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ABSTRACT - Assessing the therapeutic role of the secretome from mesenchymal stromal cells in genetic models of Parkinson's disease based on the overexpression of alpha-synuclein

The presence of aggregates containing alpha-synuclein (α-syn) and selective loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) are the main characteristics of Parkinson's Disease (PD). The secretome of mesenchymal stromal cells (MSCs) is the product of their secretory activity and is constituted by a panoply of vesicles, soluble proteins, lipids, and nucleic acids. These molecules, with broad and important roles, are essentially growth factors, anti-inflammatory and anti-apoptotic factors, able to exert protective and regenerative actions in the brain tissue. Given the complexity of the mechanisms underlying PD pathophysiology and the richness of the secretome, its application in PD has been investigated, and different studies confirmed its therapeutic potential. Despite the weight of α-syn in the progression of PD, few studies have explored the effects of secretome in models that develop α-syn-mediated pathology. This thesis explored the effects of MSCs-derived secretome in *Caenorhabditis elegans* models that overexpress α-syn. We found that secretome not only reduced α-syn aggregation but also prevented the α-syn-induced DA neuron loss. Bioreactors are essential tools for large-scale production of secretome and they can alter MSCs secretion activity. We used two dynamic culture systems with distinct design to produce secretome. The capacity of the secretomes to induce neurodifferentiation and to protect DA neurons from the effects of α-syn was compared. The secretomes had different impacts in these models, which was consistent with their distinct protein profiles. Nevertheless, we concluded that the dynamic system, as well as other conditions may have contributed to these results. Finally, we attempted to establish in our laboratory an animal model (rat) of PD, based on the viral vector-mediated overexpression of α-syn, and test the secretome with the best outcomes in the previous study. We observed that animals displayed a significant loss of DA neurons of the SNpc and striatal DA terminals. Moreover, these animals had tendentially a lower motor performance. Secretome-treated animals presented an inferior DA neuron loss, reduced levels of α-syn in the SNpc, and a slightly higher motor performance. In summary, this work provided the basis for future studies using secretome in *C. elegans* models of PD. It provided new clues regarding the effects of different culturing protocols in the therapeutic potential of secretome and it also gathered more evidence regarding the neuroprotective capacity of the secretome from MSCs.

Keywords: alpha-synuclein; mesenchymal stromal cells; Parkinson's Disease; secretome

RESUMO - Avaliação do papel terapêutico do secretoma de células estromais mesenquimatosas em modelos genéticos da doença de Parkinson baseados na sobre-expressão de alfa-sinucleína

A presença de agregados contendo alfa-sinucleína (α-syn) e a perda seletiva de neurónios dopaminérgicos na *substantia nigra pars compacta* (SNpc) constituem as principais características da doença de Parkinson (PD). O secretoma das células estromais mesenquimatosas (CEMs) é constituído por uma panóplia de vesículas, proteínas solúveis, lípidos e ácidos nucleicos. Estas moléculas, com amplas e importantes funções, são essencialmente fatores de crescimento, fatores anti-inflamatórios e anti-apoptóticos, capazes de exercer ações protetoras e regenerativas no tecido cerebral. Dada a complexidade dos mecanismos subjacentes à fisiopatologia da PD e a riqueza do secretoma, a sua aplicação na PD tem sido investigada e diversos estudos confirmaram o seu potencial terapêutico. Apesar do peso da α-syn na progressão da PD, poucos estudos exploraram os efeitos do secretoma em modelos que desenvolvem a patologia mediada por α-syn. Esta tese explorou os efeitos do secretoma em modelos de *Caenorhabditis elegans* que sobre-expressam α-syn. O secretoma de CEMs não só reduziu a agregação de α-syn, mas também evitou a perda de neurónios dopaminérgicos induzida por esta proteína. Os biorreatores são ferramentas essenciais para a produção em larga escala de secretoma e podem também alterar a atividade secretora das CEMs. Assim, usámos dois sistemas dinâmicos com *design* distintos para produzir secretoma e comparámos a capacidade dos secretomas em induzir neurodiferenciação e proteger os neurónios dopaminérgicos dos efeitos da α-syn. Os secretomas tiveram impactos diferentes nesses modelos, o que foi consistente com perfis proteicos distintos. No entanto, concluímos que não só o sistema dinâmico, mas também outras condições, poderão ter contribuído para estes resultados. Finalmente, tentámos estabelecer no nosso laboratório um modelo animal (rato) de PD, baseado na sobre-expressão de α-syn através de um vetor viral e testámos neste modelo o secretoma com os melhores resultados no estudo anterior. Os animais apresentaram uma perda significativa de neurónios dopaminérgicos na SNpc e estriado. Além disso, estes animais apresentaram tendencialmente um desempenho motor inferior. Os animais tratados com secretoma apresentaram uma perda neuronal inferior, níveis inferiores de α-syn na SNpc e um desempenho ligeiramente superior. Em resumo, este trabalho criou a base para estudos futuros usando secretoma em modelos de PD de *C. elegans*. Forneceu novas pistas sobre os efeitos de diferentes protocolos de cultura no potencial terapêutico do secretoma e reuniu mais evidências acerca da capacidade neuroprotetora do secretoma de MSCs.

Palavras-chave: alfa-sinucleína; células estromais mensenquimatosas; doença de Parkinson; secretoma

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LIST OF ABBREVIATIONS

3D Three-dimensional

6-OHDA 6-Hydroxydopamine

α-syn Alpha-synuclein

A

AAV Adeno-associated virus

ADE Anterior deirid

ALP Autophagy-lysosomal pathway

ATP Adenosine 5'-triphosphate

B

BBB Blood-brain barrier

BDNF Brain-derived neurotrophic factor

bFGF Basic fibroblast growth factor

bGH-polyA Bovine growth hormone polyadenylation sequence

BMSCs Bone marrow-derived mesenchymal stomal cells

C

CBA Chicken β-actin

CEP Cephalic

CMA Chaperone-mediated autophagy

CMV Cytomegalovirus

CNS Central nervous system

D

DA Dopamine

E

ER Endoplasmic reticulum

G

GABA Gamma-amino butyric acid

GBA1 Glucocerebrosidase 1

GCase Glucocerebrosidase

GDN

GDNF Glial cell line-derived neurotrophic factor

GFP Green fluorescent protein

GLP-1 Glucagon-like peptide-1

GMP Good manufacturing practice

H

hESCs Human embryonic stem cells

HGF Hepatocyte growth factor

hNPCs Human neural progenitor cells

I

iPSC Induced pluripotent stem cells

INF-γ Interferon-γ

IGF-1 Insulin-like growth factor I

K

KO Knockout

L

LAMP2a Lysosome-associated membrane protein type 2a

LB Lewy bodies

LN Lewy Neurites

M

MCs Microcarriers

MMP2 Matrix metalloproteinase 2

MSCs Mesenchymal stromal cells

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

N

NAC Non-amyloid-β component

NGF Nerve growth factor

NMS Non-motor symptoms

P

PD Parkinson's disease

PDE Posterior deirid

PEDF Pigment epithelium-derived factor

PFFs Pre-formed fibrils

PNS Peripheral nervous system

PTMs Post-translational modifications

R

RBD Rapid eye movement sleep behavior disorder

ROS Reactive oxygen species

S

SN Substantia nigra

SNARE N-ethylmaleimide sensitive factor attachment protein receptor

SNpc Substantia nigra pars compacta

SP Spinner flask

STR Striatum

T

TGF-β Transforming growth factor β

TH Tyrosine hydroxylase

TNF-α Tumor necrosis factor-α

U

UC-MSCs Umbilical-cord-derived mesenchymal stromal cells

UPR Unfolded protein response

UPS Ubiquitin-proteasome system

V

VEGF Vascular endothelial growth factor

VWBR Vertical-Wheel™ bioreactors

W

WPRE Woodchuck post-transcriptional regulatory element

WT Wild-type

Y

YFP Yellow fluorescent protein

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CHAPTER I

General Introduction

1. Parkinson's Disease

Parkinson's Disease (PD) is a chronic condition of the central nervous system that is manifested through motor and non-motor symptomatology (Kalia & Lang, 2015). This condition typically has a later onset affecting older individuals (more than 60 years old), but can also arise in a younger population (21-40 years) – early-onset PD (Mehanna & Jankovic, 2019). There are other neurodegenerative disorders that can mimic the clinical features of PD. The parkinsonian-like syndromes include Dementia with Lewy Bodies, Corticobasal Degeneration, Multiple System Atrophy and Progressive Supranuclear Palsy (Srivanitchapoom et al., 2018).

PD became known in 1817, when the physician after whom the disease is named published a manuscript entitled 'An Essay on the Shaking Palsy'. In this manuscript, James Parkinson describes the condition based on the symptomatology of six patients and categorizes it as a disease 'of a nature highly afflictive' (Parkinson, 1817). In 1872, Jean-Martin Charcot showed that the term 'shaking palsy' was no longer acceptable due to the identification of bradykinesia as a defining feature of PD. Furthermore, he acquainted that tremor was not necessarily present in all manifestations of the condition and named it as 'Parkinson's disease' (Goetz, 2011). Several years later, some studies started to shed light on the cerebral areas that were supposedly correlated with PD. In 1919, the remarkable work of Constantin Trétiakoff was able to link the symptomatology of the disease to the neurodegeneration of the substantia nigra (SN) (Trétiakoff, 1919). Through the use of post-mortem PD brains, he showed a substantial loss of pigmented nigral cells and the presence of, what he named as 'corps de Lewy', or Lewy bodies (LB), honouring Friederich H. Lewy, the responsible for their discovery (Lees et al., 2008). After this breakthrough, the neurochemical consequences of the degeneration of the SN remained unknown for several years. In 1957, Arvid Carlsson developed a unique work that granted him a Novel Prize for medicine. Through the administration of levodopa, a precursor of dopamine (DA) with the ability to trespass the blood-brain barrier (BBB), he counteracted the tranquilizing effect of reserpine in animals. Reserpine causes depletion of brain DA levels and he showed that levodopa administration enabled the restoration of normal brain DA levels in animals under the effect of reserpine (Carlsson, 1959; Carlsson et al., 1957). Following Carlsson's work, the depletion of DA was shown in other brain regions besides SN, namely the putamen and the nucleus caudate that together constitute the dorsal striatum (German et al., 1989). Further studies proved the beneficial effect of levodopa in PD patients (Bernheimer et al., 1973; Cotzias et al., 1967; Hornykiewicz, 2002), a fact that culminated in the approval from the Food and Drug Administration, in 1970, of levodopa as the first treatment for PD (Geibl et al., 2019).

In our days, PD is considered the second most common neurodegenerative disorder worldwide. In the past three decades, the prevalence of PD has significantly increased, with around 6 million people affected in 2016 worldwide and 211,296 deaths caused by this disease in the same year. According to this epidemiological data, PD is more prevalent in men (male-to-female ratio is 1.40) (Dorsey et al., 2018). In Portugal, the prevalence of PD in the population over 50 years of age is 180/100,000 habitants (J. J. Ferreira et al., 2017). Given the improvement of life expectancy and a rapidly aging population, the number of people with PD is expected to double by 2030, comparing with the values registered in 2005 for Western Europe's and the world's five most and ten most populous countries (Dorsey et al., 2007, 2018).

1.1. Pathophysiology of PD

As aforementioned, PD is characterized by a predominant neuronal loss in the substantia nigra pars compacta (SNpc), resulting in the depletion of DA in the striatum (STR), putamen and caudate nucleus (Bernheimer et al., 1973; German et al., 1989). Another hallmark of the disease is the widespread accumulation of cytoplasmic inclusions, named LB, that appear in brain biopsies of PD patients, but not in healthy people (Goedert et al., 2013). These inclusions can also be present within neurons' processes (Lewy neurites, LN). In 1997, the understudied protein alpha-synuclein (α-syn) was linked to PD for the first time when mutations in the SNCA gene, the gene that codifies for α-syn, were identified in familial cases of PD (Polymeropoulos, 1997). In the same year, LB and LN were shown to contain aggregated α-syn (Spillantini et al., 1997).

Firstly identified by Maroteaux in 1988, α-syn is a small protein of 140 amino acids (Maroteaux et al., 1988; Jakes et al., 1994; Shibasaki et al., 1995). Monomeric α-syn is divided into three main regions that possess distinct structural and dynamic properties (Fusco et al., 2014):

- the positively charged N-terminal region that has a role in membrane binding;
- the central hydrophobic region that includes the non-amyloid-β component (NAC) fragment, is essential for α-syn aggregation;
- a C-terminal domain which is negatively charged and has a weak association with membranes.

With an ubiquitous and predominantly distribution in the brain, α-syn is mainly expressed at pre-synaptic terminals, where it can possibly regulate neurotransmitter release and synaptic plasticity (Jakes et al., 1994). Even though the physiological function of α-syn is still unclear, different studies showed that it can act as a calcium-dependent modulator of vesicle homeostasis (Lautenschläger et al.,

2018) or even as a chaperone for the assembly of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Burré et al., 2015) by binding, for instance, to synaptobrevin-2/VAMP2 protein (Burré et al., 2010). In this regard, α-syn knockout (KO) mice display alterations mainly related to synaptic vesicles and low levels of dopamine in the STR (Abeliovich et al., 2000; Cabin et al., 2002), as well as cognitive impairments (Kokhan et al., 2011, 2012) and neuromuscular pathology (Pelkonen & Yavich, 2011). Given that other members of the synuclein family, namely β- and γ-synuclein, may compensate for the absence of α-syn, studies using double- and triple-KO animals were carried out. α/β-synuclein and triple-KO mice shared alterations in the levels of pre-synaptic proteins, such as complexins and 14-3-3 proteins (Burré et al., 2010; Chandra et al., 2004; Greten-Harrison et al., 2010). Brains of double-KO animals had decreased levels of DA compared with single-KO animals (Chandra et al., 2004). However, triple-KO mice presented a more drastic phenotype related with alterations in synaptic structure, age-dependent neuronal dysfunction and impaired survival (Burré et al., 2010; Greten-Harrison et al., 2010), suggesting that synucleins are relevant to the long-term functioning of neurons.

α-syn exists under various conformational shapes and is supposed to acquire neurotoxic potential when soluble monomers form more complex structures such as oligomers, protofibrils and ultimately insoluble fibrils that constitute LB (C. Kim & Lee, 2008). A consensus regarding the most cytotoxic α-syn species does not exist and studies point towards oligomers, fibrils and protofibrils (Gómez-Benito et al., 2020).

Additionally, post-translational modifications (PTMs), such as nitration, serine and tyrosine phosphorylation, ubiquitination and C-terminal truncation, can also trigger α-syn pathological alterations (Barrett & Timothy Greenamyre, 2015). PTMs are usually important for protein folding and can influence the subcellular localization of a folded protein or its activation or inactivation (R. G. Langston & Cookson, 2020). The most abundant PTM in α-syn found in LB is phosphorylation at the serine 129 (Anderson et al., 2006). While 90% of the α-syn found in PD patients' brains is phosphorylated, only 4% of α-syn in healthy brains is phosphorylated (Ghosh et al., 2017; Oueslati, 2016). However, despite evidence suggesting that phosphorylation and also ubiquitination may contribute to aggregation, the correlation between phosphorylation and α-syn aggregation is yet to be uncovered (Gallegos et al., 2015).

The first identified mutation of α-syn gene (in 1997) was the A53T mutation (Alanine to Threonine substitution at position 53) which is associated with early-onset PD (Polymeropoulos, 1997). Mutations

of α-syn gene can also trigger its aggregation propensity by perturbing the intramolecular interactions established between the α-syn three main regions (Ghosh et al., 2017).

Lewy pathology is supposed to play an important role in neurodegeneration in PD. However, the complexity of PD pathology extends beyond neurodegeneration caused by LB, especially because, as already mentioned, α-syn is able to adopt different conformations that might be able to generate neurotoxicity (Roberts et al., 2015; Ludtmann et al., 2018). Furthermore, other inclusions composed of different types of proteins and characteristic of other neurodegenerative diseases, including β-amyloid plaques and tau protein have been identified in brains of people suffering from PD (Irwin et al., 2012). These inclusions may interact with LB and contribute to the progression of the disease. Additionally, PD might occur without the formation of Lewy pathology as demonstrated in different reports stating that in the majority of PD patients having mutations in *LRRK2*, or the gene encoding parkin (*PARK2*), LB are sparse or even absent (Poulopoulos et al., 2012; Doherty et al., 2013; Kalia et al., 2015). Overall, the knowledge gathered until now indicates that the development of Lewy pathology is insufficient to explain the pathology of PD and that other players, such as different forms of α-syn aggregates and other types of protein inclusions have their own relevance in PD pathology.

Different mechanisms have been proposed to drive the degeneration of DA neurons, such as mitochondrial impairment, proteasomal and lysosomal dysfunction and neuroinflammation (Fig. 1) (Poewe et al., 2017). However, is still unclear which one(s) of them is the main responsible(s) for the disease process. Most likely, the dysfunction of one of them may trigger the abnormal functioning of the others, which translates into several self-perpetuating vicious cycles. What is also important to highlight is the particular vulnerability of nigral DA neurons that may be related, among other factors, with their extensive arborizations and high number of synaptic nerve terminals, which is metabolically demanding (Wong et al., 2019).

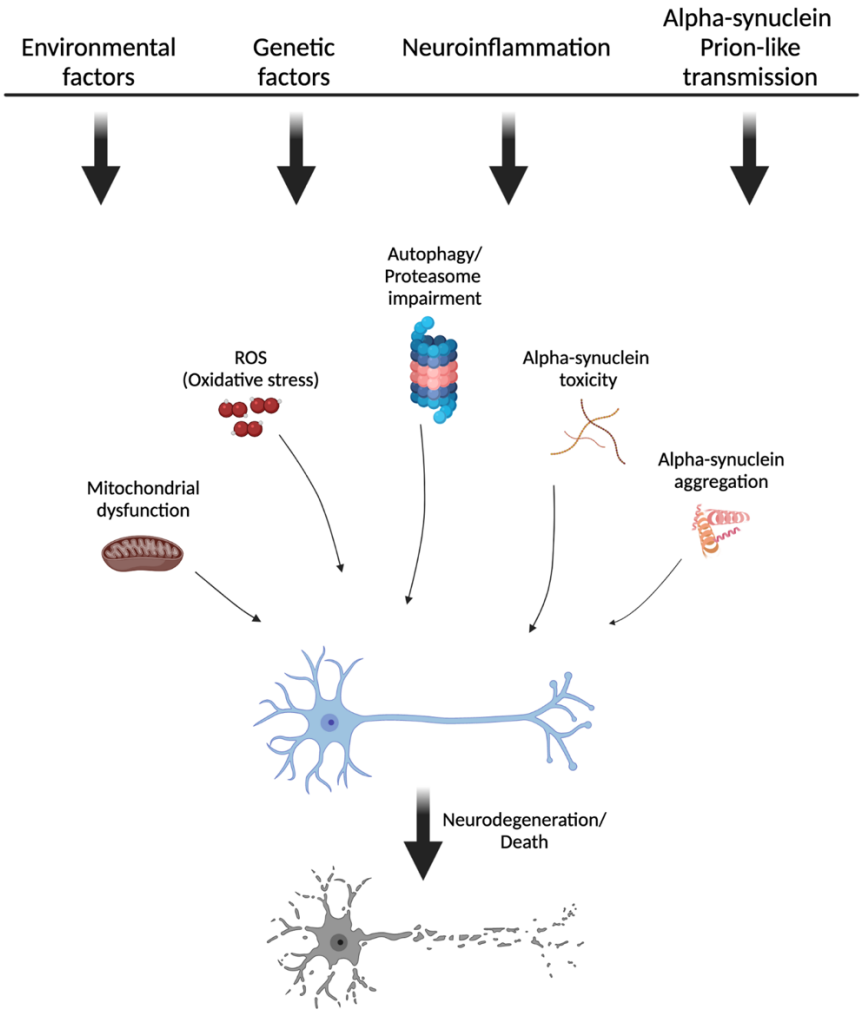

Figure 1. Representation of the main contributors to neurodegeneration in PD. Exposition to environmental factors and genetic predisposition are believed to be the basis for development of sporadic and familial PD. Additionally, other processes such as prion-like transmission of alpha-synuclein and neuroinflammation, can also influence neurodegeneration. Created with BioRender.com.

Notwithstanding, α-syn pathology is not restricted to the SNpc as hypothesized by Braak and colleagues. According to these authors, α-syn pathology initiates in the olfactory bulb or in the enteric nerves and progresses via the olfactory tract or the vagus nerve, respectively, to other brain regions (dorsal motor nucleus of vagus nerve, raphe nuclei, magnocellular portions of reticular formation, locus coeruleus, substantia nigra, and cortex) (Braak et al., 2003, 2004; Gómez-Benito et al., 2020). This

theory suggests that PD has six stages according to the spreading of α-syn pathology to other brain regions and that the presence of α-syn inclusions triggers cellular malfunctioning that leads to neuronal death and the clinical manifestation of the disease (Gómez-Benito et al., 2020). The hypothesis postulated by Braak et al. have been validated in neuropathological studies (Dickson et al., 2010; Kingsbury et al., 2010).

1.1.1. Mitochondrial dysfunction

Mitochondrial impairment has been linked with both familial and idiopathic forms of the disease (Bose & Beal, 2016). The first report showing the association between mitochondria dysfunction and PD pathogenesis came from the observation that substances capable of inhibiting the complex I of the mitochondrial respiratory chain caused nigrostriatal neurodegeneration, as well as parkinsonism (Burns et al., 1985; J. Langston et al., 1983). Another study showed that people suffering from PD presented a reduced activity of the Complex I in the SN (Greenamyre et al., 2001). Moreover, mitochondrial function can be regulated by some of the genes related with hereditary forms of PD (Giannoccaro et al., 2017).

The association between α-syn pathology and mitochondrial dysfunction has been investigated and α-syn is thought to interact with complex I, influencing mitochondria homeostasis (Faustini et al., 2017). Observations such as the evidence of complex I dysfunction due to α-syn accumulation within mitochondria supported this notion (Devi et al., 2008; Martínez et al., 2018). Even though the mechanism behind α-syn action is not yet clear, another report shows that α-syn oligomers negatively impact complex I, by inducing selective oxidation of the ATP synthase beta subunit and mitochondrial lipid peroxidation (Ludtmann et al., 2018). However, there are other possible mechanisms including defects in mitophagy, induction of mitochondria fragmentation leading to alterations in mitochondria morphology or even induction of abnormal reactive oxygen species (ROS) levels (Villar-Piqué et al., 2016).

1.1.2. Disruption of protein clearance pathways

Proteostasis is granted by the equilibrium between the mechanisms underlying protein synthesis and protein degradation. The ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) are the main mechanisms for protein degradation (Poewe et al., 2017; R. G. Langston &

Cookson, 2020). Both systems rely on target modification by ubiquitin, a small and quite conserved protein (Schmidt et al., 2021). The UPS consists of a large cylindrical protease complex, the 26S proteasome, that is more dedicated to the degradation of proteins with short half-lives. A protein that is targeted to degradation through the UPS undergoes polyubiquitylation, a process in which the protein is conjugated with ubiquitin, allowing it to be recognized by the PA700 that directs it to the proteasome to be degraded (R. G. Langston & Cookson, 2020).

ALP is responsible for the vesicle-mediated turnover of long-lived proteins and is also able to degrade cellular organelles (Xilouri et al., 2013; R. G. Langston & Cookson, 2020). The ALP encompasses three different processes of delivering the substrate to the lysosome, namely microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. Microautophagy is the most directed route, involving invagination of the lysosomal membrane. CMA requires substrate recognition by the chaperone protein Hsc70 and translocation of the substrate into the lysosome via a protein present on the lysosomal membrane, the lysosome-associated membrane protein type 2a (LAMP2a) receptor. On the other hand, macroautophagy involves the formation of an autophagosome containing the substrate, that will fuse with the lysosome (R. G. Langston & Cookson, 2020).

Data suggest that α-syn turnover can be achieved through both the UPS and ALP systems (Webb et al., 2003). As already mentioned, α-syn can adopt different conformations that can possibly be degraded by different mechanisms. Bennett and colleagues saw that both wild-type (WT) and mutant A53T α-syn were degraded by the UPS system, with the mutant form degrading at a slower rate, suggesting that the mutation may increase the propensity for accumulation of the protein and consequent aggregation (Bennett et al., 1999). On another report, WT α-syn was degraded by CMA but two mutated forms of α-syn (A30P and A53T) were not and even inhibited the CMA pathway by binding to LAMP2a (Cuervo et al., 2004). Interestingly, α-syn can also impair proteasomal performance probably by direct binding to the 26S proteasome and its substrates (Emmanouilidou et al., 2010; N.-Y. Zhang et al., 2008). Another report shows that both CMA and macroautophagy are important pathways for WT α-syn turnover in neurons, because CMA inhibition led to the accumulation of WT α-syn as insoluble and high molecular weight forms of the protein, and inhibition of macroautophagy but not the UPS, also led to the accumulation of WT α-syn (Vogiatzi et al., 2008). This is in agreement with post-mortem analysis reporting that CMA activity is reduced in PD brains, which highlights the role of autophagy in LB formation and PD (Alvarez-Erviti et al., 2010).

Overall, even though there is evidence for the involvement of both pathways in α-syn degradation, the specific mechanism for each α-syn species is still under debate (Xilouri et al., 2013). Furthermore,

as mentioned, both the presence of some α-syn species or the accumulation of the WT form, can impact both pathways and inhibit not only α-syn degradation, but also the degradation of other substrates, becoming a vicious cycle that contributes to neurodegeneration.

Aging is the principal risk factor for PD and other neurodegenerative diseases, because it impacts different cellular pathways, such as the UPS and ALP(Komatsu et al., 2006; Rubinsztein et al., 2011). There is evidence showing that LB contain ubiquitin as well as other molecules involved in UPS-related mechanisms, suggesting that malfunctioning of the UPS is probably involved in PD pathology (Bancher et al., 1989; Lowe et al., 1990; Ii et al., 1997; Kuusisto et al., 2003).

The endoplasmic reticulum (ER) also plays a role in protein degradation, even though its main function is the folding of newly synthesized proteins (M. H. Smith et al., 2011). The ER-associated protein degradation mechanism works as a quality control process for the degradation of incorrectly folded proteins. Different factors, such as an abnormal increase in protein synthesis and proteasome inhibition can disrupt ER functioning and cause the accumulation of proteins, leading to ER stress (M. H. Smith et al., 2011). The presence of toxic forms of α-syn can also disrupt ER functioning and generate ER stress (W. W. Smith et al., 2005; A. A. Cooper, 2006; Heman-Ackah et al., 2017). The unfolded protein response (UPR) aims to keep ER homeostasis by balancing the cellular folding capacity and the flux of proteins directed to the ER. When ER stress is too drastic, the UPR is activated and can induce cell apoptosis (Walter & Ron, 2011). ER stress can also trigger neuroinflammation through the UPR (Colla, 2019).

1.1.3. Neuroinflammation

Microglia are supposed to be distributed throughout the brain parenchyma where they exert their role of surveillance upon physiological conditions (Sogn et al., 2013). They are able to sense the molecular cues transmitted by the surrounding neurons, which can cause their activation (Wolf et al., 2017). When microglia are activated, their morphology changes and they can follow a protective path, being able to phagocyte cell debris and even secrete anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor β (TGF-β) (Nimmerjahn et al., 2005). However, microglia can also assume a toxic phenotype, characterized by the secretion of pro-inflammatory molecules, e.g., IL-1β, IL-6, interferon-γ (INF-γ), and tumor necrosis factor-α (TNF-α) and pro-inflammatory enzymes like nitrogen monoxide synthase (Hunot et al., 1996).

