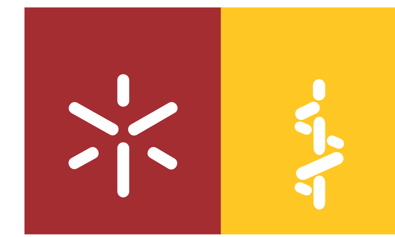

Universidade do Minho
Escola de Medicina

Ana Verónica Mendes Domingues

Finding new secretomes for Parkinson's Disease Regenerative Medicine Applications

maio de 2018

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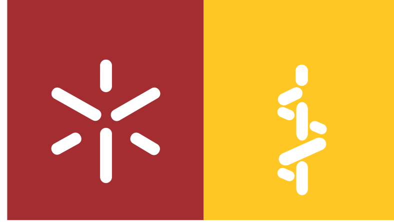

Universidade do Minho
Escola de Medicina

Ana Verónica Mendes Domingues

Finding new secretomes for Parkinson's Disease Regenerative Medicine Applications

Dissertação de Mestrado
Mestrado em Ciências da Saúde

Trabalho efetuado sob a orientação da
Doutora Ana João Gomes Rodrigues
e do
Doutor Fábio Gabriel Rodrigues Teixeira

maio de 2018

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ABSTRACT

Finding new secretomes for Parkinson's Disease Regenerative Medicine Applications

Parkinson's Disease (PD) is a progressive neurodegenerative disease that is primarily characterized by the loss of dopaminergic neurons (DAn), mostly in the nigrostriatal pathway, leading to dopamine (DA) deficiency, thereby causing the appearance of the characteristic PD motor and non-motor symptoms. Current state of the art in the field is based on the use of pharmacotherapies. However, these just mitigate motor symptomatology instead of stoping/delaying the progression of the disease, whereby imposing an urgent need for innovative therapeutical approaches.

Human Mesenchymal Stem Cells (hMSCs) secretome has been presented as a promising therapeutic option, given their ability to modulate DAn cell survival/differentiation. Apart from a direct effect in neuronal survival, recent studies showed the possibility of an interplay between hMSCs (secretome), glial cells and DAn. Indeed, glial cells have been regarded as potential targets and modulators of PD. Therefore, in the present thesis we aim to 1) determine the role of glial cells secretome and glial cells (preconditioned) with hMSCs secretome as modulators of *in vitro* neuronal survival and differentiation, and 2) investigate the effects of glial and hMSCs secretomes in a 6-hydroxydopamine (6-OHDA) rat model of PD.

Our *in vitro* data revealed that hMSCs and glial cells secretome *per se* and glial cells (preconditioned with hMSCs secretome) secretome induced higher rates of neuronal differentiation. Curiously, *in vivo*, distinct effects between hMSCs and glial cells secretomes were observed. While hMSCs secretome induced a positive impact in the amelioration of PD motor symptoms (assessed by the rotarod and staircase tests), glial cells secretome had a more pronounced effect in ameliorating PD non-motor symptomatology, namely anxious and depressive-like behaviors.

Overall, one can conclude that the use of different secretomes can differentially targets PD motor and non-motor behavioral dimensions, thereby opening new avenues for the treatment of PD.

RESUMO

Explorar novos secretomes para aplicações em Medicina Regenerativa para a doença de Parkinson

A Doença de Parkinson (DP) é uma doença neurodegenerativa progressiva caracterizada pela perda de neurónios dopaminérgicos (DAn), principalmente na via nigroestriatal, levando à deficiência de dopamina (DA), que causando perda motora e sintomas não motores. O tratamento padrão é baseado na farmacoterapia. No entanto, este apenas mitiga a sintomatologia motora em vez de parar/retardar a progressão da doença, o que leva a uma necessidade urgente de criar terapias inovadoras. O secretoma de células estaminais mesenquimatosas (MSCs) tem sido apresentado como uma opção terapêutica promissora, dada a sua capacidade em modular a sobrevivência/diferenciação dos DAn. Além de um efeito direto na sobrevivência neuronal, estudos recentes têm igualmente demonstrado uma possível interação entre as hMSCs (secretoma), as células gliais e os DAn. Na verdade, as células gliais, são hoje, consideradas potenciais alvos terapêuticos para o tratamento da DP. Assim, a presente tese teve como principais objetivos: 1) determinar o efeito do secretoma de células da glia (por si só, e precondicionadas com secretoma de MSCs) e MSCs como um modulador da sobrevivência e diferenciação neuronal *in vitro*, e 2) investigar os efeitos destes secretomas num modelo animal de degeneração dopaminérgica induzido pela injeção da neurotoxina 6-hidroxidopamina (6-OHDA). *In vitro,* verificou que o secretoma de MSCs e glias (por si só ou precondicionadas com secretoma de MSCs) induziram altas taxas de diferenciação neuronal.

Curiosamente, *in vivo*, foram observados efeitos distintos entre o secretoma de hMSCs e o secretoma de células gliais. Enquanto que o secretoma de MSCs induziu um efeito positivo na melhoria do desempenho motor (avaliada pelo rotarod e straircase), o secretoma das células gliais, apresentou, por seu lado, um impacto mais pronunciado na melhoria dos sintomas não-motores da doença, nomeadamente ansiedade e depressão.

Em suma, podemos concluir que o uso de diferentes secretomas têm impactos distintos nos sintomas motores e não motores da DP, abrindo assim novas perspetivas para o seu tratamento.

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LIST OF ABBREVIATIONS

#

6-OHDA - 6-hydroxydopamine

A

AP – Anterior Posterior

ATP – Adenosine trisphosphate

B

BBB - Blood brain barrier

BDNF - Brain-derived neurotrophic factor

bFGF - Basic fibroblast growth factor

BM-MSCs - Bone marrow mesenchymal stem cells

C

cm - centimeter

CM - Conditioned medium

CNS - Central nervous system

CO_2 - Carbone dioxide

COMT - Catechol-O-methyltransferase

CoQ10 – Coenzyme Q10

Cys C - Cystatin C

D

DA – Dopamine

DAA - Dopamine agonist

DAn - Dopaminergic neurons

DAB - 3,3-diaminobenzidine

DAPI - 4-6-diamidino-2-phenylindole-dhydrochloride

DAT - Dopamine transporter

DBS - Deep brain stimulation

DCX - Doublecortin

DMEM - Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic acid

DV – Dorsal ventral

E

EGF - Epidermal growth factor

ESCs - Embryonic stem cells

EPM – Elevated plus-maze

EVs - Extracellular vesicles

F

FA - Formic acid

FBS - Fetal bovine serum

FCS - Fetal calf serum

FGF - Fibroblast growth factor

FST – Force swimming test

G

g - g-force

GAD - Glutamic acid decarboxylase

Gal-1 - Galectin-1

GBA - Glucocerebrosidase

GDN - Glia derived nexin

GDNF - Glial cell-derived neurotrophic factor

GM1 – Monosialotetrahexosylganglioside

GSH - Glutathione

H

h - hours

H_2O_2 - Hydrogen peroxidase

HCI - Hydrochloric acid

HD - Huntington's disease

HGF - Hepatocyte growth factor

HUCPVCs - Human umbilical cord perivascular cells

I

IGF-1 - Insulin-like growth factor 1

IFN-γ - Interferon gamma

IL-10 - Interleukin 10

IL-6 - Interleukin 6

i.p. - Intraperitoneally

iPSCs - Induced pluripotent stem cells

ISCT - International Society for Cellular Therapy

IV - Intravenous

K

kDA - kilo Daltons

L

LBs - Lewy bodies

LC – Locus coeruleus

LDB – Light dark box

LDH - Lactic dehydrogenase

L- DOPA – Levodopa; L-3,4-dihydroxyphenylalanine

LNs - Lewy neurites

LRRK2 - Leucine-rich repeat kinase 2

M

m - meter

M - Molar

MAO - Monoamine oxidase

MAO-B - Monoamine oxidase-B

MAP-2 - Microtubule associated protein-2

MFB - Medial forebrain bundle

mGluR4 - Metabotropic glutamate receptor 4

mg - milligram

mg/kg - milligram per kilo

mg/ml - milligram per milliliter

MIF - Macrophage migration inhibitor factor

min - minutes

miRNAs - micro RNAs

ml – milliliter

ML – Medial lateral

mm - millimeter

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

ms - millisecond

MSCs - Mesenchymal stem cells

N

NaCl - Sodium chloride

NGF - Nerve growth factor

NMS - Nonmotor symptoms

NO – Nitric Oxide

NPCs - Neural progenitor cells

NSCs - Neural stem cells

NSF – Novelty suppress feeding

NTF-SC - Neurotrophic factors secreting cells

O

O.D. - Optical density

P

PBS - Phosphate buffered saline

PBS-T - Phosphate buffered saline-Triton

PD - Parkinson's disease

PEDF - Pigment epithelium-derived factor

PFA - Paraformaldehyde

PINK1 - PTEN-induced putative kinase 1

PPAR - Peroxisome-proliferator-activated receptor

PTEN - Parkin phosphatase and tensin homologue

R

ROS - Reactive oxygen species

rpm - Rotations per minute

RT - Room temperature

S

s - seconds

SCF - Stem cell factor

SDF-1 - Stromal cell-derived factor 1

SEM - Standard error of the mean

SNpc - Substantia nigra pars compacta

SODC - Superoxidase dismutase-cytoplasmatic

SODM - Superoxidase dismutase-mitochondrial

SPT – Sucrose preference test

STN - Subthalamic nucleus

STR – Striatum

T

TCA - Trichloroacetic acid

TGF-β -Transforming growth factor beta

TH - Tyrosine hydroxylase

Tris-HCl - Tris-hydrochloride

TrxR1- Thioredoxin reductase 1

U

UHCL1 - Ubiquitin carboxyl-terminal hydrolase L1

μl - microliter

μm - micrometer

μl/min - microliter per minute

V

V - Volts

VEGF - Vascular endothelial growth factor

VTA - Ventral tegmental area

W

W - Watts

WJ-MSCS - Wharton jelly mesenchymal stem cells

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CHAPTER 1

INTRODUCTION

INTRODUCTION
1 Parkinson's Disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder worldwide (Poewe et al., 2017). In fact, the prevalence of PD is 1 in 100 people over the age of 50 (Balestrino and Martinez-Martin, 2017), and the diagnosis is usually made in the sixth or seventh decade of life. Although, there are rare cases where the disease is found in people in their forties (Zou et al., 2013), being classified as a young onset – juvenile PD. Still, PD is more prevalent in men than in women (Van Den Eeden et al., 2003), as it has been suggested that a protective effect of female sex hormones, sex-associated genetic mechanism or sex -specific differences in exposure to environmental risk factors might be the explanation for male prevalence (Poewe et al., 2017).

Clinically, the diagnosis of PD is based on the identification of cardinal features affecting the motor system, namely, bradykinesia (slowness in the execution of voluntary movements), postural instability (a tendency to fall even in the absence of weakness or cerebellar balance disturbance), muscular rigidity (stiffness), and tremor at rest, with an asymmetric onset, which becomes bilateral along time (Poewe et al., 2017). These motor deficits, are the result of a progressive degeneration of dopaminergic neurons (DAn) in the nigrostriatal pathway at the level of the *substantia nigra pars compact* (SNpc) (Fig.1) (Langston, 2006; Lees et al., 2009), which leads to a dramatic reduction of released dopamine (DA) within the striatum (Lees et al., 2009). Nevertheless, although it has been suggested that norepinephrine and serotonin are also low in PD patients, DA is the most drastically reduced (Shannak et al., 1994), being this loss considered the responsible mechanism for the appearance of the majority of PD motor signs. Still, it has been also documented that PD motor symptoms are caused by an increased inhibitory output from the basal nuclei to the thalamus and the prefrontal motor cortex. In fact, the motor system involves a wide range of neuronal structures in the midbrain and forebrain, being DA the central neurotransmitter that plays crucial roles in the modulation of neurons of the basal ganglia. Another hallmark of PD is the formation of Lewy bodies (LBs), which are used as a post-mortem confirmation of the disease. From a broad range of proteins within its constitution, LBs are mainly composed by α–synuclein (Benskey et al., 2016). The mechanisms engaged in the formation of LBs and why they play a role on PD pathogenesis remains unclear (Dickson et al., 2009). Even though, Poewe and colleagues (Poewe et al., 2017) have recently indicated that the Lewy pathology initially occurs in cholinergic and monoaminergic brainstem neurons, as well as in the neurons of the olfactory system. This in

accordance with what was described by Braak and colleagues – 'the Braak's staging' (Braak et al., 2003, 2004), which considered such approach as the stage 1 (inclusion bodies are confined to the medulla oblongata/pontine tegmentum and olfactory bulb/anterior olfactory nucleus). Indeed, the above-referred authors have staged PD into six neuropathological disease stages, demonstrating that PD most likely progresses from an upward direction through the pons (stage 2) to the midbrain (stage 3), being followed by the prosencephalon and the mesocortex (stage 4), which could reach (in some cases) the temporal cortex and the neocortex (stage 5/6) (Braak et al., 2003, 2004; Sveinbjornsdottir Sigurlaug, 2016). Thus, when PD motor symptoms becomes evident is the indication that, at least, 60% of DAn were lost (representing Braak's stage 3) (DeMaagd and Philip, 2015). Such conclusion, is a clear indication that there is a substantive pre-symptomatic period of the disease that is hidden probably due to the existence of compensatory mechanisms (Navntoft and Dreyer, 2016). Nevertheless, the real nature of such compensatory mechanisms and how they 'hide' the early appearance of PD still remains elusive.

Concomitantly, although all PD focus is directed to its motor symptomatology, there are recent studies indicating that non-motor symptoms (NMS) are also an important feature of PD, suggesting that they could precede the appearance of the clinical PD motor symptoms (Owens-Walton et al., 2018). Such assumption is valuable, thereby indicating that, in addition of being a potential approach for an early diagnosis of the disease, PD NMS could also be a potential target to improve PD treatment (Owens-Walton et al., 2018). However, it is important to highlight that the current strategies in the treatment of PD are just being effective in the mitigation of its motor symptoms, but totally ineffective in NMS. To this date, the understanding of how the disease emerge and how it progresses is still poorly understood. Thus, multi-targeted studies should be performed in order to understand the (molecular and cellular) mechanisms that could be in the origin of PD motor and non-motor signs as a way of establishing a pathophysiology signature of PD.

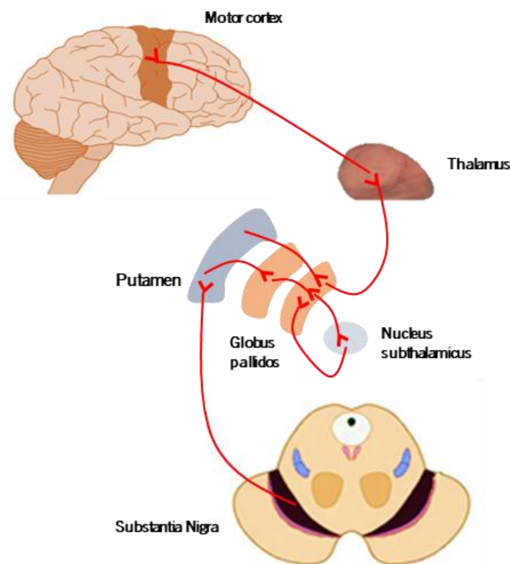


Figure 1: Neuropathology of Parkinson's Disease (PD). Representation of the nigrostriatal pathway, showing the degeneration process that occurs between the substantia nigra and the striatum in a PD brain, which leads to the reduction of dopamine production and release, and the appearance of PD motor symptomatology.

1.1 Causes/risk factors of Parkinson's Disease

Despite, the exhaustive research around PD, its causes are still poorly understood. Until now it is known that most of the cases are sporadic, and studies have shown that such prevalence might be due to a complex interaction between genetic and environmental factors (Ascherio and Schwarzschild, 2016). Aging is being described as the main predisposing factor for PD. However, although it is evident that PD prevalence increases with age, it is not well established (so far) if it is the chronological age or the aging process the responsible for PD susceptibly (Braak et al., 2003; Collier Timothy J. et al., 2017). Even though, studies have suggested that the age onset of PD significantly affects its phenotype and progression, thereby indicating that biological aging provides distinct starting points for the evolution of PD (Collier Timothy J. et al., 2017; Diederich et al., 2003).

Regarding environmental factors, farming occupation and rural living with the consequent exposure to toxic environments (e.g. pesticides such as rotenone or paraquat) have been described as promoters for the appearance and development of PD (Dallé and Mabandla, 2018). In fact, previous studies from our lab have demonstrated that by using those compounds it was possible to recreate animal models of PD (Campos et al., 2013). Nevertheless, this association between

environmental toxins and PD development remains still under discussion, as studies have been suggesting that they play a minor role in PD risk (Noyce et al., 2016). In addition to this, people's lifestyle has also been correlated with PD (Gigante et al., 2018; Lee Yoonju et al., 2017). For instance, it has been shown that cigarette smokers as well as coffee drinkers have a reduced risk of developing PD. While it has been demonstrated that caffeine is a regulator of the adenosine A2 receptor important in the regulation of DA release,it has been found that nicotine stimulates DA release and acts as an antioxidant agent (Munoz and Fujioka, 2018).

Finally, although rare, familial (genetic) forms of PD (e.g. mutations in α-synuclein, parkin phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (PINK), leucine-rich repeat kinase 2 (LRRK-2) and DJ-1 (protein deglycase) genes) are also being intensively explored, showing promising outcomes in the understanding of the pathophysiology of the disease (Bras and Singleton, 2009). Thus, although literature indicates that genetic forms of PD present different clinical and pathological phenotypes, it has been demonstrated that its mechanisms of degeneration overlap with those observed in sporadic PD, namely oxidative stress, mitochondrial dysfunction and protein aggregation (Dallé and Mabandla, 2018), also involved in PD onset and progression.

1.2 Pathophysiology of Parkinson's Disease

So far, it is known that the degeneration of DAn starts in the axonal and synaptic terminals that retrogradely progresses to the cells bodies in the SNpc. Notwithstanding, the starting point of DAn degeneration is still poorly understood. In fact, major question remain to be answered – at the cellular level, where does PD start? The development of intracellular inclusions of LBs (accumulation of α-synuclein) is one of the most characterized features of PD (Poewe et al., 2017). Moreover, mutations or overexpression of such proteins has also been associated with the formation of toxic oligomers/insoluble aggregates, which in turn has been correlated with alterations in cellular trafficking, disruption of cells morphology and impairment of mitochondrial function (DeMaagd and Philip, 2015). Nevertheless, accumulating evidences have also been indicating that in addition to abnormal protein accumulation, mitochondria dysfunction and oxidative stress are key players in PD initiation and progression (Ammal Kaidery and Thomas, 2018).

