UNIVERSIDADE
BEIRA INTERIOR

Regulation of gene expression in Parkinson's disease: microRNAs as potential therapeutic targets

Mafalda Proença

Dissertação para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos ou mestrado integrado)

Orientador: Profª. Doutora Liliana Inácio Bernardino

janeiro de 2023

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Agradecimentos

Assim termina mais uma etapa académica da minha vida. Uma etapa difícil, trabalhosa e com muitos obstáculos pelo caminho, mas dizem que o que é mais difícil de concluir é também o que mais aprendizagem nos traz. E muita aprendizagem adquiri nesta fase, tanto a nível teórico como a nível prático.

Tenho assim, de começar por agradecer à minha orientadora Profª. Doutora Liliana Bernardino pela partilha de conhecimento e também por toda a ajuda que sempre ofereceu. Sem ela nada disto seria possível.

Agradecer também, a todo o Brain Repair Group, especialmente à Marta Esteves e à Marta Pereira por sempre ajudarem em tudo quanto possível, pela paciência e generosidade que sempre demonstraram. Agradecer às colegas de laboratório Susana Ferreira, Beatriz Almeida e Tânia Silva que sempre estiveram lá para mim e que tornaram os meus dias mais alegres.

Um obrigada aos meus amigos, principalmente ao Pedro Coelho, ao Pedro Gomes à Catarina Cruz, à Joana Lebre, Gabriel Andrade e ao Jorge Baptista aqueles que já não sei há quantos anos estão na minha vida porque se eles não me aturassem não sei quem aturaria. São eles que me apoiam nos piores dias e que sabem sempre as palavras certas a dizer. Não sei o que seria da minha vida sem eles.

Agradecer à minha família por todo o apoio e paciência para os dias que não estava tão bem disposta, eles souberam sempre por um sorriso nos meus lábios e tranquilizarem-me.

E por fim, e não menos importante agradecer também à pessoa mais importante da minha vida, a minha mãe, que me ensinou a nunca desistir dos meus objetivos por mais difíceis que fossem, a batalhar sempre até ao fim.

Resumo

A Doença de Parkinson (DP) é uma doença neurodegenerativa caracterizada pela perda progressiva de neurónios dopaminérgicos na Substantia Nigra pars compacta (SNpc) e a acumulação anormal de agregados da proteína α-sinucleína (α-sin) sob a forma de corpos de Lewy e neurites de Lewy. Como não existe cura para esta doença e os tratamentos existentes apresentam sintomas secundários adversos, é importante desenvolver novas estratégias para combater esta doença neurodegenerativa. Evidências científicas recentes sugerem que os microRNAs (miRNAs) podem ser possíveis alvos terapêuticos para esta doença. A sua principal função é atuar como silenciadores na pós-tradução, emparelhando-se com RNA mensageiros (mRNA) complementares e regulando a sua estabilidade e tradução. O principal objetivo desta dissertação foi investigar se o microRNA-106a (miRNA-106a) pode atuar como um novo agente terapêutico na DP, protegendo os neurónios dopaminérgicos. Neste estudo, foi usado um modelo animal em que foi induzida a DP através de injeção estereotáxica no corpo estriado (ST) com a neurotoxina 6-hidroxidopamina (6-OHDA), seguida da injeção na Substância Nigra (SN) com o miRNA-106a. Sete dias após as cirurgias foram realizados testes comportamentais para avaliar a coordenação motora e, posteriormente, foi realizada imunohistoquímica para a tirosina hidroxilase (TH), um marcador dos neurónios dopaminérgicos, para avaliar neuroprotecção. Os resultados obtidos com os testes de comportamento no rotarod e no teste de apomorfina mostram que os murganhos tratados com a 6-OHDA e o miRNA-106a apresentam uma melhoria no comportamento motor comparativamente com os murganhos tratados apenas com a 6-OHDA. Esta melhoria motora foi acompanhada pelo aumento da sobrevivência dos neurónios dopaminérgicos, comprovada pelo aumento do número de neurónios positivos para TH na SN, assim como um aumento da intensidade e da área da região corada para TH no ST. Em suma, estes resultados sugerem que o miRNA-106a poderá ter um efeito neuroprotetor nos neurónios dopaminérgicos e é capaz de induzir melhorias motoras num modelo da DP.

Palavras-chave

Doença de Parkinson; MicroRNAs; microRNA-106a; Neurónios dopaminérgicos; Comportamento motor; Neuroproteção.

Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disease associated with the loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc) and the abnormal accumulation of α-synuclein (α-syn) aggregates in the form of Lewy bodies and Lewy neurites. As there is no cure for this disease and existing treatments often have secondary symptoms, the development of new strategies to fight this neurodegenerative disease is urgent. Recent evidence suggests that microRNAs (miRNAs) may be a possible therapeutic solution for this disease. Their main function is to act as silencers in post-translation, pairing with complementary messenger RNA (mRNA) and regulating their stability and translation. The main objective of this thesis was to investigate whether microRNA-106a (miRNA-106a) acts as a novel therapeutic agent in PD by protecting dopaminergic neurons and counteracting motor functional deficits. In this study, an animal model was used in which PD was induced by the stereotaxic injection with neurotoxin 6-hydroxydopamine (6-OHDA) into the striatum (ST), followed by the injection with miRNA-106a into the substantia nigra (SN). Seven days after the surgeries, behavioral tests were performed to assess motor coordination and immunohistochemistry was performed for tyrosine hydroxylase (TH), a marker of dopaminergic neurons, to assess neuroprotection. Results obtained from behavioral tests on the rotarod and the apomorphine test showed that mice treated with 6-OHDA and miRNA-106a had improved motor behavior compared to mice treated with 6-OHDA only. This motor improvement was accompanied by increased survival of dopaminergic neurons, evidenced by an increase in the number of TH-positive neurons in the SN, as well as an increase in the intensity and area of the stained region for TH in the ST. In summary, these results suggest that miRNA-106a may have a neuroprotective effect on dopaminergic neurons and is able to improve motor behavior in an animal model of PD.

Keywords

Parkinson’s disease; MicroRNAs; microRNA-106a; Dopaminergic neurons; Motor behavior; Neuroprotection.

Índice

Lista de Figuras

Lista de Acrónimos

6-OHDA	6-Hydroxydopamine
Ago	Argonaute
ALP	Autophagy-lysosomal pathway
ATG7	Autophagy-related gene 7
BBB	Blood-brain barrier
CICS	Health Sciences Research Center
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
DA	Dopamine
DAT	Dopamine transporter
DGAV	Direção Geral de Alimentação e Veterinária
DGCR 8	DiGeorge syndrome critical region 8
GFAP	Glial fibrillary acidic protein
Gfs	Growth factors
H_2O_2	Hydrogen peroxide
Hsc70	Heat shock cognate protein 70
IBA-1	Ionized calcium-binding adapter molecule 1
IL-10	Interleukin 10
IL-1β	Interleukin-1 beta
LAMP-2A	Lysosome-Associated Membrane Protein 2A
LB	Lewy bodies
LC3	Microtubule-associated 1 light chain 3
L-DOPA	Levodopa
LID	L-DOPA-induced dyskinesia
LN	Lewy neurites
MAO-A	Monoamine oxidase A
MAO-B	Monoamine oxidase B
miRNAs	MicroRNAs
miRNA-7	MicroRNA-7
miRNA-106a	MicroRNA-106a
miRNA-124	MicroRNA-124
$MPDP^+$	1-methyl-4-phenyl-2,3-dihydropyridium
MPP^+	1-methyl-4-phenylpiridium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger RNA
NAT	Noradrenaline transporter
NMDA	N-methyl-D-aspartate
NPs	Polymeric nanoparticles
nt	Nucleotide
ORBEA	Orgão de Bem-Estar e Ética Animal
PBS	Phosphate- buffered saline
PBS-T	Phosphate-buffered saline with Tween 20
PD	Parkinson's disease
PFA	Paraformaldehyde
pre-miRNAs	Precursor miRNAs
pri-miRNAs	Primary miRNAs
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rotations per minute
SN	Substantia Nigra

SNpc	Substantia Nigra pars compacta
ST	Striatum
TH	Tyrosine hydroxylase
TNF-α	Tumor nerosis factor alpha
UBI	University of Beira Interior
UPS	Ubiquitin-proteasome system
α-syn	α-synuclein

Chapter 1

Introduction

1. Parkinson's Disease

1.1 Pathophysiology

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder after Alzheimer's disease. It is known to affect around 0.3 % of the general population and 1 – 3 % of the population over the age of 65. It is expected that in 2030 the number of cases rises from 8.7 to 9.3 million [1]. Degeneration of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc), which results in a decrease in striatal dopamine (DA) levels, is a common neurochemical anomaly in PD. The condition can be viewed as a TH-deficiency syndrome of the striatum since tyrosine hydroxylase (TH) catalyzes the production of Levodopa (L-DOPA), the rate-limiting step in the biosynthesis of DA [2].