As already mentioned, α-syn may be a trigger for most of the processes that generate the death of dopaminergic neurons in PD. Neuroinflammation is not an exception as according to pre-clinical evidence, toxic conformations of this protein present in the extracellular space are thought to activate microglia. Activated microglia produce pro-inflammatory cytokines as a response to the stimulus that will end up harming neurons (Mount et al., 2007; Su et al., 2008; Chung et al., 2009; Q.-S. Zhang et al., 2017).

Neuroinflammation is undoubtedly involved in the pathophysiology of PD but there are questions that still need answer, regarding the moment when it begins and its relevance. Does neuroinflammation contribute to neurodegeneration in an early phase or is it a consequence of neuronal death? Data from human studies suggest that neuroinflammation is an early event in PD (Gundersen, 2021). Therefore, knowing if neuroinflammation is an important contributor to neurodegeneration will enable to understand if the use of anti-inflammatory substances can be advantageous. In this context, *in vitro* studies have been shown that microglia are essential for the neurotoxic effect of α-syn (W. Zhang et al., 2005; Acuña et al., 2019).

Besides microglia, glial cells especially astrocytes, may also be involved in PD pathogenesis. Neurotoxic reactive astrocytes can be found in brains associated with neurodegenerative diseases, including PD and its toxic phenotype is induced by activated microglia (Liddelow et al., 2017; Liddelow & Barres, 2017). Furthermore, recent reports from studies using induced pluripotent stem cell (iPSC)-derived astrocytes from PD patients, indicate that astrocytes manifest dysfunctions related with clearance of α-syn, leading to the accumulation of this protein and impairment of neuronal survival (di Domenico et al., 2019; Russ et al., 2021).

1.2. Clinical presentation of PD

PD is the most common form of parkinsonism and its diagnosis initially relies on the identification of characteristic parkinsonian motor abnormalities, namely rigidity, bradykinesia and resting tremor (Geibl et al., 2019). Rigidity comes from the stiffness of the limb, caused by an increased muscle tone and no relaxation capability (Váradi, 2020). Bradykinesia is the most occurrent primary motor symptom and is characterized by slowness of movement, reduced amplitude of movements and difficult fine motor control (Postuma et al., 2015). Resting tremor is the most pronounced characteristic of PD and is seen as an involuntary rhythmic movement as a result of a rhythmic muscle contraction and relaxation, usually affecting the upper extremities. Tremor is more perceptible during resting state but tends to

weaken or disappear when voluntary movements start (Váradi, 2020). The diagnosis of PD is based on the identification of parkinsonian motor abnormalities, as well as at least two supportive criteria, such as, clear benefit from dopaminergic therapy or presence of levodopa-induced dyskinesia. Furthermore, there must be a complete absence of absolute exclusion criteria, and if there are potential signs of other pathology, they must be counterbalanced by supportive criteria (Postuma et al., 2015).

Besides the motor symptomatology, non-motor symptoms (NMS) are also an important characteristic of PD due to the impact in the quality of life. Hyposmia, anosmia, constipation, rapid eye movement sleep behavior disorder (RBD) and depression, among other features, are just some examples of NMS that usually arise before motor symptoms and tend to worsen with the development of the disease (Pfeiffer, 2016; Schapira et al., 2017). Nowadays, it is widely recognized that given the time course for appearance of symptoms, PD can be separated into preclinical, prodromal and clinical phases (Poewe et al., 2017). The preclinical phase begins after neurodegeneration has started in the SNpc and is not correlated with clinical signs. Following this stage, prodromal PD starts and lasts for more than 10 years and involves some level of degeneration, which affects not only the central nervous system (CNS), but also the peripheral nervous system (PNS), resulting in evident NMS (Schaeffer et al., 2020). Lastly, when there is a considerable neuronal loss in the SNpc (40% to 60%), motor symptoms become perceptible, and the early stage of PD begins (Váradi, 2020). Given the prevalence and the onset of NMS before the clinical phase of PD, it has been suggested that their presence could be combined with biochemical markers and imaging to identify individuals at risk of developing the disease (reviewed in (Schapira, 2013)).

1.3. Causes of PD

PD is essentially an idiopathic disease and is nowadays thought as being the result of a complex combination of environmental and genetic risk factors. Around 15% of PD patients have family history (Deng et al., 2018). Throughout years of research, several risk factors for development of PD have been identified, with age remaining as the most significant. Other relevant risk factors include the exposition to environmental factors such as pesticides, head injury and consumption of β-blockers. Interestingly, other environmental cues were identified as contributing to decrease the chance to develop PD (e.g., consumption of tobacco, coffee and non-steroidal drugs) (Kalia & Lang, 2015).

The development of genome-wide association studies, enabled the identification of at least 10 autosomal dominant and 9 autosomal recessive disease-causing genes for parkinsonism (Deng et al., 2018).

The first gene to be associated with familial PD was *SNCA*, the gene that encodes α-syn (Kalia & Lang, 2015). Mutations in *LRRK2* and *PRKN* seem to be the most common causes of inherited PD, whereas mutations in Glucocerebrosidase 1 (*GBA1*), resulting in misfolded glucocerebrosidase (GCase), are the leading genetic risk factor for developing PD (Kalia & Lang, 2015). Misfolded GCase affects the ER, mitochondria and lysosomes, contributing to α-syn accumulation and aggregation and is associated with a more severe phenotype, compared with idiopathic PD (Cacabelos, 2017).

1.4. Animal models of PD

Animal models of PD are an essential tool to investigate disease pathogenesis and to evaluate potential therapies. Different models have been developed throughout years of research in a variety of vertebrate and also invertebrate species by using toxins, pesticides, drugs and genetic manipulation (Gomes et al., 2020). Nevertheless, neither of the available models recapitulate all the pathological and behavioral features of the disease. So, the choice for the most suited model relies on the research questions, the limitations and predictive power that each one of them presents.

1.4.1. *Caenorhabditis elegans* as a model for PD

Invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* are useful models to investigate the molecular pathways related to PD and for a high throughput analysis of novel targets and compounds.

The nematode *C. elegans* has been extensively studied and in spite of being a simple organism, it shares different conserved cellular pathways with humans, including neurotransmitter systems, receptors, axon guidance molecules and ion receptors (Maulik et al., 2017). The use of this animal involves low maintenance costs, fast reproduction and short generation time, which translates into a quick large-scale availability of animals (Fig. 1) (Riddle et al., 1997). *C. elegans* has a well-defined nervous system composed of 302 neurons from which eight are dopaminergic: six neurons in the anterior region [four cephalic (CEP) and two anterior deirid (ADE)] and two posterior deirid (PDE)

neurons in the posterior region (J. F. Cooper & Van Raamsdonk, 2018). These cells are not essential for the animal's survival neither locomotion. However, they are important for mechanosensation, enabling the worm to adapt its movement in the presence or absence of a food source (Sawin et al., 2000).

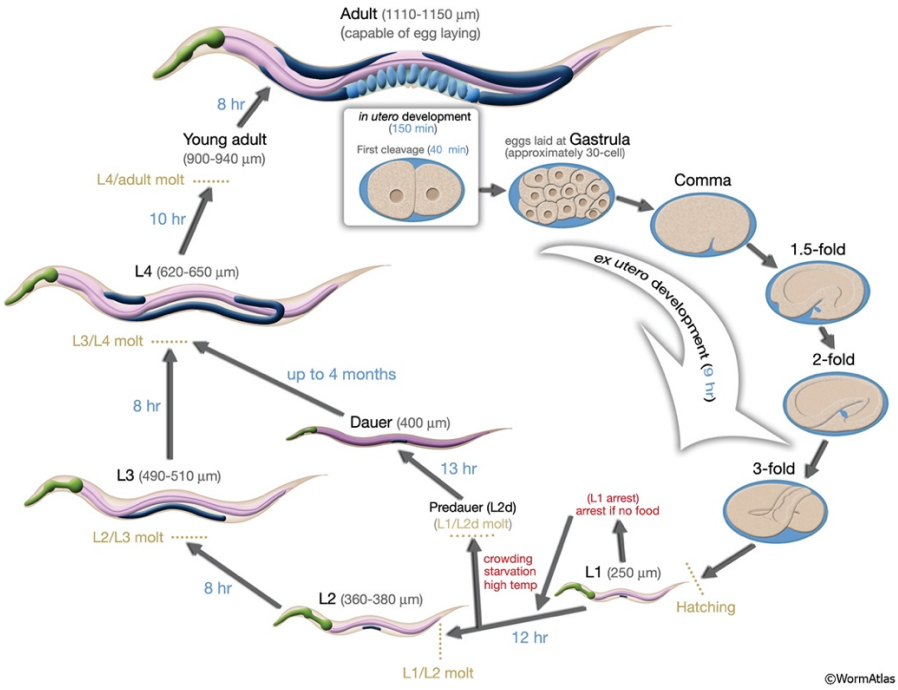

Figure 2. The *C. elegans* life cycle at 22°C. Numbers in blue along the arrows indicate the length of time spent at a certain stage. Eggs are laid outside at about 150 minutes post-fertilization and during the gastrula stage. Marked next to the stage number is the length of the animal (in µm) at that stage. Reproduced from Wormatlas with permission.

The neuronal circuitries of this animal are well explored and there is plenty of knowledge about what behaviors are modulated by a certain group of neurons (Maulik et al., 2017). Moreover, one of the great advantages of using *C. elegans* is its amenability for genetic manipulation tools (J. F. Cooper & Van Raamsdonk, 2018).

Genetic manipulation or even exposition to chemicals can trigger neuronal alterations (loss or morphological changes) which can translate into behavior abnormalities that can be identifiable and quantified (Dexter et al., 2012). Indeed, the existence of a behavior phenotype is one of the advantages of using these animals in comparison with cellular models. DA-dependent behaviors are defined as trashing (the number of body bends in a liquid environment), basal slowing (capacity to slow down in

the presence of food), swimming induced paralysis (tendency to paralyze when well-fed and after trashing for a certain period of time) and crawling (distance travelled in a solid environment) (J. F. Cooper & Van Raamsdonk, 2018).

6-Hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been extensively used to induce experimental parkinsonism, due to the induction of mitochondrial respiration impairment and oxidative stress (Caldwell & Caldwell, 2008). In analogy to what happens in other animals, *C. elegans* is susceptible to both compounds. The determination of the degree of dopaminergic neurodegeneration is very straightforward in *C. elegans* because the worms' body is transparent. Moreover, the DA neurons of some transgenic strains express green fluorescent protein (GFP) under the DA transporter promoter (Pdat-1), enabling their visualization under a microscope (Fu et al., 2014; Pu & Le, 2008).

Even though *C. elegans* lacks a homolog for *SNCA*, it presents homologs for other genes linked with familial PD, such as *LRRK2*, *PARK7*, *PARK*, *PRKN* and *PINK1*. Moreover, genetic manipulation allows the expression of human WT or mutant α-syn, ubiquitously or in particular cells (Gaeta et al., 2019). The expression of α-syn and GFP in dopaminergic neurons results in their age-dependent degeneration which can be monitored using a fluorescent microscope due to GFP expression (Cao et al., 2005). On the other hand, the expression of α-syn fused with GFP or yellow fluorescent protein (YFP) in body wall muscle cells, allows the visualization of α-syn aggregates, making this model an interesting approach to study protein aggregation and compounds that impact protein aggregation. Besides neurodegeneration, the nematodes can also manifest other perturbations, including reduction of dopamine content and alterations at the level of basal response to food, egg-laying activity, mobility or even lifespan (Fu et al., 2014; Maulik et al., 2017; Pu & Le, 2008).

As any other model, *C. elegans* presents some limitations, being the most obvious the fact that this animal is devoid of organs, particularly a defined brain (Maulik et al., 2017). Moreover, due to the worms' thick cuticle and reduced ways for drug entrance, is difficult to predict important aspects in drug discovery process, such as bioavailability or effective drug concentrations (Harrington et al., 2010).

Overall, despite the limitations, both chemical and genetic *C. elegans* models of PD have contributed to uncover different genes, proteins, chemical compounds, drugs and regulatory RNAs that impact misfolded α-syn, potentiate the clearance of α-syn, suppress the formation of α-syn inclusions and that influence dopaminergic neurodegeneration (Gaeta et al., 2019; van Ham et al., 2008). However, when the investigational question requires more advanced functional or behavioral evaluations, the tendency is to use animals that renders more complexity, such as rodents.

1.4.2. Rodents for modelling PD

The brain of rodents share some anatomical and physiological features with humans and have conserved cellular and molecular pathways (Breger & Fuzzati Armentero, 2018). Furthermore, rodents exhibit complex movements in analogy to humans, that can be similarly affected following damages in the nigrostriatal pathway and respond to DA replacement therapy (Cenci et al., 2002).

Chemical animal models of PD were developed when the genetics underlying the disease was still unknown. Different compounds have been explored and 6-OHDA, MPTP, paraquat and rotenone are the most used. Similarly to 6-OHDA and MPTP, paraquat and rotenone exert a cytotoxic effect on dopaminergic neurons through induction of oxidative stress (Przedborski & Ischiropoulos, 2005). Animal models of PD based on these chemicals reproduce some characteristics of the disease including dopaminergic neurodegeneration accompanied by striatal DA and tyrosine hydroxylase (TH) depletion, mitochondrial dysfunction, oxidative stress, neuroinflammation and motor behavioral deficits (Cenci & Björklund, 2020). One of the advantages of using rodents is the possibility they offer to study nonmotor symptomatology (Campos et al., 2013; Gomes et al., 2020; Taguchi et al., 2020). However, most of the compounds cause a severe and fast dopaminergic death which does not resemble the evolution of the disease in humans, and fail to induce α-syn accumulation or aggregation (Cenci & Björklund, 2020). Therefore, these models can be seen as holding limited translational power.

The development of genetic tools and the generation of knowledge in this field enabled the development of genetic models. Different PD-related genes, such as *SNCA*, *LRRK2*, *PRKN*, *PINK1*, and *PARK7* have been targeted. One of the most prioritized genes has been the *SNCA* gene, due to the central role of the α-syn protein in the pathophysiology of PD. Therefore, different mice transgenic lines were developed, based on the overexpression of human WT or mutant (A53T, A30P) α-syn (Visanji et al., 2016). Mice presenting these mutations usually develop key features of PD, such as decreased levels of striatal DA, α-syn inclusions, as well as motor and nonmotor phenotypes (M. K. Lee et al., 2002; Taguchi et al., 2020). Nonetheless, these animals typically lack an extensive nigrostriatal degeneration, the development of a relevant phenotype demands a long period of time and there is a high variability among animals (Trigo-Damas et al., 2018; Visanji et al., 2016).

Different animal models have been developed as an alternative to transgenic animals and consist of the injection of adeno-associated virus (AAV) particles containing WT or mutated *SNCA* or injection of α-syn pre-formed fibrils (PFFs) (Gómez-Benito et al., 2020).

The transduction of neurons with a AAV vector is a widely used method to deliver mutated or WT α-syn to dopaminergic neurons within the SNpc, because this methodology is not only efficacious and

long-lasting, but also safe (Albert et al., 2017). Indeed, AAV is capable of transducing different cell types, even post-mitotic cells, like neurons and renders long-term expression (Wu et al., 2006). In opposition to lentivirus, AAV is non-replicating and rarely integrates into the host genome (C. Li & Samulski, 2020). Moreover, AAV vectors lack 96% of the viral genome which means that the expression of viral proteins is minimized and, consequently, are the host immune responses (C. Li & Samulski, 2020).

The overexpression of WT, A53T or A30P α-syn using AAV vectors results in the progressive death of DA neurons in the SNpc, denervation of the STR, decreased levels of DA and motor deficits (Ip et al., 2017; Oliveras-Salvá et al., 2013; Koprich et al., 2011). However, when analyzing different studies that relied on this approach, there is a considerable variability within each study and between different studies (Albert et al., 2017). This is justified by the fact that different aspects can affect the parkinsonian phenotype, such as the used dosages, serotypes, α-syn species, animal species and also the period of time that has passed since the vector injections (Albert et al., 2017; Gómez-Benito et al., 2020). Another factor that can influence the efficacy of the transgene expression is the design of the vector construct and the type of promoters. The transgene expression can be improved by a strong promoter, such as cytomegalovirus (CMV), chicken β-actin (CBA) or human synapsin I, and the inclusion of post-transcriptional regulatory elements, like woodchuck posttranscriptional regulatory element (WPRE) or polyadenylation sequence (Gómez-Benito et al., 2020). An important drawback of this model is the variability that can result from the stereotaxic surgery. Moreover, as far as is known, there are no reports of the transmission of α-syn pathology to non-transduced neurons (Gómez-Benito et al., 2020). Overall, this is an approach that results in the progressive degeneration of DA neurons together with the formation of α-syn inclusions, being valuable to study the way the accumulation of α-syn influence the degeneration of DA neurons.

The administration of α-syn PFFs or brain extracts from PD patients that possess LBs, through an intracerebral or systemic injection is an approach that holds great value to study the propagation of α-syn (Gómez-Benito et al., 2020). Studies using this model have been reporting a progressive dopaminergic neuron death as a result of the aggregation of phosphorylated α-syn, a significant reduction in the levels of DA, neuroinflammation and motor abnormalities (Abdelmotilib et al., 2017; Earls et al., 2019; Patterson et al., 2019; Paumier et al., 2015). Despite the interesting outcomes provided by this model, it is also susceptible to great variability influenced by different variables, such as the type and quantity of PFFs used, the site of injection and the animal species. Nevertheless, the strength of this model to study the propagation of exogenous α-syn and its influence over endogenous

α-syn and parkinsonian phenotype is undeniable, as well as its utility for the development of compounds able to prevent or slow the aggregation of α-syn and its spreading (S. Kim et al., 2019; Recasens et al., 2014).

1.5. Current therapies and drugs in development

The available therapeutic strategies for PD consist in the management of symptomatic effects through pharmacotherapy, deep brain stimulation, and physiotherapy. Regarding pharmacotherapy, different agents are available, including DA precursors, such as levodopa; DA agonists, like apomorphine; monoamine oxidase or catechol-O-methyltransferase inhibitors, such as selegiline and entacapone, respectively (Poewe et al., 2017). However, the chronic administration of levodopa and other antiparkinsonian drugs are always accompanied by undesirable side effects, such as dyskinesia, and are ineffective for longstanding treatments, because of the wearing-off effect (Contin & Martinelli, 2010). So, the medical and scientific communities have been working to tackle the greatest unmet therapeutic need in PD, which is the development of disease-modifying drugs, that have the capacity to slow the progression of the disease or even regenerate or replace lost cells. α-syn has been one of the most relevant targets, resulting in the development of a panoply of drugs with generally the same aim, but based on different mechanisms (Stott et al., 2020). Some of them are being tested in clinical trials and rely on (Stott et al., 2020): a) antibodies targeting monomeric or aggregated α-syn; b) small molecules able to cross the BBB that directly inhibit α-syn aggregation; c) Lysosomal therapies focused on enhancing GCase activity; d) Compounds able to activate autophagy; e) Inhibitors of LRRK2 that prevent neurodegeneration and activate autophagy; f) Iron chelators for prevention of α-syn aggregation; g) Glucagon-like peptide-1 (GLP-1) receptor agonists.

Interestingly, among each of these strategies are drugs that have been used for other conditions and are now being repurposed for the treatment of PD. For instance, Nilotinib is a drug used against chronic myeloid leukemia and is being tested as an autophagy inducer (ClinicalTrials.gov Identifier: NCT03205488, NCT02281474 and NCT02954978). In the same line, Mannitol which is a diuretic agent and Ambroxol, an expectorant, are being studied in clinical trials as molecules targeting α-syn aggregation (ClinicalTrials.gov Identifier: NCT03823638) and lysosomal enhancers (ClinicalTrials.gov Identifier: NCT02941822 and CT02914366), respectively. GLP-1 receptor agonists are a class of anti-diabetic drugs, such as exenatide, that stimulate GLP-1 receptors present in the beta islet cells. This stimulation results in a glucose level-dependent release of insulin and decrease in the levels of

glucagon, enabling the control of glucose blood levels (Foltynie & Athauda, 2020). Studies in animal models of PD showed a remarkable neuroprotection capacity, with attenuation of neuroinflammation and improvement in motor function (Bertilsson et al., 2008; Y. Li et al., 2009; Yun et al., 2018). A recent randomized, double-blind, placebo-controlled, parallel-group, single-center phase 2 trial showed that PD patients submitted to a 48 weeks-treatment with exenatide experienced less decline in motor function. Moreover, the results point to a possible disease-modifying effect, because the beneficial effects were sustained after a 12-week washout period (Athauda et al., 2017).

Other potential therapies have been developed, such as gene therapy based on the viral vector-mediated delivery of growth factors or biosynthetic enzymes. Currently, there are three active phase 1 or 2 clinical trials, based on the delivery of aromatic L-amino acid decarboxylase, responsible for the synthesis of DA from levodopa (Sánchez-Pernaute et al., 2001) and two phase 1 trials that are already completed and that shown improvements in motor function and well tolerance (Christine et al., 2009, 2019). Another completed phase 1 trial aimed to deliver glutamic acid decarboxylase, an essential enzyme for gamma-amino butyric acid (GABA) synthesis that induces GABAergic inhibition of subthalamic nucleus, improving motor deficits (Kaplitt et al., 2007). Attention has also been directed to the delivery of glial cell line-derived neurotrophic factor (GDNF), as it protects dopaminergic neurons in chemical models of the disease and promotes their regeneration which translates into a functional recovery (Björklund et al., 2000). A phase 1 trial is currently ongoing based on the AAV2 vector delivery of GDNF to putamen (ClinicalTrials.gov Identifier: NCT01621581).

Cell therapy for treatment of PD was performed for the first time in 1979 (Perlow et al., 1979). Nowadays, this is becoming a potential treatment option, supported by the progress of biotechnology. Several experimental and clinical studies have used cells from various origins and sources to treat PD, namely fetal mesencephalon cells, embryonic stem cells, multipotent mesenchymal stromal cells (MSCs) or iPSCs (Shen et al., 2016; Marques et al., 2018). For different reasons that will be explored in the next section, the direct transplantation of MSCs has been earning a strong interest. At this moment, there are seven active or recruiting trials involving the transplantation of different cells in the context of PD and most of them involve MSCs (Table 1).

Table 1. Active or recruiting clinical trials based on the transplantation/infusion of cells for treatment of Parkinson's Disease.

Phase	Cell type	ClinicalTrials.gov Identifier

1/2	NSCs differentiated from MSCs	NCT03684122
1	UC-MSCs	NCT03550183
1	DA neurons derived from hESCs	NCT04802733
1/2	Fetal mesencephalic DA neuronal precursor cells	NCT01860794
2	Allogeneic BMSCs	NCT04506073
2/3	Autologous BMSCs	NCT04146519
1	Autologous peripheral nerve grafts	NCT02369003
2	AMSCs	NCT04928287

AMSCs, adipose-derived mesenchymal stem cells; BMSCs, bone marrow-derived mesenchymal stem cells; DA, dopamine; hESCs, human embryonic stem cells; MSCs, mesenchymal stem cells; NSCs, neural stem cells; UC-MSCs, umbilical cord-derived mesenchymal stem cells.

2. Mesenchymal stromal cells

Mesenchymal stromal cells are also referred to as "mesenchymal stem cells". However, given that this is a heterogeneous cell population, and only some cells have stem cell properties, the term "mesenchymal stromal cells" is seen as more adequate (Horwitz et al., 2005). MSCs are a population of plastic adherent cells that express CD73, CD90 and CD105 and lack the expression of hematopoietic and endothelial markers CD11b, CD14, CD19, CD34, CD45, CD79a and HLA-DR, with the ability to differentiate *in vitro* into adipocyte, chondrocyte and osteoblast lineages (Horwitz et al., 2005). MSCs can be obtained from different tissues such as bone marrow (Meppelink et al., 2016), adipose tissue (Alstrup et al., 2019), umbilical cord and placenta (Beeravolu et al., 2017), amniotic fluid (In`t Anker et al., 2003), cervix (Orciani et al., 2018) or menstrual blood (Dalirfardouei et al., 2018). Besides being easily retrieved from various tissues, MSCs have the capacity to migrate to the harmed site, can differentiate into different phenotypes if appropriate conditions are provided, have an intense modulatory activity towards the surrounding environment through their paracrine activity, and, importantly, do not raise ethical concerns (Hellmann et al., 2006; Eleuteri & Fierabracci, 2019; Barzilay et al., 2008). These characteristics have prompt the development of different strategies for regenerative medicine and neurodegenerative diseases, involving the direct transplantation of these cells (H. W. Kim et al., 2018; Y.-B. Park et al., 2017; Reinders et al., 2018). Clinical trials have demonstrated that transplantation of MSCs might be a safe and promising strategy in patients with diabetes (Holmes,

2014), acute myocardial infarction and heart failure (J.-W. Lee et al., 2014; Bartolucci et al., 2017), acute respiratory distress syndrome due to COVID-19 (Lanzoni et al., 2021), cerebral stroke (J. S. Lee et al., 2010), multiple sclerosis (Connick et al., 2012) and amyotrophic lateral sclerosis (Mazzini et al., 2012), among other conditions. In PD, MSCs have been studied in different animal models and those studies demonstrated that MSCs are able to stimulate the protection and survival of DA neurons and to diminish neuroinflammation, contributing to the amelioration of the motor impairments (Chi et al., 2019; Y.-J. Kim et al., 2009; Offen et al., 2007; Suzuki et al., 2015). Clinical trials have also suggested the safety of this approach and showed some motor improvements and reduction in disease severity (Boika et al., 2020; Brazzini et al., 2010; Venkataramana et al., 2010, 2012).

2.1. Secretome from MSCs

In 2005, Gnecchi and colleagues showed for the first time that the benefits seen after transplantation of MSCs were induced through their paracrine activity (Gnecchi et al., 2005). MSCs release a wide range of biological factors, such as soluble proteins, lipids, nucleic acids, and extracellular vesicles, that constitute the "secretome" (Baez-Jurado et al., 2019). These molecules can impact different biological processes, by promoting immunomodulation, angiogenesis, anti-apoptotic and anti-oxidative effects (Brown et al., 2019; Harrell et al., 2019). Given the short survival of MSCs following transplantation, some of the reported beneficial effects of MSCs might not be directly related with local engraftment, but rather be the result of their paracrine capacity. Indeed, different studies suggest that the beneficial effects of MSCs' secretome are equivalent to the effects seen when MSCs are transplanted (Imberti et al., 2007; Iso et al., 2007; Javazon et al., 2007).

What is particularly interesting is the advantages of secretome as a cell-free therapy over cell therapies. Briefly, the use of a biological product devoid of cells solves the safety concerns associated with the transplantation of cells, regarding tumorigenicity, immunogenicity and emboli formation. Furthermore, the analysis of safety, dosage and potency applied to pharmaceutical compounds can be analogously applied to secretome. Also, secretome can be easily cryopreserved without toxic agents and without loss of potency for a long period of time. Moreover, using secretome can be more cost-effective and less time-consuming, because secretome could be prepared beforehand in large-scale and be readily available for a treatment (Vizoso et al., 2017).

