Role of microRNAs on glial activity in a Parkinson's disease mouse model

Versão final após defesa

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Dissertação para obtenção do Grau de Mestre em

Ciências Biomédicas

(2º ciclo de estudos)

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Covilhã, dezembro de 2022

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Acknowledgements

The realization of this master's thesis was only possible thanks to the support and encouragement of several people to whom I will be eternally grateful.

To Professor Liliana, for her guidance, support, and comfort during the entire work.

To all the group colleagues, for their help and accompaniment during the learning of the protocols necessary for its accomplishment.

To my companion on the way, Mafalda Proença, for all the companionship, help, understanding and support to resist the frustration and difficulties inherent to the final year of the Masters.

To my classmates Beatriz Almeida and Susana Ferreira, for the help, friendship, follow-up, and love with which we shared the last few months.

To my parents for always supporting all the challenges I set myself and providing me with the resilient personality that allows me to always reach unthinkable goals.

Finally, but especially important, to my life partner, for supporting me unconditionally and making me believe that I am capable of anything. For always leading me to my best version.

I dedicate this work to them and appreciate it with all my heart.

Resumo

A doença de Parkinson (DP) é uma doença neurodegenerativa caracterizada pela acumulação da proteína α-sinucleína e, consequente, degeneração neuronal, culminando em sintomas motores e não motores. A sua etiologia pode advir de agentes ambientais ou fatores genéticos. Atualmente, não existem tratamentos eficazes para a DP, sendo assim importante identificar novos alvos e estratégias terapêuticas. Experiências realizadas em modelos animais *in vitro* e *in vivo* têm sido uma ferramenta valiosa para entender os mecanismos fisiopatológicos da DP. Modelos animais permitem o estudo de várias caraterísticas do fenótipo de DP e categorizam-se em modelos genéticos e modelos induzidos de toxinas, no qual se enquadra o modelo induzido pela 6-hidroxidopamina (6-OHDA) utilizado no presente trabalho. A 6-OHDA é uma neurotoxina que, após ser captada pelos neurónios dopaminérgicos, através dos transportadores de dopamina (DAT), induz a formação de radicais livres, disfunção mitocondrial e ativação glial, culminando na morte de neurónios dopaminérgicos. Em distintos estudos, os miRNAs (miR) foram identificados como atenuantes da DP devido ao seu potente efeito modulador da regulação da expressão de genes associados a mecanismos fisiopatológicos da DP. Estudos prévios do nosso grupo de investigação demonstraram que o miR-124 possui efeitos neuroprotetores e neurogénicos no modelo da 6-OHDA. Outros estudos relatam níveis aumentados de miR-204 e miR-224 em cérebros de pacientes com DP e modelos experimentais celulares e animais. Apesar de existirem evidências de valores aumentados destes miRs, não existem estudos sobre os seus efeitos celulares e moleculares em modelos da DP. Neste estudo, examinamos o efeito dos miR-204 e miR-224 na ativação astrocítica e microglial, avaliando os níveis de expressão proteica dos marcadores da proteína ácida fibrilar glial (GFAP) e da molécula adaptadora de ligação ao cálcio ionizado-1 (IBA-1) por western-blot. Como esperado, verificamos um aumento de expressão de GFAP e IBA-1 na substância nigra e estriado de murganhos tratados com a toxina 6-OHDA em comparação com murganhos tratados com solução salina, o que indica ativação astrocitária e da microglia, respetivamente. Em animais expostos com 6-OHDA no estriado e miR-204 ou miR-224 na substância nigra, verificamos uma diminuição da expressão de GFAP e IBA-1 relativamente aos animais tratados com a 6-OHDA, o que sugere um efeito anti-inflamatório e protetor por parte destes miRs. No entanto, estudos adicionais são necessários para compreender melhor os efeitos destes miRs na doença de Parkinson, de forma a poderem ser utilizados como alvos terapêuticos.

Palavras-chave

Doença de Parkinson;neuroinflamação;miR-204;miR-224;microglia;astrócitos

Resumo Alargado

A doença de Parkinson (DP) é uma doença neurodegenerativa com incidência em cerca de 3% da população acima de sessenta e cinco anos de idade e predominância no sexo masculino. Ao nível celular, a DP é caracterizada pela perda de neurónios dopaminérgicos na *substância nigra pars compacta* (SN) e dos seus terminais nervosos no corpo estriado (ST). As características patológicas da DP incluem a presença de inclusões citoplasmáticas de alfa-sinucleína (corpos de Lewy) em neurónios dopaminérgicos do mesencéfalo. Os principais sintomas incluem rigidez muscular, déficits funcionais, comprometimento cognitivo leve, bradicinesia, tremor e instabilidades na marcha. O desenvolvimento da DP pode ser precoce ou tardio, com sintomas geralmente categorizados em 5 categorias: precoce, motor primário, motor secundário, sintomas primários não motores e, por último, sintomas não motores.

Atualmente não existem ainda tratamentos eficazes para a DP. Assim, são vários os trabalhos com vista à identificação de novos alvos terapêuticos para esta patologia. Entre estes alvos encontram-se os microRNAs (miRs) que, devido aos seus efeitos anti-inflamatórios e neuroprotetores, têm sido um potencial alvo muito promissor. Estudos recentes indicam que os miRNAs exercem funções essenciais na etiologia e progressão de doenças neurodegenerativas, como por exemplo na DP, atuando por degradação do mRNA ou inibição da tradução de proteínas.