In his 1817 monograph "Essay on the Shaking Palsy" James Parkinson described the essential clinical characteristics of PD. Several conditions such as sleep disorders, soft speech, slight tremors, postural difficulty, impairments in normal facial expression, reduced limb movement, focus loss, general fatigue, and depression may be early signs of the disease [3]. Usually, the symptoms are divided into motor and non-motor symptoms. The motor symptoms are identified as resting tremors, bradykinesia referring to the slowness of movement, rigidity, and postural instability [4]. The non-motor symptoms include neuropsychiatric symptoms (cognitive impairments, memory loss, confusion, and hallucination), sleep disorders (insomnia, rapid eye movement disorders, and vivid dreaming), and autonomic symptoms (bladder disorders, orthostatic hypotension, and erectile impotence) [5].

There are two forms of PD: sporadic and hereditary or familial. The majority of PD cases are sporadic, and it is generally believed that the interaction of both environmental factors and genetic vulnerability plays a role in the development of the disease [6]. Historical episodes of the disease in the family are a key risk factor for PD and it's known that familial forms of PD account for 5%–15% of cases [7]. Mutations in the genes LRRK2, PARK7, PINK1, PRKN, or SNCA, as well as changes in unidentified genes, can result in familial cases of PD [8]. The first gene associated with PD was SNCA, which codes for α-synuclein (α-syn). Pink1 and PRKN are other genes implicated in familial forms of PD and they interplay in a mitochondria quality-control pathway: Pink1 'marks' damaged mitochondria and activates the mitophagy pathway with the recruitment of PRKN. In order to protect the cell from the oxidative stress caused by the pace-making activity of dopaminergic neurons and DA toxicity, DJ-1 (protein which in humans is encoded by PARK7 gene) plays a key role in controlling calcium flux in the mitochondria. An increasing body of

research connects PD to failure in cellular clearance pathways, and several autophagy-related genes have been linked to PD. Mutant LRRK2 slows down the breakdown of α-syn, interfering with autophagy and causing its accumulation [9].

This disease involves progressive degeneration of the nigrostriatal dopaminergic pathway with significant loss of the SNpc neurons and depletion of striatal DA [10]. The primary underlying reasons for SNpc neuronal loss are protein homeostatic impairments that induce protein aggregation and mitochondrial dysfunction leading to impaired bioenergetics and oxidative stress [1]. (Figure 1)

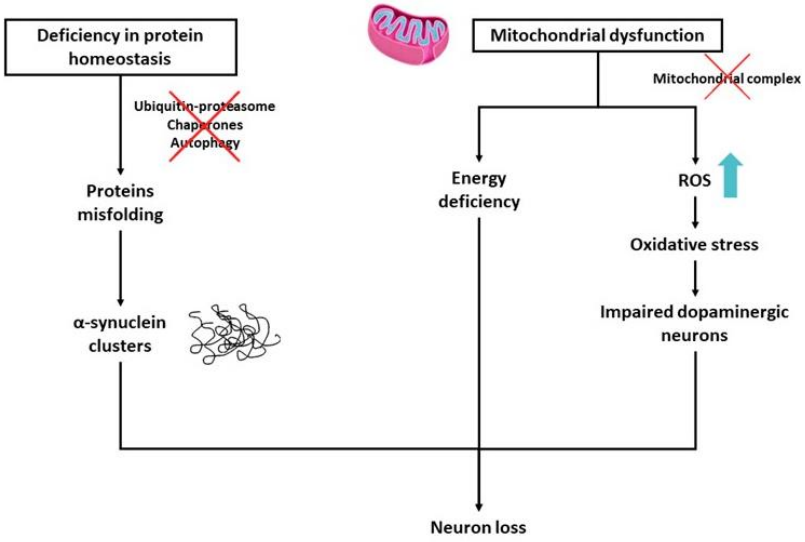

Figure 1 - Schematic illustration of the main causes of neuronal loss in PD. Aggregates of native and foreign proteins result from malfunctioning protein surveillance mechanisms, which in turn impair cellular homeostasis. On the other hand, cellular components are damaged when reactive oxygen species (ROS) accumulate due to mitochondrial malfunction and suppression of mitochondrial complex 1 activity. Adapted from [1].

The ubiquitin-proteasome system (UPS), chaperone-mediated process, and autophagy-lysosomal pathway (ALP) are examples of systems of surveillance to maintain protein homeostasis [11] (Figure 2). The failure of these systems leads to the accumulation of cytoplasmatic inclusions consisting mainly of α-syn aggregations, that are expressed primarily at presynaptic terminals in the central nervous system (CNS), in form of Lewy bodies (LB) and Lewy neurites (LN). It is well known that the three surveillance systems play a significant role in the cellular control of α-syn. The inhibition of chaperone, proteasome, or autophagy function enhances the accumulation of α-syn. It will be possible to better understand the disease by knowing the processes that lead to α-syn aggregation, as well as which α-syn-aggregated form is responsible for promoting dopaminergic cell death [12].

Chaperones facilitate protein folding, particularly for large proteins and protein complexes. The molecular chaperones Hsp60s, Hsp70s, Hsp90s, and sHsps help fold unfolded and misfolded

polypeptides by stabilizing folding intermediates and avoiding protein misfolding and aggregation [13]. Under pathological situations with significant protein misfolding and cellular stress, the consequent depletion of chaperones and the increase in aggregate-prone proteins may be higher than the chaperone system's ability to refold the proteins. Proteins that can no longer be refolded in this case might be sent to proteolytic systems to be destroyed [14].

The UPS and the ALP are the two principal proteolytic systems in neurons that contribute to normal protein turnover and assist in the elimination of misfolded proteins. The ALP is a degradation process that refers to the destruction of intracellular components, proteins, and organelles, in lysosomes, whereas the UPS destroys most short-lived soluble proteins [14].

The UPS is a major intracellular protein degradation system. Degradation of a protein occurs in two essential steps: covalent attachment of multiple ubiquitin molecules to the protein substrate and degradation of the target protein by the 26s proteasome complex with the release of free and reusable ubiquitin [15]. Many neurological disorders, including PD, are linked to UPS dysfunction. The impact of this dysfunction may be related to deficits in the clearance of misfolded proteins leading to intracellular protein aggregation, cytotoxicity, and cell death [16].

The generic name used to indicate mechanisms that come together to disintegrate intracellular proteins or organelles in lysosomes is ALP or autophagy. For each subtype of the ALP, targets are delivered to the lysosome in one of three ways: macroautophagy, chaperone-mediated autophagy (CMA), or microautophagy. The breakdown of the substrate protein in lysosomes is the last stage in each of the three autophagic processes [14]. Given the crucial function of autophagy in the removal of protein aggregates, this may mean that dysfunctional autophagy is a prevalent mechanism in neurodegenerative pathology. Autophagy also plays a crucial role in neurodegenerative illnesses because it helps clear up damaged mitochondria, which can lead to bioenergetic deficiencies or an accumulation of reactive oxygen species [17].

Numerous genes related to autophagy have been implicated in PD, and mounting evidence connects PD to malfunction in cellular clearance processes. Mutant LRRK2 slows down α-syn breakdown, interfering with autophagy and causing its buildup, according to reports. ATP132A mutations result in lysosomal dysfunction and young-onset parkinsonism, but its expression is elevated in surviving dopaminergic neurons in idiopathic PD, indicating its neuroprotective effect [7].