Concerning mitochondria dysfunction, studies have demonstrated that alterations in its bioenergetic process, mitochondrial DNA, dynamic changes in its fusion and fission processes, trafficking and presence of mutated proteins are implicated on PD (Bender et al., 2006; Thomas Bobby and Beal M. Flint, 2010; Valero, 2014). Indeed, studies have shown that under pathological conditions, mitochondrial dysfunction leads to the increase of reactive oxygen species (ROS) through the release (for instance) of cytochrome c to the cytosol (Bhat et al., 2015; Moon and Paek, 2015), thereby decreasing complex I (of mitochondrial respiratory chain) enzyme activity and causing neuronal excitotoxicity and axonal damage, contributing to the progression of PD (Chiurchiu et al., 2016).

Concerning oxidative stress, is the result of an imbalance between the production of ROS (free radicals) and the body capacity to counteract their harmful effects through neutralization by antioxidant defenses (Dallé and Mabandla, 2018). Evidences show that the increase in oxidative stress is the result of mitochondrial dysfunction in brain tissue of PD patients (Dias et al., 2013; Poewe et al., 2017). Moreover, it has also been described that nigral dopaminergic neurons seem to be more sensitive to oxidative stress. But, what makes DAn so vulnerable? Firstly, DAn are highly branched with a long axonal arbor, unmyelinated axons, with large numbers of synapses, requiring a great amount of energy to be sustained. Secondly, a distinctive pacemaker phenotype involving cytosolic calcium oscillations and calcium extrusion at the expense of energy is evident in these neurons. And thirdly, increased levels of cytosolic DA and its metabolites are also being suggested as a cause of oxidative stress and neurotoxicity, which negatively impact DAn neuronal survival (Surmeier et al., 2017). However, the cellular and molecular factors that could be involved in DAn degeneration are not exclusively confined to neurons. Actually, it has been suggested that glial cells also suffer early alterations in PD (Fig.2). From those, astrocytes and microglia have been described as key players to be involved in the tissue and cellular buildup of α-synuclein (Halliday and Stevens, 2011). In addition, post-mortem studies have shown higher concentrations of iron in SNpc region of PD patients when compared to healthy individuals (Griffiths et al., 1999). Therefore, it is now increasingly appreciated that glial cells play a critical role in the regulation of iron homeostasis (Xu et al., 2018). Impairment of these properties might lead to dysfunction of iron metabolism and neurodegeneration of DAn (Xu et al., 2018). Moreover, studies using cultured neurons, astrocytes and microglia showed that, despite all having the capability to store huge amounts of iron, glial cells are more effective on its storage (Bishop et al., 2011). Being so, due to this dual presence of

DA and higher levels of iron, DAn are thus a more susceptible to oxidative damage when compared to other neuronal cells.

Still, besides the extensive dopaminergic degeneration, currently it is also known that several other neuronal networks are affected with the disease progression. Neuronal loss of noradrenergic nuclei, like locus coeruleus (LC) were reported in PD patients (Jellinger, 1991). Serotonergic nuclei were also found to be affected, especially in the dorsal raphe nucleus, leading to serotonergic depletion in several projection areas (Jellinger, 1991). Furthermore, cholinergic denervation in cortical areas was also found to be compromised in PD patients (Poewe et al., 2017). Therefore, even not being directly correlated with the major motor symptomatology that is usually used in the diagnosis of PD, the above mentioned processes are being linked with the NMS of the disease, which in most of the cases precede the appearance of PD motor symptomatology, although such link is still under discussion.

Thus, although Parkinsonian mechanisms are (nowadays) relatively clearer, there is still a long way to find a cure. Actually, while mitochondrial dysfunction, oxidative stress, α-synuclein aggregates and glial cells (dys)function are evident on PD, the sequence in which they act and interact remains still elusive.

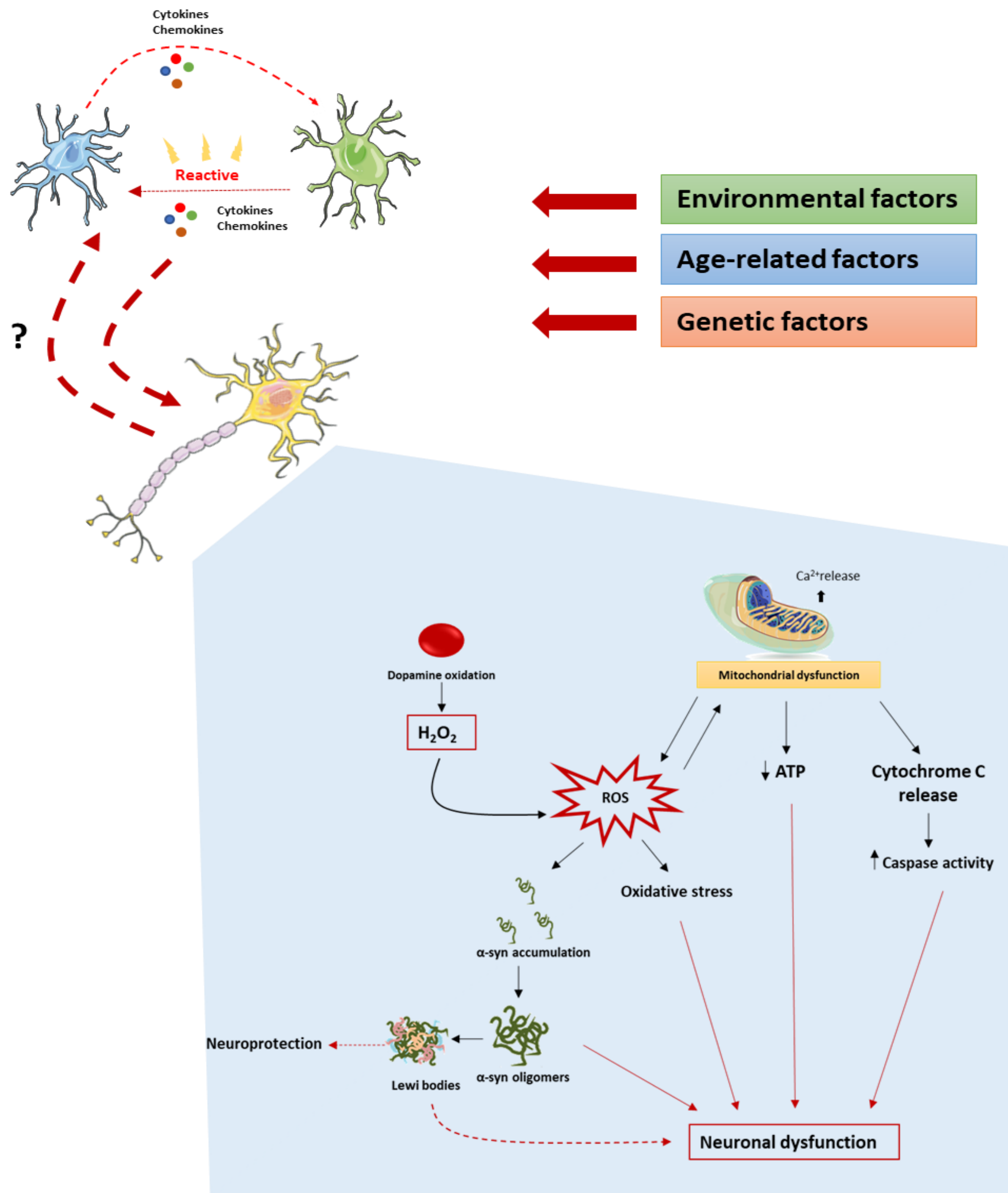


Figure 2: Mechanisms involved in the degeneration of Parkinson's Disease DAn cell death may be caused by oxidative stress, mitochondrial dysfunction and α-synuclein aggregates. However, there is some controversy on how these mechanisms can be activated. Risk factors like genetics, age-related and surrounding environment are well accepted. Still, the influence of glial cells in these processes are poorly understood and it is still unclear whether these cells are key players in the disease protection or progression. ROS production occurs through the auto-oxidation process of DA, resulting in significant amounts of H_2O_2 that can further interact with metal ions like iron, leading to DAn generation.

1.3 Parkinson's Disease therapies: What was so far achieved?

Over the last years, basic and clinical research has tried to establish management strategies involving the diagnosis and the evaluation of the condition of PD patients, in order to develop and apply personalized strategies, thereby aiming to ameliorate the patient's quality of life (Diaz and Waters, 2009, 2009; Marques de Sousa and Massano, 2013). Even being a promising approach in the care of PD patients, satisfactory approaches to relief or to slow down PD progression by protecting DAn from premature death are still missing (Onofrj et al., 2008). Still, although promising results have been experimentally and clinically obtained with several drugs and surgical processes, the challenge remains to show a clinical proof of arrest of delay of DAn loss/degeneration in PD (Teixeira et al., 2018).

As recently reviewed by Teixeira and colleagues (Teixeira et al., 2018), the current PD treatment just relies on the mitigation of its symptomatic impairments through the use of pharmacological strategies such as levodopa (L-DOPA, which is still the *gold standard* treatment), DA agonists (DAAs; *e.g.,* ropinirole or pramipexole), monoamine oxidase B (MAO-B; *e.g.,* rasagiline or selegiline) and catechol-O-methyltransferase (COMT; *e.g.,* entacapone or tolcapone) inhibitors to compensate the deficits of DA in the nigrostriatal dopaminergic pathway (Dong et al., 2016; Lindholm et al., 2016; Vijverman and Fox, 2014). However, although efficacious, studies have demonstrated that they can elicit undesirable side effects, a fact that may limit their use over long periods of time (Onofrj et al., 2008). In addition to all the pharmacological treatments, surgical interventions, such as deep brain stimulation (DBS) have also been used as a strategy for the treatment of PD (Okun, 2012). However, the apparent clinical recovery does not appear to be effective in long-term approaches, whereby PD progression is not avoided (Krack et al., 2017). Still, the use of molecular agents (*e.g.*, mGluR4 agonists; CEP-1347; GM1 ganglioside, CoQ10 and NAC) and gene engineering approaches (*e.g.,* induction of glutamic acid decarboxylase (GAD) enzyme in the striatum; delivery of synthetic enzymes to increase striatal DA levels, and local infusion of neurotrophic factors to protect and restore nigral DA neurons) have also been developed and used as potential and promising strategies in the treatment of PD (Amalric et al., 2013; Harikrishna Reddy et al., 2014; Pires et al., 2017; Seet et al., 2014). However, as far as we know, none of the current available strategies have delayed or halted PD progression. According to the literature, one of the main reasons for such failure might be the simplistic view/targeting and inadequate definition of PD (Espay et al., 2017). Indeed, and as it was previously mentioned, in addition to DAn

degeneration and DA depletion, multiple factors including oxidative stress, mitochondrial dysfunction, excitotoxicity and inflammation have also been described to be involved in the initiation and progression of PD, which makes it a multi-targeted disease in which strategies with a multimodal actions may be of particular interest (Sadeghian et al., 2016). Following such idea, safinamide (a new PD drug) was recently approved as promising multimodal therapeutic option able to modulate dopaminergic and non-dopaminergic (neuroprotective) PD dimensions (Teixeira et al., 2018). In fact, although promising results had been observed, namely through the increase in DA levels by MAO-B inhibition, or by its non-dopaminergic actions (which may lead to neuroprotective effects), including the attenuation of sodium/calcium channels and glutamate release (Caccia et al., 2006) or even through anti-inflammatory properties by blocking sodium channels in the activation of microglia (Sadeghian et al., 2016), no evidence from human studies have already confirmed these potential neuroprotective effects (Dézsi and Vécsei, 2014).

Thus, although promising, all the above-mentioned drug/surgical interventions are not fully efficacious, not avoiding PD progression avoidance. Therefore, it is based on such limitations that stem cell-based strategies have been proposed as a promising therapeutic tools for the treatment CNS neurodegenerative disorders, including PD (Anisimov, 2009).

2 New combinatory strategies

2.1 Cell-based therapies

The adult brain has the capacity to generate new cells throughout life due to the existence of pools of endogenous stem cells in specific niches (e.g. as the dentate gyrus of hippocampus and subependymal zone of the lateral ventricles), which have the ability to differentiate and replace damage cells and secrete trophic factors required for tissue repair (reviewed by Ming and Song, 2011). Nevertheless, studies have indicated that this self-repair is limited, demanding for an external intervention (Kim et al., 2013). For instance, the use of Embryonic Stem Cells (ESCs), Neural Stem Cells (NSCs), Mesenchymal Stem Cells (MSCs) and induced Pluripotent Stem Cells (iPSCs) have been investigated and used as a potential therapeutic treatments for PD (Goodarzi et al., 2015; Pires et al., 2017; Zhang et al., 2016). From these, MSCs are being suggested as a promising therapeutic tool for PD, given their ability to protect and regenerate damaged DAn, as well as to increase PD motor function due to its trophic capability (Teixeira et al., 2013, 2016). In

addition to those cells, glial cells have also been suggested as a promising cell source to tackle CNS disorders, including PD (Jha et al., 2013). Therefore, under the scope of the present thesis, we have focus our efforts in the use of MSCs and glial cells secretome as a potential therapeutic tool for PD.

2.1.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) have emerged in the last decades as promising therapeutic source for CNS regenerative medicine applications, including neurodegenerative disorders such as PD. Due to their capability of self-renewal and multiple differentiation potential, MSCs are described as a non-hematopoietic multipotent stem cell population (Gugliandolo et al., 2017). Currently, to define and characterize a population as an MSC population (and according with the International Society for Cellular Therapy (ISCT)), there are some minimal criteria for its identification, namely: 1) the adherence to plastic in standard culture conditions; 2) the positive expression of CD90, CD105, CD73 markers, and negative expression of hematopoietic markers (e.g. CD34,CD45, HLA-DR, CD14, or CD11B, CD79α or DC19), and 3) *in vitro* differentiation into at least osteoblast, adipocytes and chondroblasts (Dominici et al., 2006).

In the seventies, Friendenstein's group was pioneer in isolating MSCs from the bone marrow of mice, describing them as fibroblastoid cells with clonogenic potential and plastic culture adherence (Friedenstein et al., 1974). After such discovery, further studies have also shown that MSCs could also be isolated from various neonatal and adult tissues, like adipose tissue, dental pulp, amnion, placenta, Wharton jelly of the umbilical cord, and even the brain (Abomaray et al., 2016; Fukuchi et al., 2004; Gronthos et al., 2000; Paul et al., 2012; Shi and Gronthos, 2003; Sypecka and Sarnowska, 2015; Zuk et al., 2001, 2002). Thus, one of the reasons of (MSCs) being looked as a promising therapeutic source, relies in their widespread availability throughout the human body, along with the fact that when isolate they display a great proliferative potential (Jung Sunghoon et al., 2012; Vizoso et al., 2017). For that, it is not surprising that many groups have rapidly began to explore their therapeutic uses (Gugliandolo et al., 2017). Although under discussion, studies have defended that these cells have the ability to trans-differentiate to non-mesenchymal cell types, such as epithelial, endothelial and neuronal cells, making them of major interest in the development of PD therapeutical strategies (Barzilay et al., 2008; Gugliandolo et al., 2017). Indeed, it was already found that MSCs express neural and DAn associated genes (Blondheim et al., 2006).

For instance, when exposed to a mixture of growth factors (sonic hedgehog, fibroblast growth factor 2, FGF8 and basic FGF, brain derived neurotrophic factors), it was found that MSCs would differentiate into DAn (Bouchez et al., 2008; Jang et al., 2010; Shetty et al., 2009; Trzaska et al., 2007). Based on such assumptions, when applied in an *in vivo* model of PD, Li and colleagues (Li et al., 2001) (by using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) PD model) observed that after transplantation of bone marrow MSCs (BM-MSCs), there was an improvement in motor coordination and balance measured by the rotarod test. Moreover, they also found that MSCs survived in the transplanted areas at least 4 weeks after administration, expressing TH positive staining (Li et al., 2001). Similar results were also found by Danielyan and colleagues (Danielyan et al., 2011) in a 6-OHDA animal model of PD, in which the authors reported a neuroprotective effect of MSCs against nigrostriatal degeneration and improvements in the motor functions of 6-OHDA lesioned rats (Danielyan et al., 2011). Following the same observations, Suzuki and co-workers (Suzuki et al., 2015) showed that administration of BM-MSCs was able to inhibit methamphetamine-stimulated rotational behavior at 7, 14, 21 and 28 days after transplantation, which was positively correlated (by immunohistochemical analysis) with preservation of TH-positive neurons in the SNpc when compared to sham-operated rats (Suzuki et al., 2015).

Thus, although the transdifferentiation capacity of MSCs has been accepted over the years as one of the most probable mechanisms of its therapeutical effects (however the differentiation into functional neuronal lineages is still matter of intense debate), nowadays there has been a shift in this paradigm, in which the secretome of these cells (e.g. the release of bioactive factors and vesicles) has been proposed as the main responsible for MSCs therapeutical actions (Teixeira et al., 2013).

2.1.1.1. Mesenchymal stem cells secretome

With the increased assumption that only a small percentage of (MSCs) cells truly engraft and survive after transplantation, it is, nowadays accepted that the multipotent differentiation of MSCs represents a minimal contribution to the observed beneficial effects (Kupcova Skalnikova, 2013; Lavoie and Rosu-Myles, 2013). Therefore, it is based on such evidences that robust data has recently demonstrated that most of these (MSCs) potential effects are mainly mediated by the

secretion of bioactive molecules, which is defined as secretome (Salgado et al., 2010, 2015; Teixeira et al., 2013).

MSCs secretome has been described as a complex mixture of soluble products composed by a proteic soluble fraction (constituted by growth factors and cytokines), and a vesicular fraction composed by microvesicles and exosomes, which are involved in the transference of proteins and genetic material (e.g., miRNA) to other cells, with promising therapeutic effects (Beer et al., 2017; Marote et al., 2016; Vizoso et al., 2017). Crigler and colleagues (Crigler et al., 2006) were the first to show that MSCs were able to modulate neuronal cell survival and neurite outgrowth, through an active secretion of neurotrophic factors such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Further characterization studies have reported that MSCs are a source of neuroregulatory molecules, able to secrete (in addition to the above-mentioned factors) a wide panel of trophic factors such as glial derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and EGF, as well as cytokines like interleukin 6 (IL-6), interleukin-10 (IL-10), transforming growth factor beta (TGF-β), stem cell factor (SCF) and stromal cell-derived factor 1 (SDF-1) (Baraniak and McDevitt, 2010; Meyerrose et al., 2010; Nakano et al., 2010; Ribeiro et al., 2012) which are being described as important modulators of neuronal/glial survival/differentiation and neurite outgrowth *in vitro* (Ribeiro et al., 2011; Salgado et al., 2010; Teixeira et al., 2013; Wilkins et al., 2009). Recently, through proteomic-based analysis, our group demonstrated that, in addition to the conventional trophic factors (mentioned above), MSCs are also able to secrete other important neuroregulatory molecules such as Pigment epithelium-derived factor (PEDF), Cystatin C (Cys C), Galectin-1 (Gal-1) and Glial-derived nexin (GDN), which were also found to be important modulators of neuronal survival/differentiation and neuroprotection (Teixeira et al., 2016).Therefore, what makes (MSCs) cell secretome so special? By using the secretome as a therapeutic approach, studies have demonstrated that, rather than transplanting cells, this approach would be valuable to overcome technical concerns associated to cells transplantation, namely: 1) low survival rate of hMSCs when transplanted into the brain and 2) the significant high number of cells needed for transplantation ($7x10^6$ cells/Kg) which consequently leads to long periods of expansion of hMSCs *in vitro* prior to transplantation, that can lead to phenotypic alterations, affecting their therapeutic potential (Jung Sunghoon et al., 2012; Teixeira et al., 2015, 2016).

