this respect, PVMs/MMs have been shown to express fibronectin (Schläger et al., 2016), which is a structural protein of the BBB basement membrane (Obermeier et al., 2013). Interestingly, the depletion of PVMs leads to increased vascular permeability in peripheral mesenteric vessels (He et al., 2016), yet this function has not been addressed in the CNS.

Alternatively, since PVMs are in direct physical contact with astrocytic end-feet, it is possible that they fulfil an important role in helping astrocytes to maintain a polarized state, with aquaporin-4 positive processes lining the vascular structures. Undoubtedly, the precise analysis of structural alterations within the perivascular space, as well as the polarization/expression of aquaporin-4 in the absence of PVMs, would give essential insights to better understand their role in regulating the glymphatic pathway.

In addition to regulatory function of the glymphatic pathway, we also noticed that PVMs are important for the drainage of CSF into the deep cervical lymph nodes through the newly described lymphatic vessels of the CNS (Aspelund et al., 2015; Louveau et al., 2015). One possible explanation is that the perturbation of the glymphatic pathway may account for the reduced drainage of brain CSF observed in the same animals. Indeed, it is believed that both pathways are interconnected and form a continuous fluid circuitry. Yet, such statement still misses firm demonstration. Finally, we conclude that PVMs are important for the clearance of toxic proteins, yet probably not only due to their phagocytic capacities, but also to their implication in the regulation of the glymphatic/lymphatic systems. This would further explain the increased burden of A β in AD models that lack PVMs (Hawkes and McLaurin, 2009), but also our own data demonstrating an aggravated spreading of Lewy-like pathology in PVMs-deficient conditions (Figure 28).

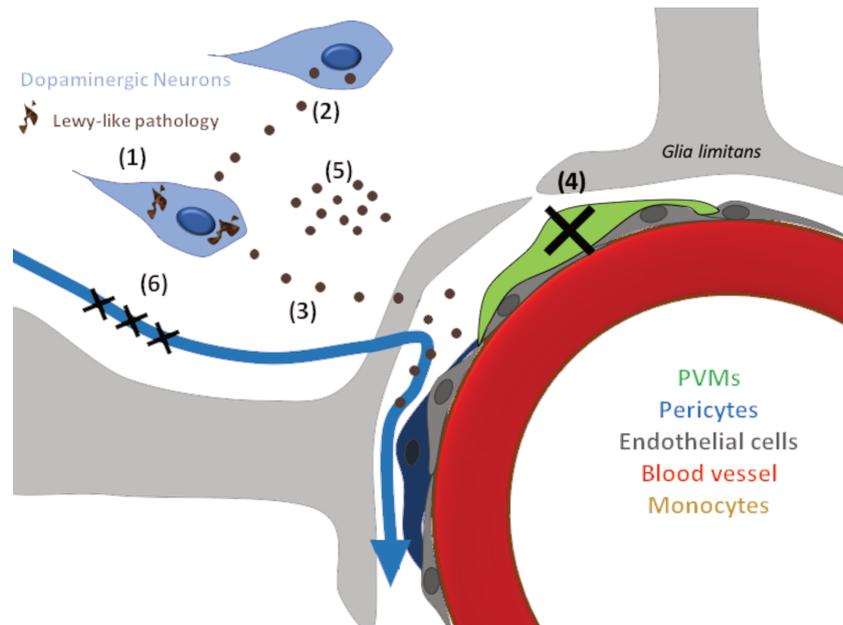


Figure 28. Hypothesis: PVMs reduce parenchymal α -Syn burden and propagation. The injection of α -Syn fibrils leads to the formation of inclusions immunoreactive for pSer129- α Syn (Lewy-like pathology) (1) that propagate into distant brain regions. Propagation requires the release of α -Syn seeds (brown dots) that are likely taken up by neighboring neurons, inducing further aggregation (2). In order to reach distant brain regions, α -Syn seeds are probably transferred transynaptically. An alternative explanation would be the brain re-distribution of α -Syn seeds following ISF/CSF flow (blue arrow) (3). The invalidation of PVMs (4) aggravates Lewy-like pathology, and we hypothesize that this occurs as a consequence of an increased brain burden of α -Syn seeds (5) caused by a reduced CSF-dependent ISF clearance (6), but also by a partial phagocytic failure within the perivascular space.

1.5 Perspectives

This thesis work provides several insights about the biology of PVMs and their potential implication in the pathogenesis of PD. For the first time, we demonstrate that PVMs can be important for the survival of dopaminergic neurons. Yet, despite this fundamental demonstration, we are still missing a profound understanding of how precisely they might confer neuroprotection. Although our data suggest that they might be important for clearance mechanisms and for regulating the adaptive immune response, additional studies will be necessary to better understand how precisely PVMs could regulate these processes in PD.

Additionally, in the present work we only analysed the consequences of depleting PVMs. We consider that assessing the impact of stimulating PVMs in the same pathological context, would be crucial to further corroborate their neuroprotective capacities and shed lights on future therapeutic strategies

Finally, we believe that two invaluable tools for studying PVMs biology in health and disease are missing: The first one is a transgenic mouse line that would not only allow the tracking of PVMs, but also achieve their specific ablation, e.g. a transgenic line that expresses inducible Cre-recombinase under the control of the CD206 promoter. Such tool would give us the chance to follow PVMs and finally figure out whether they can leave the perivascular space while losing CD206 expression, and get access to the brain parenchyma in pathological contexts, but also in steady-state conditions. It would also offer an alternative approach to deplete PVMs by, for example, driving the expression of diphtheria toxin receptor (DTR) in CD206-expressing PVMs (CD206^{CreER/+}:R26^{iDTR/+}). Although we made efforts to develop such tool, unfortunately the region of the promoter we cloned was probably missing many regulatory elements, since inducible Cre-recombinase was expressed in multiple cell populations that do not express CD206. The second tool that would be very helpful for characterizing PVMs, is an efficient protocol for preparing purified primary cultures. This protocol would be indispensable for conducting mechanistic studies to better understand how PVMs become activated in pathology (e.g. exposition to different α -Syn aggregates) or for discovering molecules with potential clinical interest through screening assays. In the laboratory, we have already started setting up the ideal conditions for culturing PVMs/MMs from new-born mice pups, with very encouraging yet preliminary results. This approach will give us the possibility to answer

several questions regarding PVMs that cannot be easily addressed *in vivo*. Finally, we believe that with these tools available, we should be able to properly characterize and potentially exploit PVMs with therapeutic purposes.

1.6 General conclusion

During this thesis work we were able to demonstrate that PVMs, a phagocytic population strategically positioned within the perivascular space, dynamically respond to neurodegeneration in PD. We show that in the context synucleinopathies, PVMs hold beneficial functions by protecting neurons from degenerating, in a mechanism that might involve the regulation of the adaptive immune response, but also the clearance of toxic molecules like α -Syn aggregates. However, besides the potential neuroprotective functions we and others have ascribed to PVMs, some reports also suggest detrimental ones. We believe that instead of determining whether PVMs might be protective or not in a particular context, it is far more important to describe specific functions with beneficial (e.g. secretion of neurotropic factors, phagocytosis) or detrimental outcomes (e.g. production of pro-inflammatory mediators). If we are able to precisely identify the effector functions displayed by PVMs, then boosting the beneficial while blocking the detrimental ones would be the ideal scenario from a therapeutic point of view.

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