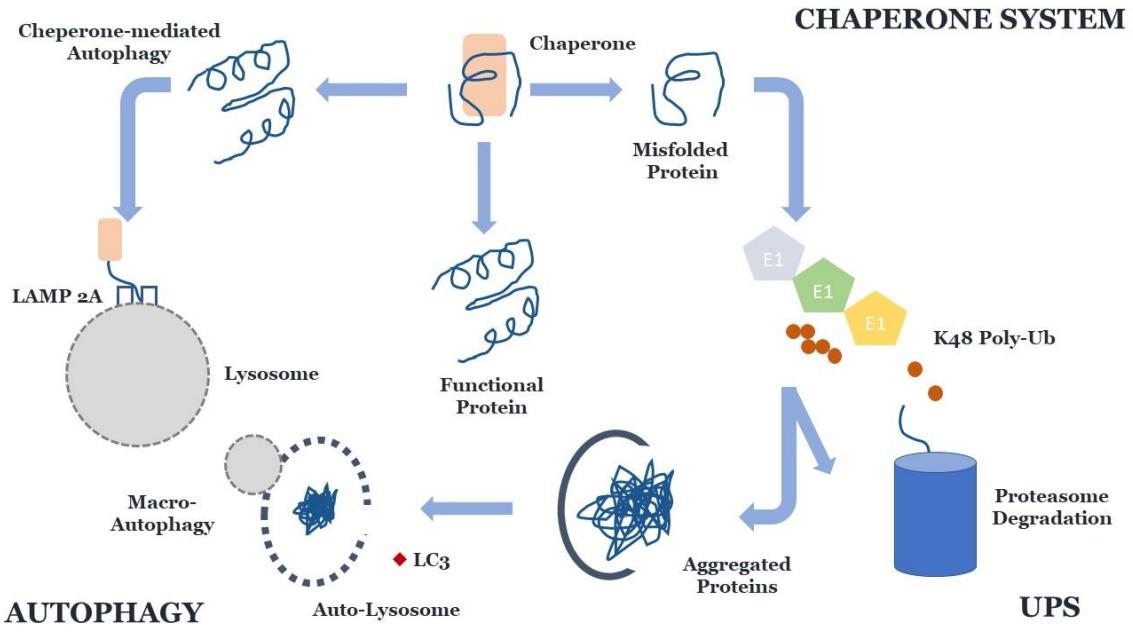

Figure 2 - The tree protein systems of surveillance: UPS, chaperone-mediated process, and autophagy. They work together to keep protein homeostasis in check. The first line of defense to guarantee proper protein folding is the chaperone-mediated mechanism. The protein will be sent to the proteasome for destruction if the proper folding is not accomplished. In some cases, proteins may be modified by the K63 polyubiquitination that shapes aggregates into inclusion bodies that will be removed by autophagy. Adapted from [18].

Mitochondria are organelles that have many functions like their important role in energy metabolism, and they are intimately involved in several key cellular processes such as the regulation of calcium homeostasis, stress response, and cell death pathways. Problems with the mitochondria function result in cellular damage and are linked to aging and neurodegenerative diseases [19].

Mitochondrial ATP generation is decreased, and ROS production is increased when complex I is inhibited. This increase disrupts redox signalling pathways and causes oxidative damage to proteins, lipids, and mtDNA. The respiratory chain may be compromised by oxidative damage to mtDNA, starting a cycle of oxidative stress and mitochondrial dysfunction [19], [20]. (Figure 3)

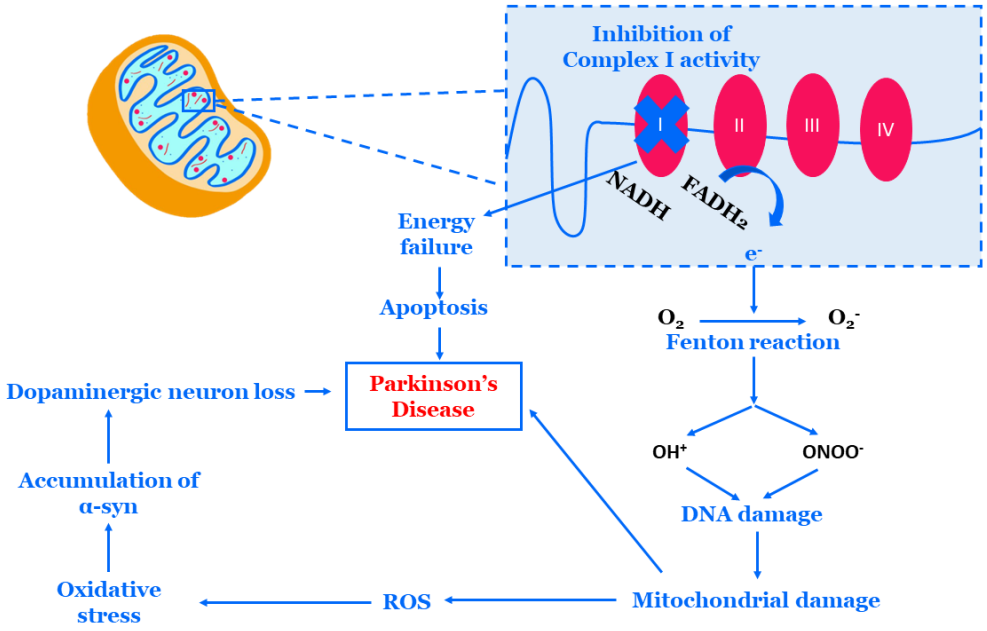

Figure 3 - Mitochondrial dysfunction in PD. The inhibition of mitochondrial complex I activity leads to energy deficiency and an increase in ROS resulting in oxidative stress and damage to dopaminergic neurons. These events are thought to lead to progressive degeneration of the nigrostriatal pathway. Adapted from [1].

Different genes connected to familial PD control mitochondrial activities. To control mitochondrial quality, PINK1, and Parkin interact in the following way: PINK1 is a serine/threonine kinase, which 'marks' damaged mitochondria and activates the mitophagy pathway through the recruitment of Parkin, an E3 ubiquitin ligase [7]. To protect the cell from the oxidative stress caused by the pace-making activity of dopaminergic neurons and DA toxicity, DJ-1 performs a key role in controlling calcium flow in the mitochondria [21].

1.2 Current Therapies

PD is still an incurable, progressive neurological disease that lowers the quality of life of patients. There have been significant improvements in the knowledge of the etiology of PD, the development of novel therapies, and the most effective ways to employ them for long-term patient management. The improvement of current therapies continues to be a top objective at the moment [22].

The symptomatic therapy is done with drug-based management techniques carefully chosen to relieve symptoms while taking the patient's tolerance and negative effects into account. The mainstay of treatment for motor symptoms continues to be medications that raise intracerebral DA levels or activate DA receptors, such as L-DOPA [23]. Suggested drugs like enzyme monoamine oxidase B (MAO-B) inhibitors such as selegiline and rasagiline, N-methyl-D-aspartate (NMDA) receptor blockers such as amantadine and anticholinergic drugs such as benztropine and ethopropazine, are used to delay the levodopa therapy for months [24]. DA agonists offer a better alternative to mild-potency medications because they directly mimic DA's

function by binding to DA receptors. DA agonist therapy such as bromocriptine and pramipexol is also considered a first-line therapy for younger patients and delays the introduction of L-DOPA [1]. L-DOPA-induced dyskinesia (LID), which may be caused by chronic overstimulation of supersensitive DA D1 receptors, may result from the long-term administration of L-DOPA to PD patients. In the future, L-DOPA might be combined with a variety of alternative techniques including gene therapy or cell transplantation [25]. The tapering of symptoms in PD patients is reduced by catechol-O-methyltransferase (COMT) inhibitor delivery, although the medication is halted if the patient has non-motor symptoms, such as hallucinations [26].

In addition to drug-based therapies, exercise also improves non-motor symptoms including cognition as well as gait-related activities like stride length and step variability [27].

As strategies and future perspectives for PD treatments some possibilities of non-drug-based strategies have been presented, namely ablative surgeries, electric stimulations, cell therapies and gene editing. These strategies are gaining increased attention [1].

A surgical approach to PD is deep brain stimulation. This technique was used to treat the distinctive tremor of PD. Deep brain stimulation at high frequency was initially utilized to replace thalamotomy in 1997. Since then, it has been used on the pallidum and the subthalamic nucleus. Clinical outcomes after deep brain stimulation are dramatically and steadily improving, according to both short-term and long-term results. There is evidence of the remarkable role of deep brain stimulation in alleviating Parkinsonian motor symptoms [28-29].

New lines of research focus on the cellular and molecular level due to variations in treatment response and adverse effects of current medications. Molecular and cellular therapy for PD includes transplantation therapy with induced pluripotent stem cells, gene editing with CRISPR-Cas9, and the use of viral vector-mediated gene delivery [30], [31]. Even before the beginning of PD symptoms, gene therapy offers a viable treatment option to address and correct the identified mutation in PD-related genes [32]. Despite their high relevance, these therapies will not be explored in detail because they are not part of the main objective of this dissertation.

2. Models of PD

2.1 Toxin-Based Models

The four neurotoxins most frequently employed to cause dopaminergic neurodegeneration are 6-hydroxidopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat (PQ), and rotenone.

MPTP is the model that has received the most attention due to being linked to several forms of human parkinsonism including tremor, rigidity, slowness of movement, postural instability, and freezing [2]. As a highly lipophilic toxin, MPTP can penetrate the blood-brain barrier (BBB) and

cause PD. First, the MAO-B converts MPTP into 1-methyl-4-phenyl-2,3-dihydropyridium ($MPDP^+$), which subsequently deprotonates to produce 1-methyl-4-phenylpiridium (MPP^+). After it is released into the extracellular space, MPP^+ is transported by the dopamine transporter (DAT) into dopaminergic cells and inhibits mitochondrial complex I activity [33-35].