Regarding *in vivo* applications, the secretome of MSCs also play a role, either by the active secretion of bioactive molecules *in situ* (after MSCs transplantation), or by the injection of the secretome itself in the form of CM (Teixeira et al., 2013, Blandini et al., 2010; Danielyan et al., 2011; Vizoso et al., 2017)). Interestingly, in a comparative study, our group has demonstrated that the injection of MSCs secretome (itself) led to the increase of neuronal densities to levels comparable to those promoted by MSCs cell transplantation (Teixeira et al., 2015). In the context of neurodegenerative diseases as PD, MSCs secretome also discloses an active role (Fig.3). Indeed, Sadan and colleagues (Sadan et al., 2009), using hBM-MSCs as neurotrophic factors secreting cells (NTF-SC) observed a significant decrease in the amphetamine-induced rotation test, as well as in the loss of TH immunoreactive nerve terminals when compared to the untreated MSCs group, correlating these effects with an active secretion of BDNF and GDNF (Sadan et al., 2009). Recently, Teixeira and colleague (Teixeira et al., 2016) demonstrated that after a local injection of hBM-MSCs secretome into the SNpc and striatum (STR), there was a partially reversion of the PD histological deficits (increase in tyrosine hydroxylase positive cells and fibers in the SNc and STR), which was correlated with evident gains in animals' motor performance (Teixeira et al., 2016).

All together, these data strongly suggests that MSCs may in fact be a promising therapeutic tool to replace stem cells transplantation strategies, and a new avenue for the treatment of PD (Blandini et al., 2010). Actually, genetic modification of MSCs to specifically release of trophic factors such as GDNF into the striatum and SNpc, have caused long-term amelioration of PD pathology (Moloney et al., 2010; Olanow, 2008). Therefore, based on such evidences, the use of secretome as a possible therapy for PD is of enormous interest to tackle DAn cell survival (Fig.3).

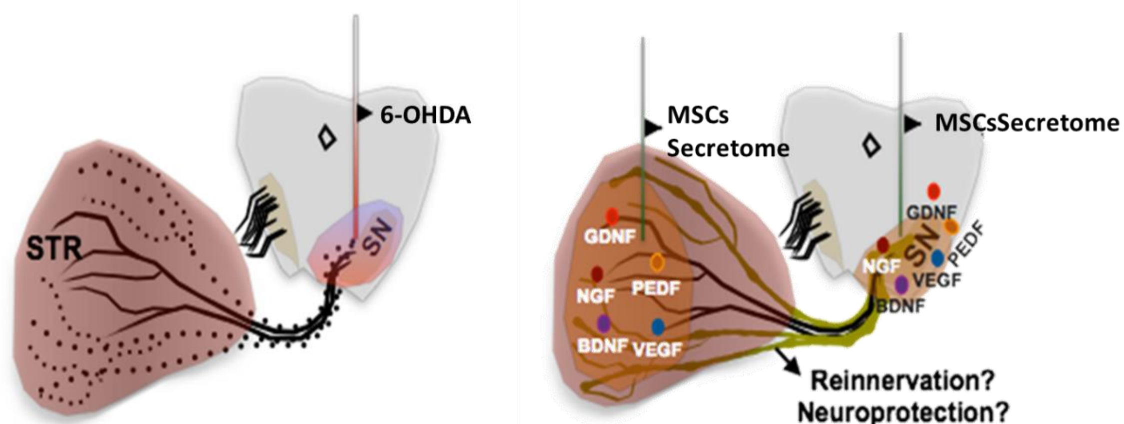


Figure 3. Mesenchymal stem cell secretomes-based therapy for Parkinson's Disease. A The release of trophic molecules by MSCs has been increasingly accepted nowadays as a new route for the treatment of PD, being

considered one of the reasons that leads to the protection of the preexisting DAn after PD onset and to its functional and motor amelioration.

2.1.2 Glial cells

For a long time, glial cells were only viewed as "glue" for neurons. However, in recent years, there has been an increasing interest in understanding the behavior and role of these cells in the CNS under normal and pathological conditions (Jäkel and Dimou, 2017). Characteristically divided into three major cell groups, namely microglia, oligodendrocytes and astrocytes, and in addition to its supportive effects to neurons, recent evidences have demonstrated that glial cells are also involved in neuronal development (Jäkel and Dimou, 2017). For instance, it was shown that glial cells are important promoters of axonal outgrowth, dendritic extension, as well as modulation of the morphological plasticity of neuronal receptive endings (Bitzer-Quintero and González-Burgos, 2012; Jha et al., 2013; Vinet et al., 2012). Additionally, glial cells have also an unique way to communicate with each other, namely through intracellular waves of calcium and through intercellular diffusion of gliotransmitters (Fields and Stevens-Graham, 2002). Although its role and function in PD still remains under intense investigation, evidences have been proposing glial cells as a promising route to tackle PD (Barker et al., 2015; Mena and García de Yébenes, 2008). In fact, glial cells transplantation has recently emerged as a promising tool to CNS regenerative approaches (White and Barry, 2015). In the context of PD, Bahat-Stroomza and colleagues (Bahat-Stroomza et al., 2009) using astrocyte-like cells derived from human bone-marrow-derived stromal cells demonstrated that, when transplanted in a PD model, there was an increase in TH-positive cells that was positively correlated with a remarkable improvement in animal behavioral outcome (Bahat-Stroomza et al., 2009). However, like MSCs, cell transplantation procedures still remain under discussion, and recent approaches have been suggesting and profiling the secretome of glial cells as a new approach for the treatment of CNS disorders, including PD (Jha et al., 2013; Mena and García de Yébenes, 2008; White and Barry, 2015). Indeed, although studies have been suggesting glial secretions as a source to provide diagnostic and prognostic biomarkers and to identify therapeutic targets, its use as a therapeutic tool still remains elusive (Chang et al., 2003; Choi et al., 2014; Suk, 2010). Nevertheless, some evidences have demonstrated that glial cells secretome may have a positive impact on CNS cells function, as it was demonstrated by Jeon and colleagues (Jeon et al., 2010) that glial cells secretome is a modulator of the phagocytic function of microglia due to the presence of pentraxin (PTX3) on it, indicating that glial cells secretome may have important implications in the regulation of CNS cells in health and disease. In line with this,

Le and colleagues (Le et al., 2016) found in patients' brains that glial cells have both neurotoxic and neuroprotective effects, depending of their activation state. Even though, the authors verified that, after glial cells activation there was an increase in neuronal survival that was correlated with the release of trophic and anti-inflammatory factors such as GDNF, which is known to be a crucial trophic factor in the enhancement of DAn cell survival and rescuing (Ding et al., 2004; Le et al., 2016; Nam et al., 2015; Schwartz and Kipnis, 2004). Additionally, it was also found that glial cells can also be involved in the up-regulation of tissue repair and regeneration genes (Le et al., 2016; Schwartz and Ziv, 2008). Following such approach, Miyazaki and colleagues (Miyazaki et al., 2016), using levetiracetam (an anti-epileptc drug that increase xCT expression, which increases GSH production in/from astrocytes via xCT), demonstrated that GSH produced by astrocytes was able to protect DAn against neurotoxicity induced by 6-OHDA, thereby indicating that (xCT) in glial cells (e.g. astrocytes) could be a potential target to prevent DAn degeneration (Miyazaki et al., 2016). Therefore, although promising, studies regarding the functional impact of glial cells secretome and its interplay under normal and pathological conditions is unexplored (Jha et al., 2013).

Thus, in addition of dissecting the role of glial cells in PD pathophysiology, explore its therapeutical potential, namely by addressing the impact of its secretome may be an opportunity for the development of new therapeutic strategies, which may open important gains to PD regenerative medicine field.

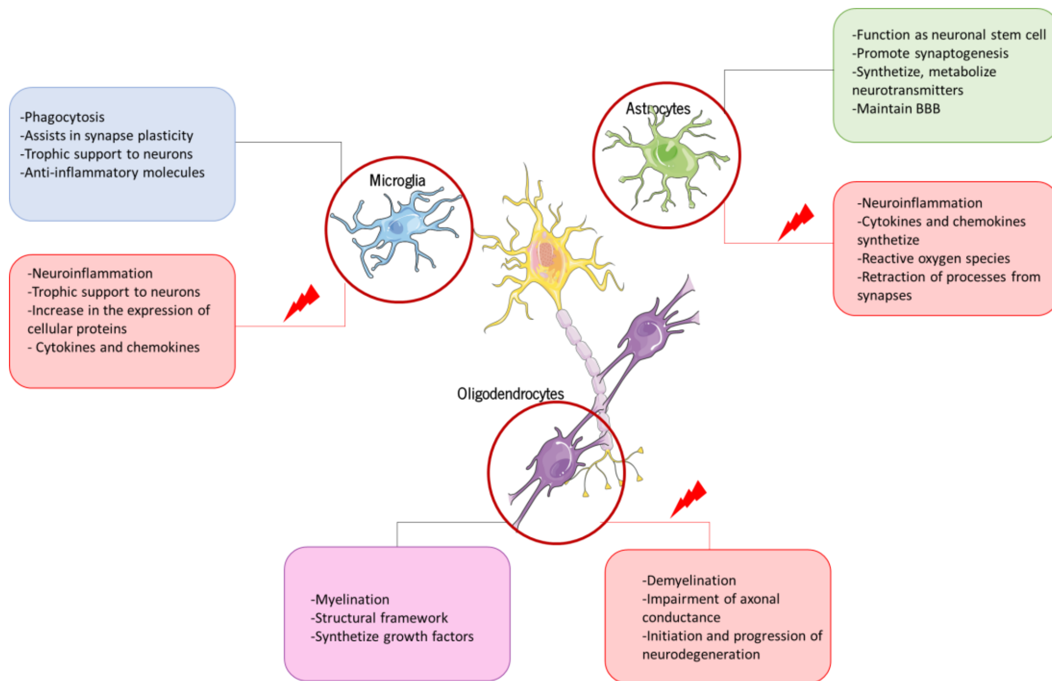


Figure 4: Glial cells in healthy and brain disease Glial cells interact with neurons in many different ways to maintain neuronal tissue health. Glia in the CNS is likely to communicate with other glial cells and neurons through secreted proteins. The scheme summarizes the actions and phenotypes of glial cells under different environmental conditions.

CHAPTER 2

RESEARCH OBJECTIVES

2 RESEARCH OBJECTIVES

Current pharmacological and surgical PD treatments are unable to cure/delay the disease. Therefore, there is the need for novel therapeutic strategies. MSCs secretome has been on the forefront of a new wave of possible therapeutic strategies for PD. In addition, glial cells have also been described as important modulators for PD regenerative medicine. Based on such evidences, this project aims to address the therapeutic potential of glial cells and MSCs secretome on DAn cell survival and protection. Therefore, the main objectives of the present project are:

I. Explore the role of glial cells' secretome and glial cells preconditioned with hMSCs' secretome on *in vitro* neuronal survival and differentiation of human neural progenitors (hNPCs).

II. Evaluate the therapeutic potential of glial and MSCs' secretome in an *in vivo* model of PD (6-OHDA-lesioned animals).

CHAPTER 3

MATERIALS AND METHODS

3 MATERIAL AND METHODS

3.1 Cell Culture Procedures

3.1.1. Expansion of human mesenchymal stem cells (hMSCs) and conditioned medium (CM) collection

hMSCs derived from bone marrow (Lonza, Switzerland) were thawed and plated into T-75 culture flasks (SPL Life Sciences, Korea) with 12 mL of α-MEM (Invitrogen/Gibco) supplemented with 10% FBS and a 1% antibiotic-antimycotic mixture. The medium was changed every 3 days and the culture maintained at 37°C in a humidified atmosphere containing 5% CO2. When the cells reached 80-90% of confluence were enzymatically dissociated using trypsin (Life Technologies, USA) during 5 min at 37°C. To stop trypsin reaction α-MEM was added. Then, cells were centrifuged at 1200 rpm (4°C) for 5 min and the supernatant was removed. The pellet was resuspended in fresh growth medium, and a small volume of cells was diluted in Trypan Blue (Life Technologies, USA) to perform cell counts. Lastly, the cells were plated into new culture flasks at a density of 5000 cells/cm^2. At passage 5 (P5), and after 72 hours of growth, the medium was removed and the cells were washed twice with Neurobasal A medium and once with phosphate buffered saline (PBS; Life Technologies, USA). Following this, Neurobasal-A medium supplemented with 1% kanamycin (Life Technologies, USA) was added to the cells, which were placed at 37°C in a humidified atmosphere containing 5% CO2. After 24 h, this medium, containing now the factors secreted by hMSCs (called conditioned medium (CM)) was collected and centrifuged at 1200 rpm for 10 min to remove any cell debris, and then stored at -80°C until it was required for further experiments.

3.1.2. Primary culture of Glial cells and preconditioning with hMSCs CM (e.g. secretome)

Glial cells were isolated from P5 newborn *Wistar Han* rats (Ethical consent for such procedure was approved by *Subcomissão de Ética para as Ciências da Vida e da Saúde (SECVS 142/2016)*). Briefly, cortices were isolated and cut into small pieces in dissociation medium (trypsin, ultrapure water with HBSS 10x, DNAse30) and incubated during 30min at 37˚C. To stop trypsin action, fetal bovine serum (FBS, Biochrom, Germany) was added followed by centrifugation; supernatant was discarded and the pellet resuspended with culture medium DMEM 10% FBS. The number of cells was counted as follows: a small volume of cells was diluted in Trypan Blue (Life Technologies, USA)

to access cells' viability. A density of 7x10^{6} cells was placed in T-75 flasks. Cells were kept in culture during 15 days (time necessary to reach confluence (Ribeiro et al., 2011)) and the growth medium was renewed every 2-3 days. After 15 days, growth medium was removed and the cells were washed twice PBS 1x and once with Neurobasal A medium (NbA; Life Technologies, USA). Then, in a first phase glial cells were conditioned by 1) MSCs secretome and 2) NbA during 24h (Fig. 5). After this 24h, the medium was collected and centrifuged at 1200 rpm for 10 min to remove any cell debris and stored at -80°C until it was required for further experiments. Still, in order to address the impact of a pre-conditioning process, glial cells were incubated during 24h with MSCs secretome, NbA and its growth medium (Fig. 5). After such pre-conditioning, mediums were removed and replaced by NbA for more 24h. After such period, the mediums were collected, centrifuged at 1200 rpm for 10 min and stored at -80°C until it was required for further experiments.

The conditions used were:

I. Neurobasal A
II. hMSCs secretome
III. DMEM

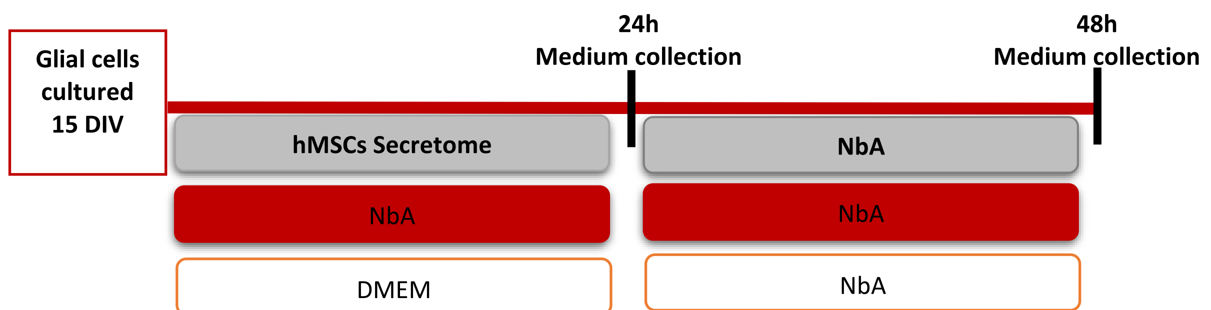


Figure 5: Schematic representation of the secretomes that were collected from Glial cells

3.1.3. Primary cultures of human neural progenitor cells (hNPCs) and incubation with hMSCs secretome and glial cells secretome

hNPCs were a kind gift from Prof. Leo A. Behie (University of Calgary, Canada). Cells were isolated from the telencephalon region of a 10 weeks post-conception fetus according with the protocols and strict ethical guidelines previously established and approved by the Conjoint Health Research Ethics Board (CHREB, University of Calgary, Canada; ID: E-18786) (Baghbaderani et al., 2010;

Mendez et al., 2002, 2005). hNPCs were thawed and the content placed in T-75 culture flasks containing 15 mL of serum-free medium PPRF-h2 (Baghbaderani et al., 2010). After 3 days, the cells were mechanically dissociated using a P1000 Pipetman set to 850µL (25-30 times) into a single cell suspension, being then cultured in fresh growth medium (PPRF-h2). Every 3 days, 40% of spent medium was replaced with fresh growth medium and the culture was maintained at 37°C in a humidified atmosphere containing 5% CO2. After 10-12 days of growth, hNPCs were centrifuged at 1000 rpm during 10 min and then enzymatically dissociated using Trypsin (1 mL) during 3 min at 37°C, being passed and placed on pre-coated (poly-D-lysine hydrobromide (100 µg/mL) and laminin (10 µg/mL) - Sigma, USA) 24-well plate at a density of 75×10^3 per well during 5 days with the 1) hMSCs secretome, 2) hMSCs secretome+glial cell secretome, 3) glial (pre-conditioned with hMSCs secretome) secretome, 4) glial cells secretomes at 37 °C, 5 % CO_2, 95 % air and 90 % relative humidity. Nba with 1% of kanamycin and a completed growth/differentiation medium were used as control groups.