Uemura and colleagues injected bone marrow-derived mesenchymal stomal cells (BMSCs) in the left ventricle of mice in the same moment that myocardial infarction was induced. After four weeks they

saw notorious improvements in mice injected with MSCs compared with control animals (Uemura et al., 2006). However, they found low numbers of stromal cells in the mice cardiac tissue, suggesting that differentiation of MSCs into cardiomyocytes is insufficient to justify the improvements.

Secretome from MSCs has shown therapeutical potential in other domains besides cardiology, including skin wound healing (Zhou et al., 2013a), diabetes (Mahdipour et al., 2019), cancer (Eiró et al., 2014), osteoarthritis (Hassan Famian et al., 2017) and chronic kidney disease (van Koppen et al., 2012). This strategy has been also applied to CNS disorders resulting from neurodegenerative processes or trauma, like PD, spinal cord injury, traumatic brain injury, and stroke (Mendes-Pinheiro et al., 2020; Pinho et al., 2020)].

In the context of PD, Teixeira and co-workers administered MSCs' secretome in 6-OHDA lesioned rats and saw an increment in DA neurons in the SNpc, accompanied by an improvement in the motor performance (Teixeira et al., 2017). Another work also showed a partial reversion of the histological deficits characteristic of this disease and motor behaviour gains in a similar animal model (Mendes-Pinheiro et al., 2019). Importantly, this work also showed that the effects of secretome seemed to be tendentially superior to the effects seen in animals injected with MSCs. Additionally, in *C. elegans* models overexpressing α-syn, secretome from BMSCs was able to protect DA neurons and to reduce the number of α-syn inclusions (Marques et al., 2021 - further discussed in Chapter II). This is in line with an *in vitro* study showing that secretome from MSCs reduced the insoluble and oligomeric forms of α-syn, which resulted in higher neuronal viability (Oh et al., 2017). Moreover, when applied to MPTP-treated animals that received injections of α-syn PFFs, the secretome decreased the number of α-syn oligomers and attenuated apoptosis, leading to an increment in the survival of DA neurons (Oh et al., 2017). What is particularly interesting in this study is that the authors determined that matrix metalloproteinase 2 protein (MMP2), a protein identified in the secretome, had a key role in the proteolysis of aggregated α-syn. However, when the injection of MMP2 alone was compared to the injection of MSCs, authors saw that MMP2 had an inferior effect, thus suggesting that other soluble factors may be important. Another report showed that MSC treatment decreased α-syn levels through the induction of M2 microglia polarization (H. J. Park et al., 2016). The authors saw that IL-4 was responsible for this effect.

In an attempt to decipher the soluble factors present in MSCs' secretome, proteomic analysis were carried out (Pires et al., 2016; Teixeira et al., 2016, 2017). Besides the presence of the classical neurotrophic factors that modulate dopaminergic neuronal survival and protection, such as vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF) and brain-derived

neurotrophic factor (BDNF), these analyses also detected various proteins including cystatin-C, galectin-1, pigment epithelium-derived factor (PEDF), parkinson disease protein 7 or DJ-1, ubiquitin-protein ligase E3A, heat shock protein beta 1, 14-3-3 proteins, among several others. These proteins might be involved in the beneficial effects of secretome, due to their known neurotrophic and neuroprotective role in the context of PD, by providing oxidative stress homeostasis, neuritic outgrowth, inducing autophagy, supressing α-syn transmission or even by targeting α-syn fibrils, reducing their cytotoxicity (Cox et al., 2018; Falk et al., 2009; Lopez et al., 2019; Oh et al., 2016; Underwood et al., 2021; B. Wang et al., 2018; C.-Y. Xu et al., 2017; Zou et al., 2017).

Besides soluble factors, the vesicular fraction has also been associated with gains in the context of PD (Vilaça-Faria et al., 2019). Vesicles, such as exosomes are important cargos for genetic material, such as miRNAs, that can also be important players in neuronal differentiation and protection. Intravenous injections of exosomes from umbilical-cord-derived mesenchymal stromal cells (UC-MSCs) led to a reduction in the loss of DA neurons, upregulation of the level of DA in the STR and contributed to an amelioration of motor dysfunction (Chen et al., 2020). However, our group recently demonstrated that the use of secretome as a whole is more advantageous than the use of its fractions (Vilaça-Faria et al., 2021).

Despite the advantages of secretome from MSCs over the transplantation of living cells and the evidence that has been gathered regarding its therapeutic potential, the number of clinical trials is still scarce. The already completed trials revealed the safety and efficacy of secretome (Fukuoka & Suga, 2015; Katagiri et al., 2016; Kordelas et al., 2014; Shin et al., 2015; Zhou et al., 2013b). Indeed, none of them reported serious adverse events. Currently, there are only nine ongoing clinical trials to evaluate the safety and efficacy of secretome therapy. None of the completed trials, neither the still ongoing ones focus on the application of secretome in the context of PD.

2.1.1. Modulation of the secretome

Given the number of proteins that can be found in the secretome, identifying the ones that are crucial for its effects could be important. Indeed, improving the know-how on this topic would contribute to the development of targeted strategies aiming to modulate the secretome. Different aspects such as the tissue source, donor or batch of MSCs can alter the secretome pattern, i.e., the concentration and diversity of its components (Heathman et al., 2016; Pires et al., 2016). There are other factors that can be manipulated during MSCs culture that can have a great impact in the final product. Gathered

evidence support the benefits of exposing MSCs to a specific condition or stimulus (preconditioning). During this process, different signaling pathways can be activated according to the preconditioning strategy. Being aware of how each stimulus impacts the cells' behavior is crucial to validate this approach as a tool to increase the safety and the therapeutic potential of MSCs' secretome (J. R. Ferreira et al., 2018).

Subjecting MSCs to hypoxic culture conditions has been associated with interesting outcomes. In this conditions of low oxygen tension, the hypoxia inducible factor is activated, leading to the transcription of angiogenic genes such as VEGF (Madrigal et al., 2014). Even though MSCs are usually cultured under normoxic conditions (21% oxygen tension), different data suggest that their physiological niches present a much lower oxygen tension (Bizzarri et al., 2006; Chow et al., 2001; Harrison et al., 2002). As already showed, MSCs benefit from being cultured under hypoxic conditions (dos Santos et al., 2010). Apart from that, Teixeira et al. saw that the secretome from cells cultured in hypoxic conditions, showed an upregulation of certain factors, compared with the secretome from normoxic conditions (Teixeira et al., 2015). Other studies using this strategy demonstrated an upregulation of several factors such as VEGF, nerve growth factor (NGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) (Ahmed et al., 2016; Chang et al., 2013; L. Liu et al., 2013).

The stimulation of MSCs with inflammatory factors, such as TNF-α and IFN-γ has shown to improve their regenerative potential and anti-inflammatory response. For instance through the upregulation of different molecules including VEGF, insulin-like growth factor I (IGF-1) and HGF (Crisostomo et al., 2008; Ragni et al., 2020).

Another pre-conditioning strategy that has been explored is the three-dimensional (3D) culture in scaffolds, encapsulated in hydrogels or as spheroids (Sart et al., 2016). Typically, *in vitro* cultures of MSCs are grown in monolayer systems but 3D cultures have been associated with higher expression of trophic factors (Carter et al., 2019; Miceli et al., 2019; Silva et al., 2013; Teixeira et al., 2016; Y. Xu et al., 2016). This may be justified by the fact that some of these strategies augment cell–cell interactions and better simulate cells' microenvironment (McKee & Chaudhry, 2017).

Dynamic cultures, using bioreactors, also create an interactive 3D microenvironment enabling a spatial distribution of macromolecules and mechanical cues. Therefore, bioreactor systems constitute an important approach to mimic the interactions between MSCs and their microenvironment, capable of changing cells secretory profile (Tandon et al., 2013).

There are other strategies to modulate the secretome but the most important would be to optimize them as a strategy to enhance the therapeutic effects of the secretome for future regenerative medicine applications.

2.1.2. Bioreactors: tools for modulation and large-scale production of secretome

Bioreactor systems are a promising technology for cell expansion. For this purpose different bioreactors have been explored for MSCs cultivation, including perfusion bioreactors (C. Liu et al., 2012; Yeatts & Fisher, 2011), rotating wall vessels (Song et al., 2008; T.-W. Wang et al., 2009), spinner flask (SP) systems (dos Santos et al., 2011; Stiehler et al., 2009; T.-W. Wang et al., 2009), stirred tank bioreactors (STBR) (dos Santos et al., 2014; Teixeira et al., 2016) and Vertical-Wheel™ bioreactors (VWBR) (Rodrigues et al., 2018; Sousa et al., 2015).

STBR have been extensively used for years. These bioreactors can be operated in batch mode, fed-batch mode, or perfusion mode and can be equipped with computer-controlled online-monitoring instruments that ensure tight control of process variables such as pH, temperature, and dissolved oxygen concentration (Taya & Kino-oka, 2011; Tsai et al., 2020). SP systems are a simpler type of STBR, that should be incubated in humidified CO_2 incubators with magnetically driven stirring provided by a bar on a central axis or a conical pendulum (Fig. 3 A) (Taya & Kino-oka, 2011). STBR were extensively used for expansion of cells that grow in suspension, but in 1967 microcarriers (MCs) were introduced for the expansion of anchorage-dependent cells as MSCs (Van Wezel, 1967). MCs are usually used in combination with SP or stirred tank bioreactors for MSCs expansion, as an important tool to increase the surface-to-volume area (Tsai et al., 2020). These small beads have a diameter that can range from 100 to 300 μm and can be made of diverse materials like gelatin, glass, and polystyrene, among others. They can be classified as porous, allowing cells to grow inside or non-porous where cells grow on the surface (B. Li et al., 2015). While porous MCs provide a higher surface area per unit of volume, retrieving cells from non-porous MCs is less difficult (Rafiq et al., 2016).

VWBR are a novel bioreactor system based on an innovative Vertical-Wheel™ technology that can also be used in combination with MCs for MSCs expansion. These bioreactors possess a vertically rotating wheel inside a U-shaped disposable vessel that provides more efficient mixing (Fig. 3 B) (Sousa et al., 2015).

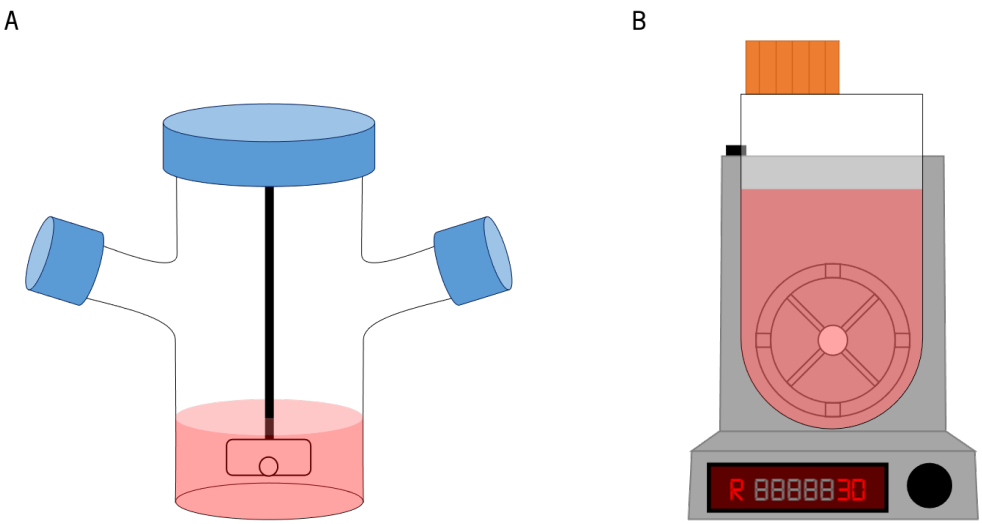

Figure 3. Schematic representation of two culture systems. (A) Spinner flask system, a glass-made system, with two openings enabling gas exchange and a bar on a central axis that provides stirring, and (B) Vertical-Wheel™ bioreactor, a U-shaped vessel with a vertical wheel that occupies a considerable area of the bottom of the vessel and its universal base unit for magnetically controlled rotation.

One important factor besides oxygen supply, that should be contemplated when culturing cells inside bioreactors is the stress caused by the hydrodynamic shear. Cells adherent to a surface are exposed to shear forces from the fluid in movement. So, shear stress is defined as the "force per unit area acting on and parallel to a surface" (Jossen et al., 2014; Panchalingam et al., 2015). Shear stress can have a negative impact in different aspects, such as MSCs growth. Increasing this parameter can even induce osteogenic and chondrogenic differentiation (Jossen et al., 2014; Z. Li et al., 2010; Yourek et al., 2010; Zhao et al., 2007). While cultures using spinner flasks or stirred tank bioreactors have been associated with high levels of shear stress, the design of VWBR induces less hydrodynamic shear even at higher agitation rates (Ismadi et al., 2014; Sousa et al., 2015). This is particularly important because reducing the agitation rate to decrease the shear stress can result in an inefficient suspension of MCs (Takahashi et al., 2016).

MSCs have been expanded in different types of bioreactors but few studies have focused on the impact of dynamic cultures in the modulation of the secretome. Hupfeld and co-workers addressed the effects of the expansion of MSCs from two sources in bioreactors and they concluded that the paracrine and gene expression profile significantly differed from cells cultured in static cultures. Curiously, VEGF was not secreted in static cultures of UC-MSCs and was significantly up-regulated in dynamic cultures of AM-MSCs (Hupfeld et al., 2014). On another study, Teixeira and colleagues identified a higher number

of soluble factors in dynamic cultures of BMSCs and an up-regulation of important factors such as GDN, Cystatin-c, Galectin-1, PEDF, BDNF, VEGF, NGF and IGF-1 in dynamic secretome, i.e. the secretome produced in the bioreactor (Teixeira et al., 2016). These findings may explain why they saw an enhancement of differentiation of human neural progenitor cells (hNPCs) after incubation with dynamic secretome and a similar trend in the enhancement of survival and differentiation of neurons and astrocytes in the hippocampal dentate gyrus of rats after intracranial injection of secretome.

Despite the proved efficacy of secretome from MSCs in different preclinical models, there is still a need for the development of large-scale good manufacturing practice (GMP)-grade secretome-based pharmaceuticals. GMP is based on a set of guidelines that guarantee the consistent and quality-controlled production of pharmaceuticals. Assuring product efficacy, safety, reproducibility, and quality through a validated production process can be a reasonable compensation for the lack of a complete definition of all the components that constitute the secretome (Laggner et al., 2020). As part of a validated production process different quality-assessment tests must be established. These tests can encompass physical, chemical, and biological assessments to guarantee product physical state (color, pH, etc.), sterility, the presence of bacterial endotoxins or the efficacy of the final product. These assessments should be tailor-made, especially the potency assays (to determine the efficacy), that will vary according to the disease in which the secretome will be applied (Laggner et al., 2020).

Another obstacle for the translation of secretome to the clinical setting are the current regulatory requirements because secretome is not consider an Advanced Therapy Medicinal Product, neither a biological medicinal product, which means that cell-derived secretomes fall into a normative gap. Notwithstanding, to date and as a result of the lack of a better definition, secretome is classified as a biological medicinal product, which demands extensive product characterization (Laggner et al., 2020).

3. Aims

Bearing in mind the importance of tackling the most important PD therapeutic unmet medical need, the potential of secretome as a cell-free therapy in this context and the value of bioreactors as a tool to produce large quantities of secretome (enabling compliance with regulatory requirements and quality standards), and as a modulation strategy was assessed in this thesis. Therefore, the following aims were defined:

- Evaluate the effect of secretome in *C. elegans* models of PD (Chapter II);

- Investigate the effects of secretome produced in two dynamic systems in a cell model of neurodifferentiation and in a *C. elegans* model of PD (Chapter III);
- Establishment of a rodent model of PD, based on the overexpression of α-syn (Chapter IV);
- Assess the impact of secretome produced in one dynamic system in a rodent model of PD (Chapter IV).

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CHAPTER II

Mesenchymal stem cell secretome protects against alpha-synuclein-induced neurodegeneration in a *Caenorhabditis elegans* model of Parkinson's disease

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Mesenchymal stem cell secretome protects against alpha-synuclein-induced neurodegeneration in a *Caenorhabditis elegans* model of Parkinson's disease

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ABSTRACT

Background aims: The capacity of the secretome from bone marrow-derived mesenchymal stem cells (BMSCs) to prevent dopaminergic neuron degeneration caused by overexpression of alpha-synuclein (α-syn) was explored using two *Caenorhabditis elegans* models of Parkinson's disease (PD).
Methods: First, a more predictive model of PD that overexpresses α-syn in dopamine neurons was subjected to chronic treatment with secretome. This strain displays progressive dopaminergic neurodegeneration that is age-dependent. Following chronic treatment with secretome, the number of intact dopaminergic neurons was determined. Following these initial experiments, a *C. elegans* strain that overexpresses α-syn in body wall muscle cells was used to determine the impact of hBMSC secretome on α-syn inclusions. Lastly, in silico analysis of the components that constitute the secretome was performed.
Results: The human BMSC (hBMSC) secretome induced a neuroprotective effect, leading to reduced dopaminergic neurodegeneration. Moreover, in animals submitted to chronic treatment with secretome, the number of α-syn inclusions was reduced, indicating that the secretome of MSCs was possibly contributing to the degradation of those structures. In silico analysis identified possible suppressors of α-syn proteotoxicity, including growth factors and players in the neuronal protein quality control mechanisms.
Conclusions: The present findings indicate that hBMSC secretome has the potential to be used as a disease-modifying strategy in future PD regenerative medicine approaches.

Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease in developed societies, causing debilitating motor symptoms, cognitive changes and behavioral/neuropsychiatry alterations [1]. The pathological hallmarks of PD consist of the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of Lewy bodies, mainly composed of alpha-synuclein (α-syn) aggregates [2].

PD is still an incurable disorder, and the classical therapeutic approaches—namely, pharmacotherapy and deep brain stimulation—mainly rely on the amelioration of psychomotor symptomatology, aiming at improving patients' quality of life and resulting in considerable side effects [3].

Given the limitations of conventional therapies, different disease-modifying strategies have been proposed over the last two decades [4,5]. Among these, the secretome from mesenchymal stem cells (MSCs) has gained interest as a possible cell-free therapy for different conditions [6–8]. MSCs are multipotent cells with the ability to self-renew and the potential to differentiate into different cell lines [9,10]. Their strong paracrine interactions with neighboring cells and tissues led the development of cell therapy strategies involving direct transplantation to the affected areas [11].

By contrast, different studies have been exploring the effects of the secretome from MSCs in the context of PD. The authors' group has shown that injection of the secretome from MSCs is able to rescue dopaminergic neurons within the SNpc in a rat model of PD [12–14]. Moreover, secretome showed a superior effect regarding neuronal survival and motor function compared with the transplantation of MSCs [13]. In another study, administration of the secretome led to an improvement in animals' skilled motor function that was corroborated by incremental growth in tyrosine hydroxylase-positive

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neurons and fibers in the SNpc and striatum, whereas injection of levodopa was unable to achieve such benefits [14]. These results, together with results from different groups, point toward the potential of the MSC secretome in the stimulation of neuronal survival and differentiation but also angiogenesis, modulation of apoptosis and immunomodulation [15]. Furthermore, these results highlight the possibility of using the MSC secretome, rather than MSC transplantation, as a therapeutic tool in PD regenerative medicine.

The aforementioned studies have been developed in acute models of PD based on the injection of 6-hydroxydopamine. Even though toxin-based models have been important for the development of treatments for PD and in studying side effects underlying dopamine replacement therapies, they do not mimic some of the key aspects of the pathophysiology of the disease (e.g., α-syn proteotoxicity) [16]. Therefore, the use of genetic models displaying, for instance, α-syn overexpression might be a valuable strategy for modeling the disease.

In vitro approaches have advanced with the aid of induced pluripotent stem cell cultures or organoids, but among all the limitations regarding reproducibility, costs or even ethical issues, the conclusions that can be drawn from these strategies are limited. Indeed, they are devoid of a functional nervous system and are therefore not able to recapitulate behavioral phenotypes [17].

Given that humans share physical, genetic and cellular analogies with other mammals, studies conducted using mammal models of PD have a greater chance of being translated to humans. However, maintenance of mammalian models is expensive and requires considerable space and conditions. Furthermore, the use of these animals is subject to ethical considerations. These constraints might be the reason for using an inadequate number of animals, which translates into a negative impact on the experiment's statistical power.

The nematode *Caenorhabditis elegans* has a well-characterized nervous system with 302 neurons, of which eight are dopaminergic [18]. This is an organism with a short life span, high reproductive rate and small size that is easy and affordable to maintain, considering the abundance of animals it is possible to obtain from a single hermaphrodite (around 300 progeny), and amenable to genetic manipulation [19]. The body of this animal is permeable to dissolved molecules and lacks a blood–brain barrier [20]. Moreover, different *C. elegans* models of PD exhibit several phenotypic deficits, such as age-dependent aggregation of α-syn, loss of dopaminergic neurons, disruption of dopamine-dependent behaviors and even deficits in movement [21]. Consequently, in the search for disease modifiers, all of these phenotypes can be valuable tools.

Despite the limitations, including the absence of an α-syn homolog, dissimilarities in neuronal connectivity compared with humans and difficulty in performing molecular biology on the eight dopaminergic neurons, the use of *C. elegans* models of PD has contributed to identifying modulators of neuronal dysfunction due to α-syn cytotoxicity, potential targets and neuroprotective agents and, especially, novel factors with disease-modifying potential [22]. Given the exploratory strengths of using *C. elegans* as a model of PD, the authors aimed to investigate the effect of the secretome from human bone marrow-derived mesenchymal stem cells (hBMSCs) in two *C. elegans* models of PD that overexpress α-syn.

Methods

Human bone marrow mesenchymal stem cell culture

The hBMSCs (Lonza, Basel, Switzerland) were thawed and cultured in Minimum Essential Medium Eagle, alpha modification (Alpha MEM) (Life Technologies, Carlsbad, CA, USA), with 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany) and 50 U/mL of penicillin and streptomycin (PenStrep) (Life Technologies). The medium was replaced every 3 days, and cells were kept at 37°C with 95% air and 5% carbon dioxide. When cells reached passage four and 80% confluence, they were harvested using 0.05% trypsin/ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, CA, USA) and plated again at a density of 4000 cells·cm^{-2}.

Secretome collection and preparation of drug assays

Secretome from hBMSCs was collected when cells reached passage five following a procedure already established by the authors' group [23,24]. Briefly, cells cultured at a density of 4000 cells·cm^{-2} for 72 h were washed three times with phosphate-buffered saline without calcium and magnesium ions (Invitrogen) and once with Alpha MEM and 50 U/mL PenStrep without FBS and incubated with Alpha MEM and 50 U/mL PenStrep without FBS. After 24 h, the medium containing all factors secreted by the cells within this period was collected and centrifuged at 300 g for 10 min to remove cell debris. Afterward, the medium was concentrated by centrifugation using a Vivaspin™ 5 kDa cutoff concentrator (GE Healthcare Bio-Sciences, Uppsala, Sweden) to achieve a 2× concentrated secretome. Aliquots of 2× concentrated secretome were snap-frozen with liquid nitrogen and stored at −80°C.

Nematode strains and culture conditions

All strains were maintained in nematode growth media containing agar plates seeded with *Escherichia coli* OP50 as a food source at 20°C as previously described [25]. Strains BZ555 *egfs*1 (*dat-1*p::green fluorescent protein [GFP]) and NL5901 *pkls*2386 (*unc-54*p::alpha-synuclein::yellow fluorescent protein [YFP] + unc-119[+]) were acquired from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). Strain BZ555 expresses GFP in the eight dopaminergic neurons and strain NL5901 overexpresses α-syn fused to YFP in body wall muscle cells, resulting in α-syn inclusions that are easily observable under the microscope and that tend to increase with an animal's age. Strain UA44 (*baln*1, P*dat-1*::α-syn high, P*dat-1*::GFP) was generously provided by Guy Caldwell (University of Alabama). This strain expresses GFP and overexpresses α-syn in the eight dopaminergic neurons. The overexpression of α-syn causes age-dependent dopaminergic neurodegeneration.

Two types of plates were prepared. One was seeded with 2× concentrated secretome diluted in inactivated OP50 (secretome final concentration = 1x) and the other was seeded with 2× concentrated Alpha MEM diluted in inactivated OP50 (secretome final concentration = 1x) as control. To guarantee intake of the secretome or Alpha MEM, both were diluted in the animals' food source (*E. coli* OP50). However, to prevent any metabolization of both solutions by the bacteria and to ensure that the effects seen in animal phenotypes were due to secretome supplementation and not due to its metabolization by live bacteria, inactivated OP50 was used. To prepare inactivated OP50, bacteria were grown overnight at 37°C and 150 rpm in Luria broth medium, pelleted by centrifugation, inactivated by three cycles of freeze/thawing, frozen at −80°C and then resuspended in S medium supplemented with 25 U/mL of PenStrep and 50 U/mL of nystatin (Sigma-Aldrich, St Louis, MO, USA).

Quantitative analysis of dopaminergic neuronal loss

Age-synchronized BZ555 and UA44 worms were obtained by egg-laying. For this, adult worms were plated in freshly prepared plates for 2 h and removed from the plates after this period (day 0). After 4 days, worms were transferred to freshly prepared plates every day until day 7. On day 7, nematodes (N = 20 per condition) from each condition were washed in M9 buffer and mounted on a 3% agarose pad. A solution of 5 mM levamisole (Sigma-Aldrich) was used to immobilize animals, enabling live imaging 2 h later. Representative confocal microphotographs were obtained with an Olympus LPS

FV1000 confocal microscope (Olympus, Tokyo, Japan) under a ×60 oil (numerical aperture = 1.35) objective. Z-series imaging was acquired for all vehicle- and secretome-treated animals using a 488-nm laser excitation line for GFP visualization. The pinhole was adjusted to 1.0 Airy unit of optical slice, and a scan was acquired every 0.5 μm along the z-axis. For quantification purposes, dopaminergic neurons were visualized with an Olympus BX-61 fluorescence microscope (Olympus) because of GFP expression. Intact dopaminergic neurons were scored and the experiment independently repeated four times.

Quantitative determination of α-syn inclusions

Age-synchronized NL5901 nematodes were obtained by egg-laying, as described earlier, except for the fact that a laser excitation line at 488 nm was used for YFP visualization. Four days later, worms were mounted on a 3% agarose pad, immobilized with 5 μL of 5 mM levamisole and observed 2 h later under confocal microscopy to visualize fluorescent α-syn inclusions in the head region. Confocal microphotographs were obtained with an Olympus LPS FV1000 confocal microscope (Olympus) as described earlier. For more accurate and unbiased quantification of individual inclusions, z-stack overlay images were analyzed using ImageJ software. Represented values are the mean (normalized to vehicle-treated control) of at least nine animals per group.