Due to its lipophilic nature, rotenone can also cross the BBB and is independent of transporters, unlike MPP^+. Rotenone is one of the most widely used insecticides and pesticides around the world. Exposure to this toxin may be a risk factor for PD pathogenesis [36]. Rotenone inhibits complex I of the mitochondrial electron transport chain pushing the cell to glycolysis and loss of energy reserve leading ultimately to cell death. In addition, rotenone induces *in vivo* aggregations of α-syn that are found in the brain of PD patients in the form of LB and LN, being another hallmark of PD neuropathology. Furthermore, rotenone also mimics peripheral PD symptoms including decreased intestinal motility and peripheral α-syn aggregation, which are thought to occur before the classic PD symptoms in humans [37-38].

PQ is also an herbicide that has been associated with PD through epidemiological research and experimental studies in rodents. Superoxide radical production appears to be a mediator of paraquat toxicity in dopaminergic neurons of SNpc [39].

6-OHDA is a neurotoxin that is not capable of crossing BBB. The toxicity in the CNS is only obtained when this toxine is injected by stereotaxic surgery into the brain. Due to its similarity to endogenous catecholamines, 6-OHDA binds to DAT with a high degree of affinity, allowing the toxin to enter the dopaminergic neurons [40]. 6-OHDA neurotoxic effects are caused by a two-step process that involves the accumulation of the toxin in catecholaminergic neurons followed by changes in cell homeostasis and neuronal damage. Once inside the neuron, 6-OHDA accumulates in the cytoplasm and quickly undergoes auto-oxidation and degradation by Monoamine oxidase A (MAO-A). This increases the rate at which free radicals (mainly hydrogen peroxide (H_2O_2)) are formed resulting in damage to the cell. This neurotoxin can also accumulate in the mitochondria, where it blocks complex I to inhibit the electron transport chain from functioning [41-42]. (Figure 4)

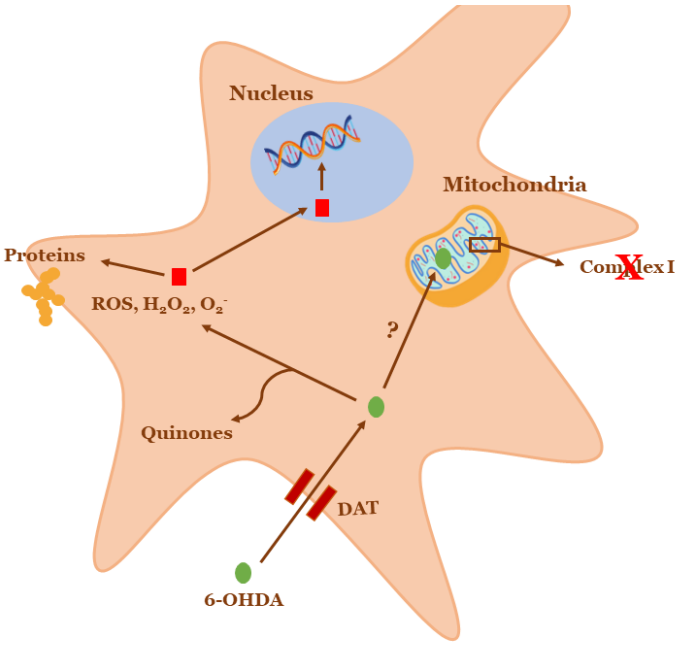

Figure 4 - Mechanisms of neurotoxicity caused by 6-OHDA which is taken up from the extracellular environment and then stored in catecholaminergic neurons via DAT or noradrenaline transporter (NAT). Inside neurons, 6-OHDA is subjected to autoxidation as well as an enzymatic breakdown by MAO-A. This produces several cytotoxic molecules that cause neuronal death. Additionally, 6-OHDA may cause neurotoxicity by reducing mitochondrial complex I ability to function. Adapted from [42].

3. MicroRNAs

3.1 Role of microRNAs

Short non-coding RNAs (ribonucleic acid) called microRNAs (miRNAs) have a length of 19–25 bp and negatively control the expression of genes at the messenger RNA (mRNA) and protein levels [43]. MiRNAs are essential for the normal development and survival of different neuronal populations [44-45].

The two stages of miRNA synthesis take place in the nucleus and cytoplasm. RNA polymerase II, which transcribes miRNA genes into lengthy, capped, polyadenylated RNA molecules known as primary miRNAs (pri-miRNAs), starts the synthesis of miRNAs. A microprocessor complex in the nucleus that includes the RNA-specific ribonuclease Drosha and its binding protein, DiGeorge syndrome critical region 8 (DGCR8), is able to detect pri-miRNAs. This complex cleaves the pri-miRNAs into RNAs of 60–100 nucleotide (nt), known as precursor miRNAs (pre-miRNAs). Exportin-5 transporter transfers the pre-miRNAs from the nucleus to the cytoplasm. In the cytoplasm, the RNAse III endonuclease Dicer process the pre-miRNAs into a form of 18–25 nt double-strand RNA [46]. One strand of the duplex is destroyed while the other is integrated into the RNA-induced silencing complex (RISC) composed of Argonaute (Ago) proteins, that bind the mature miRNA and orient it to facilitate the interaction with its target mRNA. Target mRNA is mainly degraded or repressed as a result of RISC hybridization [47]. (Figure 5)

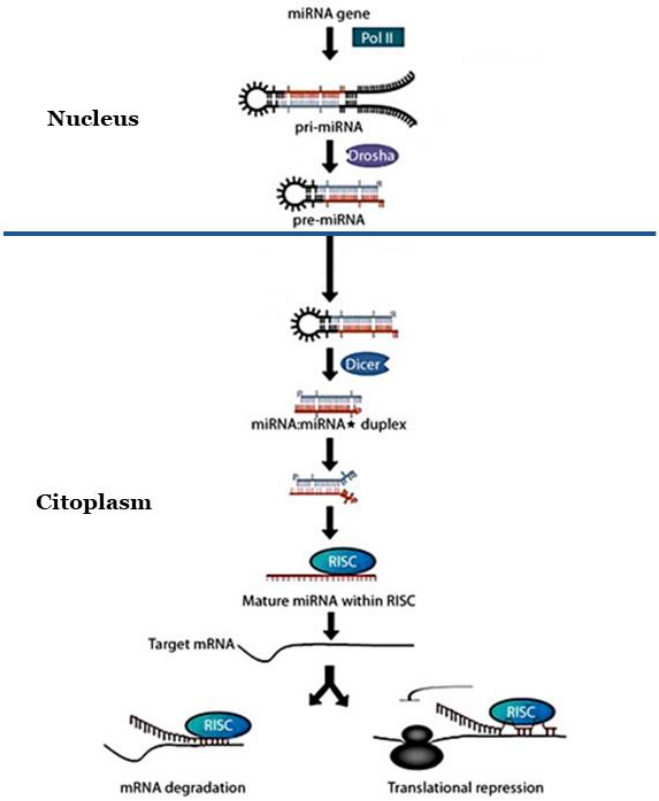

Figure 5 - Molecular mechanisms of miRNA biogenesis. The miRNA biogenesis begins in the nucleus with its transcription by RNA polymerase II. DGCR8 recognizes this double-stranded RNA structure and connects with the Drosha enzyme forming the pre-miRNAs. Pre-miRNAs are then exported from the nucleus to the cytoplasm by exportin-5. Then, the RISC made up of Ago proteins, binds mature miRNA and orientates it to assist the interaction with its target mRNA leading to its degradation or translational repression. Adapted from [47].

The α-syn aggregates known as LB are a characteristic of PD neuropathology. It is suggested that its degradation has been compromised by the decreased levels of CMA proteins like lysosome-associated membrane protein 2A (LAMP-2A) and heat shock cognate protein 70 (Hsc70). The decrease in the levels of these proteins was associated with the decreases in their respective mRNA levels. As referenced above miRNAs are involved in the regulation of gene expression by degrading their target mRNA [46]. MiRNA dysregulation has been reported in PD brains, and several miRNAs like microRNA-106a (miRNA-106a) are thought to regulate the expression of LAMP-2A or Hsc70 and were shown to be upregulated in PD. In addition, it has been shown that miRNA-106a could potentially affect colorectal cancer apoptosis by inhibiting autophagy-related gene 7 (ATG7), a critical component of the microtubule-associated 1 light chain 3 (LC3) conjugation system that activates autophagy and apoptosis (Figure 6) [48-49].

These findings open a new line of investigation into PD pathogenesis and give credibility to the idea that diminished CMA brought on by miRNA-induced, plays a significant role in the α-syn pathology linked to PD [50]. The 3′-untranslated region (UTR) of α-syn mRNA, which is mostly produced in neurons, regulates the levels of α-syn protein. Importantly, α-syn is downregulated

by microRNA-7 (miRNA-7), protecting cells from oxidative stress. Additionally, miRNA-7 expression is downregulated in animals and neuronal cells treated with MPTP, which may be responsible for elevated expression of α-syn [51].