3.1.4. *In vitro* immunostaining

After 5 days in culture, hNPCs were fixed in 4% paraformaldehyde (PFA, Merck, Portugal) for 30 min at room temperature (RT), to retain the antigenicity of the target molecules and preserve cells morphology. Cells were permeabilized in 1X PBS with 0.1% Triton X-100 (Sigma, USA) (PBS-T) for 5 min at RT and washed three times with 1X PBS. Blockage of non-specific binding sites was performed using 1X PBS with 10% Fetal calf serum (FCS; Biochrom, Germany) for 1h at RT. hNPCs were then incubated with the primary antibodies: Microtubule associated protein-2 -MAP 2 mouse (1:500; Sigma) and Doublecortin (DCX) – Rabbit (1:500; Abcam) diluted in 1X PBS with 10% FCS for 1h at RT, after which they were washed with 1X PBS with 0.5% FCS and incubated with the secondary antibodies: Alexa Fluor 488 - Goat anti-rabbit (1:1000; Life Technologies) and Alexa Fluor 594 - Goat anti-mouse (1:1000; Life Technologies) diluted in 1X PBS with 10% FCS for 1h at RT. Finally, the cells were incubated with the nuclear counterstain 4-6-diamidino-2-phenylindole-dhydrochloride (DAPI, 1:1000; Life Technologies, USA) for 10 min at RT. Samples were then observed under a fluorescence microscope (BX61, Olympus, Germany). For this purpose, six coverslips per condition and ten representative fields were chosen and analyzed to quantification. To normalize the data between the different sets, the results are presented in percentage of cells.

This was calculated by counting the positive cells for the respective staining markers, dividing this value by the total number of cells/field (DAPI-positive cells).

3.2. Stereotaxic surgeries

3.2.1. 6-OHDA lesions

All the experiments were performed after the consent from the Portuguese national authority for animal research, *Direcção Geral de Alimentação e Veterinária* (SECVS 142/2016), and conducted in accordance with the local regulations on animal care and experimentation (European Union Directive 2010/63/EU). Nine-weeks old Wistar-Han male rats (Charles River, Barcelona) were housed in pairs, in appropriate cages, under standard controlled conditions (12 h light/12 h dark cycles; RT at 22-24°C and 55% humidity; food and water ad libitum). Animals were handled for 1 week before the beginning of the injections to reduce the stress induced by the surgical procedures. For surgical procedures, animals were anesthetized with ketamine (Imalgene, Merial, USA)-medetomidine (Dorbene, Zoetis, Spain) [75 mg/kg; 0.5 mg/kg intraperitoneally (i.p)], placed on a stereotaxic frame (Stoelting, USA), and unilaterally injected using a 30-gauge needle Hamilton syringe (Hamilton, Switzerland), with either vehicle (Sham group, n=8) or 6-OHDA hydrochloride (Sigma, USA) (6-OHDA group, n=60) directly into the medial forebrain bundle (MFB) [coordinates related to Bregma: AP= -4.4 mm, ML= - 1.0 mm, DV= -7.8 mm; (Paxinos and Watson, 2007)]. At a rate of 1 μl/min, Sham animals received 2 μl of 0.2 mg/ml of ascorbic acid in 0.9% NaCl and the 6-OHDA animals were injected with 2 μl of 6-OHDA hydrochloride (4 μg/μl) with 0.2 mg/ml of ascorbic acid in 0.9% NaCl. After each injection the needle was left in place for 2 min in order to avoid any backflow up the needle tract. Behavioral assessment was performed three weeks after surgery.

3.2.2. Surgical treatment: Injection of hMSCs secretome and Glial cells secretome

Six weeks after the unilateral injection of 6-OHDA, animals were unilaterally injected into the striatum and SNc with hMSCs and glial cells secretome. As previously described for the surgical procedure, animals were anesthetized with ketamine-medetomidine [75 mg/kg; 0.5 mg/kg, i.p.] and placed on a stereotaxic frame, and unilaterally injected using a 30-gauge needle Hamilton syringe, with either vehicle (NbA medium: 6-OHDA control group; n=14), hMSCs secretome (n=15),

glial cells preconditioned with hMSCs secretome (n=15) or glial cells preconditioned with NbA (n=15) directly in the SNc (coordinates related to Bregma: AP= - 5.3mm, ML= -1.8 mm, DV=-7.4mm) and striatum (coordinates related to Bregma: AP= -1.3 mm, ML= 4.7 mm, DV= -4.5 mm; AP= -0.4 mm, ML= 4.3 mm, DV= -4.5 mm; AP= 0.4 mm, ML= 3.1 mm, DV= -4.5 mm; AP= 1.3 mm, ML= 2.7 mm; DV= -4.5 mm) (Paxinos and Watson, 2007). 6-OHDA-control group received 4 μl of NbA medium in the SNc and 2 μl in each coordinate of striatum at a rate of 1 μl/min. The secretomes-injected animals received 4 μl in the SNc and 2 μl in each coordinate of striatum at a rate of 1 μl/min. After each injection the needle was left in place for 2 min in order to avoid any backflow up the needle tract. At one week and four weeks following surgery, behavioral assessment was performed.

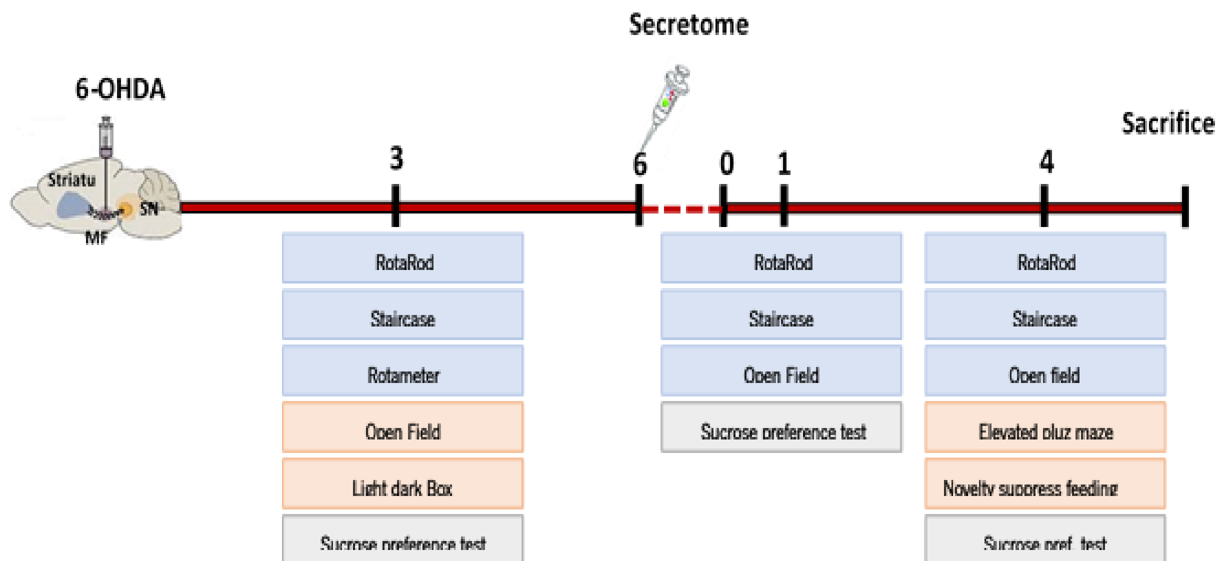


Figure 6: Experimental design. Parkinson's disease model was induced by an unilateral injection of a neurotoxin (6-OHDA) into the medial forebrain bundle (MFB). After three weeks, and in order to validate the PD model motor and non-motor behavioral tests were performed. After such validation, animals were then treated with MSCs and glial cells secretome, and at 1 and 4 weeks after treatment, animal behavior assessment was performed.

3.3. Behavioral assessment

3.3.1 Rotarod

To access to the motor coordination and balance of animals was used the TSE RotaRod System (catalog no. 3376-4R; TSE Systems, Chesterfield, MO, http://www.tse-systems.com) with 7 cm diameter drums (which are machined with grooves to improve grip) in order to get a stable performance. The first 3 days of testing was only to train the animals and the training consisted in 4 trials under an accelerating protocol starting at 4 rpm and reaching 40 rpm in 5 min. Animals were allowed to rest for at least 20 minutes between each trial. The testing day was on day 4, using the same protocol, the animal latency to fall was recorded.

3.3.2. Skilled paw reaching test (Staircase)

The skilled paw reaching test, usually named as staircase test, was assessed using double staircase boxes (80300, Campden Instruments Ltd., UK) as previously described (Montoya et al., 1991). This test provides the basic assessment of the independent forelimb use in skilled reaching and grasping test. Briefly, this test consists of a clear chamber with a hinged lid (285 x 90 x 60 mm). A narrow compartment, with a central platform running along its length, is connected to the chamber. The removable double staircase with 7 steps on each side can be inserted in the space between the platform and the box walls. Five pellets were placed into each well of the double staircase, so in total 35 pellets in each side. In the first day, the rats were familiarized with the test, with pellets available for 10 min. In the test session, animals were placed inside the box, having 15 min to reach, retrieve and eat the food pellets present on the steps. All sessions were performed at the same time of the day (namely by 7 consecutive days) and with food-restricted animals. After each test interval, animals were removed from the staircase boxes and the remaining (left over) pellets were counted.

3.3.3. Open field

The open field was used to teste anxiety and the locomotion of the animals (Leite-Almeida et al., 2009). To assess exploratory activity, the animals were placed in the center of the arena with transparent acrylic walls (ENV-515, Med Associates inc. USA) for 5 minutes with the aid of 16-beam infrared assays to automatically recorded animal movements. Total distance travelled and

number of ambulatory episodes were recorded using a tracking software (SOF-811, Med Associates).

3.3.4. Light dark box

The light dark box test (LDB) was used to measure anxiety-like behavior and was conducted in an arena with transparent acrylic walls and white floor (Med Associates Inc., St. Albans, VT, USA), with a black box over one half of the apparatus (Dark) (Arrant et al., 2013). Rats were placed in the center of the light box and their movement was monitored over a period of 10 min with the aid of two 16-beam infrared arrays. Time spent in the light part of the box was used as a measure of anxious behavior. Total distance traveled was used as an indicator of locomotor activity.

3.3.5. Novelty suppress feeding

Anxiety-like behavior was assessed though the Novelty suppress feeding (NSF) test (Bessa et al., 2009). Animals were food-deprived for 18h and then placed in an open-field arena for a maximum of 10 min, where a single food pellet was positioned in the center as previously described (Patricio et al., 2014). The latency to feed was used as an index of anxiety-like behavior. After reaching the pellet, animals were returned to the home cage and allowed to feed during 10 min. Thus, food consumption levels was used a measure of appetite drive.

3.3.6. Elevated plus maze

The elevated plus maze (EPM) paradigm was used to assess anxious-like behavior as a consequence of 6-OHDA lesion (Pêgo et al., 2006). The behavioral apparatus (ENV-560; Med Associates Inc., St. Albans, VT, USA) consisted of two opposite open arms (50.8 cm × 10.2 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm) elevated 72.4 cm above the floor and weakly illuminated. Animals were individually placed in the center of the maze and allowed to freely explore it during 5 min. Each trial was video-recorded and the percentage of time spent in the open arms was measured using EthoVision XT 11.5 tracking system (Ethovision, Noldus Information Technologies, Wageningen, The Netherlands) as an index of anxiety-like behavior.

3.3.7. Sucrose preference test

Anhedonia was assessed by the sucrose preference test (SPT) at several timepoints throughout the experimental protocol (Bessa et al., 2009).To test sucrose preference, animals were food and water deprived for 12 h and then presented with two pre-weighed bottles containing 2% sucrose solution or tap water for a period of 1 h (starting at the beginning of the dark period). Sucrose preference (SP) was calculated according to the formula: SP = (sucrose intake / (sucrose intake + water intake)) x 100. Baseline SP value was assessed before the model induction (6-OHDA injections). Anhedonia was defined as a reduction in SP relative to baseline levels.

3.3.8. Forced swim test

Depressive-like behavior was assessed at the end of all the experiments by using the force swimming test (FST) (Bessa et al., 2009). To do so, test trials were conducted 24 h after a 5-min pretest session. Rats were individually placed in transparent cylinders (62 cm height and 25.4 cm diameter) filled with water (25°C; 50 cm depth) for 5 min. Trials were video-recorded and the total time animals spent immobile (immobility time) was measured using the EthoVision XT 11.5 tracking system (Noldus Information Technology). An increase in immobility time was taken as a measure of depressive-like behavior. Behavioral despair was defined as an increase in the immobility time.

3.3.9. Apomorphine turning behavior (Rotameter)

In order to test apomorphine-induced turning behavior, animals' necks were subcutaneously injected with a solution of 0.05 mg/kg apomorphine hydrochloride (Sigma, USA) dissolved in 1% of ascorbic acid in 0.9% of NaCl, and then placed on metal testing bowls (MED-RSS, Med Associates) for 45 min. After this, the number of contralateral rotations was digitally recorded, which allowed assessment of the effects of the injection vehicle (0.2 mg/mL of ascorbic acid in 0.9% of NaCl) and 6-OHDA (with 0.2 mg/mL of ascorbic acid in 0.9% of NaCl). This test was just used for the validation of the model (after 6-OHDA injections) as previously described by our lab (Teixeira FG et al. 2017). As apomorphine is a strong dopamine agonist, its repeated use could lead to an overstimulation of the dopaminergic system, which could impair the adequate interpretation of the impact of hMSC secretome on the functional outcomes of the animals (Bibbiani et al., 2005; Poewe and Wenning,2000; Trenkwalder et al., 2015).

3.4. Histological procedures

After 13 weeks (including the development of the lesion and its consequent treatment) animals were sacrificed with sodium pentobarbital (Eutasil, 60 mg/kg i.p., Ceva Saúde Animal, Portugal), and transcardially perfused with 4% paraformaldehyde (Merck, Lisbon, Portugal) in 0.1 M of PBS. Afterward, the brains were stored in 30% sucrose solution with 0.1% azide before going to histological processing.

3.4.1 Tyrosine hydroxylase immunohistochemistry

Striatal and mesencephalon coronal sections (including SNc) with a thickness of 50 μm were obtained using a vibratome (VT1000S, Leica, Germany). Five series of consecutive slices were obtained and and one was processed as free-floating TH-immunohistochemistry. First, the slices were immersed for 20 min into PBS with 3% of H_2O_2 for the inhibition of endogenous peroxidase activity. Then, the slices were followed by blocking 2 h with 5% fetal calf serum (FCS, Life Technologies) in PBS. After this, slices were incubated overnight (at 4°C) with rabbit TH primary antibody diluted in PBS 1X with 2% FCS (TH, 1:2000, Millipore, USA). After this period, the slices were washed with 0.1% PBS-T (three times for 10 min), and followed by incubation for 30 min with a biotinylated secondary anti-rabbit antibody (LabVision, USA), and another 30 min incubation with an Avidine/Biotine complex (LabVision). The antigen visualization was performed using 3,3í-diaminobenzidine tetrahydrocloride (DAB, Sigma, USA) (25 mg of DAB in 50 mL of Tris-HCL 0.05 M, pH 7.6 with 12.5 μL of H_2O_2) and stopped at the desired time. Then, slices were mounted on superfrost slices and thionin counter-coloration was performed.

3.4.2 Stereological analysis

To assure a representative sampling between all the animals, four identical TH-labeled slices covering the entire mesenphalon were selected, including all the portions of the SNc. Using a brightfield microscope (BX51, Olympus, Japan) equipped with a digital camera (PixeLINK PL-A622, CANIMPEX Enterprises Ltd., Canada), and with the help of Visiopharm integrator system software (V2.12.3.0, Denmark), the boundaries of SNc area was drawn. The delineation of this region was performed through identification of anatomic standard reference points and with the help of the rat brain atlas (Paxinos and Watson, 2007). Counting of total TH-immunopositive cells in the SNc area

was performed on both hemispheres (40 x magnification), and the data were presented as the percentage (%) of remaining TH+ cells in the lesioned side compared to the control side. All the counting and analysis was performed under blind conditions

3.4.3 Striatal fiber density measurement

Total immunoreactivity of TH-positive fibers was measured by densitometry as described by Febbraro et al. (Febbraro et al., 2013). To do so, TH-immunostained striatal sections (four sections per animal) representing the coordinates of injection sites (within the striatum) were selected and photographed (1 x magnification) under brightfield illumination (SZX16, Olympus, Japan) fitted with a DP-71 digital camera (Olympus, 32 Japan). All image analysis was completed using the ImageJ software (ImageJ v1.48, National Institute of Health, USA). Although this method provided a gross estimation of Parkinsonian pathology on lesioned side, micrographs were converted to grey scale and analyzed for grey intensity after calibrating ImageJ program. This was done using the "optical density step tablet" to determine the optical density (O.D.) of the selected sections and performed according to program instructions. From here, striatum O.D. values were determined in both hemispheres using a 1.0 mm^2 rectangular grid, encompassing injection sites, as determined by anatomical references and rat brain atlas (Paxinos and Watson, 2007). Corpus callosum (internal control) O.D. was also measured in both hemisphere sides, to avoid nonspecific background. TH striatal fiber densities were determined by calculating the O.D. difference between the lesioned side and the corpus callosum, as well as, between the intact striatum and corpus callosum. The extent of the immunostaining on lesioned side was expressed as a percentage of the intact side (contralateral striatum).

3.5 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics ver.22 (IBM Co., USA) and graph's representation using GraphPad Prism ver.6 (GraphPad Software, La Jolla, USA). To perform the evaluation of the in vitro assay a one-way ANOVA was used in order to compare the mean values for the seven groups. Statistical evaluation for animal behavior tests after 6-OHDA injections was performed using an independent sample t-test, and repeated measures ANOVA if an evaluation along time was desired. After treatments, the behavior and histological data was analyzed using one-way ANOVA to compare the mean values for the five groups. To evaluate along, a mixed design

factorial ANOVA was performed. Normality was measured using the Kolmogorov-Smirnov and Shapiro-Wilk statistical tests and taking into account the respective histograms and measures of skewness and kurtosis. Equality of variances and Sphericity were measured using the Levene's and Mauchly's tests, respectively, and was assumed when $p>0.05$. Multiple comparisons between groups were accomplished through the Bonferroni statistical test. Values were accepted as significant if the p-value was higher than 0.05 and all results were expressed as group mean±SEM (standard error of the mean). Effect size was calculated using the Cohen's d or $\eta 2$partial.

CHAPTER 4

RESULTS

4 RESULTS

4. *In vitro* assay

3 Neuronal differentiation of hNPCs induced by MSCs and Glial cells conditioned medium

As previously described by our group, hNPCs grow as neurospheres in PPRF-h2 serum-free growth medium (Teixeira et al., 2015). However, when this medium is removed and replaced by MSCs and Glial cells secretomes, hNPCs lose its neurosphere-like form, adhere and start to differentiate.