Protein functional associations

Previous work developed by the authors' team enabled the identification, using mass spectrometry, of several proteins secreted by MSCs [12,24,26]. The authors aimed to explore the potential mechanisms and players that might be responsible for the effects of the secretome. For this, the authors started by searching on PubMed for work that showed the efficacy of a given protein against α-syn pathogenesis. Afterward, the authors cross-referenced the proteins retrieved from the literature search with the proteins present in the secretome of hBMSCs (according to our proteomics databases). In the end, the authors concluded that 13 proteins identified on our previous proteomics analyses were already described in the literature as playing a role in blocking α-syn pathogenesis. The authors then intended to understand whether the 13 proteins were related and possibly involved in cooperating biological pathways. Therefore, the existence of functional associations between them was assessed using STRING v.11.0 [27]. Finally, the homology between humans and *C. elegans* regarding these proteins was determined using OrthoList 2 [28] or Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To verify the conservation of each of the 13 proteins between species, their sequences were aligned with the respective homologs found in *C. elegans* using the T-Coffee multiple sequence alignment server [29]. This alignment was used to determine the percentage of similarity through Sequence Manipulation Suite 2.0 [30].

Statistical analysis

Statistical analysis was performed using SPSS Statistics 25 software (IBM, Armonk, NY, USA). The normal distribution of continuous variables was analyzed according to Shapiro–Wilk or Kolmogorov–Smirnov normality tests. Homogeneity of variances was assessed with Levene's test. When the assumptions were confirmed, groups were compared through an independent samples *t*-test (Figure 2). The Pearson's chi-square test was used for proportion analysis, with a follow-up *z*-test for independent proportions with Bonferroni correction (Figure 1). Appropriate effect size measures were used for each test (Cohen's *d* for Student's *t*-test and φ for Pearson's chi-square test). Detailed statistics are available in supplementary Table 1.

Results and Discussion

During the last decade, the use of MSC secretome has been studied for a wide range of conditions and applications [31–34]. Some of

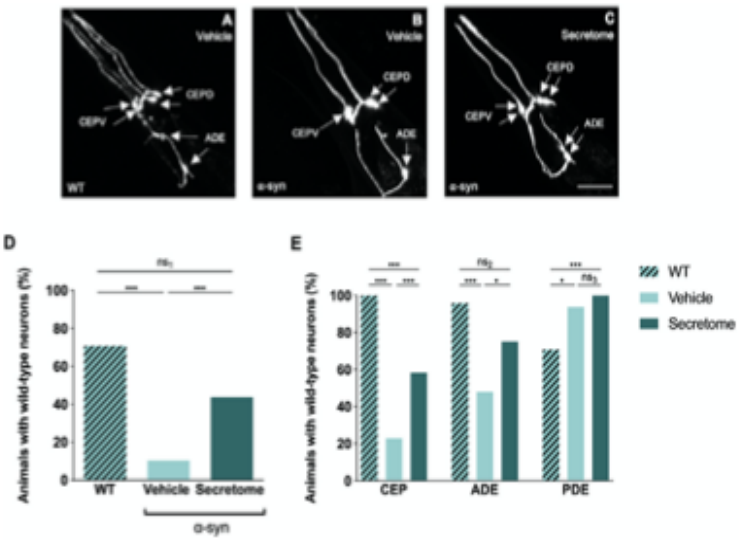

Fig. 1. The hBMSC secretome treatment protects dopaminergic neurons from α-syn-associated effects. GFP expression pattern in dopaminergic neurons of transgenic *C. elegans* at adult day 7 obtained by confocal live imaging in (A) untreated control (BZ555), (B) untreated control (UA44) and (C) treated nematode (UA44). Scale bar = 100 μm. (D) The proportion of animals with intact dopaminergic neurons for each condition was determined by counting the number of animals with WT neurons. (E) The proportion of animals with intact CEP, ADE or PDE neurons was determined by counting the animals with WT dopaminergic neurons belonging to each subpopulation. A total of 24–48 animals were assayed per group across four independent experiments. There is no representation of error bars because of the statistical test used, which is a comparison of categorical data (having or not having all WT dopaminergic neurons). *$P < 0.05$, ***$P < 0.001$, $ns_1 = 0.06$, $ns_2 = 0.07$, $ns_3 = 0.24$ (Pearson's chi-square test). ADE, anterior deirid; CEP, cephalic; CEPD, distal cephalic; CEPV, ventral cephalic; ns, non-significant; PDE, posterior deirid; WT, wild-type.

those studies highlighted the potential of the secretome as a cell-free therapy that can be made readily available as an "off-the-shelf" product. However, in the context of PD, most of the studies that contributed to proving the potential of the secretome were based on neurotoxin-induced models that do not recapitulate some of the most important pathogenic processes of idiopathic PD [12–14,35–38]. Hence, efforts should be made at exploring other approaches based on models that better recapitulate the pathophysiology of the disease and can thus make a greater contribution to deciphering the mechanisms involved in the effects of the secretome. Simpler genetically engineered model organisms, such as invertebrates, can be a more reliable and exploratory approach for comparing with *in vitro* studies. Additionally, such organisms have shown a tendency to replicate neuronal cell loss and the development of motor symptomatology in a more consistent way than other animals, such as rodents [39].

Herein the effect of the secretome on α-syn overexpression was analyzed using two *C. elegans* models of PD. Initially, a strain that overexpresses human wild-type α-syn in dopaminergic neurons was used. Notably, not only do these animals display dopaminergic neurodegeneration, but this neurodegeneration is also progressive and worsens with age [40]. When this strain was subjected to chronic treatment with the secretome, the authors observed reduced loss of dopaminergic neurons. Indeed, about 44% of the animals treated with the secretome had all dopaminergic neurons intact compared with 10% in the group treated with the vehicle (Figure 1D). If we specifically consider subpopulations of dopaminergic neurons, secretome treatment showed a significant protective effect in cephalic and anterior deirid neurons (Figure 1E).

Following this, the authors aimed to understand the effect of the secretome in an animal that overexpresses α-syn in the body wall muscle cells, resulting in α-syn misfolding and aggregation. This is an interesting model for studying the impact of different compounds on α-syn aggregation. Given the transparent body of the animal and the expression of α-syn fused to YFP, α-syn inclusions can be easily observed. Chronic treatment with the secretome led to a 13% reduction in α-syn inclusions when results in the treatment arm were normalized to the control arm (Figure 2). Despite not demonstrating a drastic decrease in α-syn inclusions, this value may be sufficient to translate into a relevant biological effect, especially if taking into account the obtained effect size for this experiment ($d = 0.506$) (see supplementary Table 1). However, given that PD is an age-related condition and that this strain presents more inclusions throughout time, it might be interesting to study in the future the effect of the secretome in older animals.

Next, the authors aimed to study the possible effects of secretome supplementation on α-syn proteotoxicity in neuronal cells using an in silico approach. The secretome is a complex mixture of bioactive soluble proteins, lipids and microvesicles that can contribute to neuroprotection and enhancement of protein homeostasis through different pathways. The authors' group has already shown that the MSC secretome is constituted by several soluble factors that can aid in enhancing the function and signaling of existing nigrostriatal dopaminergic neurons [12,14,24,26].

Taking advantage of the proteomic data gathered by the authors' group with regard to the proteome of the secretome, 13 proteins were selected that, according to the existing literature, were linked to an ability to block α-syn proteotoxicity and that could potentially be correlated with the current experimental findings [12,24,26]. Interestingly, with the use of STRING bioinformatics-based analysis, the authors found that these molecules are functionally interconnected (Figure 3). Importantly, most of these proteins and respective signaling pathways are highly conserved in *C. elegans* (Table 1) [41–51]. Moreover, all of them, being growth factors and involved in

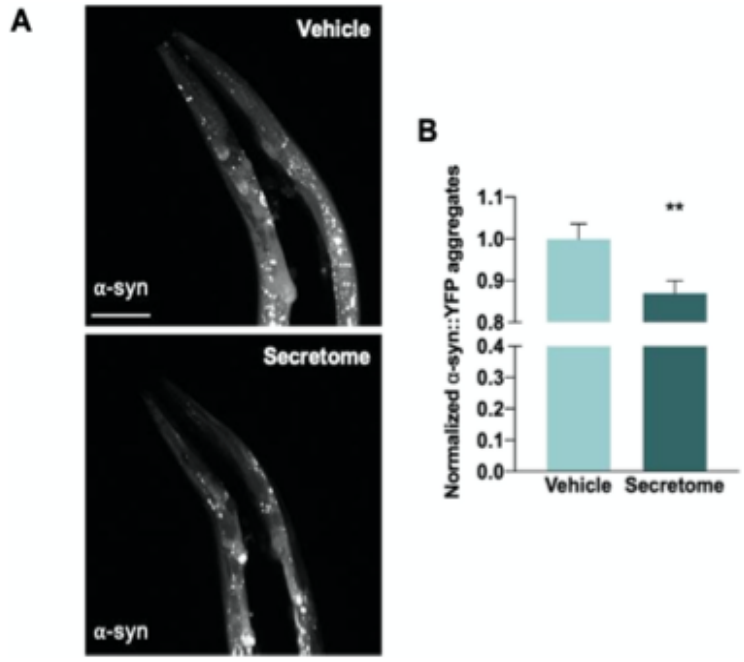

Fig. 2. The hBMSC secretome treatment leads to a reduction in α-syn inclusions. (A) The α-syn expression pattern in the head region of NL5901 animals obtained by confocal live imaging. Scale bar = 100 μm. (B) Number of aggregates from a pool of five individual experiments (mean ± SD, n = 60–61) normalized to the average number of aggregates in control animals for each experiment. $^{**}P < 0.01$ (independent samples *t*-test). SD, standard deviation.

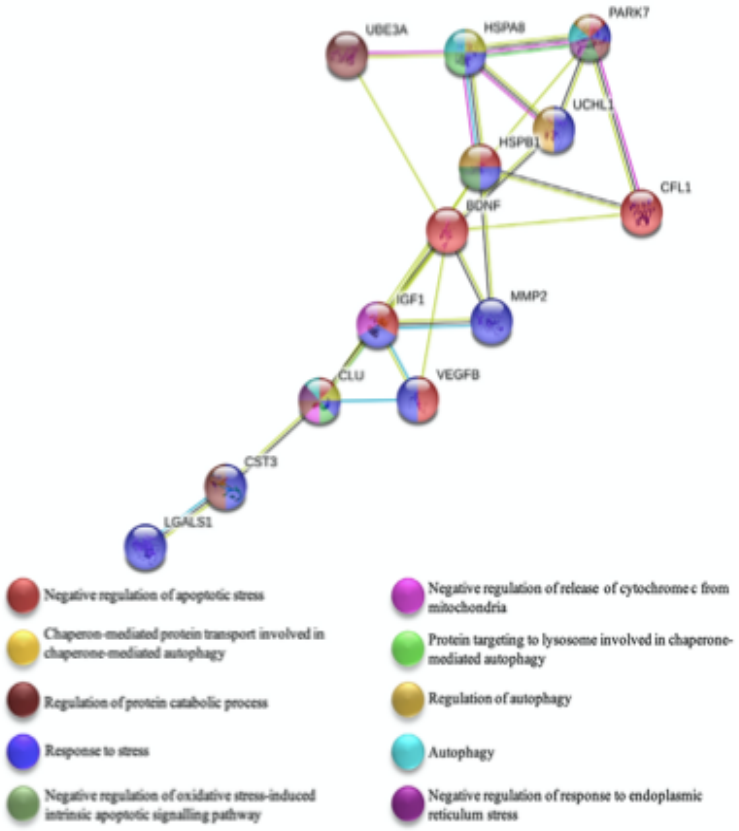

Fig. 3. MSCs secrete several molecules involved in different biological processes that can impact PD. Based on previously generated proteomic databases derived from Bio-Plex Luminex and mass spectrometry-based analysis, the authors selected 13 proteins indicated by the literature to play a role in blocking α-syn toxicity. The 13 selected proteins—namely, MMP2, BDNF, PARK7, CFL1, HSPA8, CST3, CLU, VEGFB, IGF1, UCHL1, HSPB1, UBE3A and LGALS1—are functionally interconnected, according to STRING bioinformatics analysis (https://string-db.org/) and are involved in molecular pathways important to PD modeling and repair. https://blast.ncbi.nlm.nih.gov/Blast.cgihttp://ortholist.shaye-lab.org/ BDNF, brain-derived neurotrophic factor; CFL1, cofilin 1; CLU, clusterin; CST3, cystatin c; HSPA8, heat shock cognate 71 kDa protein; HSPB1, heat shock protein beta 1; IGF1, insulin-like growth factor 1; LGALS1, galectin 1; MMP2, matrix metalloproteinase 2; PARK7, parkinson disease protein 7 or Dj-1; UBE3A, ubiquitin-protein ligase E3A; UCHL1, ubiquitin carboxyl-terminal hydrolase isozyme L1; VEGFB, vascular endothelial growth factor B.

the autophagy pathway, play important roles in biological processes that may be disrupted in PD and were found to be enriched in this analysis (Figure 3).

For instance, brain-derived neurotrophic factor (BDNF) is a potent dopaminergic neurotrophin distributed throughout the central nervous system, including the striatum. However, it is downregulated in the SNpc of PD patients, which might indicate involvement in the pathogenesis of PD [52]. Indeed, overexpression of α-syn leads to BDNF downregulation, contributing to neurodegeneration. By contrast, BDNF expression induced by the transplantation of neural stem cells in a progressive transgenic animal model of dementia with Lewy bodies contributed to the survival of still intact nigrostriatal dopaminergic neurons, leading to a dramatic improvement in cognitive and motor function [53]. Vascular endothelial growth factor B (VEGFB) is a member of the VEGF family, representatives of which are known as key regulators of vascular biology. VEGFB is a protective factor that mediates the crosstalk between oxidative metabolism and mitochondrial biogenesis. Evidence suggests that VEGFB may potentiate the effects of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha, a neuroprotective factor that attenuates the effects of α-syn overexpression [54,55].

Parkinson disease protein 7 or DJ-1 is well known as a redox-regulated protein that acts as a sensor of oxidative stress [56]. However, it can also inhibit α-syn aggregation, and it possesses chaperone activity against α-syn by regulating chaperone-mediated autophagy, a very important catabolic pathway for wild-type α-syn [57,58]. Molecular chaperones, such as the small heat shock proteins, are important players in the proteostasis network, and at least some of them have been linked to the prevention of α-syn aggregation [59]. For instance, heat shock protein beta 1 is a small heat shock protein present in the MSC secretome that can bind α-syn fibrils, decreasing cytotoxicity [60]. In this context, the heat shock cognate 71 kDa protein is involved in chaperone-mediated autophagy and is also the main constituent of a disaggregation system that efficiently disassembles α-syn amyloid fibrils in non-toxic monomers [61]. Similar effects are also seen with clusterin, an extracellular chaperone that, in addition to the capacity to inhibit the aggregation of different proteins, including α-syn [62], can suppress the α-syn-induced formation of reactive oxygen species in neuronal cells [63].

Cofilin 1 has an essential role in controlling actin dynamics and influencing cell proliferation and motility [64]. Additionally, it has been shown to prevent α-syn-driven defects in axon elongation and

Table 1
Protein homologues found in C. elegans, showing the percentage of similarity for each of the 13 selected proteins.

Human protein	Accession number	*C. elegans* homolog	Similarity (%)	References
MMP2	P08253	ZMP-1	29.45	[41]
		ZMP-2	26.89	
		ZMP-3[a]	28.07	
		ZMP-4	27.79	
		ZMP-5[b]	27.65	
		ZMP-6	21.34	
BDNF	P23560	Not found	-	[42]
PARK7	Q99497	DJR-1.1[b]	70.53	[43]
		DJR-1.2[b]	60.21	
CFL1	P23528	UNC-60[b]	41.90	[44]
UCHL1	P09936	UBH-1[b]	54.67	[43]
		UBH-2[b]	49.35	
		UBH-3[b]	51.72	
CLU	P10909	Not found	-	[45]
CST3	P01034	CPI-1[a]	37.97	[46]
		CPI-2[a]	40.00	
VEGFB	P49765	PVF-1	24.84	[47]
LGALS1	P09382	LEC-6[b]	42.11	[48]
HSPB1	P04792	HSP-16.2[b]	32.68	[49]
UBE3A	Q05086	Not found	-	-
IGF1	P05019	INS-1[a]	18.75	[50]
HSPA8	P11142	HSP-1[b]	90.11	[51]

[a] Based in BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi)
[b] Based on Ortholist2 tool (http://ortholist.shaye-lab.org/)
BDNF, brain-derived neurotrophic factor; CFL1, cofilin 1; CLU, clusterin; CST3, cystatin c; HSPA8, heat shock cognate 71 kDa protein; HSPB1, heat shock protein beta 1; IGF1, insulin-like growth factor 1; LGALS1, galectin 1; MMP2, matrix metalloproteinase 2; PARK7, parkinson disease protein 7 or DJ-1; UBE3A, ubiquitin-protein ligase E3A; UCHL1, ubiquitin carboxyl-terminal hydrolase isozyme L1; VEGFB, vascular endothelial growth factor B.

turning [65]. Cystatin c has several biological roles in different cellular systems. Recent studies attributed a neuroprotective role to cystatin c in some diseases, including PD, because of the capacity to induce autophagy [66,67]. Insulin-like growth factor 1 is a neurotrophic factor involved in fostering cell survival, preventing apoptosis and promoting neurogenesis. Additionally, it is linked to the suppression of α-syn cytotoxicity and protein aggregation through the activation of the PI3K/Akt pathway [68]. Ubiquitin carboxyl-terminal hydrolase isozyme L1 is a neuron-specific deubiquitinating enzyme that induces the elimination of misfolded proteins, including α-syn [69]. Ubiquitin-protein ligase E3A (UBE3A) is an E3 ubiquitin ligase that targets proteins for degradation through the ubiquitin-proteasome system [70].

A study by Mulherkar *et al.* [71] suggested that UBE3A plays a role in the degradation of cytoplasmic misfolded proteins like α-syn. The researchers showed that overexpression of UBE3A enhanced the degradation of wild-type and mutant forms of α-syn in a proteasome-dependent manner. Galectin 1 is a multifunctional protein that plays roles in the proliferation of neural stem cells, neuritic outgrowth and oxidative stress homeostasis. Moreover, it has been shown to suppress α-syn transmission by competing with this protein for N-methyl-D-aspartate receptor binding, inhibiting N-methyl-D-aspartate receptor-mediated endocytosis [72]. Finally, matrix metalloproteinase 2 participates in diverse biological processes through the degradation of extracellular and non-extracellular matrix molecules. In fact, matrix metalloproteinase 2 was recently identified as the main factor responsible for the degradation of oligomeric α-syn following incubation with the MSC secretome [73].

Conclusions

Overall, the results reported in this study show that the secretome from hBMSCs can act as a neuroprotective agent, protecting and preserving dopaminergic neurons from the effect of α-syn overexpression. To the authors' knowledge, this is the first study reporting a neuroprotective effect of the secretome from hBMSCs in different *C. elegans* models of PD, which is characterized by the overexpression of human α-syn. Furthermore, the authors suggest candidate molecules and possible interconnected mechanisms that might be involved in the effect seen in this study.

Despite the evidence showing the potential for using the secretome from MSCs in the context of PD, more knowledge should be gathered regarding its main players and associated mechanisms of action before proceeding to clinical trials. By demonstrating that the secretome from human MSCs can exert a neuroprotective action in these models, the authors expect to leverage the development of different studies using *C. elegans* to further depict the cellular and molecular pathways involved in the effects of the secretome before proceeding to studies in more complex animal models.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: CRM, JPS, ATC, AJS. Acquisition of data: CRM, ATC. Analysis and interpretation of data: CRM, JPS, ATC, AJS. Drafting or revising the manuscript: CRM, JPS, ATC, FGT, RAS, AJS. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2021.04.002.

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1. Supplementary material

Supplementary Table 1. Statistical report. Effect sizes calculated using Lenhard, W. (2016) and Uanhoro, J. O. (2017). Effect size calculators available online at: https://www.psychometrica.de/effect_size.html and https://effect-size-calculator.herokuapp.com/, respectively.

Figure	Statistical report	Sample size
Fig. 1D	χ^2 (2) = 27.581, $p < 0.001$, φ = 0.479	24 to 48 (per group)
Fig. 1E	CEP χ^2 (2) = 39.215, $p < 0.001$, φ = 0.572 ADE χ^2 (2) = 18.620, $p < 0.001$, φ = 0.394 PDE χ^2 (2) = 18.273, $p < 0.001$, φ = 0.390	24 to 48 (per group)
Fig. 2B	t(119) = 2.781, p = 0.006, d = 0.506	60 to 61 (per group)

CHAPTER III

Neurodifferentiation and neuroprotection potential of mesenchymal stromal cell-derived secretome produced in different dynamic systems

Manuscript in preparation

Neurodifferentiation and neuroprotection potential of mesenchymal stromal cell-derived secretome produced in different dynamic systems

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1. Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized by the degeneration of the dopamine (DA) neurons in the substantia nigra pars compacta, leading to a loss of DA in the basal ganglia, resulting in the debilitating motor symptoms characteristic of the disease. The presence of aggregates of alpha-synuclein (α-syn) is seen as the main contributor to the pathogenesis and progression of PD. Evidence suggests that the secretome of mesenchymal stromal cells (MSCs) could be a potential cell-free therapy for PD. However, to accelerate the integration of this therapy in the clinical setting, there is still the need to develop a protocol for the large-scale production of secretome under good manufacturing practices (GMP) guidelines. Bioreactors have the capacity to produce large quantities of secretome, as well as to surpass other limitations of planar static culture systems. However, there are several types of bioreactors, and even though much is known about the expansion of MSCs in these systems, few studies focused on the profile of the secretome that can be obtained in these platforms. Therefore, in this work, we studied the capacity of the secretome from bone marrow-derived mesenchymal stromal cells (BMSCs) produced in a spinner flask (SP) and in the Vertical-Wheel™ bioreactor (VWBR), to induce neurodifferentiation of human neural progenitor cells

(hNPCs) and to prevent dopaminergic neuron degeneration caused by the overexpression of α-syn in one *Caenorhabditis elegans* model of PD. In the conditions studies, results show that the secretomes were able to induce neurodifferentiation, but the secretome collected in the SP system had a greater effect. Additionally, only the secretome produced in SP had a neuroprotective potential. Lastly, the secretomes had different profiles regarding the presence and/or specific intensity of different molecules, namely interleukin (IL)-6, IL-4, matrix metalloproteinase-2 (MMP2) and 3 (MMP3), tumour necrosis factor-beta (TNF-β), Osteopontin, nerve growth factor (NGFβ), granulocyte colony-stimulating factor (GCSF), Heparin-binding (HB) epithelial growth factor (EGF)-like growth factor (HB-EGF), and IL-13.

Overall, our results suggest that the culture conditions might have influenced the secretory profiles of cultured cells and, consequently, the observed effects. Moreover, more studies should explore further the effects that different culture systems have in the secretome potential in the context of PD.

2. Introduction

Mesenchymal stromal cells (MSCs) are a population of multipotent progenitor cells with the ability to self-renew and potential to differentiate into different cell lines, and that can be retrieved from different tissues (Bianco, 2014; Mushahary et al., 2018). Despite the advantages and limitations of each tissue source, bone-marrow is still the gold standard and more widely used tissue source. Gathered evidence suggests that the beneficial effects of MSCs' secretome are equivalent to the effects of the administration of MSCs and extends to different domains, including hepatic, skeletal, cardiovascular and nervous system, among others (reviewed in (Harrell et al., 2019)). The benefits of secretome have been studied for a large spectrum of conditions of the nervous system, including PD, where it showed to outperform MSCs transplantation related approaches (Chudickova et al., 2019; Elia et al., 2019; Muhammad, 2019; Teixeira et al., 2017a).

Parkinson's disease (PD) is characterized by the decrement in DA levels due to the degeneration of dopaminergic cells present in the substantia nigra pars compacta (SNpc) (Poewe et al., 2017). The molecular pathways responsible for neurodegeneration in PD are not well clarified but accumulated evidence suggests that mitochondrial dysfunction is highly linked to the development of the disease. This pathway leads to oxidative stress, accumulation of oxidized dopamine, being also correlated with lysosomal dysfunction, as well as alpha-synuclein (α-syn) accumulation and aggregation (Burbulla et al., 2017; Poewe et al., 2017). Specifically, α-syn is a protein mainly located at the pre-synaptic terminal, that is supposed to be involved in vesicular packaging, trafficking and synaptic transmission (Wong & Krainc, 2017). However, accumulation of α-syn and presence of oligomerized or aggregated forms of α-syn can greatly impact different cellular mechanisms, leading to oxidative stress and neurodegeneration (Villar-Piqué et al., 2016).

Different disease modifying strategies for PD have been studied, including the use of secretome from MSCs, due to its neuroprotective and neurodifferentiation potential. In fact, MSCs' secretome has protected cultured rodent cortical neurons from death, a process that was dependent on the PI3kinase/Akt survival pathway. Brain-derived neurotrophic factor (BDNF) was defined as particularly responsible for the neuroprotective effect (Wilkins et al., 2009). The same factor is required for the existence of an adequate number of dopamine neurons in the SNpc (Baquet et al., 2005). Moreover, our group have been showing the beneficial effects of secretome from MSCs on the survival of DA neurons and stimulation of neurodifferentiation in different models of the disease (Mendes-Pinheiro et al., 2019; Teixeira et al., 2016, 2017b, 2020).

Bioreactors are an important platform to enable the establishment of dynamic cultures, that better recreate MSCs' microenvironment (Das et al., 2019). As already shown by our group, the production of secretome in a bioreactor system can impact the expression of different factors and even enhance the expression of others, compared with secretome produced in static conditions (Teixeira et al., 2016). Bioreactors also provide the means to produce large volumes of secretome, fundamental for application in large groups of patients. Nevertheless, there is a wide range of bioreactor configurations available in the market, with different characteristics and the effects of their particularities in the secretory profile of MSCs is poorly understood.

In this work, two different mechanically agitated systems were used to produce secretome from bone marrow-derived MSCs (BMSCs). The widely known spinner flask (SP) system has a cylindrical shape and is harnessed with 90° paddles and a magnetic stir bar. Its agitation mechanism greatly contrasts with the Vertical-Wheel™ bioreactor (VWBR) in which agitation is generated by a large vertical impeller and a U-shaped bottom, providing mixing and suspension of particles with low agitation speeds (Croughan et al., 2016). Both systems are available in a single-use format, which could facilitate translation to clinics. Indeed, single-use systems are important for biopharmaceutical manufacturing, to eliminate the need for cleaning and sterilization between runs and, thus, to significantly reduce the contamination rates and production costs (Shukla & Gottschalk, 2013). Herein, the capacity of MSCs-derived secretomes produced in both systems to induce neurodifferentiation and neuroprotection was assessed and compared.

3. Materials and methods

Human Bone Marrow Mesenchymal Stromal Cell (BMSCs) Cultures

BMSCs were isolated from bone marrow aspirates obtained from healthy donors after written informed consent at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal, according to the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells (Portuguese Law 22/2007, June 29), with the approval of the Ethics Committee of the respective clinical institution, according to the Portuguese Regulation (Law 21/2014, April 16). MSCs were retrieved according to the established protocols as described by dos Santos *et al.* (2010) (dos Santos et al., 2010). Cells with 4 and 5 passages and from the same donor were used.

BMSCs Cultures Under Static Conditions

Cryopreserved BMSCs were thawed and plated at a cell density of 3,000 cells·cm^{-2}, on T-75 flasks with low glucose (1 g·L^{-1}) Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 5% v/v human platelet lysate (hPL) UltraGRO™-PURE (AventaCell, USA). Alternatively, cells were plated at the same cell density in CELLstart™ substrate (Thermo Fisher Scientific, USA) pre-coated T-75 flasks with StemPro™ MSC SFM XenoFree medium (Thermo Fisher Scientific). Cells cultured in DMEM medium supplemented with hPL were destined to being cultured in VWBR, whereas cells destined to the SP were cultured in StemPro™ MSC SFM XenoFree medium. At 70% cell confluence, MSCs were harvested with 1x TrypLE™ Select Enzyme solution (Thermo Fisher Scientific) for 5 min at 37°C. Cell number and viability were determined using the Trypan Blue (Thermo Fisher Scientific) exclusion method.