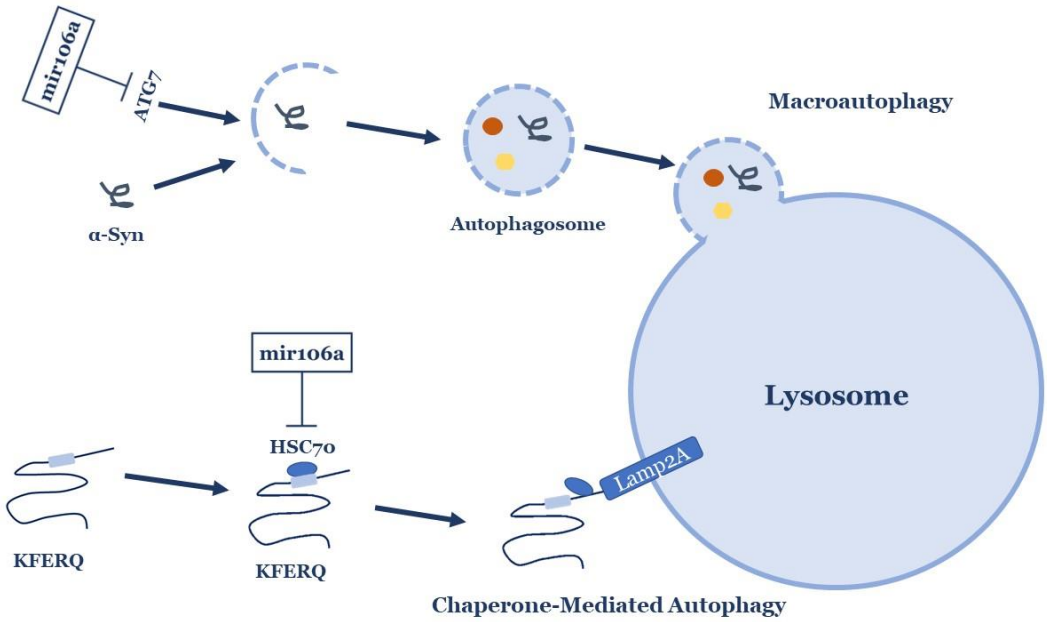

Figure 6 - The representation of CMA: Chaperone proteins such as hHSC-70 interact with cytosolic proteins destined for degradation. This complex is recognized by LAMP-2A, resulting in the translocation of the unfolded cytosolic protein into the lysosome. Hsc70 protein facilitates the proper folding of newly translated and unfolded proteins, as well as stabilizing or degrading altered proteins. MiRNA-106a targets Hsc70 by reducing their expression and causes the increasing accumulation of α-syn in SH-SH5Y cells. This miRNA also targets ATG7 an LC3 processor by inhibiting autophagy. Adapted from [49-50].

Chapter 2

Objectives

PD is a progressive neurodegenerative disorder associated with the degeneration of dopaminergic neurons in the nigrostriatal pathway. Small, non-coding RNAs called miRNAs have regulatory properties and are crucial in the pathophysiology of many human disorders, including PD. MiRNA expression levels have diagnostic and prognostic significance, and research is currently being done on their potential for brain disease therapeutics. The treatments available for PD are not fully effective and they could have several side effects, so the use of cellular and molecular therapies offers an improved way to treat the neurodegenerative disorder. In this thesis, it was hypothesized that miRNA-106a can act as a potential neuroprotective agent to prevent the death of dopaminergic neurons and thus may counteract motor deficits in the 6-OHDA *in vivo* toxin model for PD.

To confirm this hypothesis, we aimed to:

- determine if miRNA-106a can improve motor behavior by performing the rotarod and apomorphine-induced behavioral tests;

- determine if miRNA-106a can protect dopaminergic neurons by analyzing the staining for TH in the SN and ST.

Chapter 3

Materials and Methods

1. *In vivo* studies

All experiments were performed according to protocols approved by the Direção Geral de Alimentação Veterinária (DGAV) and Orgão de Bem-Estar e Ética Animal (ORBEA) from the Health Sciences Research Center (CICS) of the University of Beira Interior (UBI). Furthermore, taking into account the animal's welfare, the principles of the 3 R's were followed.

In this study, male C57BL/6J mice at approximately 6 months of age were used. All of them were under monitored conditions (12-hour light/dark cycles with an ambient temperature of 22°C) in cages with food and water *ad libitum*.

1.1 Stereotaxic Injections

Handling was performed before the stereotaxic injections for the habituation of animals to the handler. After that, the stereotaxic injections were performed, and for this, the animals were anesthetized through an intraperitoneal injection of a mixture of ketamine (90 mg/kg of mouse weight) and xylazine (10 mg/kg of mouse weight) dissolved in 0.9% NaCl (Figure 7). Afterward, the animals were positioned on the STOELTING 51900 stereotaxic table where an incision was made in the middle of the skull using a scalpel after disinfection of the area with Betadine® and local anesthesia with lidocaine (Lidonostrum®). With the skull exposed, the bregma was then defined as the zero-coordinate point. Stereotaxic injections of 7 µg of 6-OHDA and 0.2 µg/µL of miRNA-106a were given using Hamilton™1700 series Gastight™ Gel Loading Syringes, 10 µL at a rate of 0.2 µL/min. The 6-OHDA was injected into the ST in the right hemisphere with the coordinates: anteroposterior (AP) -0.6 mm; dorsolateral (DL) -2.0 mm; dorsoventral (DV) – 3.0 mm. The injection of miRNA-106a was performed in the SN in the right hemisphere with the coordinates: AP -3.0 mm; DL -1.4 mm; DV -4.2 mm. The syringe was left in place for additional five minutes before gradually retracting. The incision was sutured, and the animals were kept in to the incubator at a temperature of 37°C until full recovery.

1.2 Rotarod Performance test

The rotarod performance test involves a spinning rod and forced motor activity, which is often applied by a rodent. One week after the stereotaxic injections, the animals performed the rotarod test, to evaluate motor impairments. Before the test, the animals had training for 2 successive days, using on the first day a velocity of 12 rotations per minute (rpm) and on the following day a velocity of 24 rpm. This training was performed for the habituation of the animals to the apparatus (Figure 7). This test consists in placing the animals on five machine (Rotarod Ugo basile)

individual compartments with a velocity between 4-40 rpm for about 5 minutes. Four trials were performed for each animal with a 20-minute break between trials to allow the animals to rest. After these fourth trials, the animal values were averaged and compared between groups.

1.3 Apomorphine - Induced Rotation Test

The most widely used behavioral test to evaluate unilateral striatal damage is the apomorphine-induced rotation test. Apomorphine is an agonist of DA and its administration to rodents induces several behaviors such as locomotion, sniffing, and stereotypies (repetitive movements).

The rotation of the animals induced by apomorphine was tested on the seventh day after the stereotaxic injections (Figure 7). This test consists of the administration of 0.5 mg/kg apomorphine by subcutaneous injection. Then the animals were placed in a hemispheric rotational bowl for 45 minutes and the number of rotations was recorded and then counted. The results were then calculated using the number of net contralateral turns = contralateral turns – ipsilateral turns. After that the average per group was calculated and compared between groups.

1.4 Perfusion and Tissue Fixation

Perfusion is defined as the passage of fluid through the lymphatic system or blood vessels to an organ or tissue. The objective was to clean and fixate the brain for further analysis. After the apomorphine-induced rotation test, mice were anesthetized with a mixture of 10 mg/kg xylazine and 90 mg/kg ketamine according to the mouse weight (Figure 7). Then, the heart was exposed through an incision in the thoracic midline. The right aorta was cut after a needle was inserted into the left ventricle and, the animals were quickly perfused using sterile 1X phosphate- buffered saline (PBS), followed by 4% Paraformaldehyde (PFA) using the Pharmacia LKB P-1 Peristaltic Pump. The brains were later collected post-fixed overnight at 4°C on a 4% PFA solution, and on the next day the brains were transferred and immersed in a 30% sucrose solution for cryoprotection at 4°C. The last step was freezing with liquid nitrogen and storing the brains at -80°C until they were needed.

2. Brain Slices Preparation

The previously stored frozen brains were cut into coronal sections of 40 µm using a cryostat microtome (Leica CM 3050S, Leica Microsystems). The brain slices were collected sequentially to a 24-weel plate and kept in an antifreeze solution (30% of ethylene glycol, 30% glycerol, 30% water, and 10% phosphate buffer solution) at 4°C. The ST and SN from each animal were collected for immunochemistry.