Immunohistochemistry analysis revealed that when hNPCs were incubated for 5 days with MSCs' and glial cells' secretomes, there was a clear increase in DCX (immature neurons, $F_{(6,223)}$=22.0, p=0.0001; Fig.7I) and MAP-2 positive cells (mature neurons; $F_{(6,223)}$=25.20, p=0.0182) when compared to the control (incubation with Neurobasal A medium; Fig.1H). Additionally, it was possible to observe that MSCs and glial cells secretome induce a trend in increasing neuronal differentiation of hNPCs.

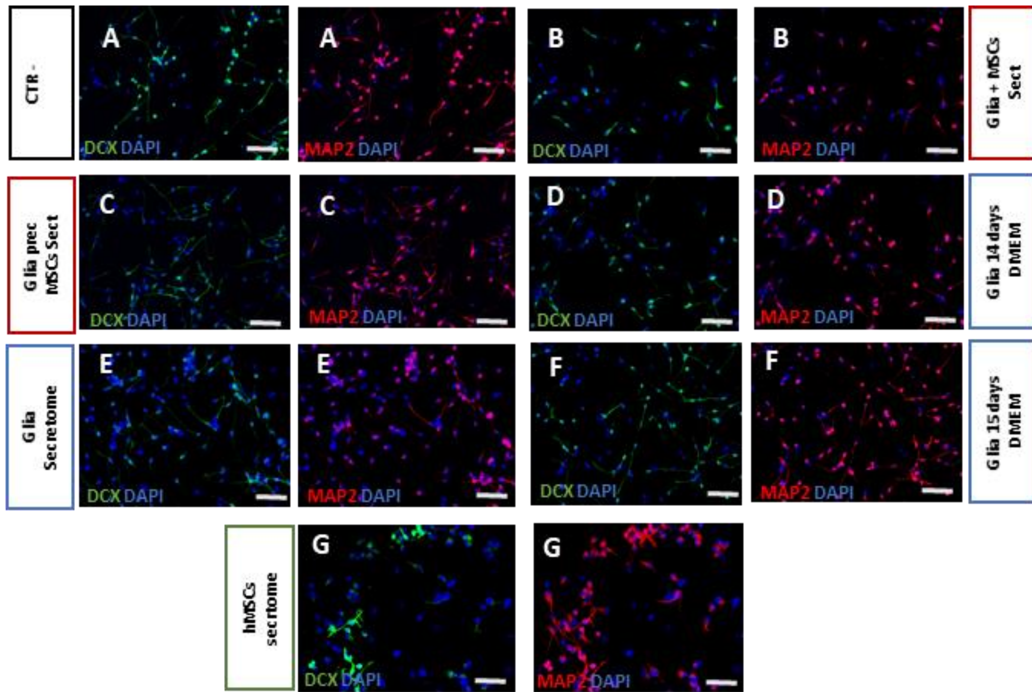
In addition, MSCs secretome (Fig7G) induced higher rates of neuronal differentiation into MAP-2-positive cells (p<0.0001), in comparison to the negative control. Concerning glial cells secretome (preconditioned with MSCs secretomes or the growth medium DMEM), we have also verified distinct effects of such: glial cells (secretome) plus MSCs secretomes (Fig. 7B) comparatively to the control group (Fig.7A) induced higher rates of neuronal differentiation into MAP-2 (p<0.0001) and DCX (p=0.0001) (Fig.7H,I); this increase in neuronal differentiation was also significantly different from glial cells 14 Days DMEM to DCX-positive cells, p=0.0047 (Fig.7D,I), although not significant for Glial cells 15 Days DMEM (MAP-2, p=0.2411; DCX, p=0.9985) (Fig.7F,I) and for MSCs secretomes (MAP-2, p=0.9884; DCX, p=0.0548) (Fig.7G,H,I). Similar effects were observed in glial cells (preconditioned with MSCs secretome) secretome, where it was possible to observe a significant increase in the densities of MAP-2-positive cells (p<0.0001) and DCX (p=0.0001) (Fig.7C,H,I) when compared to the control group.

Moreover, glial cells (preconditioned with MSCs CM) secretome significantly induced the neuronal differentiation when compared to the glial cells plus MSCs CM (MAP-2, p<0.0001; DCX, p<0.0001) (Fig.7C,B,H,I) glial 14 days DMEM (MAP-2, p<0.0001) (Fig.7C,D,H), glial 15 days DMEM (MAP-2,

p<0.0001); DCX, p=0.0077) (Fig.7C,F,H,I), and MSCs secretome (MAP-2, p<0.0001; DCX, p<0.0001) (Fig.7C, G,H,I), but not to glial cells secretome (MAP-2, p=0.0561; DCX, p=0.9835)(Fig.7C,E,H,I). Concerning glial cells 14 Days DMEM secretome, this was able to induce neuronal differentiation (MAP-2, p<0.0001; DCX, p=0.0001) when compared to the control group (Fig.7A,D,H,I), however, no significant differences were found when compared to the glial cells secretome (MAP-2, p=0.2214; DCX, p=0.9999) (Fig.7D,E,H,I) glial cells 15 days DMEM (MAP-2, p=0.3639; DCX, p=0.0863) (Fig.7D,F,H,I) and MSC secretomes (to MAP-2 positive cells, p=0.9955) (Fig.7D,G,H,I).

Regarding glial cells secretome (Fig.7E), it was also able to induce higher rates of neuronal differentiation (MAP-2, p<0.0001; DCX, p<0.0001) when compared to the control group (Fig.7A,E,H,I). Compared to the other secretome groups, glial cells secretome was statistical different from glial cells 15 Days DMEM (secretome) (MAP-2, p=0.0002; DCX, p=0.0467) (Fig.7E,F,H,I) and from MSCs secretome (to DCX-positive cells, p<0.0001)(Fig.7E,G,I). Finally, glial cells 15 Days DMEM (secretome) (Fig.7F) has also induced a significant level of neuronal differentiation (MAP-2, p=0.0014; DCX, p=0.0001) when compared to the control group (Fig.7A,F,H,I). This group was also statistically different to MSCs secretome, concerning DCX-positive cells (p=0.0358) (Fig.7F,G,I).

In summary, all the secretomes increased the number of MAP-2- and DCX-positive cells in comparison to control conditions, albeit the effect was different in magnitude. Glial cells (preconditioned with MSCs CM) secretome was the condition that elicited the highest increase of MAP-2- and DCX-positive cells.



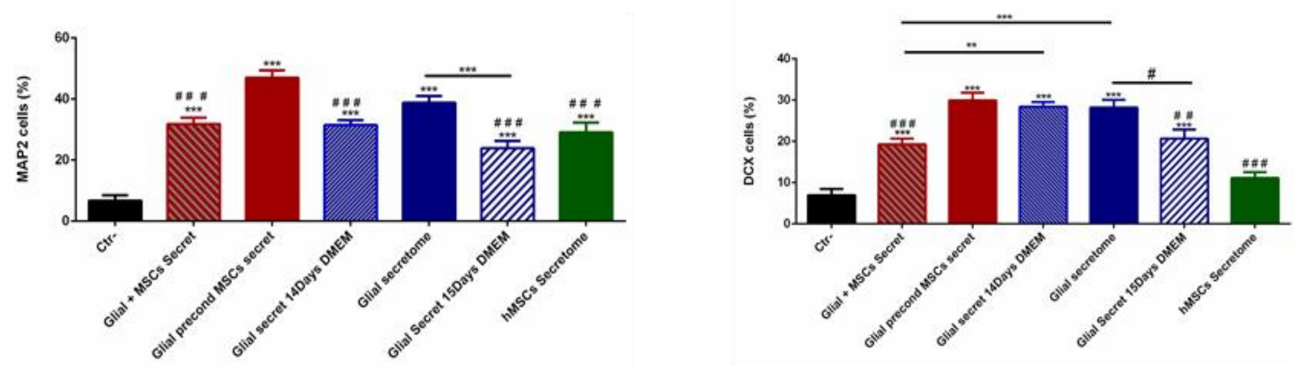


Figure 7 – Impact of different cells secretome (in the form of conditioned medium) on the differentiation of hNPCs. Glial cells preconditioned with MSCs secretome was able to significantly induce the differentiation of hNPCs into MAP-2 positive cells and DCX positive cells (C,H,I). In parallel, the same effect was found in the secretome of glial cells secretome (E,H,I). Data presented as mean±SEM. n=3. *p<0.05, ***p<0.001. Control group statistically different from all the other groups, *; Glial cells precond MSCs secretome statistically different from all the other groups, # (scale bar: 50μm).

4.2 *In vivo* assay

4.1. Phenotypic characterization of 6-OHDA lesioned animals

We used a model of 6-OHDA lesion to mimic loss of dopaminergic neurons observed in PD, as previously described by our team and others ((Carvalho et al., 2013; Deumens et al., 2002; Teixeira et al., 2016). We have evaluated these animals in terms of motor function but also at other dimensions of PD such as emotional behaviors.

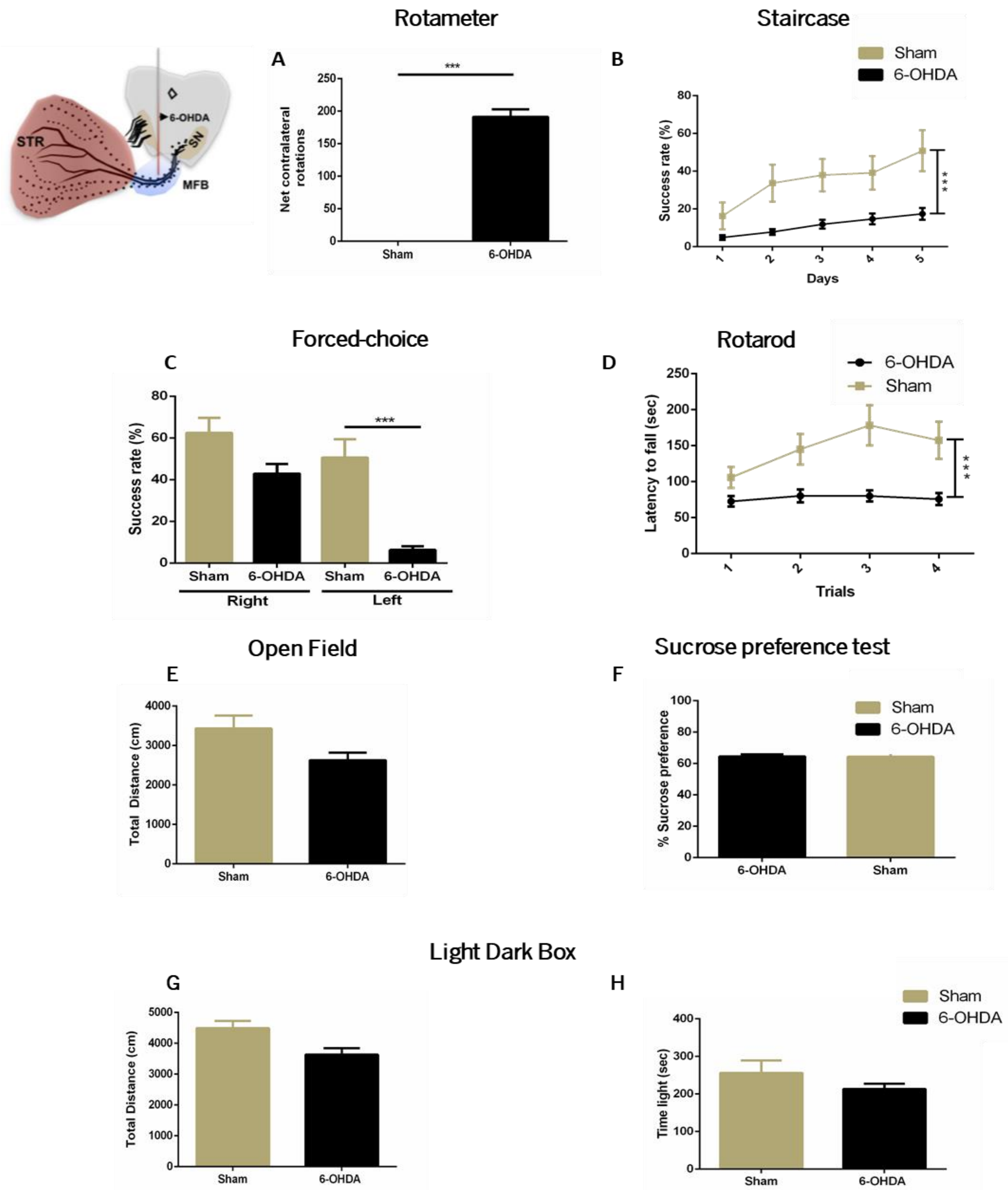
In order to dissect the functional integrity of DAn system upon injection of 6-OHDA, the apomorphine-induced turning test was performed at the end of the other behavioral tests (rotarod and staircase). Three weeks after the injection of 6-OHDA, we observed that there was a significantly higher number of apomorphine-induced turning rotations in the 6-OHDA-injected animals when compared to the sham group (Fig. 8A; $p<0.0001$).

In the staircase test, used to assess the forelimb and skilled motor function, 6-OHDA injected animals were found to be clearly affected when compared to the control group (Sham, Fig.8B; $F_{(4,176)}=18.567$, $p<0.0001$, $\eta^2_{partial}=0.297$). In a staircase forced-choice task, where the animals are forced to choose one of the steps side, 6-OHDA-injected animals were found to be significantly impaired in the contralateral lesioned side (left) (Fig. 8C; $t_{(44)}=8.181$, $p<0.0001$) when compared to the sham group, as anticipated. In the right side, this effect was relatively diluted, although there is a trend for this side to be affected as well (Fig. 8C; $t_{(44)}=1.734$, $p=0.0899$).

Regarding motor coordination and balance, measured by the rotarod test, 6-OHDA-injected animals showed a clear impairment (Fig. 8D; $p<0.0001$) when compared to the sham group. Still, in the Open Field test, also used to assessed to the motor activity of the animals, we have found a trend for a slight impairment in the freely moving performance of 6-OHDA-injected animals when compared to the Sham group (Fig. 8E; $t_{(44)}=1.903$, $p=0.0636$).

In addition,, we have also analyzed non-motor PD dimensions, namely anxious-like behavior and anhedonic-like behavior through the light dark box test (LDB) and sucrose preference test (SPT), respectively. Regarding SPT, no differences were found between 6-OHDA injected animals and sham group (Fig.8F, $t_{(44)}=0.8835$, $p=0.3818$). Concerning LDB, statistical analyses shown that

although not significant, 6-OHDA-injected animals presented a trend to travel relatively less than control group (Sham) (Fig. 8G, $t_{(44)}$=1.849, p=0.0713). Concerning the time that animals spent in the dark side, no differences were observed between the two groups (Fig. 2G; $t_{(44)}$=1.411,



p=0.1654).

Figure 8: Behavioral characterization of 6-OHDA-lesioned animal model. (A) Apomorphine-induced turning behavior (rotameter) revealed that 6-OHDA-injected animals exhibited intense turning behavior when compared to Sham group. 6-OHDA-injected animals also presented significant impairment in motor coordination on the (B,C) the paw-reaching test performance and in (D) rotarod , and in a lesser extent in the Open Field (E), where a positive trend in the distance traveled was found. The lesioned animals also exhibited a tendency to develop an anxious-like phenotype (G, H) but not anhedonic-like behavior (F). Sham: n=8, 6-OHDA: n=38. Data presented as mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.2.2 Transplantation of MSCs and Glial cells secretome modulates 6-OHDA-lesioned animal behavioral performance

After confirming the behavioral deficits of 6-OHDA-injected animals, we aimed to evaluate the effects of MSCs and glial cells (preconditioned) secretome in the motor and non-motor performance. So, we injected these secretomes in the striatum and SNc, evaluating motor and non-motor behavioral performance at 1 and 4 weeks after treatment by using the rotarod, staircase, Open Field, Elevated plus-maze, Novelty suppressed feeding and Forced-swimming test. The selection of the tests was based on the fact that some of the previous tests cannot be repeated, so we had to find alternative measures for that behavioral dimensions.

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4.2.3 Rotarod test

ANOVA analysis showed a significant effect for treatment and time, but no interaction between these factors (Table 1). Further comparison shown that lesioned animals treated with MSCs secretome had a significant improvement in their motor balance when compared to the control group at 1W and 4W (6-OHDA+NbA; p=0.021, p=042 respectively; Fig. 9). Such effect was also observed with the application of glial secretome, at 1W (p=0.014). Sham was different for all groups (p<0.001).

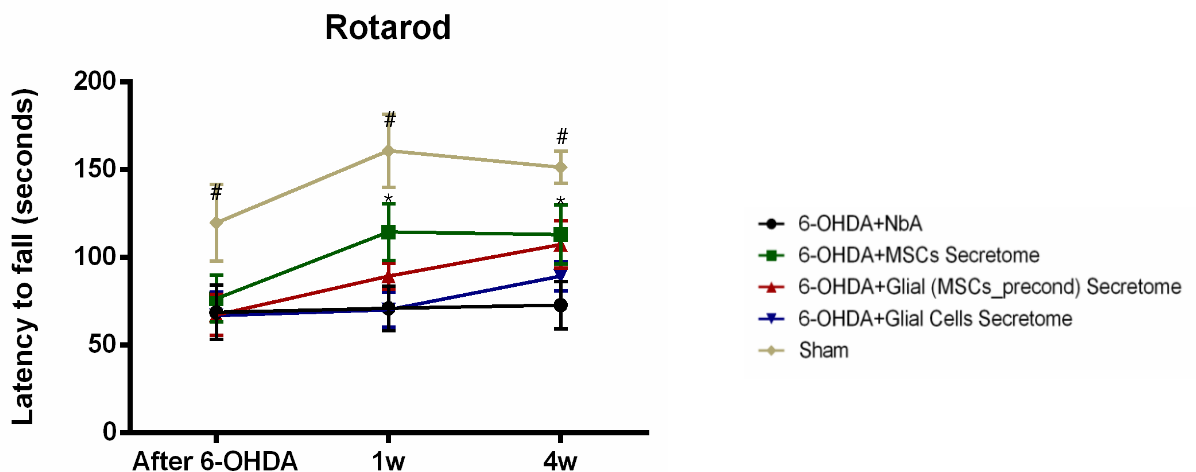

Figure 9: Motor coordination performance 1 and 4weeks after the transplantation of different conditioned mediums in the SNpc and striatum of 6-OHDA-lesioned animals. Latency to fall was measured in the accelerating rotarod test, demonstrating that the MSCs CM-injected animals had a significant improvement in their motor coordination when compared with non-treated group. Sham n=7; 6-OHDA control: n=10, MSCs: n=12, Glia cells precond MSCs secretome: n=13; Glial cells secretome: n=12. Data presented as mean±SEM. MSCs animals statistical differences, *p<0.05; Sham animals statistically different from all the other groups, #p<0.001.