Microcarrier preparation

SoloHill plastic microcarriers (MCs, Pall, USA) of 360 cm^{2}·g^{-1} superficial area, were used for BMSCs culture in both systems. The preparation of the MCs varied according to the culture medium used in each system.

For inoculation of the Vertical-Wheel Bioreactor (VWBR)

Following sterilization by autoclave (120°C, 20 min), coating of MCs with a solution of DMEM supplemented with 50% hPL UltraGRO™-PURE was performed using a Thermomixer® comfort (Eppendorf AG, Germany) following a protocol consisting of cycles of 2 min at 750 rpm agitation and 10 min without agitation, during 1h. Prior to cell inoculation, MCs were resuspended in DMEM supplemented with 5% hPL UltraGRO™-PURE (de Almeida Fuzeta et al., 2020).

For inoculation of the spinner flask (SP)

After sterilization by autoclave (120°C, 20 min), MCs were coated with a CELLstart™ substrate (diluted 1:100 in 1x PBS) for 1 h at 37°C, with an intermittent agitation (cycles of 2 min at 750 rpm, 8 min without agitation) using a Thermomixer® comfort, and afterwards equilibrated in pre-warmed StemPro® MSC SFM XenoFree medium (Carmelo et al., 2015).

BMSCs culture on VWBR

PBS MINI vertical-wheel bioreactor, namely PBS 0.1 (PBS Biotech, USA) was operated at its full working volume (100 mL). Previously prepared MCs were used at a concentration of 20 $g \cdot L^{-1}$. Following the introduction of the MCs into the bioreactor, BMSCs previously expanded under static conditions for 2 passages, were transferred at a cell density of 5×10^5 $cells \cdot mL^{-1}$ to the bioreactor. DMEM supplemented with 5% hPL UltraGRO™-PURE was added to reach 60 mL of culture medium inside the bioreactor. The culture was maintained at 37°C and 5% CO_2. During the first 6h the agitation was set to cycles of 25 rpm for 1 min, followed by 20 min without agitation. In the end of this regime, the agitation was set to 25 rpm and then 30 rpm after 24h. After 48h, 40 mL of fresh culture medium with a glucose pulse (3 g/L) was added to the VWBR, to achieve a final volume of 100 mL. Feeding was performed from the third day of culture on a daily basis, by replacing 25% of volume with fresh culture medium supplemented with a glucose pulse (3 g/L).

Daily sampling of the culture was performed to determine total cell number and metabolites concentration. Briefly, when the MCs settled, supernatant was harvested, centrifuged, transferred to a new tube and stored at -20°C until further analysis of glucose and lactate concentrations. In feeding days, the concentration of both metabolites was assessed before and after medium replacement. Afterwards, MCs were incubated with TrypLE™ Select Enzyme solution at 37°C for 7 min and 750 rpm, using a Thermomixer® comfort. After stopping the reaction by diluting with culture medium, mechanical dissociation was performed by pipetting up and down and the mixture was then filtered using a 100 µm cell strainer (BD Biosciences, USA) to remove the MCs. Cells were centrifuged at 349 g for 7 min, and

total cell number and viability were determined throughout time using the Trypan Blue exclusion method. The determination of the growth rate was based on an exponential fitting to experimental data that correspond to the exponential growth phase. The doubling time was calculated by the ratio between the logarithm of the growth rate by the logarithm of 2. Adhesion efficiency to the MCs was calculated as the ratio between the total number of cells at day 1 and the number of inoculated cells.

To monitor cell adhesion and growth along the culture an additional sample was daily collected. Following cell fixation using 4% paraformaldehyde (PFA; Sigma-Aldrich, USA), MCs containing cells were incubated with 4-6-diamidino-2-phenylindole-dihydrochloride (DAPI, Sigma-Aldrich) at 1.5 $mg \cdot L^{-1}$ for 10 min and observed in a fluorescence inverted microscope (Leica DMI 3000 B).

BMSCs culture on SP

Bellco® spinner flasks (Bellco Glass, USA) offer a working volume of 100 mL but were operated at 80 mL. Prior to use, spinner flasks were autoclaved and treated with Sigmacote® (Sigma-Aldrich) to prevent microcarrier adhesion to the surface of the flask. MCs prepared following the aforementioned protocol were added at a concentration of 20 $g \cdot L^{-1}$. MSCs previously expanded under static conditions in StemPro® MSC SFM XenoFree medium for 2 passages, were seeded at a cell density of 4×10^6 $cells \cdot mL^{-1}$ in the spinner flask with a total volume of 40 mL and incubated at 37°C and 5% CO_2. Following cell inoculation, agitation was set to 30 rpm. After 24 h, agitation was increased to 40 rpm. The necessary volume of medium to attain 80 mL was added after 96h post inoculation. Feeding was performed from the fourth or fifth day of culture on a daily basis, by replacing 25% of volume with pre-warmed medium or by the addition of a glucose pulse (from a glucose stock solution of 20 $g \cdot L^{-1}$) to maintain the levels of glucose at a non-limiting concentration (superior to 1 mM) (Schop et al., 2009).

Daily sampling was performed according to the methodology followed for the culture on the VWBR. Adhesion, growth rate and population doubling were calculated for both cultures.

BMSCs conditioning and secretome collection

BMSCs were cultured in both dynamic systems until stationary cell growth phase. At this point, the medium was removed from the culture systems and MCs containing cells were washed once with 100 mL of Neurobasal-A (Gibco) or AlphaMEM (Gibco) medium. Afterwards, 100 mL for the VWBR or 80 mL for the SP of AlphaMEM (SP1 and VWBR1) or Neurobasal-A (SP2 and VWBR2) medium, with 1% Antibiotic-Antimycotic (Gibco) were added to the culture systems. After 24 h, the medium was harvested and centrifuged at 300 g for 10 min to remove cell debris.

The secretome collected using AlphaMEM medium was destined to be used for *C. elegans* assays and was concentrated by centrifugation to 2x using a Vivaspin 5 kDa cut-off concentrator (GE Healthcare, UK). The secretome collected in Neurobasal-A medium was used for the differentiation of human neural progenitor cells (hNPCs) and was not concentrated. Aliquots of secretome were flash frozen with liquid nitrogen and stored at −80 °C.

Metabolite analysis throughout cultures

The consumption of glucose and the production of lactate were monitored throughout the culture period. Glucose and lactate concentrations were analysed using a YSI 7100MBS equipment (YSI Incorporated, USA). The yield of lactate from glucose (YLac/Glc), was calculated for each day as the ration between the specific metabolic rates, qmet ($mol \cdot day^{-1} \cdot cell^{-1}$), which corresponds to the production of lactate during that day and the consumption of glucose during the same period. These parameters were determined as described in the literature (Serra et al., 2010).

Immunophenotypic analysis of BMSCs

At the end of the culture, cells were harvested from the MCs and analysed for the expression of specific surface antigen by flow cytometry. Approximately 1 x 10^5 cells were resuspended in 1x PBS and incubated for 15 minutes, in the dark with the antibodies. After a washing step with 1x PBS, cells were fixed with 1% PFA and stored at 4°C until analysis using a BD FACSCalibur™ platform, equipped with the CellQuest™ software (BD Biosciences). The antibodies used were CD105 PE (phycoerythrin), CD90 FITC (Fluorescein isothiocyanate), CD80 PE, CD73 PE, CD45 FITC, CD34 FITC, CD14 PE and HLA-DR PE. All of them were purchased from BioLegend (USA), except for CD105 PE (Thermo Fisher Scientific, USA). Non stained cells were also prepared for every experiment. A minimum of 10,000 events was collected for each sample. Analysis was performed using FlowJo™ v10 software (BD Biosciences).

Multilineage Differentiation Assays of BMSCs

Upon dynamic culture, multilineage differentiation assays were performed for BMSCs as previously described (de Soure et al., 2017). Briefly, BMSCs were retrieved from the MCs (as previously described) and to assess differentiation toward an adipocytic phenotype, staining was based on the Oil Red-O solution to visualize the existence of lipidic vacuoles. For osteogenic differentiation, cells were prepared for alkaline phosphatase (ALP) and von Kossa staining. ALP enables the visualization of osteogenic progenitors and von Kossa stains calcium deposits. For chondrogenic differentiation, Alcian

Blue was used to stain the proteoglycan aggrecan (an indicator for cartilage formation), which will be dark-blue stained.

Expansion of human neural progenitor cells and incubation with hBMSCs secretome

The procedure for isolation of hNPCs was already described by our group and followed the strict ethical guidelines established and approved by the Conjoint Health Research Ethics Board (CHREB, University of Calgary, AB, ID: E-18786) (Teixeira, Panchalingam, et al., 2015). hNPCs were thawed and placed onto a T-75 flask containing 10 mL of Complete NeuroCult™-NS-A Proliferation Medium (STEMCELL Technologies, Canada). After two days, cells were harvested and mechanically dissociated into a single cell suspension and plated at a density of 1 x 10^4 viable cells·cm^{-2} in new culture flasks. The feeding regime was performed every 2 days, by adding 10% of fresh complete medium. After 10-12 days of culture, coverslips (Marienfeld, Germany) were placed inside a 24-well plate and were pre-coated with poly-D-lysine hydrobromide (100 μg/mL) (Sigma-Aldrich, USA) during 2h at room temperature (RT) and laminin (10 μg/mL) (Sigma-Aldrich) for 3h at 37°C. hNPCs were mechanically dissociated and plated at a density of 5 × 10^4 cells·cm^{-2}. Cells were incubated at 37 °C, 5% CO_2, 95% air and 90% relative humidity, for 5 days with secretome collected in Neurobasal-A medium from each culture system (SP2 and VWBR2) supplemented with 1% GlutaMAX (Gibco). Neurobasal-A medium with 1% Antibiotic-Antimycotic and 1% GlutaMAX was used as a control.

Immunocytochemistry analysis of human neural progenitor cells

Following incubation with secretome, the induction of differentiation was evaluated. Therefore, cells were fixed with 4% PFA (PanReac, Barcelona), washed with 1x PBS and blocked as already described (Teixeira, Carvalho, et al., 2015). Following blocking, cells were incubated for 1h at RT with the primary antibodies: rabbit anti-doublecortin (DCX; 1:300, Abcam, USA) to detect immature neurons and mouse microtubule associated protein-2 (MAP-2; 1:500, Sigma) to detect mature neurons. After a washing step, cells were incubated for 1h at RT with secondary antibodies (1:1000): Alexa Fluor 488 goat anti-rabbit (Thermo Fisher Scientific) and Alexa Fluor 594 goat anti-mouse (Thermo Fisher Scientific). Following this procedure, DAPI (Life Technologies) was added for 5 min. Coverslips were observed under an Olympus BX-61 Fluorescence Microscope (Olympus, Japan). Briefly, four coverslips per condition and ten representative fields per coverslip were analysed and the experiment was independently repeated four times. Results are presented as percentage of cells positive for MAP-2 or DCX markers divided by the total number of cells/field (DAPI-positive cells).

Nematode strains and culture conditions

All strains were kept in nematode growth media (NGM) containing agar plates seeded with *Escherichia coli* OP50 at 20°C, as previously described (Brenner, 1974). Strain BZ555 *egIs1* (P*dat-1*::green fluorescent protein [GFP]) was acquired from the Caenorhabditis Genetics Center. Strain UA44 (*baIn1*; P*dat-1*::α-syn high, P*dat-1*::GFP) was gently provided by Guy Caldwell (University of Alabama).

Secretome collected in AlphaMEM medium was used for *C. elegans* assays. Briefly, three types of plates were prepared: two were seeded with 2x concentrated secretome in AlphaMEM from VWBR or SP diluted in inactivated OP50 (secretome final concentration = 1x) and the other was seeded with 2x concentrated AlphaMEM diluted in inactivated OP50 (secretome final concentration = 1x) as control. To prepare inactivated OP50, bacteria were grown overnight at 37°C and 150 rpm in Luria Broth medium, pelleted by centrifugation, inactivated by three cycles of freeze/thawing, frozen at -80°C and then resuspended in S-medium supplemented with 25 U/mL PenStrep (Thermo Fisher Scientific) and 50 U/mL Nystatin (Sigma-Aldrich).

Quantitative analysis of dopaminergic neuronal loss

Age-synchronized BZ555 and UA44 worms were obtained by egg-laying, by plating adult worms in freshly prepared plates followed by their removal from plates after 2h (day 0). Worms born on the treated plates were maintained until day 10 and prepared for scoring of dopaminergic neurons according to the procedures described by Marques et al. 2021 (Marques et al., 2021) (Chapter 2). Intact dopaminergic neurons were scored, and the experiment was independently repeated for three times (n = 12 animals/condition).

Membrane antibody arrays

Some soluble factors present in all produced secretome were identified and quantitatively compared using Human Cytokine Antibody Array 5 (AAH-CYT-5, RayBiotech, USA) and Human Neuro Discovery Array C1 (AAH-NEU-1, RayBiotech). Each antibody array matrix can simultaneously detect 80 cytokines and 20 neurologically relevant molecules, respectively. Briefly, antibody arrays were incubated with the four secretome overnight at 4°C. Membranes were then processed according to the manufacturer's instructions. Relative expression levels were evaluated by comparing signal intensities, which were obtained with the Sapphire Biomolecular Imager (Azure Biosystems, USA) and quantified by

densitometry. A positive control was used to normalize the results from the different membranes being compared, which resulted in a normalized intensity value corresponding to each factor.

Statistics

Statistical analysis was performed using the IBM SPSS statistics 25 software. The normal distribution of continuous variables was analysed according to Shapiro-Wilk or Kolmogorov-Smirnov normality tests. Homogeneity of variances was assessed with Levene's test. When both assumptions were not met, a robust ANOVA with Welch correction and bootstrap with BCA were performed. Bootstrap sampling was followed by one-way ANOVA with Sidak post-hoc test with bias correction (Fig. 4 B, C and Fig. 5 A). The Pearson's chi-squared test was used for proportion analysis, with the follow-up z test for independent proportions with the Bonferroni correction (Fig. 5 B, C). Appropriate effect size measures were used for each test (ω^2_p for ANOVA with Welch correction and V for Pearson's chi-square test). Detailed statistics are available in Supplementary Table 1.

4. Results

BMSCs were successfully expanded in both dynamic systems

In this study, we employed each one of the culture systems making use of previously established and optimized culture conditions aimed at optimizing cell expansion (Carmelo et al., 2015; de Almeida Fuzeta et al., 2020; dos Santos et al., 2014; Fernandes-Platzgummer et al., 2016; Pinto et al., 2019). The cells were successfully expanded in both systems (Fig. 1A, B). The highest cell number was obtained at day 9 in the VWBR (VWBR1) but there was more heterogeneity in cultures using this system, because cell number ranged between $9.57 \pm 1.11 \times 10^6$ and $19 \pm 1.42 \times 10^6$. MCs colonization increased throughout time with MSCs expansion, and the higher occupancy of MCs translated into a boost in MCs aggregation (Fig. 1 C). In VWBR1 culture, the cell number determined after cell conditioning was higher than before medium change. Given that at this stage MCs aggregation makes it difficult to estimate the cell numbers, this discrepancy might be explained by a poor homogenization of the cel/microcarrier suspension before sampling or by a non-representative sampling.

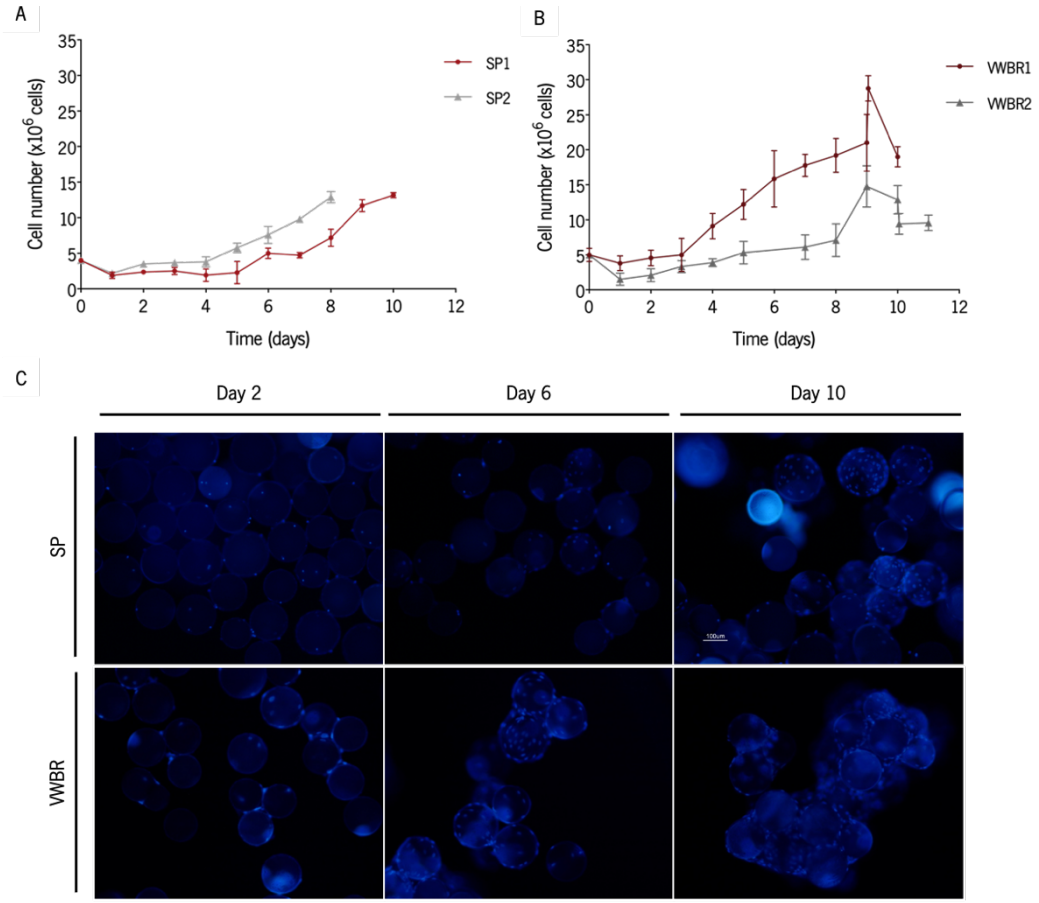

Figure 1. Ex vivo expansion of BMSCs in the two dynamic culture systems. Representation of cell growth for cultures in the (A) SP and in the (B) VWBR. SP1 and VWBR1 represent the secretomes collected in AlphaMEM medium and SP2 and VWBR2 represent the secretomes collected in Neurobasal-A medium. Results are presented as mean ± SD of cell count for each time point. (C) Representative images of BMSCs attached to MCs at 2, 6 and 10 days of culture in SP and VWBR. Cell nuclei were stained with DAPI and images were acquired using a fluorescence microscope. Scale bar = 100 μm. SP, Spinner flask system; VWBR, Vertical-Wheel™ bioreactor; SD, standard deviation.

The percentage of cells that adhered to the MCs within the first 24 h was also determined. The higher and lower values for adhesion efficiency were obtained with VWBR but the average of both systems is similar (Table 1). The highest values for cell growth rate and the lowest doubling times were attained in both cultures with SP; the VWBR2 culture had the lowest growth rate, probably as a result of the low initial adhesion efficiency (31% versus 76% for VWBR1, 48% for SP1, and 55% for SP2).

Table 1. Characteristics of BMSCs expansion in both dynamic systems.

Dynamic system	Culture medium	Medium for conditioning	Agitation rate (rpm)	Adhesion efficiency (%)	Growth rate (day^{-1})	Doubling time (day)
VWBR1	DMEM 5% UltraGRO™-PURE	AlphaMEM	30	76	0.309	2.24
VWBR2	DMEM 5% UltraGRO™-PURE	Neurobasal-A	30	31	0.239	2.90
SP1	StemPro MSC SFM	AlphaMEM	40	48	0.362	1.92
SP2	StemPro MSC SFM	Neurobasal-A	40	55	0.311	2.23

The determination of nutrient consumption and production of metabolites is a relevant procedure to ascertain the availability of nutrients or accumulation of waste products. Therefore, the concentration of glucose and lactate was daily monitored (Fig. 2 A-D). Glucose and lactate profiles were similar between both cultures in each system. The lowest values for glucose were detected in VWBR cultures (Fig. 2A, C). The levels of glucose in all cultures were kept within non-limiting (over 1 mM) levels for cell proliferation (Fig. 2 A, C). (Schop et al., 2009). For all cultures, the highest values for lactate concentration were registered at the conditioning day and were always bellow 35 mM, described in the literature as a minimum inhibitory lactate concentration (Fig. 2B, D) (Schop et al., 2009). The evaluation of the consumption of glucose and production of lactate enables the determination of the yield of lactate from glucose (YLac/Glc). For all cultures the value of (YLac/Glc) was approximately 2 mol lactate·mol^{-1} glucose, which is a typical value when cells rely on glycolysis for energy metabolism (Rodrigues et al., 2018).

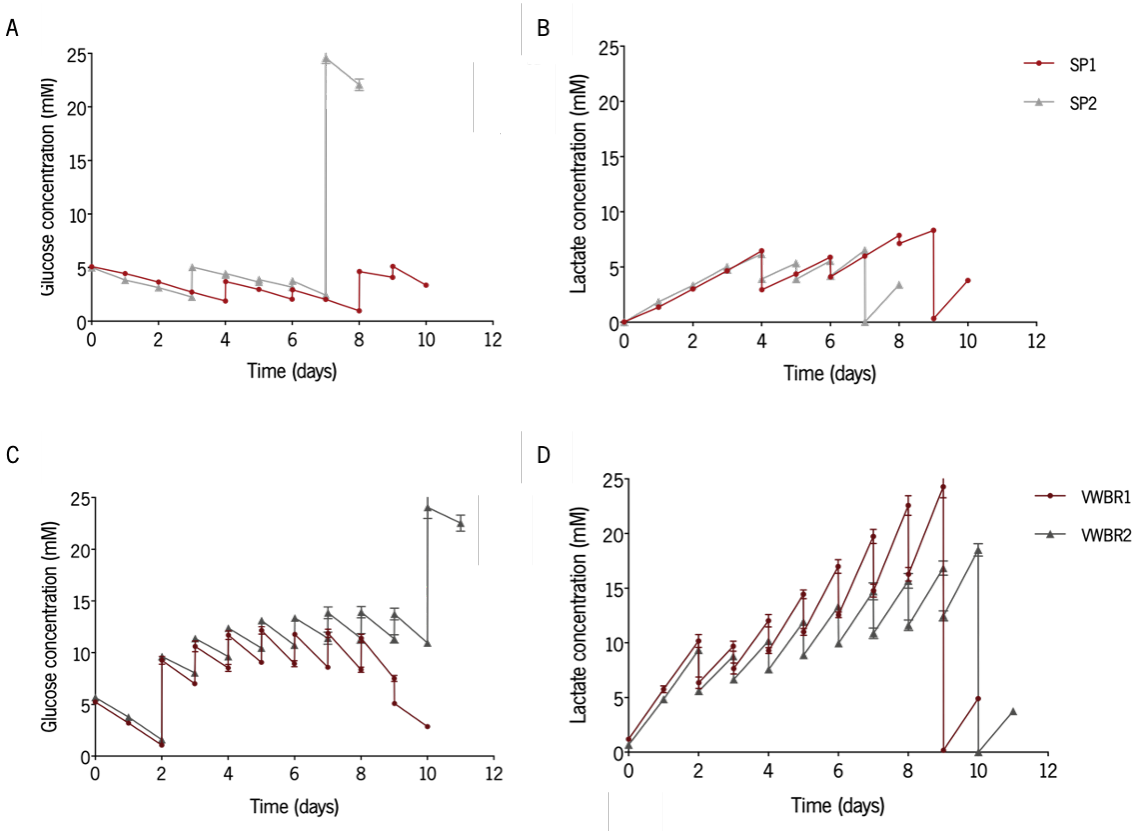

Figure 2. Metabolic analyses of the expansion of BMSCs in the SP and VWBR. Concentration profiles of glucose and lactate during the culture in (A, B) SP and in (C, D) VWBR. SP1 and VWBR1 represent the secretomes collected in AlphaMEM medium and SP2 and VWBR2 represent the secretomes collected in Neurobasal-A medium. Results are presented as mean ± SD (n = 2).

Following BMSCs expansion, immunophenotypic assays were performed in order to guarantee that their immunophenotype was not affected by culture in each of the dynamic systems (Fig. 3A) (Dominici et al., 2006). Due to technical concerns, we were not able to analyse the immunophenotype of one of the cultures in VWBR (VWBR1). Following culture in SP, CD73, CD90 and CD105 biomarkers were expressed in more than 95% of the cells (Fig. 3 A). Regarding MSCs "negative" markers, we observed that a considerable percentage of cells expressed CD14 and CD80, especially cells from the first culture in SP. Overall, cells from VWBR1 kept the characteristic MSC immunophenotype after culture in VWBR system (Fig. 3 B). Moreover, cells cultured in both systems preserved the multilineage differentiation ability toward adipogenic (Figure 3 C), osteogenic (Figure 3 D), and chondrogenic (Figure 3 E) lineages.