3. Immunochemistry for Tyrosine Hydroxylase

The brain slices of the ST and SN were initially washed several times with PBS and PBS–Tween 20 0.1% (PBS-T) to remove the cryopreservation solution. The sections were then incubated for 1

hour at room temperature with a permeabilization/blocking solution of 10% Fetal Bovine Serum (FBS) and 0.1% Triton X-100 in PBS to prevent nonspecific bindings. After that, the slices were washed two times with PBS-T. Then, the sections were incubated with 3% H_2O_2 in water protected from the light for 10 min to inhibit endogenous peroxidase activity. After washing again with PBS-T, the sections were then incubated in the primary antibody-rabbit anti-TH (1:2500) diluted in 5% FBS serum overnight at 4°C. On the next day, sections were washed three times with PBS-T for 10 minutes. After the washing process, the slices were incubated with the biotinylated secondary antibody- anti-rabbit (1:200) diluted in 5% FBS serum in PBS for 1 hour at room temperature. The sections were then washed three times for 10 minutes with PBS-T, followed by incubation with Avidine/Biotine (AB) solution (1:500 in PBS) for at least 30 minutes at room temperature. Then the sections were washed three times for 10 minutes with PBS-T and one time for 10 minutes with PBS. This step was followed by the incubation with 3,3'Diaminobenzidine (DAB) solution until color developed approximately for 5-10 minutes. The final step was mounting the sections on microscope slides and allowing them to dry at least overnight. The tissue dehydration was then performed by incubating the sections in different percentages of ethanol (50%, 75%, 35% and 100%), cleared using xylene for 5 minutes and finally, covering the sections using Entellan mounting medium.

4. Statistical analysis

GraphPad Prism 8.0 software was used for the statistical analysis, which included one-way ANOVA and Dunnett's multiple comparisons test. Values of p values < 0.05 were considered significant and data are shown as the mean ± standard error of the mean (SEM).

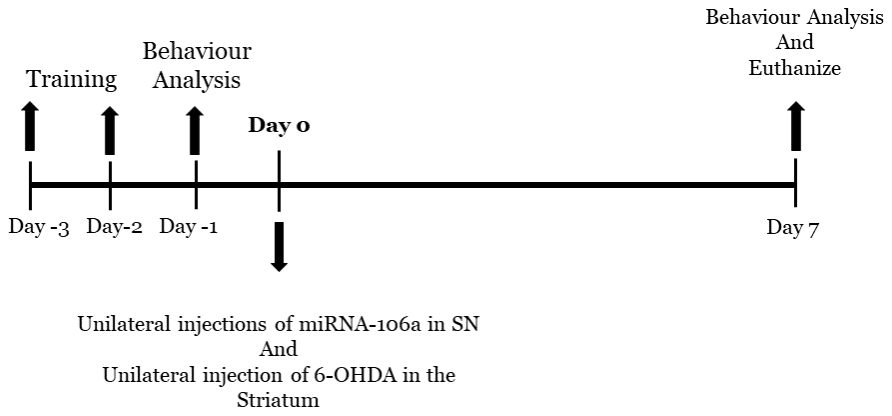

Figure 7 - Schematic representation of the experimental treatments and assays performed *in vivo*.

Chapter 4

Results

1. The effects of miRNA-106a on motor behavior

1.1 Rotarod Performance Test

First, we analyzed the impact of miRNA-106a on motor behavior by performing the rotarod test. This behavioral test was performed one week after the stereotaxic injections with 6-OHDA in the ST and miRNA-106a in the SN. The saline animals were subjected to an injection of 0.02% ascorbic acid dissolved in 0.9% NaCl. The animals were evaluated for four trials for 5 minutes to analyze which ones could stay standing up, without falling off.

As expected, the animals that received 6-OHDA held on to the rack less and had a lower latency time to fall, while the animals treated with 6-OHDA and the miRNA-106a had greater motor endurance, thus holding on to the rack for longer, therefore having a higher latency time to fall (Figure 8; Saline: 267.8 ± 28.3, n=2; 6-OHDA: 228.9 ± 31.6, n=4; 6-OHDA+miRNA-106a: 291.3 ± 3.4, n=4). Despite the differences presented between these groups, these results were not statistically significant. Even so, the animals treated with 6-OHDA and miRNA-106a showed a tendency to improve their motor behavior in comparison to the group of animals treated with 6-OHDA.

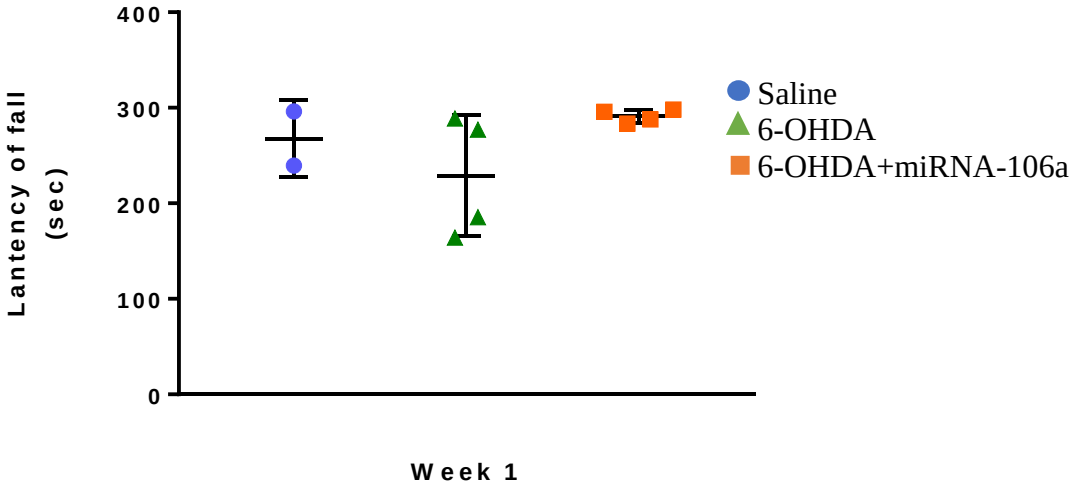

Figure 8 - Evaluation of motor behavior using the rotarod performance test. The animals underwent stereotaxic surgery where the 6-OHDA was injected in the ST and the miRNA-106a was injected in the SN. The control group was injected with 0.02% ascorbic acid dissolved in 0.9% NaCl. One week after surgery the animals performed the rotarod performance test. The graph indicates the seconds that the mice held on the

rack without falling off. The data is shown as the mean ± SEM. Statistical analysis was performed using One-way ANOVA, followed by Tukey's multiple comparison test.

1.2 Apomorphine-Induced Rotation Test

The apomorphine-induced rotation test is a motor test often used when there is a unilateral lesion. This test was performed after the rotarod performance test. The mice were injected subcutaneously with 0.5 mg/kg of apomorphine one week after surgery. Rotations that were made to the side where no lesion was induced (contralateral rotations) were then measured for 45 minutes.

As expected, the 6-OHDA-injured animals showed increased contralateral rotations when compared to the saline animals. Interestingly, a significant decrease in the number of contralateral rotations was seen in mice treated with 0.2µg/µl miRNA-106a + 6-OHDA when compared to the animals injected with 6-OHDA (Figure 9; Saline: -2 ± 8, n=2; 6-OHDA: 49 ± 9.5, n=4; 6-OHDA+miRNA-106a: 8 ± 5.0, n=4). This may suggest that miRNA-106a has a protective function in dopaminergic neurons.