Table 1: Statistical analysis of the rotarod test after treatments (Data presented as mean±SEM)

Group	After lesion	1 week	4 weeks	Statistical test, significance, effect size
6-OHDA control	68.73±15.55	71.02±13.45	72.86±14.28	Treatment effect: $F_{(4,49)}$ =351.403, p=0.0001, $\eta^2_{partial}$=0,878 Time effect: $F_{(2,98)}$ =6.012, p=0.003, $\eta^2_{partial}$=0,109 F Interaction time-group: $F_{(8,98)}$ =1.251, p=0.278, $\eta^2_{partial}$=0,093
MSCs CM	85.96±14.19	114.59±12.32	113.15±13.04	
Glia (precon) CM	67.31±13.64	89.37±11.84	107.39±12.53	
Glia CM	66.88±14.19	70.35±12.32	89.5±13.04	
Sham	119.83±18.58	160.88±16.13	129.63±17.07	

4.2.4 Staircase test

To assesses the forelimb fine motor coordination, the skilled paw reaching test (also known as staircase) was performed. Statistical analysis demonstrated a significant effect for treatment and time, but no interaction of these two factors (Table 2, Fig.10). Concerning the effects of secretome injections, although a trend was observed to the MSCs secretome-injected animals when compared to the untreated group (6-OHDA), post-hoc analysis revealed that it was not significant (p= 0.279). Moreover, it was also possible to observe that MSCs CM presented a trend to ameliorate the performance when compared to the glial cells secretome (p=0.193) and glial cells (preconditioned with MSCs secretome) secretome (p=0.125) (Fig.10A).

Regarding the forced-choice task, in which the animals were forced to choose one of the steps side of the double staircases, statistical analysis revealed an effect for treatment but not for time nor interaction (Table 3, Fig.10). Relatively to the left side (the affected side), animals injected with MSCs secretome displayed a slightly positive trend on the success rate of eaten pellets when compared to the untreated 6-OHDA group (p=0.063) (Fig. 10B). A similar effect was also observed

when we compared MSCs secretome treatment with the glial cells (preconditioned with MSCs secretome) secretome (p=0.093) and, into a lesser extent, with the glial cells secretome (p=0.185). Concerning the forced-choice at the right side, statistical analysis revealed an effect for treatment and for time, but no interaction between these factors (Table 3, Fig.10C).

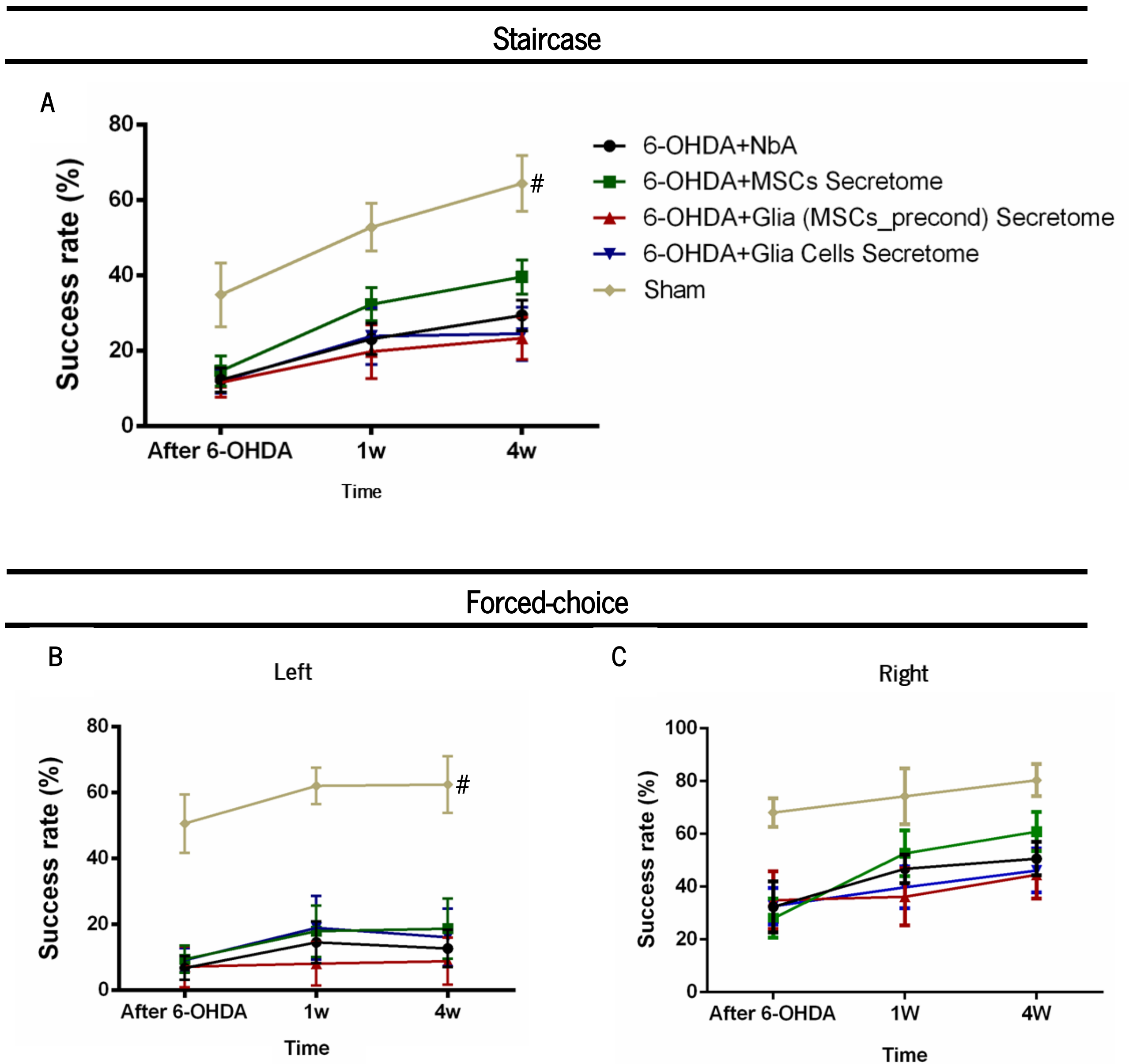

Figure 10: Impact of different secretomes in fine motor performance at 1 and 4 weeks after treatment. (A) Paw reaching performance of rats (through staircase test) revealed a slightly improvement (although not significant) of the forelimb coordination of the MSCs secretome-injected animals comparatively to non-treated group. (B, C) When animals were submitted to the paw reaching forced performance task, the group injected with MSCs CM displayed a positive trend in skilled motor performance when relatively to the untreated group 6-OHDA regarding the left side (the affected side). Performance of rats is expressed as success rate of eaten pellets. Sham n=7; 6-OHDA control: n=11, MSCs: n=11, Glia cells (precond MSCs

secretome) secretome: n=10; Glial cells secretome: n=11. Data presented as mean ± SEM. Sham animals statistically different from all the other groups, #p<0.001.

Table 2. Statistical analysis of the staircase test after treatments (Data presented as mean±SEM)

Group	After lesion	1 week	4 weeks	Statistical test, significance, effect size
6-OHDA control	12.27±3.18	23.38±4.14	29.42±4.07	Treatment effect: $F_{(4,45)}$ =5.728, p<0.0001, $\eta^2_{partial}$=0,776 Time effect: $F_{(1.62,72.77)}$ =60.55, p<0.0001, $\eta^2_{partial}$=0,574 Interaction time-group: $F_{(6.47,72.77)}$ =1.867, p=0.093, $\eta^2_{partial}$=0,147
MSCs CM	14.70±4.00	32.38±4.43	39.61±4.54	
Glia (precon) CM	11.62±32.82	19.81±7.16	23.34±5.65	
Glia CM	11.95±3.20	23.84±7.52	24.55±7.10	
Sham	34.88±8.50	52.86±6.30	64.46±7.43	

Table 3. Statistical analysis of the forced choice task for the left side after treatments (Data presented as mean±SEM)

Group	After lesion	1 week	4 weeks	Statistical test, significance, effect size
6-OHDA control	6.75±3.58	14.54±6.32	12.73±5.69	Treatment effect: $F_{(4,45)}$ =6.339, p<0.0001, $\eta^2_{partial}$=0,522 Time effect: $F_{(2,90)}$ =2.790, p=0.067, $\eta^2_{partial}$=0,058 Interaction time-group: $F_{(8,90)}$ =0.580, p=0.792, $\eta^2_{partial}$=0,049
MSCs CM	16.62±8.11	17.95±7.82	18.71±9.10	
Glia (precon) CM	7.14±6.22	8.09±6.62	8.86±7.16	
Glia CM	16.63±8.18	18.96±9.70	16.10±8.71	
Sham	50.61±8.56	62.04±5.57	62.45±8.62	

Table 4. Statistical analysis of the forced choice task for the right side after treatments (Data presented as mean±SEM)

Group	After lesion	1 week	4 weeks	Statistical test, significance, effect size
6-OHDA control	35.06±8.29	45.20±5.85	45.59±5.50	Treatment effect: $F_{(4,45)}$ =8,268, p<0.0001, $\eta^2_{partial}$=0,948 Time effect: $F_{(1.60,72.18)}$ =7.956, p=0.002, $\eta^2_{partial}$=0,150 Interaction time-group: $F_{(6.42,72.18)}$ =0.623, p=0.722, $\eta^2_{partial}$=0,052
MSCs CM	37.40±6.63	49.74±6.89	68.83±6.52	
Glia (precon) CM	39.14±7.12	22.87±8.21	55.14±7.10	

Glia CM	38.18±7.57	41.95±5.55	54.55±7.21
Sham	62.45±7.28	74.28±10.60	80.40±6.10

4.2.5 Open Field

To assess exploratory activity, animals were placed in the Open Field arena. One week after treatment no differences were found between groups ($F_{(4,47)}$ =0.8018, p=0.5302, $\eta^2_{partial}$=0,06388; Fig. 11A). Following the same tendency, 4 weeks after treatment no differences were also observed in the freely moving performance of the animals ($F_{(4,47)}$ =0.4930, p=0.7408, $\eta^2_{partial}$=0,04027; Fig. 11B).

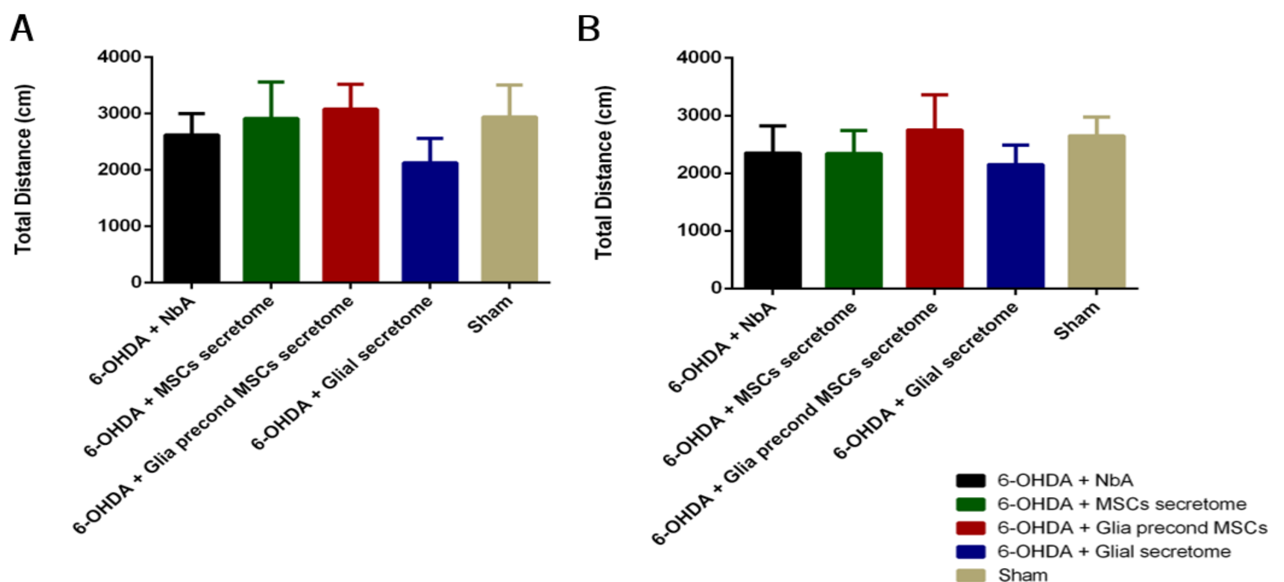

Figure 11: Impact of different secretomes in exploratory activity at 1 and 4 weeks after treatment. Both at (A) 1week and (B) 4weeks after treatment there was no alteration on total distance traveled on the Open Field arena. Sham n=8; 6-OHDA control: n=10, MSCs: n=11, Glia cells precond MSCs secretome: n=11; Glial cells secretome: n=12. Data presented as mean±SEM.

4.2.6 Novelty suppressed feeding

To assess anxious-like behavior, we performed the novelty suppressed feeding (NSF) test. Statistical analysis revealed an effect for treatment ($F_{(4,46)}$ =3.627, p=0.012, $\eta^2_{partial}$=0,240). P*ost-hoc* analysis shown that animals injected with glial cells secretome attenuated the anxious-like behavior of 6-OHDA animals, since these animals behave similarly as sham group (control group; p=0.096). On the other hand, both MSCs secretome (p=0.028) and glia (preconditioned with MSCs secretome)

cells secretome (p=0.006) animals presented anxious-like behavior when compared to the sham group. The same effects were also found when MSCs secretome (p=0.028) and glia cells (preconditioned with MSCs secretome) secretome (p=0.004) were compared to glial cells secretome (Fig. 12A). After the test, animals were left in a box with free access to food, being food intake used as a measure of motivation to eat, from which no differences were observed between groups ($F_{(4,46)}$ =1.160, p=0.341) (Fig. 12B).

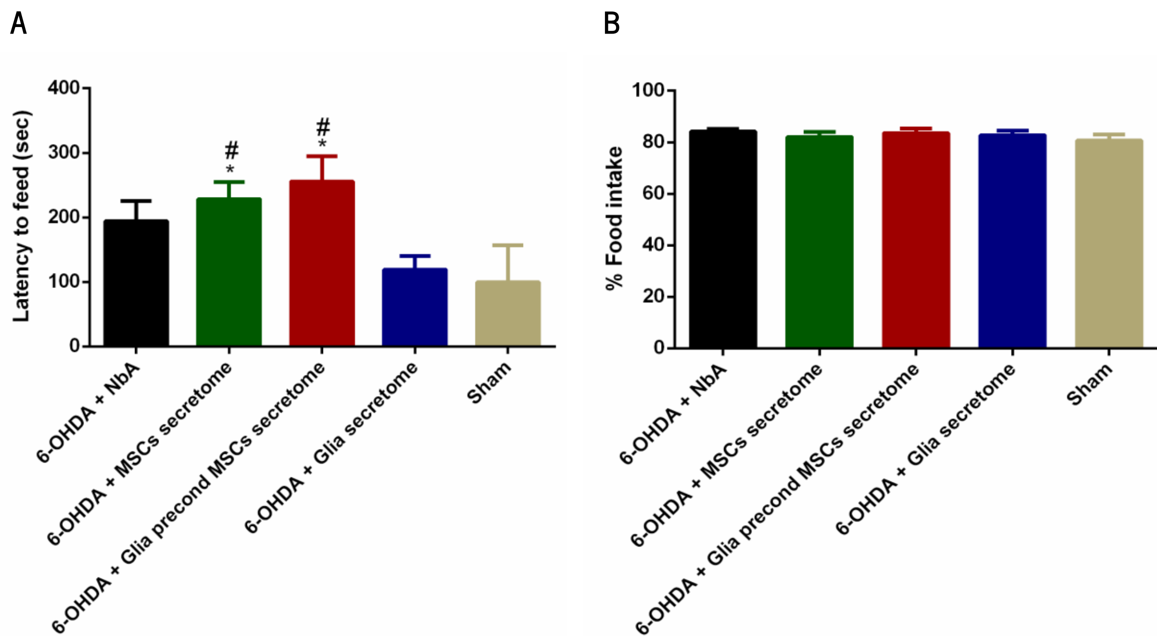

Figure 12- Impact of different secretomes in anxious behavior at 1 and 4 weeks after treatment. Upon 4 weeks of treatment surgeries, the anxiety-like behavior was determined by the NSF. 6-OHDA enhances latency to feed in all lesioned groups except the group treated with glia secretome, which is similar as sham group. Number of animals: Sham n=6; 6-OHDA control: n=11, MSCs: n=10, Glia cells precond MSCs secretome: n=13, Glial cells secretome: n=11. Data presented as mean±SEM.*Refers to differences with glial secretome; # Refers to differences with Sham group

4.2.7 Elevated plus-maze

To further analyze anxious-like behavior, we also used the Elevated plus-maze test (EPM). From the results, statistical analysis revealed an effect for treatment ($F_{(4,49)}$ =7,675, p<0.0001). Through the time that the animals spent on the open arms, it was possible to observed that the treatment with glial cells secretome was able to partially revert the anxious phenotype of 6-OHDA animals when compared to the glial cells (preconditioned with MSCs secretome) secretome (p=0.044; Fig. 13A), further confirming the data in the NSF.

Regarding the number of entries on the open arms, all groups revealed a smaller number of entries in the open arms when compared to the control group (sham; p<0.0001; Fig. 13B).