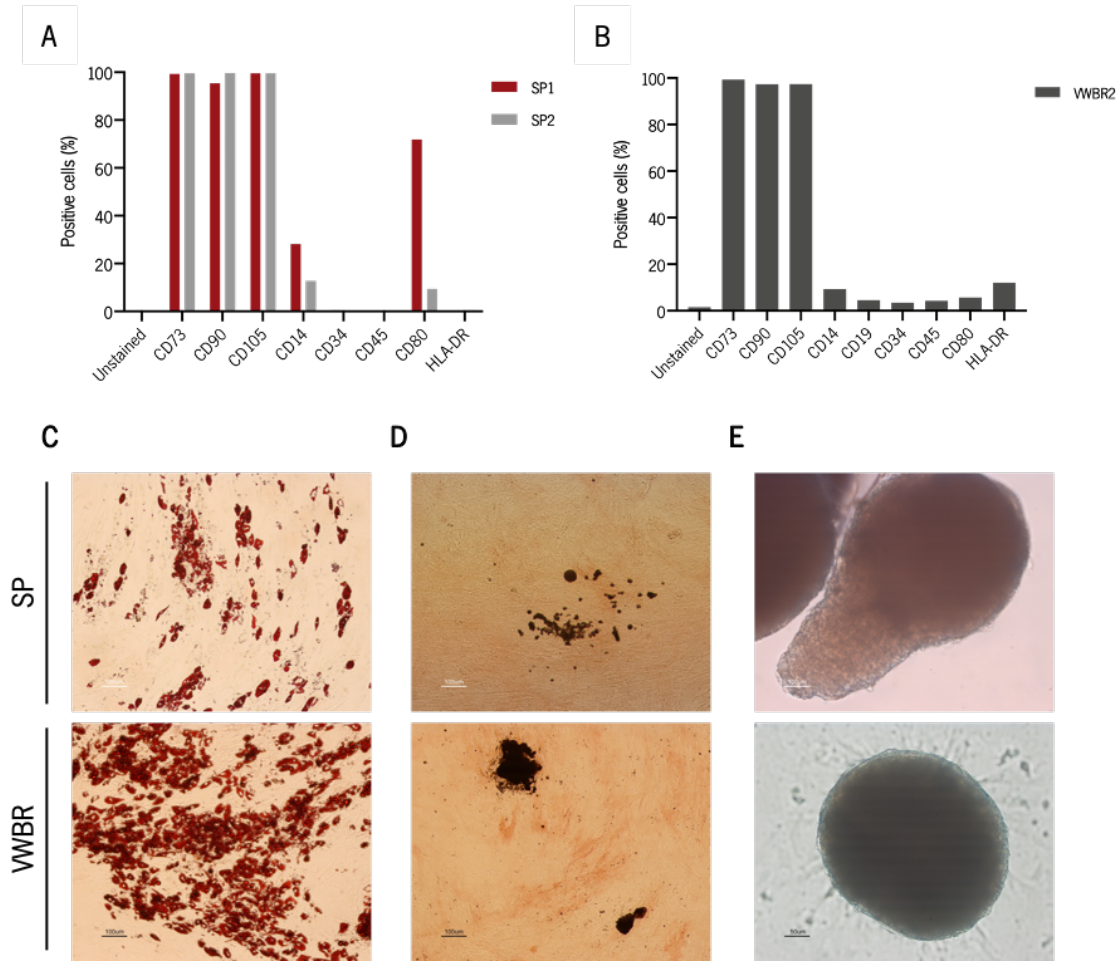

Figure 3. Immunophenotypic analysis and multilineage differentiation potential of BMSCs after expansion under dynamic conditions. The percentage of expression of each surface antigen (CD14, CD19, CD34, CD45, CD73, CD80, CD90, CD105, and HLA-DR), analysed by flow cytometry is represented for one of the cultures in the (A) SP and (B) VWBR. Representative images of multipotency characterization of BMSCs cultured in SP and VWBR through multilineage differentiation assays, upon 22 days under (C) adipogenic, (D) osteogenic, and (E) chondrogenic differentiating conditions. Scale bar = 50 µm (for E) and 100 µm (for C and D). SP, Spinner flask system; VWBR, Vertical-Wheel™ bioreactor

Secretomes produced in both dynamic culture systems were able to induce neurodifferentiation

The capacity of SP2 and VWBR2 secretomes to induce neurodifferentiation was explored by incubating hNPCs with both secretomes, followed by the analysis of the expression of DCX and MAP-2. After the incubation period, both secretomes induced cells toward a differentiated state, which was confirmed by the expression of both markers (Fig. 4 A, B). We observed that SP2 secretome had a

superior effect regarding the expression of DCX, an early neuronal marker, comparing with VWBR2 secretome and the positive control (Seki et al., 2019). The same was not seen for MAP-2 expression, because both secretomes induced the same level of MAP-2 expression, a characteristic marker of mature neurons (Soltani et al., 2005).

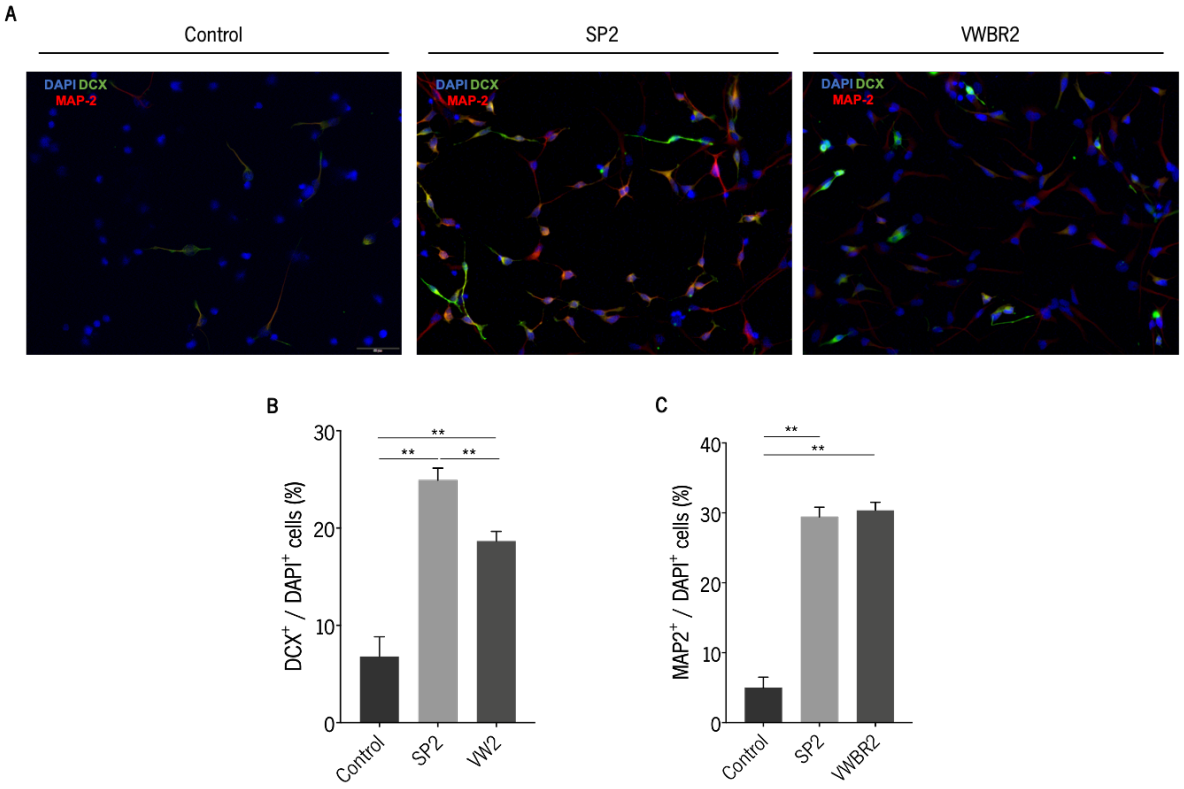

Figure 4. Secretomes collected in SP and VWBR were able to significantly increase the survival and differentiation of hNPCs. *In vitro* differentiation of hNPCs was assessed by counting immature (DCX^+ cells) and mature ($MAP\text{-}2^+$ cells) neurons and dividing by the total number of cells ($DAPI^+$). (A) Representative photographs for each condition showing cells positive for DCX and/or MAP-2. Scale bar = 50 µm. Graphical representation of the percentage of cells expressing (A) DCX and (B) MAP-2. SP2 and VWBR2 represent the secretomes collected in Neurobasal-A medium. A total of four coverslips per condition and ten representative fields per coverslip were analyzed and the experiment was independently repeated four times. **P < 0.01 (ANOVA, SIDAK test corrected for BCA). DAPI, 4-6-diamidino-2-phenylindole-dihydrochloride; DCX, doublecortin; MAP-2, microtubule-associated protein-2.

Distinct capacity to induce neuroprotection was shown with secretomes from different systems

The secretomes produced in both systems (SP1 and VWBR1) were applied to a *C. elegans* model that overexpresses WT α-syn in the eight dopaminergic neurons. This model of PD is characterized by

an age-dependent loss of dopaminergic neurons that can be quantified microscopically due to the expression of GFP in the same cells. Eggs from adult *C. elegans* were plated in petri dishes containing secretome diluted in their food source, and after 10 days DA neurons were scored. We observed that only animals treated with secretome produced in the SP were less susceptible to the effects of α-syn, because they presented a higher number of intact DA neurons (Fig. 5 A). When we compared the percentage of animals with WT DA neurons the same tendency was observed, though there are not statistically significant differences between groups (Fig. 5B). Lastly, the differences between groups regarding the three populations of DA neurons [cephalic (CEP), anterior deirid (ADE) and posterior deirid (PDE)] were analysed. Animals treated with SP1 showed a higher number of ADE neurons compared with animals treated with the other secretome (Fig. 5C). Both groups clearly displayed a higher number of ADE neurons compared with untreated animals, but this difference was not statistically significative.

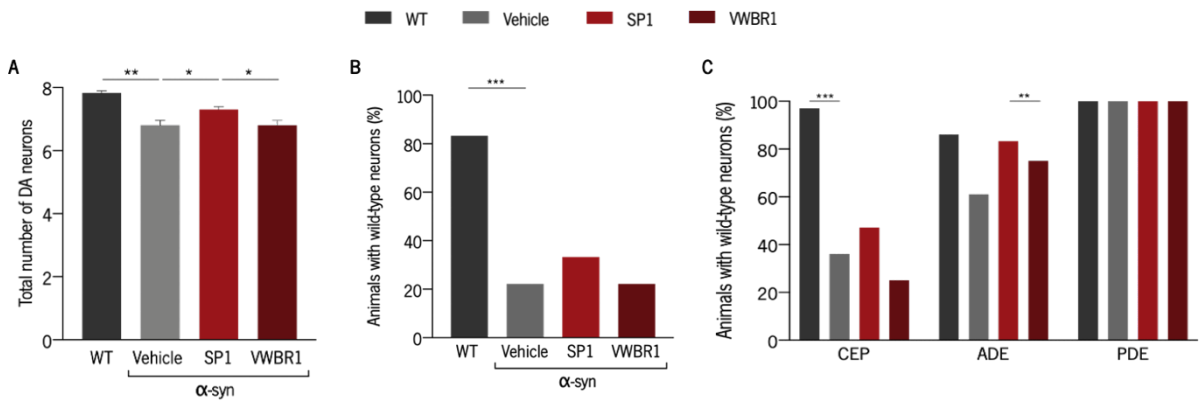

Figure 5. BMSCs secretome from SP protects dopaminergic neurons from α-syn-associated effects. (A) Graphical representation of the total number of intact dopaminergic neurons for each condition (ANOVA, SIDAK test corrected for BCA). (B) The proportion of animals with intact DA neurons for each condition was determined by counting the number of animals with WT neurons. (C) The proportion of animals with intact CEP, ADE or PDE neurons was determined by counting the animals with WT DA neurons belonging to each subpopulation (Pearson's chi-square test). SP1 and VWBR1 represent the secretomes collected in AlphaMEM medium. A total of 36 animals were assayed per group across three independent experiments. *P < 0.05, **P < 0.01 ***P < 0.001. ADE, anterior deirid; CEP, cephalic; DA, dopamine; PDE, posterior deirid; WT, wild-type.

Cells cultured in both systems had distinct secretory profiles

The expression of specific factors was compared among the different secretomes, to depict possible players that can be contributing to the observed effects. Antibody arrays were incubated with secretome and the analysis of the signal intensity enabled the determination of the relative intensity of each factor (Fig 6 A-F). Most of the molecules assessed presented higher relative intensities in SP1 secretome. Two molecules were clearly highly expressed in all secretomes, namely the chemokines Interleukin 8 (IL-8) and Monocyte Chemoattractant Protein-1 (MCP-1) (Fig. 6A). The relative intensities of IL-6, MMP3 and TNF-β were higher in both secretomes produced in SP, whereas Osteopontin relative intensity revealed a higher value in VWBR2 secretome and NGFβ, GCSF, HB-EGF, IL-13 in VWBR1.

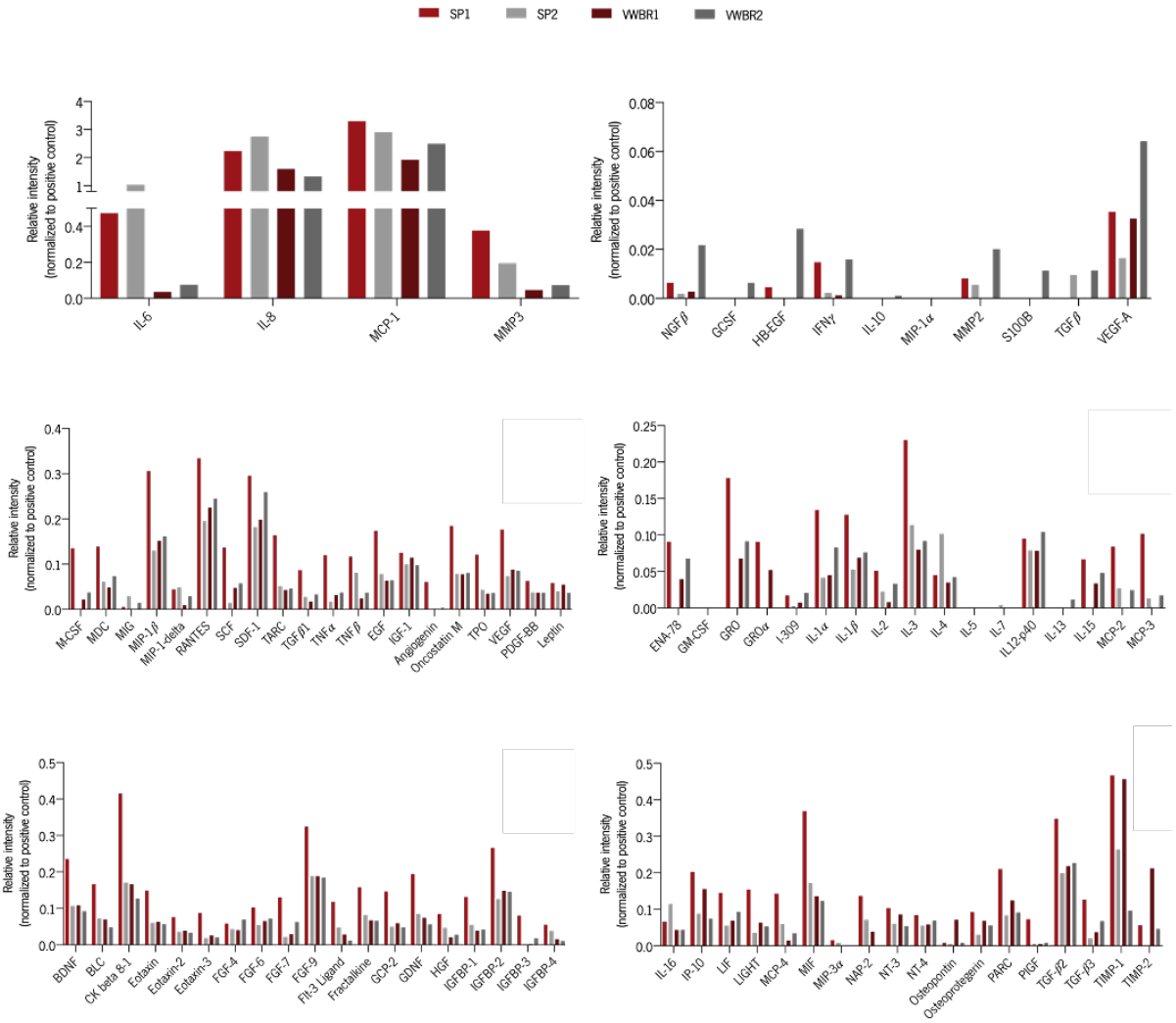

Figure 6. Antibody array identification of neurologically relevant proteins and cytokines in the secretomes from both dynamic systems. Antibody arrays were incubated with secretomes produced in SP and VWBR and collected in AlphaMEM (SP1 and VWBR1) and Neurobasal-A (SP2 and VWBR2) medium. Signal intensity of arrays were analyzed by densitometry, and the relative intensities of

individual proteins were calculated after normalizing to the positive controls on each array. The experiment was performed once for each secretome.

5. Discussion

The development of a protocol for the large-scale, clinical-grade, production of secretome is an important step towards bringing secretome-based therapies to the clinics. Bioreactors are a valuable tool for this process, due to their capacity to produce large volumes of secretome. Moreover, they can be implemented not only for secretome production, but also as a preconditioning strategy of functional enhancement of MSCs and their secretome.

In this work, two different dynamic systems were used to culture human bone marrow-derived MSCs and collect their secretome. Cells were successfully expanded in both systems, and despite the attained differences in maximum cell number, there were no relevant differences regarding cell metabolism. Moreover, cells retrieved from MCs in the end of culture in both systems retained the capacity to multilineage differentiation and the characteristic immunophenotype, except for cells from the first culture in SP (SP1). In fact, these cells showed the expression of CD80, which is expressed on activated B-cells, macrophages and dendritic cells (Mir, 2015). We can exclude the hypothesis that cells already expressed this marker before culturing, because cells cultured in static conditions did not display this marker (data not shown). Given that previous studies from the group using the same culture conditions and the same cell donor did not see this alteration, we believe that it should be the result of experimental error (Carmelo et al., 2015; dos Santos et al., 2014; Fernandes-Platzgummer et al., 2016).

The *in vitro* application of SP2 and VWBR2 secretomes led to the differentiation of human CNS-derived cells, which was shown by the expression of markers for both immature (DCX-positive cells) and mature (MAP-2-positive cells) neurons. The induction of neurodifferentiation can be supported by the identification in all secretomes of trophic factors and other molecules involved in neurogenesis, neuronal survival and maintenance, such as stromal cell-derived factor-1 (SDF-1), BDNF, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF)-BB, glial cell line-derived neurotrophic factor (GDNF), and hepatocyte growth factor (HGF), among others (Allen et al., 2013; Guyon, 2014; Liu et al., 2014; Sil et al., 2018). Some of them were approached in Chapter II. Nonetheless, in the conditions of our study, SP2 secretome had a superior performance as it was able to significantly increase the survival and differentiation of hNPCs into immature neurons, compared with VWBR2 secretome and control medium (Neurobasal-A medium). According to our results, the relative intensity of interleukin-4 (IL-4) cytokine was greater in SP2 secretome, compared to VWBR2 secretome. This is particularly interesting because IL-4 was shown to significantly induce neural stem/progenitor cells proliferation and neurogenesis when injected in healthy zebrafish (Bhattarai et al., 2016).

The effects of the prolonged exposure to SP1 and VWBR1 secretomes in the survival of dopaminergic neurons overexpressing α-syn in a *C. elegans* model revealed that the secretome collected in SP had a superior neuroprotective potential. We have previously shown an apparently superior effect of secretome from static conditions in the same model (discussed in Chapter II). However, throughout the present work, we observed that control animals presented a reduced neuronal loss. This is the reason for increasing the period for incubation with secretome from 7 to 10 days. We hypothesise that, under these conditions, the secretome might induce a greater neuroprotective effect, in a situation characterized by an increased DA neuron death. Based on our array analysis, MMP2 was not detected in VWBR1 and seems to be more present in VWBR2 secretome, compared to SP1 and 2. As already mentioned, MMP2 was identified in the secretomes and considered a molecule involved in the cleavage of preformed fibrils of α-syn (Oh et al., 2017). So, we hypothesize that the reduced expression of MMP2 in SP1 and absence in VWBR1 might be a possible contribution to our results.

The MSCs used in this work were isolated from the same tissue and the same donor and cultured in different dynamic systems. We employed for each culture system, previously established and optimized culture conditions aimed at optimizing cell expansion (Carmelo et al., 2015; de Almeida Fuzeta et al., 2020; dos Santos et al., 2014; Fernandes-Platzgummer et al., 2016; Pinto et al., 2019). Therefore, the difference of culture conditions in both systems is significant, namely the culture media used in the cell expansion stage. Still, the culture medium used for conditioning was the same. Consequently, in this study, different elements should be considered as possible contributors to the different potential of the secretomes. As previously discussed in the last part of Chapter I, cells cultured in a mechanically agitated environment are subjected to hydrodynamic shear stress and the SP is reported to induce higher levels of shear stress, whereas the design of VWBR enables the reduction of agitation needed to keep the homogenization of the system, contributing to a less shear stress (Jossen et al., 2014). Even though shear stress is known to negatively impact MSCs growth, we still do not know how the hydrodynamic shear modulates the secretory pattern of MSCs (Ismadi et al., 2014). In line with this, Diaz and co-workers showed that when exposed to fluid shear stress caused by vascular flow, BMSCs immunomodulatory function is activated, suggesting that mechanical preconditioning of MSCs could be an effective strategy to modulate the secretome (Diaz, Evans, et al., 2017; Diaz, Vaidya, et al., 2017). Curiously, our analysis showed that IL-6, oncostatin M, transforming growth factor (TGF)-β1 and IL-4, cytokines involved in the regulation of anti-inflammatory responses, presented higher relative intensities in both secretomes produced in SP or at least in one of them (Jones & Jenkins, 2018; Zhang & An, 2007).

The use of culture medium and other solutions containing animal-derived products, such as fetal bovine serum, for cell expansion purposes involves risks if the cells or their products are going to be applied in the clinical setting. Indeed, these products do not have a defined number of components and present the risk of transmission of unknown infectious agents. Thus, large-scale expansion of MSCs in defined medium that is FBS-free is critical for translational research (Swamynathan et al., 2014). In this work, the culture medium used in VWBR cultures was a xeno-free medium supplemented with human platelet lysate and the medium used in SP cultures is a commercially available xeno- and serum-free formulation. A study that compared MSCs grown in xeno- and serum-free conditions with FBS containing medium concluded that MSCs grown in xeno- and serum-free conditions had a higher immunosuppression activity (Swamynathan et al., 2014). More recently, Yoshida and colleagues showed that serum-free culture conditions can improve the immunosuppressive capacity of MSCs, which is aligned with our results (Yoshida et al., 2018). However, this is a debatable question because other studies suggested the opposite (Abdelrazik et al., 2011; Oikonomopoulos et al., 2015).

Another contrast between cultures in SP and VWBR was the concentration of glucose throughout the expansion period. The average concentration of glucose for SP cultures before conditioning was 3.4 ± 1.1 mM whereas for VWBR cultures was almost three times higher (9.5 ± 3.4 mM). There is evidence showing that the expansion of MSCs in low (5.5 mM) and high (20 mM or 30 mM) glucose concentrations does not alter the secretion of VEGF, HGF, and basic fibroblast growth factor (bFGF) (Weil et al., 2009). However, cells were subjected to those glucose concentrations for only 24h and 48h. So, the hypothesis that a longer exposure to different concentrations of glucose might impact the secretion of specific factors cannot be put aside.

6. Conclusions

The results from the present work suggest that the culture conditions might have contributed to the distinct secretory profiles and, consequently, to their action regarding the stimulation of neurodifferentiation and neuroprotection. However, our results need further confirmation through the replication of the experiment and comparison of a pull of different secretome batches produced in both culture conditions. Moreover, more studies should focus on the effects of each dynamic system in the secretome profile using uniformized protocols.

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10. Supplementary material

Supplementary Table 1. Statistical report. Effect sizes calculated using Lenhard, W. (2016), Uanhoro, J. O. (2017), and https://www.psychometrica.de/effect_size.html. Effect size calculator available online at https://effect-size-calculator.herokuapp.com/.

Figure	Statistical report	Sample size
4 B	$F(2,155.566) = 29.911$, $p < 0.001$, $\omega^2 p = 0.267$	160 (per group)
4 C	$F(2,183.822) = 105.599$, $p < 0.001$, $\omega^2 p = 0.528$	160 (per group)
5 A	$F(3,73.496) = 24.690$, $p < 0.001$, $\omega^2 p = 0.478$	36 (per group)
5 B	$\chi^2 (3) = 38.223$, $p < 0.001$, $V = 0.297$	36 (per group)
5 C	CEP $\chi^2 (3) = 43.923$, $p < 0.001$, $V = 0.319$ ADE $\chi^2 (3) = 17.542$, $p < 0.001$, $V = 0.201$	36 (per group)

CHAPTER IV

Impact of the secretome of dynamically cultured mesenchymal stromal cells in an alpha-synuclein rat model of Parkinson's Disease

Manuscript in preparation

Impact of the secretome of dynamically cultured mesenchymal stromal cells in an alpha-synuclein rat model of Parkinson's Disease

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1. Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder. The etiology of the disease remains largely unknown, but evidence have suggested that the overexpression and aggregation of alpha-synuclein (α-syn) play key roles in the pathogenesis and progression of PD. Mesenchymal stromal cells (MSCs) have been earning attention in this field, mainly due to their paracrine capacity. The bioactive molecules secreted by MSCs i.e., their secretome, have been associated with enhanced neuronal survival as well as a strong modulatory capacity of the microenvironments where the disease develops.

The selection of the appropriate animal model is crucial in efficacy assessment studies. Given the involvement of α-syn in the pathogenesis of PD, the evidence generated from the use of animal models that develop a pathologic phenotype due to the action of this protein is extremely valuable. Therefore, in this work, we established an animal model based on the viral vector-mediated overexpression of A53T α-syn. Ten weeks after lesion induction, the animals received secretome injections in the substantia nigra pars compacta (SNpc) and striatum (STR). The secretome used was produced in a spinner flask (SP) system. Nine weeks later, animals that received the viral vector containing the gene for A53T α-syn

and treated with vehicle (Neurobasal-A medium) presented dopaminergic cell loss in the substantia nigra pars compacta (SNpc) and denervation in the STR. The treatment with secretome, significantly reduced the levels of α-syn in the SNpc and protected the DA neurons within the SNpc and STR.

Our results are aligned with previous studies in both α-syn *C. elegans* models, as well as 6-OHDA rodent model, revealing that secretome exerted a neuroprotective effect. This further supports the development of new studies exploring the effects and the mechanism of action of secretome from MSCs against the α-syn-induced neurotoxicity.

2.Introduction

PD is characterized by the progressive degeneration of dopaminergic neurons in several dopaminergic networks, particularly in the substantia nigra pars compacta (SNpc) and by the presence of Lewy bodies and Lewy neurites, mainly composed by the protein alpha-synuclein (α-syn) (Poewe et al., 2017). α-syn can acquire different conformations, and even though the doubt remains regarding the most toxic species, it is widely recognized that the assembly of toxic species of α-syn actively potentiates mitochondrial dysfunction, endoplasmic reticulum stress, impairs protein clearance and degradation, disrupts microtubule formation and neurotransmitter release, and enhances neuroinflammation (Bengoa-Vergniory et al., 2017; Rocha et al., 2018).

The development of animal models of PD based on the overexpression of α-syn was suggested at the end of the last century as an innovative and appealing strategy for animal model development and a better approach to model the human disease (Gómez-Benito et al., 2020; Koprich et al., 2017). Models based on the viral vector-mediated overexpression of α-syn can overcome some of the limitations of transgenic models, such as lack of consistent nigrostriatal dopaminergic loss, by targeting specifically the nigrostriatal system, thereby being a potentially more effective approach (Campos et al., 2013; Koprich et al., 2011). Moreover, these models recapitulate many of the neuropathological hallmarks of PD, such as degeneration of nigral dopaminergic neurons, intracellular α-syn inclusions, parkinsonian behavioral deficits, and also neuroinflammation (Gombash et al., 2013; Koprich et al., 2017).

Mesenchymal stromal cells (MSCs) are a promising tool for therapeutic purposes, particularly for regenerative therapies (Luzzani & Miriuka, 2017). Besides their multilineage differentiation capacity, it is well accepted that their beneficial actions are mostly mediated by their secretome. In line with this, different *in vitro* (Assunção-Silva et al., 2018; Egashira et al., 2012; Nakano et al., 2010) and *in vivo* (Chen et al., 2020; Chudickova et al., 2019; Elia et al., 2019; Lin et al., 2011) studies revealed that MSCs-derived secretome displays relevant neuroprotective actions in different pathophysiological contexts, inhibits apoptosis and glial scar formation, promotes neurogenesis, glial cell survival, and has an immunomodulatory and angiogenic role (reviewed in (Baez-Jurado et al., 2019)). Moreover, it may potentiate the integration of local progenitor cells in processes of neuronal regeneration (Lin et al., 2011). As a consequence, MSCs secretome may be the basis of a future cell-free neuro-regenerative strategy for Parkinson's Disease (PD). Indeed, our team showed that the secretome from MSCs triggered neuronal differentiation *in vitro* and *in vivo* and stimulated the increase of DA neurons, reverting the phenotype of the disease in 6-Hydroxydopamine (6-OHDA) lesioned animals (Mendes-

Pinheiro et al., 2019; Teixeira et al., 2015, 2016, 2017, 2020). Moreover, using a genetic model of the disease characterized by the overexpression of α-syn, we concluded that secretome from MSCs was able to reduce α-syn aggregation and protected DA neurons from the effects of this protein (Marques et al., 2021) (Chapter II).