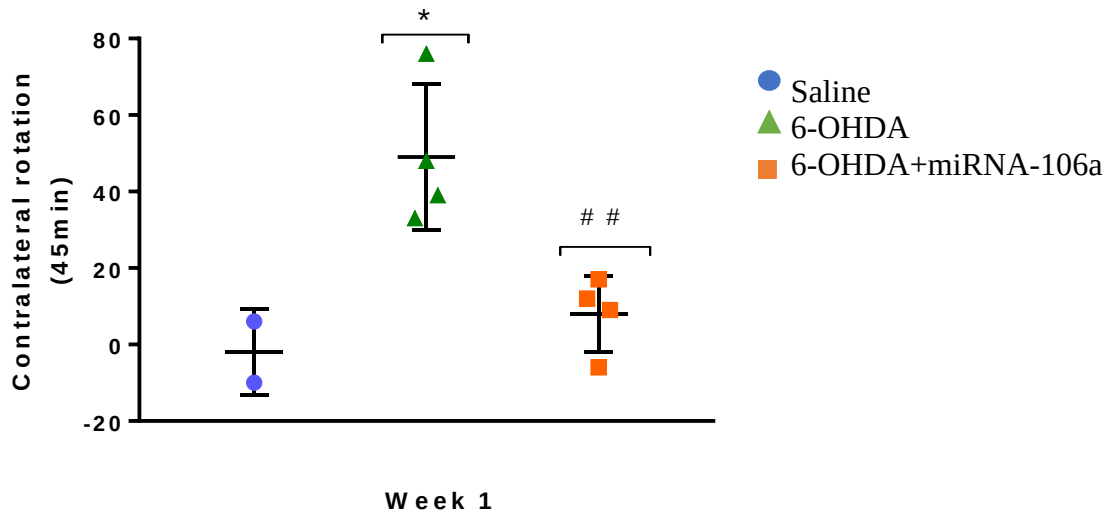

Figure 9 – Evaluation of motor behavior using the apormophine test. MiRNA-106a is able to counteract 6-OHDA-induced motor deficits evaluated by the apomorphine test *in vivo*. Mice underwent stereotaxic surgery where 6-OHDA was injected in the ST and miRNA-106a in the SN. The control group was injected with the solution used to prepare the toxin (0.02% ascorbic acid dissolved in 0.9% NaCl). One week after surgery the mice received a subcutaneous injection with 0.5 mg/kg of apomorphine followed by the apomorphine-induced rotation test. The graph indicates the number of contralateral rotations, over 45 minutes in the experimental group of mice treated with saline, 6-OHDA, and 6-OHDA together with miRNA-106a. Data are shown as the mean ± SEM. Statistical analysis was performed using One-way ANOVA, followed by Tukey's multiple comparison test. *p<0.05 when compared with the saline group and ##p<0.01 when compared with the 6-OHDA group.

2. The effects of miRNA-106a on dopaminergic survival

Following the previous behavioral experiments, mice were then perfused, brain collected, and brain slices were made to proceed with immunohistochemistry against TH. TH was analyzed in the two most affected brain regions in PD: the SN and the ST. In the case of the ST, two variables

were studied: the intensity of the staining and the area of the stained region. In the case of the SN, TH-positive neurons were counted to evaluate dopaminergic survival. Representative images of each staining are shown in Figure 10.

As expected, 6-OHDA-lesioned mice showed a decrease in the area and intensity of TH staining in the ST, as well as in the number of TH-positive neurons in the SN when compared to the saline animals (Figure 11, A: 6-OHDA: 86.7 ± 1.0, n=2; B: 6-OHDA: 76.6 ± 9.6, n=3; C: 6-OHDA: 64.0 ± 5.9, n=3, respectively; saline was set to 100%). Mice treated with miRNA-106a and 6-OHDA showed a recovery in the area and intensity of TH staining in the ST when compared with 6-OHDA-lesioned mice (Figure 11, A: 6-OHDA+miRNA-160a: 133 ± 3.3, n=3; B: 6-OHDA+miRNA-160a: 94.3 ± 1.0, n=4; C: 6-OHDA+miRNA-160a: 67.2 ± 3.7, n=4; respectively; saline was set to 100%). In contrast, no protective effect was seen regarding the number of TH neurons in the SN. These results suggest that miRNA-106a may have a protective effect on dopaminergic striatal terminals without a robust effect on the number of neurons in the SN.

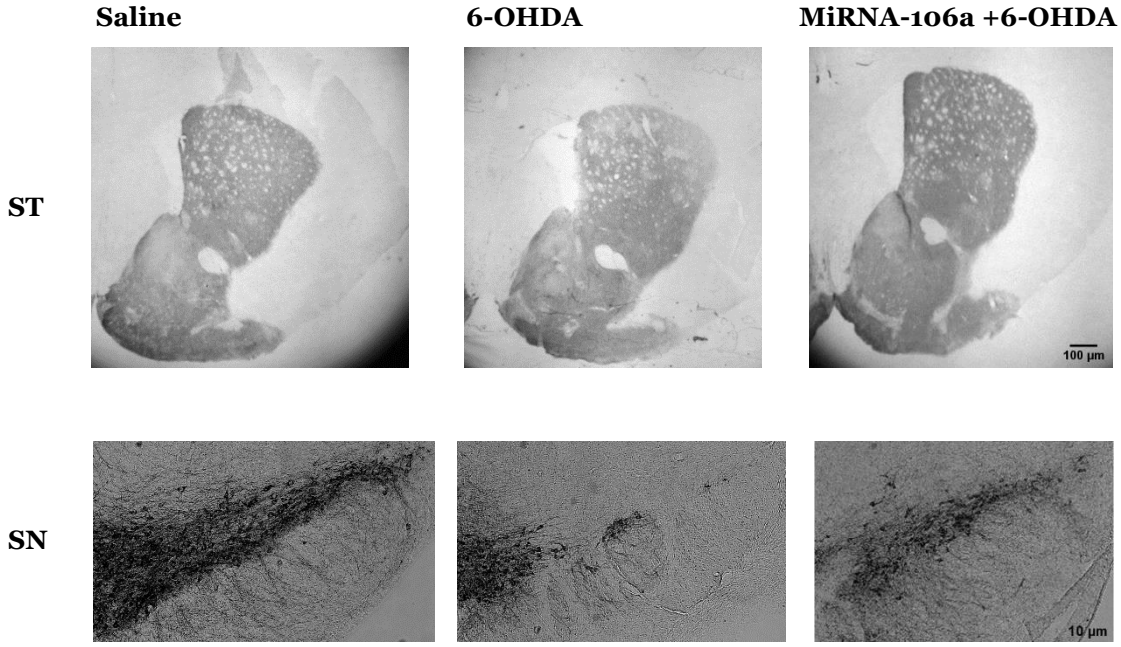

Figure 10 - Representative images of TH staining in the ST and SN. Immunohistochemistry against TH in the SN and ST, one week after stereotaxic surgeries with saline, 6-OHDA and/or miRNA-106a. Scale bar: 100 µm for the images above and 10 µm for the images below. By analyzing the images, we could see that mice injected with 6-OHDA had a decrease in the intensity/area of the staining and the number of TH-positive neurons. Recovery is seen in mice injected with 6-OHDA and miRNA-106a.