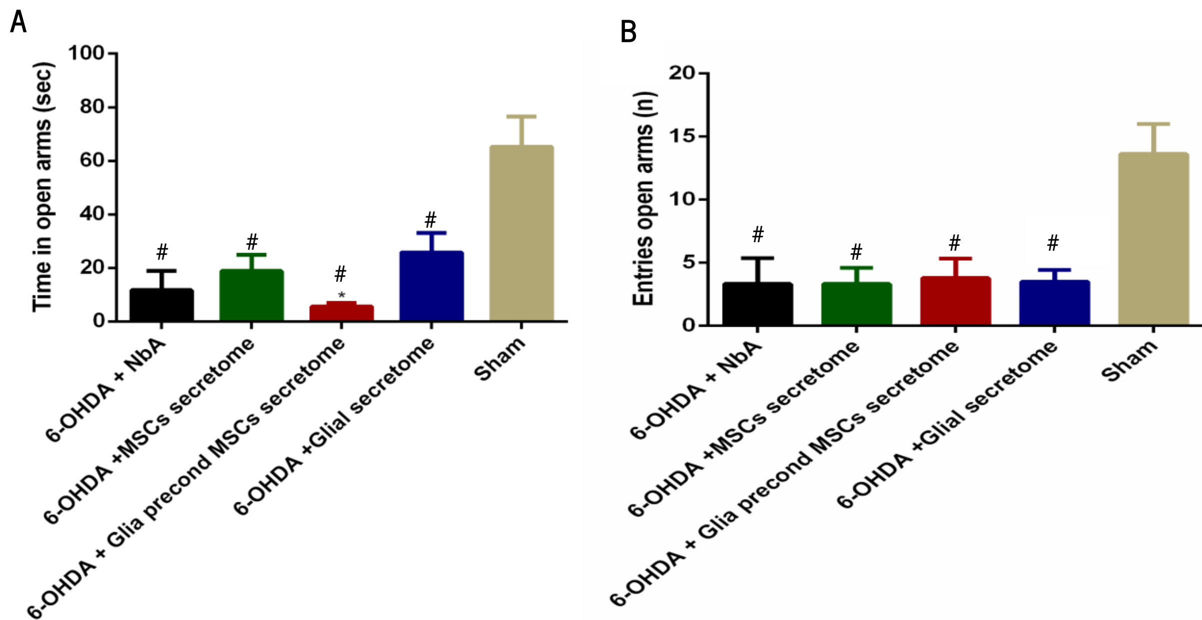

Figure 13– Glial cells secretome attenuated the anxious phenotype of 6-OHDA-lesioned animals. Anxious-like behavior was assessed after 4 weeks of treatment surgeries. Injection with glial secretome revealed an improvement in anxious behavior given by time spent in open arms (A), but had no effect in the number of entries in the open arms which is still smaller than sham (B). Number of animals: Sham n=8; 6-OHDA control: n=10, MSCs: n=11, Glia cells precond MSCs secretome: n=12, Glial cells secretome: n=13. Data presented as mean±SEM. #p<0.0001,Sham animals statistically different from all the other groups,* Differences for glial CM

4.2.8 Sucrose Preference test

Anhedonic-like behavior, a key feature of depressive-like behavior, was tested by using the sucrose preference test (SPT). This test was assessed at 1week (A) and 4 weeks (B) after secretome injections. We verified that one week after secretome injections, it was possible to observe an effect for treatment ($F_{(4,47)}$=11.56; p=0.0001, $\eta^2_{partial}$=0,4959; Fig.14). Surprisingly, no differences were observed between the untreated group (6-OHDA+NbA) and the control group (Sham; p=0.9708; Fig. 6A). However, animals injected with MSCs secretome seems to present an anhedonic-like behavior when compared to the control group (Sham; p=0.0001), to glial cells secretome (p=0.0002), to glia (precond) CM (p=0.0406) and even when compared to the untreated group (6-

OHDA; p=0.0001) (Fig. 6A). We also observed that the preconditioning of glial cells with MSCs secretome presented an anhedonic-like behavior when compared to the untreated group 6-OHDA (p= 0.0283) and control group (sham; p=0.0280), but not with glia secretomes (p=0.3237). After 4 weeks later, the above-mentioned results were diluted, as no differences were observed between groups ($F_{(4,47)}=1.953$, $p=0.1425$, $\eta^2_{partial}=0,1172$).

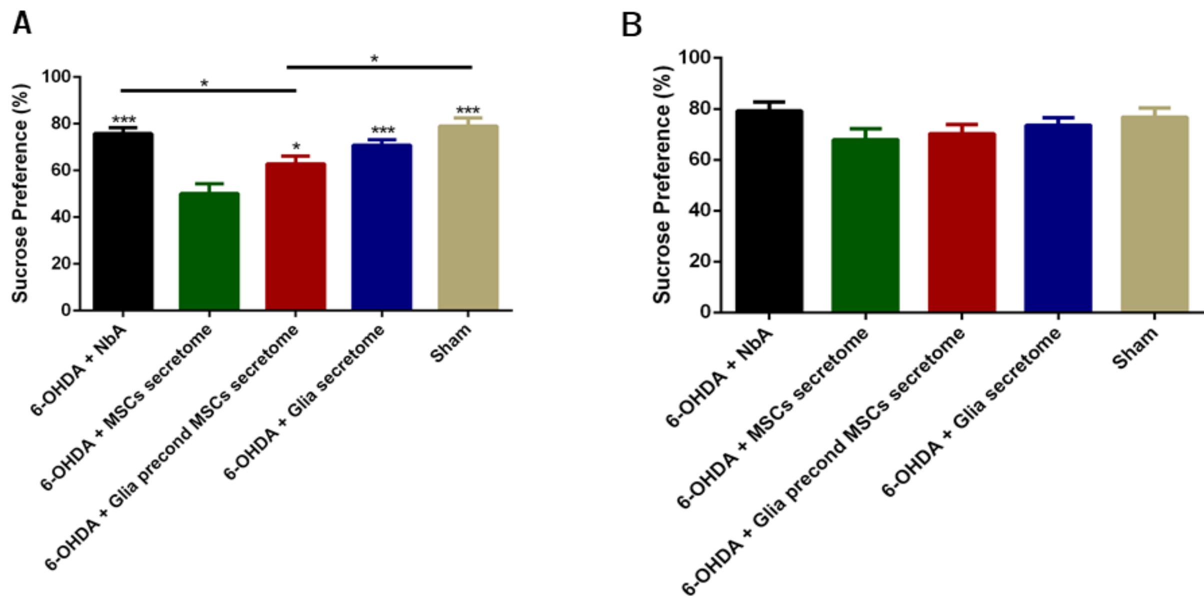

Figure 14: Impact of different secretomes on anhedonic-like behavior of 6-OHDA-lesioned animals. After 1W (A) upon treatment surgery, MSCs seretome seems to develop an anhedonic-like behavior comparatively to the sham group, lesioned group, glia precond MSCs CM and glial cells secretome. However, 4W (B) after treatment these effects diluted. Number of animals: Sham n=6; 6-OHDA control: n=12, MSCs: n=11, Glia cells precond MSCs secretome: n=12; Glial cells secretome: n=11; Data presented as mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; * Refers to the differences for 6-OHDA+MSCs secretomes with other

4.2.9 Forced-swimming test

To evaluate the effects of secretome treatment on depressive-like behavior, the forced-swimming test was also performed. Statistical analysis revealed an effect for treatment ($F_{(4,51)}=3,407$, $p<0.015$). Also, we have also observed that 6-OHDA injected animals (p=0.032) and the glial (precond) secretome-injected group (p=0.008) appear to develop a very subtle depressive-like behavior when compared to sham group. However, these effects were not verified in the MSCs CM (p=0.072) and glia CM (p=0.071), though there is a similar trend.

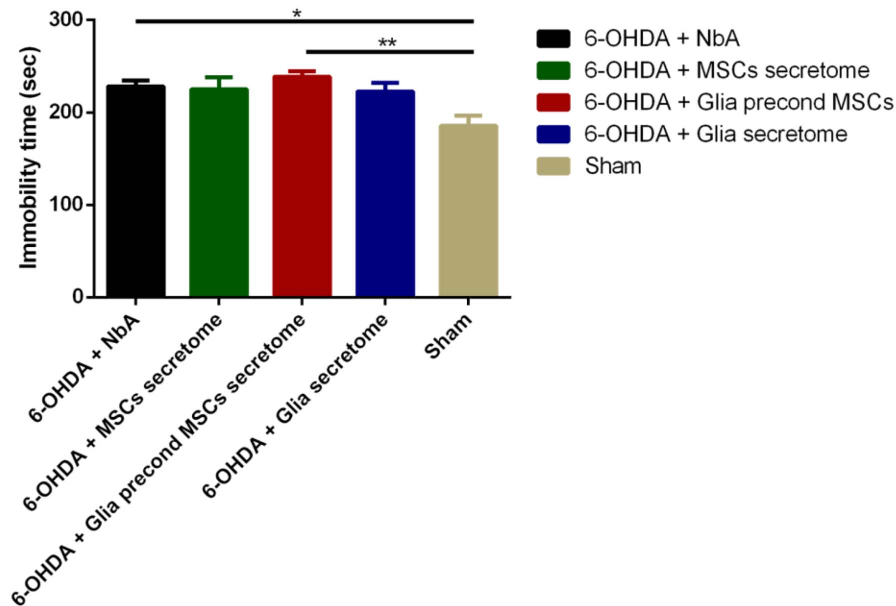

Figure 15– Impact of different secretomes on depressive-like behavior of 6-OHDA-lesioned animals. Depressive-like behavior was assessed after 4 weeks of treatment surgeries. Number of animals: Sham n=8; 6-OHDA control: n=12, MSCs: n=10, Glia cells precond MSCs secretome: n=13, Glial cells secretome: n=13. Data presented as mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001;

4.3 Assessment for the extension of the lesion

In order to analyze the effects of the 6-OHDA injections as well as the resulting treatments, histological analyses for TH was performed in the SNpc and STR and MFB. From the results, statistical analysis revealed that there was a significant decrease on TH-positive staining after the injection of 6-OHDA into the MFB (Fig.16; Table 5). Regarding the injection of the secretomes, statistical analyses demonstrated that the injection of the MSCs CM most likely play a role in the survival of DAn, leading to an increase of TH-positive cells in the SNpc when compared to the untreated group 6-OHDA (p=0.084, Fig.16). The same observations were also found in the striatum, by assessing TH-positive fibers through densitometry analysis (Fig.17; Table 6), in which it was observed significant differences into densitometries between non-treated group with MSCs CM (p=0.004), glial precond with MSCs CM (p=0.003), glial CM (p=0.012) and sham group (p<0.0001).

Table 5. Statistical analysis of the TH-positive cells in the SNpc (Data presented as mean±SEM)

Group	Mean±SEM	Statistical test, significance, effect size
6-OHDA control	12.32±2.78	
MSCs CM	24.62±7.46	
Glia (precon) CM	12.50±3.90	$F_{(4,51)}=35.569$, $p<0.0001$, $\eta^2_{partial}=0.752$
Glia CM	20.51±3.69	
Sham	28.11±4.50	

Table 6. Statistical analysis of the TH-positive fibers in the striatum (Data presented as mean±SEM)

Group	Mean±SEM	Statistical test, significance, effect size
6-OHDA control	13.11±0.77	
MSCs CM	33.63±5.50	
Glia (precon) CM	33.16±3.22	$F_{(4,52))}=40.220$, $p<0.0001$, $\eta^2_{partial}=0.770$
Glia CM	30.65±4.32	
Sham	84.15±1.90	

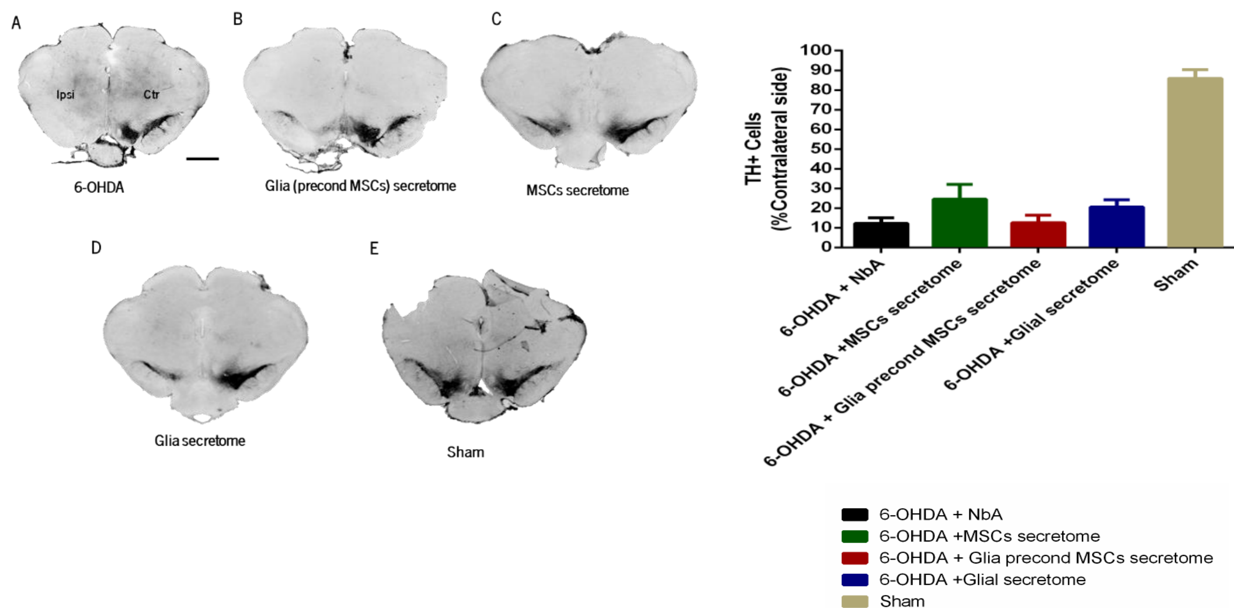

Figure 16. Representative micrographs of SNpc slices stained for TH. Compared to the Sham group (E), all the animals that were submitted to 6-OHDA injection presented a reduction of TH cells. However, animals injected with MSCs CM (C) present a subtle increase in TH-positive cells when compared to 6-OHDA-control (A) group. Number of animals: Sham n=8; 6-OHDA control: n=9, MSCs: n=11, Glia cells precond MSCs secretome: n=13, Glial cells secretome: n=13. Data presented as mean±SEM. (Scale bar: 2000 µm).

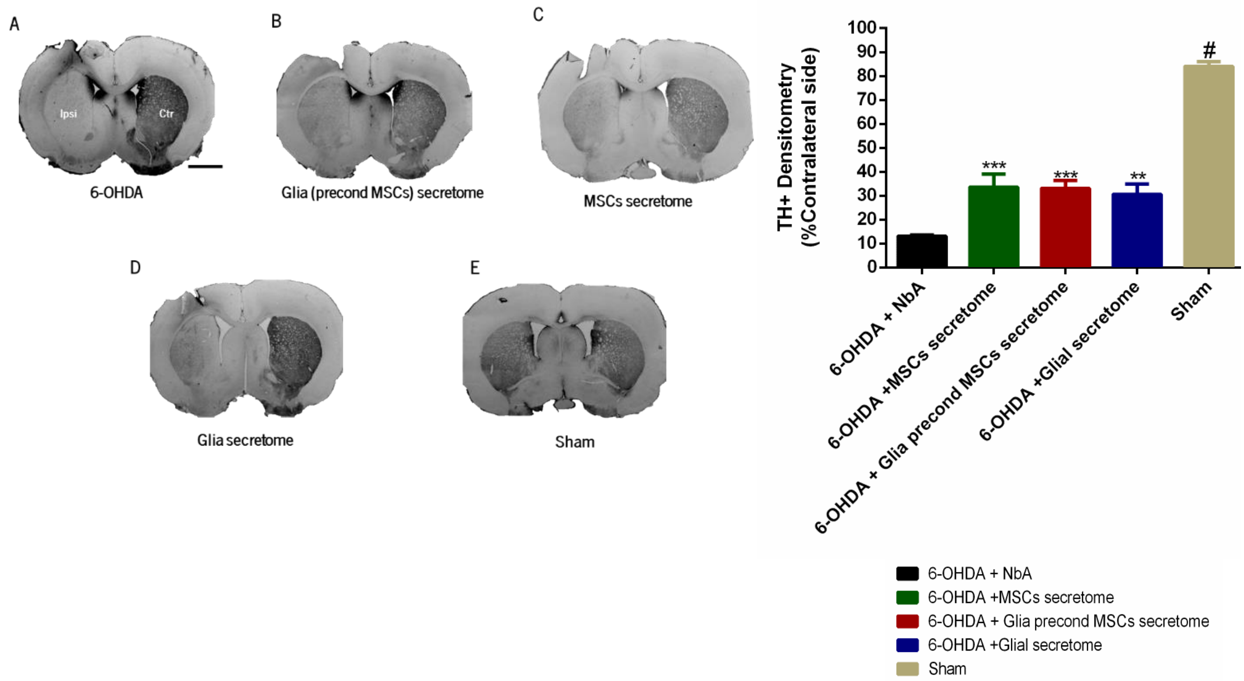

Figure 17. Representative micrographs of striatum slices stained for TH. Compared to the Sham group (E), all the animals that were submitted to 6-OHDA injection presented a reduction of the TH positive fibers. However, animals injected with MSCs CM (C), glial precond MSCs CM (B) and glial CM (D) presented a significant increase in TH-positive cells when compared to 6-OHDA-control group. Number of animals: Sham n=8; 6-OHDA control: n=10, MSCs: n=12, Glia cells precond MSCs secretome: n=13, Glial cells secretome: n=13. Data presented as mean±SEM. #p<0.0001 Sham group different to all groups; *p<0.05 differences in comparison to 6-OHDA control animals. (Scale bar: 2000 μm).

CHAPTER 5

DISCUSSION

5 DISCUSSION

Neurodegenerative disorders are a growing health concern with the aging of the population. In fact, CNS repair is probably one of the biggest challenges of the pharmacological and regenerative medicine fields (Chitnis and Weiner, 2017). Concerning PD, no cure exists, and the current pharmacological and surgical treatments just mitigate motor symptomatology, instead of promoting its regeneration (Behari and Singhal, 2011; Muramatsu, 2010; Oh et al., 2017). Therefore, in the last years, efforts have been made to design and develop therapeutical strategies that can tackle the protection and repair of PD (Behari and Singhal, 2011; Dantuma et al., 2010; Lu et al., 2017; Vizoso et al., 2017). Based on such view, studies have recently been suggesting that the success to obtain a functional recovery cannot just be obtained by using a single (drug, surgical) therapeutic approach, but most likely through the combination of integrative multiple strategies (Pires et al., 2017). As recently reviewed by Pires and colleagues (Pires et al., 2017), adult stem cells can exert therapeutically effects when applied in CNS neurodegenerative disorders as PD (Pires et al., 2017). Indeed, throughout the years, several research groups have attributed the beneficial effects of stem cells transplantation to their transdifferentiation into neural cells (Dantuma et al., 2010; Wang et al., 2010). Nevertheless, recent approaches have presented a shift concerning this hypothesis, claiming that the main responsible mechanism by which these cells mediate improvements in the CNS, including PD, is based in an active production and release of trophic molecules, known as secretome (Teixeira et al., 2013, 2015, 2017).