In this study, we aimed to establish a viral vector-based rat model overexpressing α-syn and evaluate the effect of the secretome collected in a dynamic system against α-syn-induced neurotoxicity. We have previously investigated the neurodifferentiation and neuroprotective potential of secretomes from MSCs produced in different culture systems, namely the spinner flask (SP) and the Vertical-Wheel™ bioreactor (VWBR) (Chapter III). Based on our results, we selected the secretome produced in SP to further explore its capacities in an animal model of increased complexity.

3. Materials and methods

Cultures of human bone marrow-derived mesenchymal stromal cells (BMSCs)

BMSCs were isolated from bone marrow aspirates obtained from healthy donors after written informed consent at Instituto Português de Oncologia Francisco Gentil, Lisboa, Portugal, according to the Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells (Portuguese Law 22/2007, June 29), with the approval of the Ethics Committee of the respective clinical institution, according to the Portuguese Regulation (Law 21/2014, April 16). MSCs were retrieved according to the established protocols as described by dos Santos *et al.* (2010) (dos Santos et al., 2010). Cells with 4 and 5 passages and from the same donor were used.

Microcarrier preparation

SoloHill plastic microcarriers (MCs, Pall, USA) of 360 $cm^2 \cdot g^{-1}$ superficial area, were used for BMSCs culture. After sterilization by autoclave (120°C, 20 min), MCs were coated with a CELLstart™ substrate (diluted 1:100 in 1x PBS) for 1 h at 37°C, with an intermittent agitation (cycles of 2 min at 750 rpm, 8 min without agitation) using a Thermomixer® comfort, and afterwards equilibrated in pre-warmed StemPro® MSC SFM XenoFree medium (Carmelo et al., 2015).

BMSCs culture on spinner flask (SP)

Bellco® spinner flasks (Bellco Glass, USA) offer a working volume of 100 mL but were operated at 80 mL. Prior to use, spinner flasks were autoclaved and treated with Sigmacote® (Sigma-Aldrich) to prevent microcarrier adhesion to the surface of the flask. MCs prepared following the aforementioned protocol were added at a concentration of 20 $g \cdot L^{-1}$. MSCs previously expanded under static conditions in StemPro® MSC SFM XenoFree medium for 2 passages, were seeded at a cell density of 4×10^6 $cells \cdot mL^{-1}$ in the SP with a total volume of 40 mL and incubated at 37°C and 5% CO_2. Following cell inoculation, agitation was set to 30 rpm. After 24 h, agitation was increased to 40 rpm. The necessary volume of medium to attain 80 mL was added after 96h post inoculation. Feeding was performed from the fourth or fifth day of culture on a daily basis, by replacing 25% of volume with pre-warmed medium or by the addition of a glucose pulse (from a glucose stock solution of 20 $g \cdot L^{-1}$) to maintain the levels of glucose at a non-limiting concentration (superior to 1 mM) (Schop et al., 2009).

BMSCs conditioning and secretome collection

BMSCs were cultured until stationary cell growth phase. At this point, the medium was removed from the culture system and MCs containing cells were washed once with 100 mL of Neurobasal-A (Gibco) medium. Afterwards, 80 mL of Neurobasal-A medium, with 1% Antibiotic-Antimycotic (Gibco) were added to the system. After 24 h, the medium was harvested and centrifuged at 300 g for 10 min to remove cell debris. Aliquots of secretome were flash frozen with liquid nitrogen and stored at −80 °C.

Prior to use, the secretome was concentrated by centrifugation to 100x using a Vivaspin 5 kDa cut-off concentrator (GE Healthcare, UK).

Viral vectors

Two vectors were used in this work, namely an adeno-associated viral vector (AAV) of 1/2 serotype containing the gene for human A53T α-syn and the same empty vector (without any gene for a specific protein) as a control (GeneDetect, New Zealand). The AAV1/2 capsid expresses AAV1 and AAV2 serotype proteins in a 1:1 ratio. α-syn expression was under the control of a chicken β-actin (CBA) promoter hybridized with a cytomegalovirus (CMV) enhancer sequence. In addition, a woodchuck post-transcriptional regulatory element (WPRE) and a bovine growth hormone polyadenylation sequence (bGH-polyA) were incorporated to further enhance transcription following transduction. AAV1/2 used the AAV2 inverted terminal repeats (ITRs) under the following scheme: CMV/CBA promoter––human A53T α-syn ––WPRE-bGH-polyA––ITR.

Stereotaxic surgeries

Viral vector injection

21 male 11 and 12-week-old CD® Sprague Dawley IGS Rats (Crl:CD(SD)), were housed in pairs and maintained in a controlled environment at 22°C, 55% humidity under a 12-hour light-dark cycle with food and water ad libitum. Animal experimentation was consented by the Portuguese national authority for animal research, Direção-Geral de Alimentação e Veterinária (DGAV005453) and Ethical Subcommittee in Life and Health Sciences (SECVS 142/2016) and was conducted in accordance with the local regulations on animal care and experimentation (European Union Directive 2010/63/EU).

Under combined ketamine and medetomidine anesthesia (75mg/kg:0.5mg/kg, i.p.), animals were placed on a stereotaxic frame (Stoelting, USA) with non-traumatic ear bars and bilaterally injected with either empty AAV1/2 vector (AAV1/2-EV, n = 5), AAV1/2 containing the gene for human A53T α-syn

(AAV1/2-A53T-α-syn, n = 12), or 0.9% NaCl solution (sham group, n = 4). 2 µL of viral vector were infused to the SN at a rate of 0.5 µL·min^{-1} to the following coordinates from bregma: AP, -5.2 mm; ML, ±2.0 mm; DV, -7.5 mm and using a 30-gauge needle Hamilton syringe (Hamilton, Switzerland) (Koprich et al., 2011). After injection, the needle was left in place for an additional 5-minute period to allow diffusion. The AAV1/2 stock concentration for each vector was 5.1 x 10^{12} genomic particles (gp)·mL^{-1}. Both vectors were diluted (1:3 dilution, 1.7 x 10^{12} gp·mL^{-1}). Control (sham) animals received an equal volume of 0.9% NaCl solution in the same brain region and following the same procedure.

BMSCs-derived secretome and vehicle injection

Injection of BMSCs-derived secretome or vehicle (Neurobasal-A medium) was conducted 10 weeks after viral vector injection. Two homogeneous groups of animals previously injected with AAV1/2-A53T-α-syn were defined based on the results of the staircase test performed 2 weeks earlier. After anesthesia, animals were placed on a stereotaxic frame, as aforementioned, and bilaterally injected with the vehicle [AAV1/2-EV + vehicle group (n = 5) and AAV1/2-A53T-α-syn + vehicle group (n = 6)] or secretome [AAV1/2-A53T-α-syn + secretome group (n = 6)] in previously mentioned coordinates in the SN and into two different coordinates of the striatum (STR): AP, -1.3 mm; ML, ±4.7 mm; DV, -4.5 mm and -4.0 mm; AP, 0.4 mm; ML, ±3.1 mm; DV, -4.5 mm and -4.0 mm. Animals received 24 µL of 100× concentrated secretome or vehicle (4 µL in the SN and in each coordinate of the STR for both hemispheres) at a rate of 1 µL·min^{-1}. The needle was left in place for 4 min after each injection in order to avoid any backflow up the needle tract. Sham animals were subjected to the same procedures but were not injected with any solution.

Staircase test

The skilled paw reaching test (staircase test) was performed 4, 8, 11, 14 and 17 weeks after viral vector injection to evaluate animals' fine motor control and independent forelimb extension and grasping skills (Figure 1). A double staircase box (80300, Campden Instruments, UK) was used as previously described (Montoya et al., 1991). Briefly, in each step, five small pellets were placed in each step of a double staircase apparatus with 7 steps on each side (total number of pellets = 70). Before the first staircase test, animals were familiarized with the test and pellets, which were available for 10 minutes. During five consecutive days of the test session, the animals were placed inside the box and had 15 minutes to reach, retrieve and eat the food pellets on the steps. In the last 2 days of testing, animals were subjected to a forced-choice (FC) task, with food pellets restricted to one side (e.g., left

(FCL) and right (FCR)). All sessions were held at the same time of the day and animals were restricted to food prior to the test and during the test (animals had access to only three food pellets daily). After each test, animals were removed from the staircase boxes and the remaining pellets were counted.

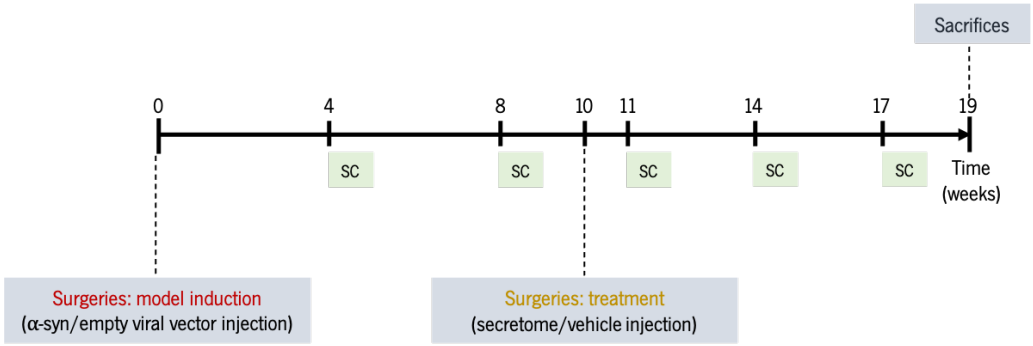

Figure 1. Experimental design. The PD model was induced by a bilateral α-syn viral vector injection into the SNpc. Animals were submitted to a behavioral analysis using the staircase test to characterize the model 4 and 8 weeks later. At week 10, animals received bilateral injection of secretome or vehicle (Neurobasal-A medium) in the SNpc and STR. Behavior analysis was performed 1, 4 and 7 weeks after treatment surgery (weeks 11, 14, and 17 respectively), using the staircase test. Animals were sacrificed 19 weeks after the beginning of the experiment. SC, staircase.

Tissue processing and immunohistochemistry

The extent of dopaminergic neurodegeneration was evaluated through immunohistochemical staining for tyrosine hydroxylase (TH). Briefly, animals were injected with a solution of sodium pentobarbital (Euthasol, 200 mg/kg, i.p.; Ceva Saúde Animal, Portugal). When animals were unresponsive to stimuli, they were perfused through the ascending aorta with 0.9% NaCl solution followed by 4% paraformaldehyde (PFA; PanReac AppliChem, USA) in 1x PBS. Brains were post-fixed in 4% PFA during 48h, followed by 30% sucrose in 1x PBS. Afterwards, brains were embedded in Tissue-Tek® O.C.T.™ Compound (Sakura, The Netherlands) and frozen using a platform embedded in isopentane, placed on liquid nitrogen. 40 µm thick coronal striatal and mesencephalon sections were obtained using a Cryostat Leica CM 1900 (Leica, Germany). For immunohistochemical staining, the activity of endogenous peroxidases in the free-floating sections was stopped using 3% hydrogen peroxidase in 1x PBS for 20min at room temperature (RT), followed by permeabilization in 0.1% Triton X-100 (Sigma-Aldrich, USA) in 1x PBS for 30min and blockage in 10% fetal-calf serum (FCS; Gibco) in 1x PBS for 1h. After these steps, the sections were incubated with anti-TH (mouse monoclonal IgG, 1:1,000; Merck Millipore) overnight at 4°C. In the next day, sections were incubated with a biotinylated

secondary antibody (goat anti-polyvalent, TP-125-BN, Thermo Fisher Scientific) for 30 min at RT, followed by streptavidin-peroxidase solution (TP-125-HR, Thermo Fisher Scientific) for 30 min at RT. Antigen revelation was performed using of 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) (25 mg DAB in 50 mL 0.05 M Tris–HCl with 12.5 µL H_2O_2, pH 7.6). Sections were mounted on slides, and after 24h drying in the dark, mesencephalon sections were counterstained with hematoxylin.

Cell counting and optical densitometry analysis

The determination of the total number of DA neurons in the SNpc was performed by scoring the total number of TH-positive cells in both hemispheres, using an upright microscope BX51 (Olympus, USA), equipped with a digital camera (PixeLINK PL-A622; CANIMPEX Enterprises, Canada), and with the help of Visiopharm Integrator System software (V2.12.3.0; Visiopharm, Denmark), as already described in detail (Teixeira et al., 2017). Four to six sections covering the entire extent of the SN were used in this analysis. Data are presented as the mean of remaining TH-positive cells (as a percentage) normalized to sham (for AAV1/2-EV group) and normalized to AAV1/2-EV group (for both groups injected with α-syn).

Quantification of TH-positive fibers in the STR was performed by densitometry analysis using the ImageJ software (Version 1.52q, NIH, USA), of all the STR area of four to six sections covering the entire extent of the STR. Images were captured under uniform light conditions using a SZX16 stereo microscope (Olympus). The determined values were corrected for non-specific background through the subtraction of values obtained from the cortex. Represented values are the mean (as a percentage) normalized to sham (for AAV1/2-EV group) and normalized to AAV1/2-EV group (for both groups injected with α-syn).

Immunofluorescence

α-syn and TH expression were analyzed by double staining for human α-syn in combination with TH. Briefly, four sections covering the entire extent of the STR and SN were permeabilized using 0.3% Triton X-100 in 1x PBS for 30min and blocked with 10% fetal-calf serum in 1x PBS for 1h. Then, sections were incubated with anti-TH antibody (rabbit polyclonal IgG, 1:1,000; AB152, Merck Millipore) and anti-α-syn (human) antibody (rat monoclonal IgG, 1:100; ALX-804-258, Enzo Life Sciences, USA) overnight at 4°C. In the next day, sections were incubated with goat anti-rabbit IgG and goat anti-rat secondary antibodies (1:1,000; A11008 and A11007, respectively; Invitrogen, USA) for 2h at room temperature, followed by DAPI for 10 minutes.

Immunofluorescence image acquisition

Two imaging workflows were developed for the scanning of SNpc and striatal areas, respectively. Photomicrographs from double immunofluorescence stains were acquired with a confocal laser scanning microscope (Olympus FV3000, Germany) and are representative of 4 slices per animal. To standardize image acquisition and minimize inter-sample variation, all photos were taken at a sampling speed of 2 μs/pixel, using lasers 488 (Green) and 594 (Red). Striatal images were acquired along the dorsal-ventral and medial-lateral axis, and tiles encompassing the whole SNpc area were imaged using a motorized XY scanning stage equipped with a 10x air objective (UPlanSApo-NA 0.40). SNpc images had resolution of 0.628px/micron and a voxel size of 1.5915x1.591x8 μm^3 producing tiles with dimensions of 2481x1272 μm (width/height). Striatal images had resolution of 0.804px/micron and a voxel size of 1.243x1.243x10 μm^3. Individual acquisition settings for each marker of interest can be found in supplementary table 1.

Image analysis of TH-positive and α-syn-positive labeling

Photomicrographs containing both channels were analyzed using a semi-automated workflow on FIJI (NIH, USA). Measurements of the area occupied by dopaminergic neurons and their processes (TH-positive labeling) and α-syn-positive labeling were assessed in both the SNpc and STR. Images were first processed to produce projections along the Z-axis. Manual intensity-based thresholding was applied to segment TH-positive cells and processes as well as α-syn-positive inclusions in separate channels. The area occupied by the positive labeling was measured and normalized against the control from both the SNpc and STR. A minimal of four images per animal were assessed and brain sections that were cracked, folded or sections washed off during immunostaining procedure were excluded from analysis.

Statistical analysis

Statistical analysis was performed using the IBM SPSS statistics 25 software. All data sets were tested for normality distribution with the Shapiro–Wilk test prior to any statistical analysis. Data are expressed as mean ± standard error of mean (SEM). Equality of variances and sphericity were measured using the Levene's and Mauchly's tests, respectively. A mixed between-within subjects analysis of variance (mixed design ANOVA) was performed with a Greenhouse-Geisser correction for staircase test (Fig. 2). A one-way ANOVA was used to assess the differences between groups regarding the levels of TH-positive cells and TH-positive densitometry (Fig. 3 I, J). A one-way ANOVA with Welch

correction was employed to evaluate the differences in the areas occupied by TH-positive cells and processes and α-syn. For all analysis, upon confirmation of significant main effects, differences among group means were analyzed using the Tukey post-hoc test. A value of $P < 0.05$ was considered statistically significant. Detailed statistics are available in supplementary table 2.

4. Results

In the previous chapter we have compared secretomes from BMSCs produced in two dynamic culture systems, regarding their ability to induce neurodifferentiation and neuroprotection. Based on that work, we selected one of the secretomes for application in a more complex animal model of PD. The secretome used in this work was produced in a SP system.

Injection of α-syn tendentially led to a reduction in motor performance

Fine motor control and independent forelimb extension and grasping skills were evaluated at five moments: four, eight, eleven, fourteen and seventeen weeks after AAV1/2-A53T-α-syn injections. Ten weeks after the surgeries for model induction, animals received the treatment with secretome (or vehicle). Given the position of the animals and the dimensions of the apparatus, the access to the test food pellets is difficulted. So, animals cannot use both front paws at the same time and should be able to extend them, reach the pellets, grab them, and bring them to their mouth. The success rate is determined by the percentage of eaten pellets in relation to the total available pellets.

In what concerns the success rate when animals had access to food pellets in both sides of the apparatus, we observed that sham animals and animals that received the empty vector had a similar performance, which was superior to the performance of the animals that were injected with α-syn. Moreover, the group that was treated with secretome had a slightly superior performance than the group treated with vehicle (Neurobasal-A medium). However, none of these differences were statistically significant. Additionally, regardless of the groups, there was a significant difference between time point 1 and the other time points and between time point 2 and time points 4 and 5 (Fig. 2 A).

In the FCR, animals of the sham and empty vector groups showed a similar behaviour. Animals that received the AAV1/2-A53T-α-syn and were treated with secretome tendentially increased their success rate over time, whereas animals that received the same viral vector but were treated with vehicle did not show the same level of improvement. Moreover, there was a significant difference between time point 1 and the other four time points and between time point 2 and 5 (Fig. 1 B).

Lastly, in the FCL, sham and empty vector groups had again similar and superior performance compared to the other groups. Regarding the AAV1/2-A53T-α-syn groups, the tendency was the opposite to the FCR, which means that the group treated with secretome showed a worse performance. In this test, we observed again a significant difference between time point 1 and the other four time points and between time point 2 and 5 (Fig. 1 B).

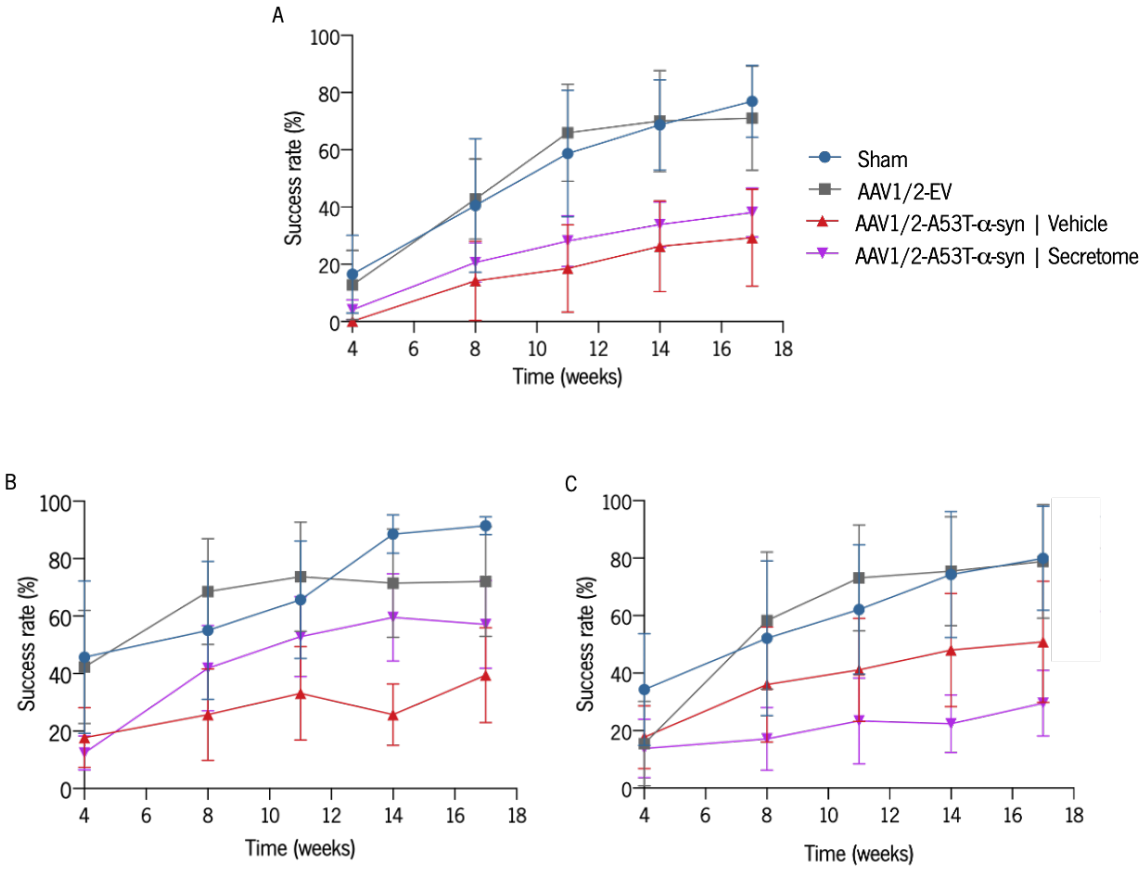

Figure 2. The effect of AAV1/2-A53T-α-syn and secretome injections on animal's fine motor control and independent forelimb extension and grasping skills. Staircase test was performed at 4, 8, 11, 14 and 17 weeks post viral vector injections. (A) During the first five days, animals had access to test food pellets in both sides of the box. At the sixth- and seventh-day, animals had access to test food pellets on the (B) right side and on the (C) left side of the apparatus. Results are presented as mean ± SEM (n = 4 – 6). α-syn, alpha-synuclein; EV, empty vector; SEM, standard error of mean.

BMSCs-derived secretome displays a neuroprotective effect

Following the motor behaviour analysis, we assessed the level of neurodegeneration in the SNpc and STR, caused by the presence of mutated α-syn and the impact of secretome in the model. The α-syn viral vector was injected in the SNpc and the secretome was administered in the SNpc and STR. Figure 3 A-D shows representative images of the SNpc for each of the four groups. In this brain region, we observed that animals injected with mutated α-syn and treated with vehicle presented a lower number of cells expressing TH. This reduction was statistically significant compared with the sham group. Moreover, animals treated with secretome presented a higher percentage of TH-positive cells compared with the group treated with vehicle (Fig. 3 I).

Regarding the level of denervation in the STR, Figure 2 E-H shows representative photographs of the STR of all groups. In this area, the level of denervation in animals injected with α-syn and treated with vehicle was significantly superior to both sham and empty vector groups. The levels of TH-positive terminals of the AAV1/2-A53T-α-syn group treated with secretome was inferior in comparison to the AAV1/2-EV group, but was still superior to the AAV1/2-A53T-α-syn group treated with vehicle, despite not statistically significant (P = 0.052) (Fig. 3 J).

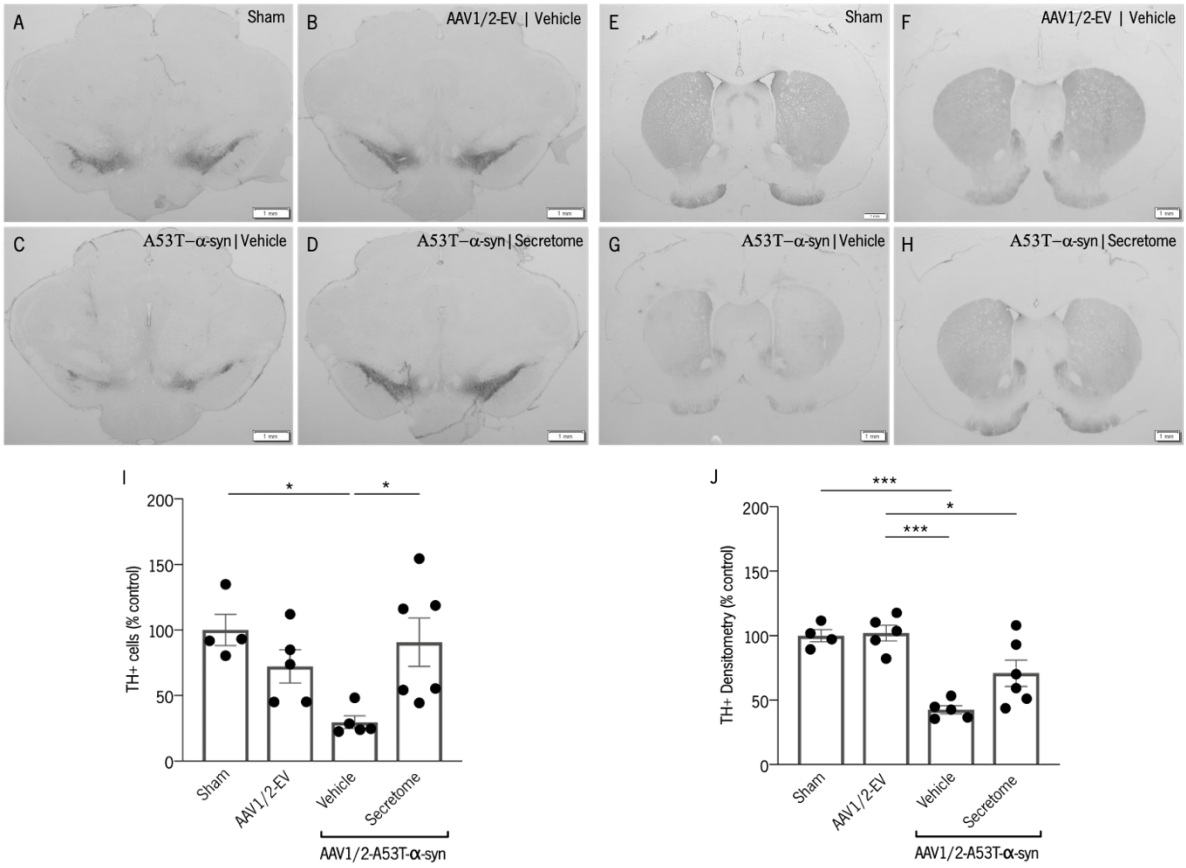

Figure 3. Striatum and substantia nigra brain slices photomicrographs stained for TH and quantification of TH-positive cells and terminals. (A-D, I) TH-positive cells in the SNpc were scored and the group treated with secretome showed a higher number of these cells compared with group injected with vehicle (Neurobasal-A medium). (E-H, J) TH-positive terminals determined by densitometry showed that secretome tendentially protects striatal dopaminergic innervation. Data are presented as the mean ± SEM of remaining TH-positive cells or TH-positive DA terminals (as a percentage) normalized to sham (for empty group) and normalized to empty group (for both groups injected with α-syn) (n = 4 – 6). *P < 0.05, ***P < 0.001 (One-way ANOVA). Scale bar = 1 mm. α-syn, alpha-synuclein; EV, empty vector; SEM, standard error of mean; TH, tyrosine hydroxylase.