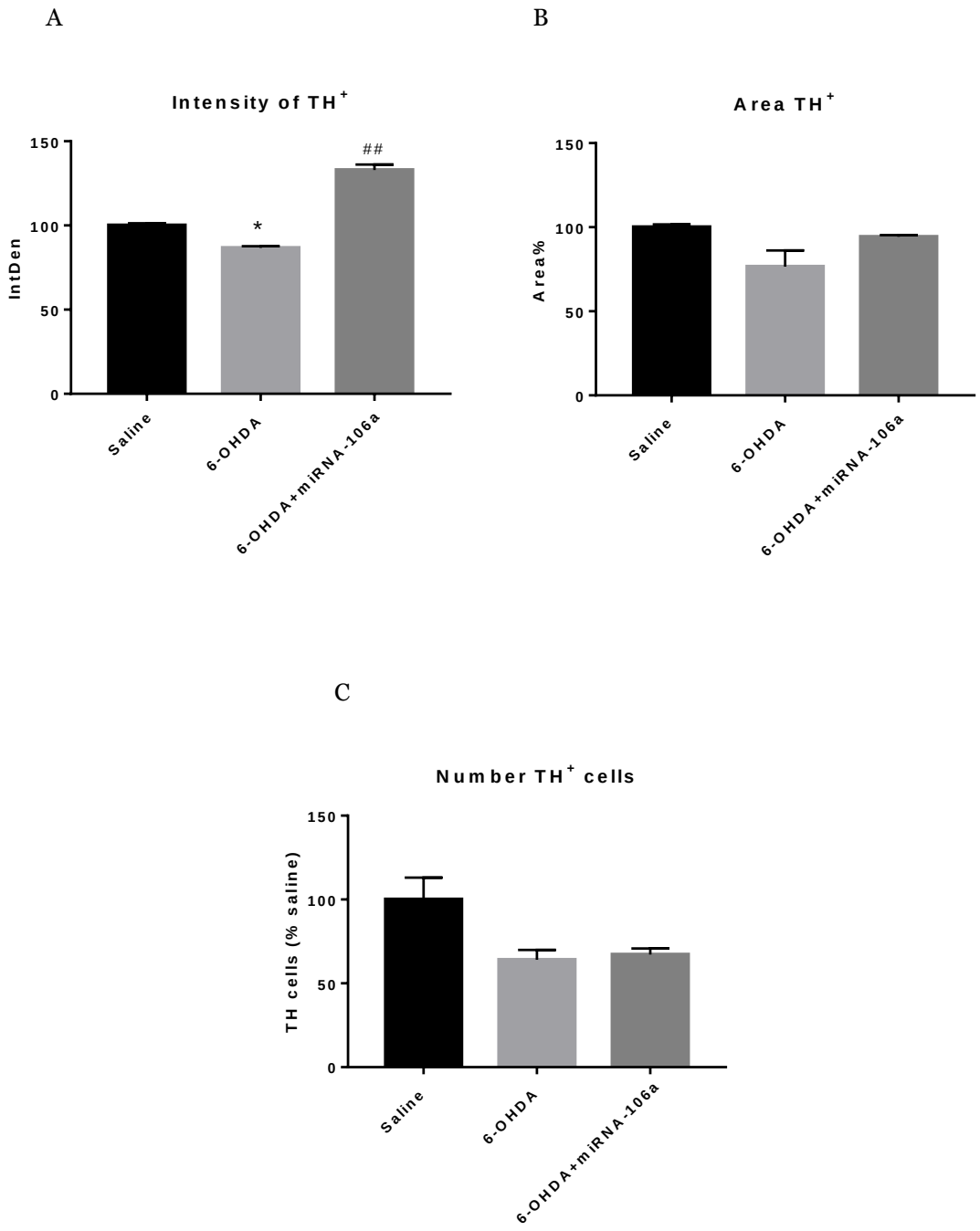

Figure 11 - MiRNA-106a counteracts the reduction induced by 6-OHDA on striatal TH-positive fibers but not on the number of TH+ neurons in the SN. Bar graph representing TH staining intensity (A) and % area (B) in the ST. (C) Bar graph of the number of TH-positive neurons in the SN. The control group was set to 100%. Data are shown as the mean ± SEM. Statistical analysis was performed using One-way ANOVA, followed by Tukey's multiple comparison test. *p<0.05 when compared with control conditions, and ##p<0.01 when compared 6-OHDA group.

Chapter 5

Discussion

MiRNAs are small endogenous RNAs that regulate several biological functions such as cell proliferation, differentiation, apoptosis, and tumor growth by modulating post-transcriptional programs [43]. Recent evidence showed that they could be potential therapeutic molecules to counteract pathogenic processes in PD [44]. The already existing therapies like drug-based therapies try to relieve symptoms at an early stage of the disease [23]. It has been shown that the combination of physical exercises with drug-based therapy improves the motor activity of the patients [27]. In addition to these strategies, other approaches include ablative surgeries, electric stimulation, cell therapies, and gene editing. Since PD is a multifactorial disease, current treatments must modulate several targets and molecular pathways. A whole disease phenotype might be affected by manipulating a single miRNA molecule considering that a single miRNA can influence numerous target genes, making these RNA molecules particularly attractive from a therapeutic standpoint. Furthermore, finding unregulated miRNAs in patients may help with earlier diagnosis and disease progression monitoring [52]. Therefore, research on the role of miRNAs in neurodegeneration is growing quickly.

Previous research showed the critical role of the miRNA machinery in DA neurons. In Drosophila and mouse models, disruption of this network results in the progressive loss of midbrain dopaminergic neurons, which degenerate in PD, generating movement signs and symptoms [45]. For example, it is shown that miRNA-7, which is expressed mainly in neurons represses α-syn protein levels. In the MPTP-induced neurotoxin model of PD, miRNA-7 expression decreases, perhaps contributing to increased α-syn expression. MiRNA-7 causes the downregulation of α-syn protecting cells against oxidative stress [51].

Since there are no reports that associate miRNA-106a with PD, this dissertation aimed to investigate the effects of this miRNA on dopaminergic neuron survival and animal motor behavior. First, we evaluate the effect of miRNA-106a on animal motor behavior with the rotarod performance test and apomorphine-induced rotation test. These two tests demonstrated that miRNA-106a counteracts motor behavior performance as compared to 6-OHDA-lesioned animals. Next, we evaluated the effect of miRNA-106a on dopaminergic survival through immunohistochemistry against TH. Altogether, our results suggest that miRNA-106a has a neuroprotective effect on dopaminergic neurons and a positive functional effect on motor behavior. Some evidence shows that miRNA-106a expression is increased in PD and given that it leads to a decrease in the levels of LAMP2A and Hsc70 this will cause a dysregulation in the autophagy process. This is an important process in the control of cellular homeostasis that when

altered may become harmful [48]. The results obtained in this thesis may be contradictory. Therefore, in subsequent trials, it will be necessary to increase the number of mice *per* experimental group to acquire more accurate results and also to assess the molecular mechanisms behind the observed effects.

MiRNA transport into the brain is still a significant barrier to the effective development of new miRNA-based therapeutic approaches. Exosomes, viruses, liposomes, polymeric nanoparticles (NPs), and other techniques have been explored to get around their drawbacks [53]. Our research group showed that NPs loaded with microRNA-124 (miRNA-124) were able to stimulate the migration of newly born neurons into the 6-OHDA-induced lesion striatum *in vivo*. Most importantly, miRNA-124 NPs were able to counteract the motor deficits induced by 6-OHDA. Overall, these results showed that miRNA-124 NPs can be used as a novel therapeutic strategy to enhance endogenous brain repair in PD [54]. Then, we also showed that extracellular vesicles loaded with the same miRNA protected dopaminergic neurons in the SN and ST, ultimately counteracting motor deficits induced by 6-OHDA. Our research demonstrates a unique and therapeutically promising use of miRNA specifically the miRNA-124-3p, for PD [55]. In the future, we may also envisage loading miRNA-106a into these delivery systems to boost its beneficial effects.

In this study, miRNA administration was performed by intracerebral delivery. This procedure has the advantage that the molecules are delivered to the precise region of interest but is an invasive procedure with a slow postoperative recovery. Other delivery strategies may be used in the future, such as intranasal administration, to maximize the neuroprotective effect of this molecule and avoid invasive procedures. This kind of administration has the advantages of being rapid, safe, and non-invasive, it is reproducible in patients and could circumvent the BBB, which prevents some drugs from entering the brain. Recently, this alternative of a non-invasive method has been used to improve brain targeting in PD. The delivery of growth factors (GFs) like glial cell-derived neurotrophic factor (GDNF) using this kind of delivery system has gained attention in recent years as a potentially effective treatment strategy [56].

It will be also relevant to compare the results obtained with other toxins or genetic animal models of PD. For example, other models like the intranasal administration of MPTP in rats may also offer fresh insights into the underlying processes of PD pathogenesis because of the time course of the olfactory, cognitive, and motor deficits. Previous research has shown that intranasal infusion of viruses, cadmium, or aluminium, or inhalation of manganese or aluminium, can all cause a deposition inside the CNS and might induce neurotoxic effects that may be related to the etiology of PD [57]. These models could also be envisioned for future studies.

To understand the effective role that miRNA-106a plays it would be important to evaluate the signaling pathways responsible for the functional effects and other parameters related to the disease like autophagy. For example, it could be interesting to evaluate the expression of the

proteins LC3, Lamp2 and p62 wich are involved in autophagy. Neuroinflammation is also a common event in neurodegenerative disorders. Glial cells play an important role in neuroinflammation. Therefore, it could be interesting to measure the expression of glial markers, such as GFAP (Glial fibrillary acidic protein), DAT, and IBA-1 (ionized calcium-binding adapter molecule 1), and also the expression of pro-inflammatory and anti-inflammatory cytokines like TNF-α (tumor nerosis factor alpha), IL-1β (interleukin-1 beta), and IL-10 (interleukin 10).

Pharmaceutical companies may view the discovery of therapeutic miRNAs as one of the most significant and exciting medical advancements. However, to join the market, they must pass various stages of clinical trials. MiRNA-based medicines have shown promise as potential next-generation treatments for several illnesses. Currently, several pharmaceutical companies are working on the creation of therapeutic miRNAs and cutting-edge delivery technologies to transport miRNAs to the desired place. It is possible to anticipate that therapeutic miRNAs currently in development will reach the clinics as next-generation therapies. The ideal choice for future medicine is the ability to regulate, specifically therapeutic miRNAs [58]. The development of therapeutic miRNAs is now moving to a new stage that combines pharmacological drug delivery, preclinical toxicity, and regulatory guidelines after the completion of target validation for several candidates.

Chapter 6

Conclusions

PD is a neurodegenerative disorder with no cure. Current therapies attenuate the symptoms only and often induce side effects. Therefore, it is urgent to find new therapeutic targets for this disease. The use of miRNAs considering their putative neuroprotective effect would be an alternative to the existing therapies.

The results of this dissertation suggest that the intracerebral administration of miR-106a had a neuroprotective effect against 6-OHDA-induced lesion *in vivo*. MiRNA-106a was able to improve motor behavior activity, as detected by the rotarod performance test and apomorphine-induced rotation test, and also enhance dopaminergic neuronal survival, analyzed by immunochemistry for TH.

For further and more effective conclusions, more animals and experimental approaches are needed to prove the obtained results presented in this thesis. It will also be important to test alternative drug delivery systems to boost clinical translation.

Chapter 7

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