Thus, the work described on the scope of the present thesis was centered in the establishment of a new route to restore, maintain or improve PD tissue function through a cell-free based strategy by using the secretome of hMSCs and glial cells. Based on such purposes, in our *in vitro* experiments that aimed to address the impact of hMSCs and glial cells secretome on neuronal survival and differentiation of hNPCs, we found that both secretomes were able to induce neuronal differentiation (Fig.7). When hNPCs were incubated with hMSCs secretome, Glial cells (preconditioned by hMSCs secretome) and Glial cells secretome *per se*, there was a significant differentiation into neuronal linages, namely immature (DCX-positive cells) and mature neurons (MAP-2-positive cells) (Fig.7). Concerning hMSCs secretome, the results herein presented are in line with what was previously shown by our group, which showed that different hMSCs secretomes (obtained from different cell sources) were able to induce higher rates of neuronal survival and

differentiation both *in vitro* (on hNPCs) and *in vivo* (in the DG of hippocampus) (Teixeira et al., 2015, 2016, 2017). Supporting such observations and through proteomic analysis our group has demonstrated that apart from the traditional growth factors associated with neural survival and differentiation (e.g BDNF, GDNF, VEGF, SCF and others), other neuroregulatory molecules that regulate neuroprotection, neural differentiation and axonal growth/reinnervation such as DJ-1, PEDF, Cys C, GDN, Gal-1 and miR16 were also found in MSCs secretome (Pires et al., 2016; Teixeira et al., 2016). Similar results, were also observed by Sart and colleagues (Sart et al., 2014), which demonstrated that MSCs secretome had the capability to induce hNPCs neuronal differentiation into different stages of maturation, by correlating these observations with the presence of bioactive molecules as FGF-2, BDNF and TGF-β1 on hMSCs secretome.

In addition to the above-referred results, we have also observed that glial cells secretome (by using different conditions) was able to induce higher rates of neuronal differentiation, in which the preconditioning with MSCs secretome led to more efficient results (Fig.7). Such observations were quite challenging and puzzling, as there are no specific references on the literature supporting the impact and therapeutical effect of glial cells secretome in neuronal differentiation. Still, although we know that glial cells had beneficial effects on the CNS (Jha et al., 2013, 2017; Parkhurst et al., 2013), as far as we know, there are also no studies regarding the integrative combination or preconditioning strategies by using MSCs secretome and glial cells. Therefore, although the exact mechanism by which MSCs secretome together or by preconditioning glial cells, or even glial cells secretome (*per se*) modulate the behavior of neuronal progenitor cells is poorly understood, the application of such cells in the CNS have already revealed promising therapeutical effects (Drago et al., 2013; Lu et al., 2017). Nevertheless, although a deep proteomic analysis is missing (to identify regulatory molecules) to glial cells secretome in order to dissect how this secretome induce such beneficial effects (such as we have done in previous works (Teixeira et al., 2016)), studies has been describing that these cells are also able to secrete important trophic molecules with impact in CNS functionality (Keene et al., 2009). In fact, and as described for MSCs, glial cells were also found to secrete classical neurotrophic factors and cytokines like GDNF, BDNF, FGF, and IL-1 (important on neurogenesis and neuronal survival, (Borsini et al., 2015)), as well as other important neuroregulatory molecules suc as pentraxin 3 (PTX3), thrombospondin (TSP), important molecules involved in the development of new synapses (Jeon et al., 2010).

After these *in vitro* assays, our next step was to perform an *in vivo* experiment in order to address the impact of hMSCs and glial cells secretome in a rat model of dopaminergic degeneration that mimics part of PD pathology. This model is obtained by an unilateral injection of 6-OHDA into the MFB (Carvalho et al., 2013), and resembles the progressive and extensive loss of DAn in the SNpc and their terminals in the striatum, leading to a decrease in DA production that results in the appearance of debilitating motor problems (Poewe et al., 2017). However, it is important to refer that in early phases (preceding motor symptomatology), there is also the occurrence of non-motor symptoms, like depression and anxiety (Fontoura et al., 2017; Owens-Walton et al., 2018). Importantly, this rat model of 6-OHDA lesion also presents emotional deficits (Carvalho et al., 2013).

First, we have validated the model of 6-OHDA-induced lesions by showing that in the rotameter test after apomorphine injection, animals have disclosed and intense turning behavior when compared to the sham group (Fig.8A). We further evaluated motor coordination and balance (measured by the rotarod and staircase tests), and observed that animals' motor function was affected, presenting a clear impairment when compared to control group – sham (Fig.8B).
Besides motor evaluation, we have also evaluated PD non-motor symptomatology. By using the SPT (Fig. 8F; to anhedonic-like behavior), we have found that animals do not develop anhedonic behavior, in contrast to previous work from our team which has shown that this model presents anhedonia (Carvalho et al., 2013). Yet, one has to refer that the preference ratios of the SPT were clearly inferior to those of previous data (±90% of preference in Carvalho et al., 2013 versus ±65% in this work - in sham animals). This low preference may indicate that this test did not work well in this work, as usually we consider only SPT with preferences above 80% in control animals. So, this test should be repeated to disclose if these animals present anhedonia. In contrast, although not significant, in the Light Dark Box test, it seems that 6-OHDA injected animals tend to an increase in anxious behavior (Fig.8G).

After this characterization of the PD model, we aimed to address the impact of MSCs and glial cells secretome (*per se* and preconditioned by hMSCs secretome) on motor performance and on the DAn cell survival.

Regarding the effects on motor coordination and balance, assessed by rotarod, it was possible to observe that MSCs and glial secretomes showed a tendency to improve the motor performance. However, at one and four weeks after treatment, MSCs secretome have induced a better motor performance in 6-OHDA-injected animals when compared to the untreated group (6-OHDA; Fig.9). Similar observations were also obtained in the staircase test, in which hMSCs secretome-injected animals present a slightly trend for improvement when compared to untreated group (6-OHDA; Fig.10). In contrast, glial cells secretome effects in (staircase) motor performance were not so evident when compared to the untreated group (Fig.10A). Concerning the forced-choice test, and regarding the affected side (left side) we have observed a positive effect induced by the MSCs secretome in the success rate of eaten pellets when compared to the non-treated group (6-OHDA; Fig.10B). This motor amelioration promoted by MSCs secretome may be due to the increase in the number of TH-positive cells and fibers in the SNpc and STR, respectively, observed in treated animals when compared to the untreated group 6-OHDA (Fig. 16,17). This is in line with what was recently described by our lab, showing that injection of hMSCs into the striatum and SNpc of 6-OHDA lesioned animals potentiated the recovery of DAn, thereby supporting the motor performance amelioration that was observed and indicating that the action of MSCs secreted factors is correlated with beneficial actions on PD (Teixeira et al., 2016, Vizoso et al., 2017). Several factors have been presented as potential candidates to explain the therapeutical effects of MSCs secretome, namely BDNF, GDNF, VEGF and SDF-1α, which were found to improve motor and histological outcomes of PD animal models (Cova et al., 2010; Sadan et al., 2009; Wang et al., 2010; Tian et al., 2007). Nevertheless, in addition to these conventional trophic molecules, other neuroregulatory molecules have also been proposed as promising candidates to PD. For instance, Prosaposin (SGP-1) is being described as an intriguing multifunctional protein that plays important roles both intracellularly (as regulator of lysossomal enzyme function), and extracellularly, presenting remarkable neuroprotective effects (Meyer et al., 2014). Also, SGP-1 was found to be an up-regulator of the anti-apoptotic factor Bcl-2, and a down-regulator of the pro-apoptotic factor BAC, inhibiting MPTP neuronal-induced toxicity both *in vitro* and *in vivo* (Gao et al., 2013). Recently, Teixeira and colleagues (Teixeira et al., 2016) found PEDF in MSCs secretome, which has been described as a multimodal protein with important actions in migration, differentiation and neuroprotection on PD (Falk et al., 2010; Yabe et al., 2010). Indeed, by using a MPTP PD mouse model, Yasuda and colleagues (Yasuda et al., 2007) have shown that in response to MPTP administration there was an up-regulation of PEDF, thereby indicating that PEDF

may act as an endogenous neuroprotective molecule, triggering DAn survival and behavioral improvements in PD animal models.

Concerning Glial cell secretome effects, as far as we know, there are no studies focusing its application as a therapeutical strategy, as well as in combination with MSCs secretome. Yet, studies have suggested that in early stages of PD, glial cells can act as modulators of neuroprotection by the release of trophic factors (Jha et al., 2014; Mena and García de Yébenes, 2008). However, with disease progression it has also been suggested that glial cells shift their behavior, starting to release pro-inflammatory and ROS, further contributing to the progression of PD (Block et al., 2007; Halliday and Stevens, 2011; Liddelow et al., 2017), although this is an issue that is under controversy. Regarding our data, we have observed that glial cells secretome either preconditioned with hMSC secretome or itself was not able to impact in a significant way on the motor functionality of 6-OHDA injected animals (Fig.9,10). However, histological data show that glia cells secretome (preconditioned with hMSC secretome or *per se*) led to an interesting increase on TH positive cells and fibers (Fig. 16,17). These were, indeed interesting observations, which led us to make some questions, namely why does the animals injected with glial cells secretome exhibit an improvement/protection of TH-positive cells and fibers, and this is not correlated with a better motor performance (as it is observed to MSCs secretome)? One possibility is that though there is a small improve in the dopaminergic system, and this is still not sufficient to improve motor symptoms. However, to better answer to such questions, proteomic-based analysis should be performed in order to correlate the secretome profile with with the behavioral and histological data herein described. Even though, studies have shown that these cells have also the capability to secrete important trophic molecules with protective roles on DAn, like GDNF. For instance, Safi and colleagues (Safi et al., 2012), have demonstrated that GDNF-enriched conditioned medium from engineered astrocytes, was able to induce DAn neuronal survival *in vitro* (Safi et al., 2012). However, these authors have also observed that higher levels of GDNF was hazardous to the cells, thereby indicating that the concentration of the factors (as GDNF) could an important issue in the mediation of secretome beneficial effects. Therefore, glial cells' secretome did not seem to have a positive impact on motor performance (Figure 9,10). However, we do observed an increase of TH-positive cells and fiber densities in the SNpc and STR. In line with these findings, Polazzi and colleagues (Polazzi et al., 2009), observed in primary cultures of cerebellar granule neurons (CGNs) exposed to 6-OHDA, that microglial secretome was able to protect CGNs from the neurotoxin,

correlating such observations with the presence of TGF-β2 on microglial secretome. Overall, these results indicate that *in vivo*, the secretome of hMSCs and Glial cells acts as a modulator of DA neuronal survival.

In addition to the impact of MSCs and glial cells secretome on motor behavior, we have also evaluated their impact on the non-motor symptoms of PD. We observed that all secretome-treated groups and untreated group 6-OHDA revealed an anxious-like behavior when compared to the control group Sham. These results are in line with what is already described about the connections between brain areas involving emotional anxiety and those controlling posture: amygdala and associate limbic structures play a pivotal role in the acquisition, modulation and expression of emotions, like anxiety, and have widespread efferent connections to areas involved in posture, like basal ganglia and nucleus accumbens (Balaban and Thayer, 2001; Prediger et al., 2012), which are known to be impaired in PD. In addition, noradrenergic and serotonergic systems may also be another players contributing to the manifestation of PD-related anxiety and depression (Eskow et al., 2011; Prediger et al., 2012). Indeed, serotonergic cell loss in the raphe nuclei is evident prior to nigrostriatal DAn degeneration (Braak et al., 2004; Del et al., 2002), leading to the decrease of serotonin, an important neurotransmitter that provides a massive input to the corticolimbic structures, which are involved in the control of anxious state (Millan, 2003; Prediger et al., 2012). Moreover, in PD patients, there are reports describing that the loss of the noradrenergic (NA) cells in the locus coeruleus, leads to significant changes in the expression of NA receptors and transporters, which has been correlated with a prompt development or exacerbation of anxiety (Millan, 2003; Prediger et al., 2012).

In contrast, on the elevated plus-maze test, we have observed an improvement on anxious-like behavior promoted by the glial cells secretome when compared to the glial secretome (preconditioned with hMSCs secretome) (Fig.13), as well as a trend to the non-treated group 6-OHDA. The secretion of trophic molecules such as, D-Serine, ATP, FGF2 and BDNF may explain the above-referred results, as it has been suggested that they disclose anxiolytic and anti-depressant effects (Birey et al., 2015; Sild et al., 2017). Curiously, similar results were expected to hMSCs secretome, as they also secrete FGF2 (Vizoso et al., 2017). A possible cause for such observation may reside in its factor concentration, or in the interplay that could be established after injection, as in the preconditioning strategy the impact of the secretome collected was potentiated

(Fig.12,13). Even being quite interesting, it is also important to ask if is this anxious phenotype really arises from the denervation of DAn or other circuits such as the serotoninergic.

Regarding the results obtained in SPT, we found that one week after treatment animals injected with hMSCs secretome presented an anhedonic-like behavior when compared to the all other groups (Fig.14A). Four weeks later, this effect was diluted (Fig.14B). So, one has to confirm if this also occurs in other set of animals, in order to better explore this effect of MSCs. In the other test of depressive-like behavior, the FST, we observed that non-treated 6-OHDA group and glia cells (preconditioned with hMSCs secretome) secretome developed a depressive-like phenotype when compared to the sham group (Fig. 15). This is in line with what was previously described by our group, where the injection of 6-OHDA lead to an depressive-like phenotype (Carvalho et al., 2013). However, the results obtained with secretome injections are interesting, as to date, there are no studies exploring this. Even though, the pathophysiology of depressive symptoms on PD are complex and probably includes dopaminergic, but also serotonergic and noradrenergic mechanisms (Kamińska et al., 2017), similarly to what occur with anxious-like behavior. Nevertheless, what can lead to this improvement? BDNF is being described as a possible candidate to explain the observed effects, as it has been described that is action is critically involved in the recovery of depression due to its ability of BDNF to modulate neuronal plasticity (Rial et al., 2016). Moreover, depressive post-mortem brains have found decrease levels of BDNF, thereby supporting the above mentioned results (Sild et al., 2017).

In summary, the injection of MSCs and glia cells secretome act as modulator of neuronal cell survival and differentiation *in vitro*. *In vivo* and in the context of PD, hMSCs secretome revealed to be able to increase the TH-positive cells and fiber densities, leading to motor improvements. On non-motor behavior, MSCs secretome also reveal to have a positive impact on depressive-like behavior. Concerning glial cells secretome, although no positive effects were observed in PD motor performance, surprisingly, histological results revealed a positive impact in TH-positive cells and fibers in the SNpc and STR. In contrast, on PD non-motor dimension, glial cells secretome have positive impact on anxiety and depressive-like behavior. The effects verified on this work might be due to the secretions of each individual cell type. For instance, while MSCs secretome have molecules such as DJ-1, PEDF and Cys C, glial cells secret molecules like TGF-β2, PTX3 and TSP, although, both cells have some molecules in common, which is the case of GDNF, BDNF and

FGF2. Still, the effects of these cells secretomes are different and the final action of the secretome can be influenced by different concentrations of its components and/or by its combinations.
Overall, our results strongly suggest that (stem) cells secretome can be a potential therapeutic tool to treat PD, since they can modulate DAn survival and animal motor and emotional performance. Even being promising, future studies including proteomic identification of the (glial) secretome molecules, should be performed. We hypothesize that different cell secretomes may act in different ways, by which new insights about the release and interplay of different cells populations and its trophic factors may lead to a rational design of new therapeutical strategies for the functional recovery of neurodegenerative disorders like PD.

CHAPTER 6

CONCLUDING REMARKS

6 CONCLUSION AND FUTURE PERSPECTIVES

As a final conclusion, this work provided important gains on the potential use of (stem) cells secretome as promising therapeutical tool for CNS neurodegenerative disorders, as PD (Figure 18). In fact, we have observed that the injection of hMSCs secretome was able to modulate the DAn survival and ameliorate PD motor deficits. Interestingly, and tackling PD non-motor symptomatology, glial cells secretome appears to be a promising approach. Although proteomic analysis was not performed, this should be considered in the future in order to understand and correlate the data obtained with the secretory profile of each cell population, as previous studies have already pointing out that hMSCs and even glial cells are a source of important neuroregulatory molecules with impact in different CNS mechanisms (e.g. anti-inflammatory effects, reduction of oxidative stress, stimulation of neurogenesis, neuronal cell survival and differentiation, as well as neurite outgrowth). Moreover, it will also allow a better understanding of the mechanisms behind the secretome effects, as well as a possible elucidation of the activation or inhibition of molecular pathways, and its temporal effects.

Therefore, future studies should be performed in order to improve and validate this data, since as far as we know, this work shows for the first time the application of Glial cells secretome as possible therapeutic tool for PD.

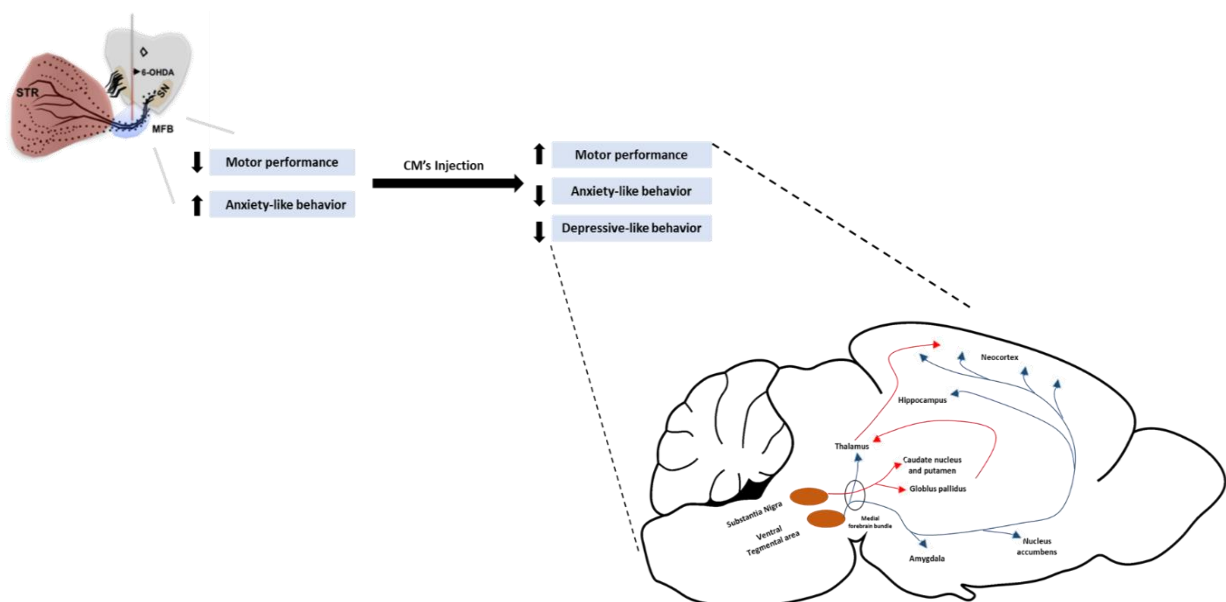

Figure 18. Possible mechanism explaining the results observed. The injection of 6-OHDA into MFB lead to the degeneration of DAn, not only in the SN but also into VTA. Over time, both degenerations lead to the motor (loss of DA into STR) and non-motor (loss of DAn in VTA and SN) symptoms. However, upon

secretomes injections it was verified an improvement in the animal's behavior. These effects may be due to the presence of important trophic factors such as BDNF, FGF, GDNF in hMSCs and glial cells secretome.

CHAPTER 7

REFERENCES

7 REFERENCES

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