Reduced α-syn expression levels in the SNpc of animals injected with BMSCs-derived secretome

An immunofluorescence assay was employed to immunolabel the dopaminergic neurons (TH-positive cells) as well as the human α-syn expressed in the SNpc and STR of animals. Photomicrographs showed the neurotoxic effect induced by the overexpression of α-syn in these brain regions (Fig. 4 A-H). We were able to determine the area occupied by the TH-positive cells and processes, as well as by the human α-syn, which were normalized to the controls (Fig. 4 I-L). The area occupied by TH-positive labeling correlates with the amount of DA neurons and their processes. Our analysis demonstrates that the overexpression of human α-syn was responsible for the reduced levels of TH expression in the groups injected with α-syn. The levels of TH expression were higher in animals treated with secretome (Fig. 4 I, J). Indeed, even though there is not a statistically significant difference between both groups injected with α-syn, the fold-difference is almost 50% in both the SNpc and the STR.

Overall, the expression levels of α-syn were higher in the SNpc, which was the injection site (Fig 4. K, L). However, the levels of α-syn expression in the SNpc are significantly decreased in animals treated with BMSCs-derived secretome (Fig. 4 K).

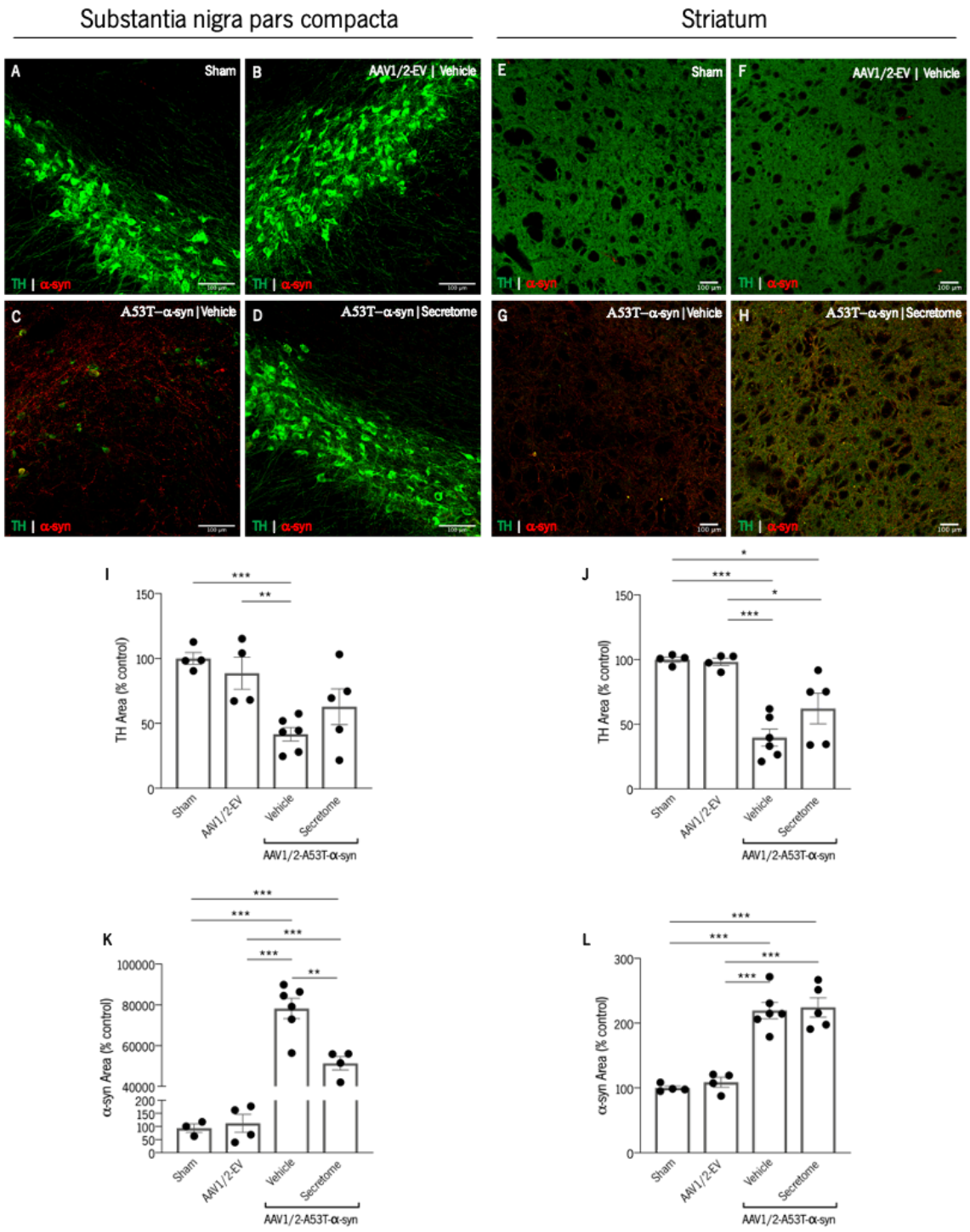

Figure 4. Expression of TH and α-syn in the SNpc and STR of rats, 19 and 9 weeks after α-syn and BMSCs-derived secretome injections, respectively. Photomicrographs of double immunofluorescence staining for α-syn and TH in (A-D) SNpc and (E-H) STR. Area occupied by dopaminergic neurons and their processes in (I) SNpc and (J) STR, and area occupied by α-syn in (K) SNpc and (L) STR were determined. Data are presented as the mean ± SEM of area occupied by TH-positive labeling or α-syn-positive labeling (as a percentage) normalized to sham (for empty vector group) and normalized to

empty vector group (for both groups injected with α-syn) (n = 3 – 6). *P < 0.05, **P < 0.01, ***P < 0.001 (One-way ANOVA). Scale bar = 100 μm. α-syn, alpha-synuclein; EV, empty vector; SEM, standard error of mean; TH, tyrosine hydroxylase.

5. Discussion

Given that PD is an α-synucleinopathy, the most appropriate animal model for efficacy assessments, must develop the pathology through mechanisms that involve α-syn (Koprich et al., 2017). Animal models based on the viral vector-mediated overexpression of α-syn usually develop accumulation of this protein, LBs and LNs, and also DA neuron loss in the SNpc (Ip et al., 2017; Koprich et al., 2011; Oliveras-Salvá et al., 2013). Therefore, in this study, we aimed to establish in our laboratory a bilateral PD animal model based on the viral vector-mediated overexpression of α-syn and study the effects of the intracranial injection of secretome from BMSCs produced in a dynamic system.

The majority of studies involving the use of animal models based on the viral vector-mediated overexpression of α-syn, consist of unilateral injections of this protein, which offers the advantage of using each animal as its own control for individual differences in motivation, exploration and activation (Brooks & Dunnett, 2009). However, in our study we opted to induce a bilateral lesion as it better reflects the clinical condition.

Animals injected with the viral vector containing the gene for A53T α-syn and treated with vehicle had a worse performance in the paw reaching test. Even though this difference was not statistically significant, it is corroborated by our immunohistochemical results. Indeed, this group displayed a lower percentage of TH-positive cells and terminals in the SNpc and STR, respectively. The onset of behavioral impairments is variable in viral vector-based models, with studies that used newer generations of hybrid serotype adeno-associated virus reporting experimental periods of 3 to 26 weeks (Koprich et al., 2017; Van der Perren et al., 2015). Therefore, a longer period might have been relevant to follow-up the evolution of the motor behavior of this model.

The treatment with secretome was not able to completely recover the motor phenotype but, based on the immunohistochemical and immunofluorescence results, secretome was able to protect DA neurons. Our results are aligned with other studies that observed significant DA neuron loss in the SNpc but did not find significant differences between groups in the staircase test 6 and 24 weeks after vector injection (Kirik et al., 2002; Van der Perren et al., 2015). Contrary to traditional toxin based rodent models [based on 6-Hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)] that display a compromised motor phenotype when more than 50% of SNpc DA neurons and more than 80% of DA terminals are lost, the animal model based on the viral vector-delivery of α-syn can display motor impairments at a lower level of dopaminergic neuron loss (Decressac et al., 2012; Koprich et al., 2011, 2017). Nevertheless, the existence and extent of motor deficits can be highly

variable most likely because not all viral injections result in the same degree of axonal degeneration and striatal DA depletion necessary to prompt motor deficits (Gombash et al., 2013). Indeed, our results show a significant variability between animals, which led us to present the results as mean ± SEM. Several features can contribute to differences in the extent of neurodegeneration and, consequently, the existence of a parkinsonian phenotype, such as the injection site and the volume injected, the titer of the virus, vector serotype, the promoter used to control the transgene expression, the type of α-syn (WT or mutant), and the time-course after injection (Carta et al., 2020; Eslamboli et al., 2007; Gombash et al., 2013; Gómez-Benito et al., 2020). In this study, we used a recombinant AAV1/2, considered as having high levels of transduction efficiency and has a preferential tropism for dopaminergic neurons (Gómez-Benito et al., 2020). Moreover, the vector construct used in this work has a CBA promoter, a CMV enhancer sequence and post-transcriptional regulatory elements that improve transgene expression, such as WPRE and bGH-polyA (Gómez-Benito et al., 2020; Koprich et al., 2017). However, there are other parameters that can also impact the vulnerability of DA neurons towards α-syn, including the specie of the animal, the strain, and the age of the animals. Moreover, the stereotaxic surgeries are technically demanding and can influence the end results (Gómez-Benito et al., 2020). Herein, we used Sprague-Dawley rats that were 30 to 31 weeks of age in the end of the experiment. This strain was reported as more prone to DA neuron degeneration compared to Wistar rats, but it would have been interesting to use older animals, since elderly animals are associated with faster and more robust DA neuronal death, as well as higher dysfunctions on skilled motor function (Barata-Antunes et al., 2020; Bourdenx et al., 2015; Rybnikova et al., 2018). On the other hand, the use of elderly animals has the disadvantage of generating more costs and/or requiring more time.

The staircase test involves a refined motor function that determines the dexterity of the animal, hence requiring a learning process (Brooks & Dunnett, 2009). This may be the reason for observing a significant increase in success rate between the first and the later time points.

To the best of our knowledge, this is the first time that the effect of secretome from BMSCs was explored in this animal model. We concluded that the secretome was able to protect the DA neurons within the SNpc and STR. Moreover, the treatment with secretome led to a reduction in the levels of α-syn in the SNpc, suggesting that the secretome might stimulate the α-syn turnover. This effect is possibly mediated by proteins with chaperone activity present in MSCs secretome, such as DJ-1 and small heat shock proteins, that regulate chaperone-induced autophagy (Jia et al., 2019; Mak et al., 2010; Xu et al., 2017). Additionally, other proteins present in secretome can also contribute to this effect, such as matrix metalloproteinase 2 (MMP2), which has been reported as being one of the main

contributors to the cleavage of α-syn preformed fibrils, following treatment with MSCs-derived secretome (Oh et al. 2017).

Given that the group treated with secretome presented higher levels of TH-positive labeling in the SNpc and STR, and that reduction in the expression levels of α-syn was only seen in the SNpc, we believe that other mechanisms are involved in the neuroprotective effect exerted by the secretome.

Animals injected with α-syn and secretome displayed a slightly superior success rate compared with animals injected with α-syn and vehicle, but this difference was not statistically significant. The results of a study from Lundblad and his team that used a model based on the viral vector-mediated overexpression of α-syn, suggest that overexpression of α-syn triggers an early defect in the handling of synaptic DA which results in a vicious circle that drives a progressive degenerative process that impacts the axons and dopaminergic terminals in the first place (Lundblad et al., 2012). Additionally, the authors observed partial loss of the striatal dopaminergic innervation, more abundant signs of axonal damage, and a reduction in striatal DA levels at 5 weeks post α-syn injections. According to these results, we hypothesize that by the time we performed the secretome injections, striatal denervation was in an advanced stage, which is aligned with the absence of a more robust impact of secretome treatment in the animals' motor performance.

When animals were subjected to the FCR and FCL, even though we did not find any statistical differences between groups, the control groups had tendentially a better performance with both paws. Interestingly, animals injected with α-syn did not showed the same performance with both paws: the group treated with secretome had a closer performance to the control groups in the FCR but presented a lower success rate in the FCL. There are, at least, two possibilities to justify these tendencies. On the one hand, the more obvious is the preference for the right paw in the secretome-treated group and for the left paw in the vehicle-treated group. However, this hypothesis does not justify the same level of performance with both paws in the control groups. Moreover, at the first time point (4 weeks after the lesion induction), both groups have the same performance in the FCR and FCL. On the other hand, we believe that animals injected with α-syn did not present the same lesion extent in both hemispheres. Therefore, to validate this theory, it would have been interesting to perform the cylinder test, which is useful to detect asymmetries, prior to the lesion induction and in the end of the experiment.

6. Conclusions

Overall, in this work, we were able to establish an animal model that presented α-syn-induced death of DA neurons in the SNpc and loss of dopaminergic terminals in the STR. However, even though the control groups displayed a better performance in the staircase test, there were no statistically significant differences between groups. We believe that this is the result of the variability of the model.

The injection of secretome from human BMSCs was able to protect DA neurons within the SNpc and STR and led to a reduction in the expression levels of α-syn in the SNpc. However, these effects did not translate into a significant improvement in the motor performance of animals treated with secretome. Therefore, the effect of an injection of secretome administered in an earlier time point should be further explored. Future studies must also use older animals and monitor at different time points the level of α-syn expression, the evolution of the lesion, and the effect of secretome throughout time.

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9. Supplementary material

Supplementary Table 1. Confocal microscopy: acquisition parameters.

	Antigen	PMT Voltage (V)	Laser Transmissivity (%)	Emission Wavelength (nm)	Detection Wavelength (nm)	Detector Gain	Detector Offset (%)
SNpc	TH	447	1.05	520	500-540	1.0	10.0
	α-syn	409	2.15	618	570-620	1.0	10.0
STR	TH	497	1.87	520	500-540	1.0	8.0
	α-syn	450	2.79	618	570-620	1.0	15.0

α-syn, alpha-synuclein; SNpc, substantia nigra pars compacta; STR, striatum; TH, tyrosine hydroxylase.

Supplementary Table 2. Statistical report. Effect sizes calculated using Lenhard, W. (2016) and Uanhoro, J. O. (2017). Effect size calculators available online at https://effect-size-calculator.herokuapp.com/.

Figure	Statistical report	Sample size
2 A	Within subjects effects (Time point) $F(1.967,29.498) = 24.868$, $P < 0.001$	4 to 6 (per group)
2 B	Within subjects effects (Time point) $F(1.992,31.867) = 11.027$, $P < 0.001$	4 to 6 (per group)
2 C	Within subjects effects (Time point) $F(2.071,33.132) = 11.414$, $P < 0.001$	4 to 6 (per group)
3 I	$F(3,16) = 11.996$, $P < 0.01$, $\omega^2 p = 0.623$	4 to 6 (per group)
3 J	$F(3,16) = 14.035$, $P < 0.001$, $\omega^2 p = 0.662$	4 to 6 (per group)
4 I	$F(3,7.426) = 19.975$, $P < 0.01$, $\omega^2 p = 0.833$	4 to 6 (per group)
4 J	$F(3,7.909) = 24.698$, $P < 0.01$, $\omega^2 p = 0.856$	4 to 6 (per group)
4 K	$F(3,6.465) = 135.173$, $P < 0.01$, $\omega^2 p = 0.975$	3 to 6 (per group)
4 L	$F(3,15) = 32.196$, $P < 0.01$, $\omega^2 p = 0.831$	4 to 6 (per group)

CHAPTER V

General discussion, future perspectives and conclusions

1. Discussion and future perspectives

Different pathogenic mechanisms associated with genomic, epigenetic and environmental factors are involved in the development of PD (Cacabelos, 2017). A deeper understanding of the molecular and cellular pathways involved in the neurodegenerative process will be essential to the establishment of relevant biomarkers and to tackle the most relevant unmet therapeutic need in PD, which is the development of disease modifying therapies (Kalia & Lang, 2015; Lang & Espay, 2018). PD is a complex condition and not all PD patients present the same mechanisms of disease (Lang & Espay, 2018). So, future disease-modifying therapies will probably need to be combined and even individualized to yield the best outcomes (Kalia & Lang, 2015).

The protein α-syn is the main constituent of LBs and LNs, and the occurrence of different mutations, as well as duplication and triplication of the *SNCA* is correlated with different forms of PD (Ibáñez et al., 2004; Polymeropoulos, 1997; Singleton, 2003). Moreover, genome-wide association studies consistently linked α-syn to sporadic PD (Edwards et al., 2010). Therefore, α-syn is a pathologic marker for both genetic and idiopathic forms of PD, which makes it an attractive target for the development of animal models and for the screening of potential drugs.

Given the diversity of molecules that constitute the secretome of MSCs, it is considered a very versatile biological product, and, thus, a valid therapeutic strategy for such a complex disease (Baez-Jurado et al., 2019). The effects of the secretome from MSCs has already been explored in different CNS disorders, including PD (Mendes-Pinheiro et al., 2019; Pinho et al., 2020; Teixeira et al., 2017). Despite the encouraging results, most of the studies that addressed the effects of the secretome in the context of PD, relied on chemical models of the disease. Given the relevance of α-syn in the pathophysiology of PD, more should be done to explore the gains of secretome therapy in genetic models of PD, particularly models that display α-syn-induced pathology.

We envisioned the use of genetic models of *C. elegans* as a platform for both high-throughput screening of different secretomes and for exploring the secretome-mediated mechanisms. Even though *C. elegans* lack the expression of endogenous α-syn, transgenic expression of human α-syn in different tissues, including DA neurons and muscle cells, enables the recapitulation of the primary cellular phenotypes of PD in humans (Gaeta et al., 2019). Indeed, *C. elegans* models of PD have aided the discovery of several potential targets, neuroprotective compounds, as well as genes and related proteins, able to modulate DA neuron degeneration, such as glucocerebrosidase (GBA) and 14-3-3 proteins (Gaeta et al., 2019; Mazzulli et al., 2011; Yacoubian et al., 2010). We used a transgenic *C.*

elegans strain that overexpresses human WT α-syn in the body wall muscle cells. The size of these cells enables the visualization of α-syn inclusions, that tend to increase in size and number with animals ageing (Chapter II). Our results regarding the application of secretome from human BMSCs produced in static conditions in this α-syn aggregation model, showed a significant reduction in the number of α-syn inclusions. According to proteomic studies developed by our team (Pires et al., 2016; Teixeira et al., 2016, 2017), the secretome contains molecular chaperons, able to prevent α-syn aggregation, such as HSPB1 and UCHL1, as well as proteins with the ability to degrade α-syn toxic species, such as MMP2 (Cox et al., 2018; Oh et al., 2017; Sarkar et al., 2016). Therefore, secretome might have exerted its action through different mechanisms, such as α-syn clearance, suppression of its aggregation or by disassembling α-syn oligomers and fibrils. In opposition to the size of body wall muscle cells, the nematode's DA neurons are smaller, which hampers the visualization of α-syn inclusions. Regardless that limitation, the overexpression of human WT α-syn in these cells drives an age-dependent DA neuron loss (Hamamichi et al., 2008). Our results show that when these animals were subjected to treatment with secretome since the egg stage of development, secretome prevented the characteristic dopaminergic cell loss observed in the untreated animals. In this chapter, we also provided an *in silico* approach, involving other potential players besides HSPB1, UCHL1 and MMP2, which indicates that the thirteen explored proteins have functional interactions, i.e., they interact in certain cell types or physiological conditions. Furthermore, they are highly conserved in *C. elegans*. As a conclusion, the secretome can display a neuroprotective effect in *C. elegans* models of PD that overexpress α-syn, encouraging further studies using these animals to explore the secretome-mediated pathways that are underlying these effects. Moreover, the nematode can also be a useful platform to investigate factors that are essential to the secretome neuroprotective effects, for instance by knocking down or inactivating specific molecules and assessing the effects of the resulting secretomes. Despite advances in cellular models, like induced-pluripotent stem cells and organoid cultures, *C. elegans* is a living organism with DA neurons integrated in a circuitry that regulates behavioral responses, that are susceptible to gene overexpression, various mutations, or knockdown. The behavioral responses are quantifiable and can be investigated over time, providing unique and complementary advantages in the research for PD modifiers (Gaeta et al., 2019). Thus, it would be relevant in future studies to assess the impact of the secretome on pathologic pathways that trigger behavioral deficits.

Cell cultures usually rely on two-dimensional/planar static culture systems. However, monolayer cultures require a laborious work, due to routine cell passaging and medium change, being highly inefficient for large-scale production of secretome (McKee & Chaudhry, 2017). The application of

secretome in the clinical setting will demand the production of high volumes, which requires more efficient culture methods (Das et al., 2019). A valid and widely known alternative to planar static cultures is the use of bioreactors, that enable the reduction of labor and enable more space-effective production (Das et al., 2019). MSCs paracrine activity is influenced by the stimuli they receive from the microenvironment. Studies have shown the modulation of the composition of the secretome by preconditioning strategies, such as the use of dynamic culturing conditions provided by bioreactors (Sart et al., 2016; Teixeira et al., 2016). Harnessing the knowledge regarding the precise impact of the bioreactor-mediated cues will be extremely useful to the design of protocols for large-scale production of secretome. Therefore, we aimed to compare neurodifferentiation and neuroprotection potential of secretomes produced in two dynamic systems with different mixing mechanisms, namely a spinner flask (SP) system and a Vertical-Wheel™ bioreactor (VWBR) (Chapter III). For that, we used human neural progenitor cells (hNPCs) as a model for neurodifferentiation and one of the previous used *C. elegans* strains (Chapter II) to assess neuroprotection. Interestingly, we observed that the secretomes produced in the SP rendered the best outcomes in both models. According to our analysis regarding the presence of several cytokines and neurologically relevant proteins, the induction of neurodifferentiation and neuroprotection was supported by the presence of several factors, some of them discussed in Chapter II. However, the secretomes have distinct patterns and one of the secretomes produced in the SP displayed higher intensities for most of the molecules. The design of the VWBR, provides a more efficient homogenization at a lower rotation, which implies less shear stress. The SP requires higher agitation levels, involving superior shear stress. The secretomes produced in the SP presented higher intensities of certain anti-inflammatory molecules, such as IL-4, IL-6, oncostatin M, and TGF-β1. We believe that this can be the result of the insult induced by an increased hydrodynamic shear, but further studies should explore this hypothesis. In this work, we used previously established and optimized culture conditions aimed at optimizing cell expansion in each system (Carmelo et al., 2015; de Almeida Fuzeta et al., 2020; dos Santos et al., 2014; Fernandes-Platzgummer et al., 2016; Pinto et al., 2019). Therefore, features like culture medium and supplementation, might also have contributed to the differences between secretomes. Thus, to ascertain the contribution of each system to the final secretome, a standardized protocol for both dynamic systems should be used. In fact, this is the major limitation of the work developed in this thesis and future studies should focus on that issue.

The BMSCs were conditioned when cell growth attained the stationary phase/late log phase. Given that in the stationary phase cell growth reaches a plateau, what if the secretome was collected sooner? Would it present a higher number of soluble factors? Future studies should compare the potential of

secretomes collected when cells are in different growth phases and be supported by proteomic analysis.

We consider that the outcomes of this work, as well as the raised questions are relevant for the design of future protocols for the large-scale production of MSCs-derived secretome. Chapter III was also important to select the secretome that was used in the following work (Chapter IV).

Efficacy assessment studies using mammalian models are highly like to yield results with increased probability of directly translating to humans. Therefore, we aimed to test the effects of the previously selected secretome from the SP system, on a mammalian model of PD (Chapter IV). We decided to proceed with this study using a model based on the AAV-mediated expression of α-syn, because animals usually develop the phenotype in weeks to months and present DA neuron degeneration, accumulation of α-syn resembling LNs, and PD-like motor deficits (Jellinger, 2019; Koprich et al., 2017). Moreover, the viral vector contained the gene for A53T α-syn. This mutated form of the protein manifests a considerable propensity toward rapid misfolding and oligomerization (Conway et al., 1998; Lázaro et al., 2014). Our behavior results demonstrate a clear difference between control animals and animals that received α-syn (despite the inexistence of statistical significance), regarding the success rate in the staircase test. The histological results confirm that animals injected with α-syn present higher levels of dopaminergic neurodegeneration in the SNpc and STR. A study from Koprich and colleagues showed a more evident motor phenotype at 6 weeks after the injection of the viral vectors (Koprich et al., 2011). Nevertheless, in that study, authors induced a unilateral model and used the cylinder test which enables the detection of asymmetries. So, neither the used motor tests, neither the induced model (unilateral versus bilateral) can be directly compared. The extent of neurodegeneration obtained with this type of models depends on several parameters and has been variable among studies, which highlights the importance of implementing standardized experimental parameters (Koprich et al., 2017).

The treatment with BMSCs-derived secretome was able to reduce the levels of α-syn in the SNpc and to prevent part of the neurodegenerative process. These observations are aligned with the results obtained in Chapter II and III. Furthermore, the reduction in the α-syn levels can also be justified by the presence of proteins with chaperone activity within the secretome. However, given that secretome was able to protect DA neurons within the SNpc and STR and that we observed a reduction in α-syn levels only in the SNpc, we believe that secretome might exert its action through different mechanisms. Despite the evident histological benefits due to secretome treatment, the difference between treated and untreated animals regarding motor performance was not significant. Given that the degenerative

process may start in the axons and DA neuron terminals (Lundblad et al., 2012), we wonder if a secretome injection in a sooner time point would have a greater impact in animals' motor performance and future studies should tackle this question. In this study, it would have been interesting to evaluate the differences in the levels of striatal DA between groups. Finally, bearing in mind the challenges that come with the translation of secretome to the clinics, it would be important to explore and compare the effects of intravenous injections of secretome in mammalian models.

2. Conclusions

Despite the evidence that has been generated throughout the years regarding the potential of secretome therapy in PD, there is still a long way to go to take the secretome from the bench to the bedside of PD patients. The work developed in this thesis addressed some relevant points in this field. We explored the efficacy of BMSCs-derived secretome in different genetic models of PD that relied on the overexpression of WT or mutated α-syn. By showing a neuroprotective effect in *C. elegans* models, we paved the way to the development of studies using nematodes as a platform for screening of different secretomes, as well as to investigate, in a faster and economically affordable fashion, the secretome-mediated mechanisms and the factors that are key PD modifiers. Moreover, we showed that the MSCs culturing process can have distinct impacts in completely opposite models and highlighted some of the parameters that could influence the secretome potential and that must be considered in the future for the design of protocols for large-scale production of MSCs-derived secretome.

Finally, by demonstrating the reduction of α-syn aggregation and the amount of expressed α-syn in different models, we gathered more evidence that can contribute to unravel the mechanisms involved in secretome-mediated benefits against α-syn neurotoxicity.

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