Vários estudos mostraram que vários miRs estão desregulados na DP e, que essa desregulação, pode contribuir para a patogénese da DP. Inclusivé, estudos *in vitro* e *in vivo* recentes comprovaram a importância dos miRs na regulação da expressão proteica e génica de moduladores com efeitos neuroprotetores e anti-inflamatórios tornando os miRs uma das novas formas terapêuticas mais promissoras na DP. Estudos prévios do grupo de investigação identificaram o miR-214 como alvo terapêutico relevante para a DP, atuando como agente neuroprotetor e neurogénico, capaz de contrariar os déficits motores presentes no modelo animal da DP induzido pela neurotoxina 6-hidroxidopamina (6-OHDA). Neste trabalho de mestrado, pretendeu-se avaliar o efeito dos miR-204 e o miR-224 na reatividade glial associada à DP.

Em particular, o miR-204 possui efeitos nas vias de sinalização, incluindo a P53 e FoxO, que se encontram alteradas na DP. A proteína p53, com expressão e atividades alteradas associadas à morte neuronal, e a proteína FoxO, com funções pró-apoptóticas, quando superexpressa conduz à apoptose de neurónios dopaminérgicos. Relativamente ao miR-224, estudos prévios mostram que se encontra aumentado na SN e que tal poderia conduzir à inibição da expressão de LAMP-2a, resultando na acumulação de α-sinucleína e inibição da sua degradação. Os fatores referidos sobre os miR-204 e miR-224, em conjunto com resultados promissores do nosso grupo de investigação referentes ao miR-124, levaram ao estudo destes miR-204 e -224 a nível da atividade glial, de forma a procurar mais respostas acerca dos seus efeitos numa das vertentes da fisiopatologia da DP.

Assim, o objetivo principal deste trabalho foi avaliar o papel dos miR-204 e miR-224 na atividade glial utilizando o modelo animal *in vivo* de DP através da injeção intraestriatal da neurotoxina 6-OHDA. A injeção intracerebral de 6-OHDA é um modelo experimental amplamente utilizado para estudar a fisiopatologia e os déficits motores da DP. Murganhos adultos foram injetados com 6-OHDA no ST e com o respetivo miR na SN. 1 e 5 Dias após as cirurgias fomos avaliar a expressão do marcador astrocitário proteína ácida fibrilar glial GFAP e do marcador microglial adaptadora do cálcio ionizado (IBA-1) no ST e na SN por western-blot (WB) e por imunohistoquímica.

A expressão quantitativa de GFAP analisada por WB foi mais elevada nas amostras de ST e SN de murganhos tratados com 6-OHDA comparativamente aos animais tratados com a solução salina ou tratados com 6-OHDA+miRs. Analisando a morfologia celular obtida pela imunohistoquimica, é notório que fatias de SN e ST de murganhos tratados com 6-OHDA apresentam maior intensidade da expressão de GFAP e maior reatividade que as restantes condições experimentais. Assim, murganhos tratados com as condições 6-OHDA+miR-204 e 6-OHDA+miR-224 apresentam menor expressão e intensidade de marcação de GFAP, o que sugere um efeito anti-inflamatório por parte dos miRs em estudo.

A exposição à 6-OHDA no ST, parece induzir um aumento nos níveis de expressão de GFAP em relação aos animais salinos tanto 1 quanto 5 dias após as cirurgias. Este efeito não é estatisticamente significativo devido ao baixo número de murganhos testados (n).

Um dia após a cirurgia, observamos que o miR-204 conferiu efeito protetor, reduzindo os níveis de expressão de GFAP em comparação aos animais tratados com 6-OHDA.

Aos 5 dias pós-cirurgia, por meio de imunohistoquímica, é notório que a 6-OHDA induziu uma coloração de GFAP mais reativa e com maior intensidade do que as demais condições experimentais.

Relativamente aos níveis de expressão de GFAP em amostras de SN em 1 e 5 dias após a cirurgia, a expressão de GFAP foi encontrada aumentada em animais tratados com 6-OHDA, 6-OHDA+ miR-204 e 6-OHDA+miR-224 em comparação com animais tratados com solução salina. Aos 5 dias pós-cirurgia, encontramos um aumento significativo da expressão de GFAP em animais tratados com 6-OHDA em comparação com solução salina. Curiosamente, ambos os miRs foram capazes de reduzir essa expressão aumentada de GFAP induzida por 6-OHDA. A partir da imunohistoquímica observamos que 6-OHDA induziu uma morfologia de GFAP mais reativa e com maior intensidade de coloração do que os animais tratados com a solução salina. Além disso, parece que tanto o miR-204 quanto o miR-224 reduziram a reatividade do GFAP, parecendo o miR-204 mais eficaz.

Numa segunda etapa do estudo, fomos avaliar a ativação da microglia através da deteção da proteína IBA-1 em cortes cerebrais de murganhos tratados com as mesmas condições experimentais. Análise microscópica qualitativa sugere que a microglia apresenta uma morfologia amebóide em fatias de SN e ST de animais tratados apenas com 6-OHDA e nos tratados com 6-OHDA +miR-204 e miR-224. No entanto, esta forma típica do seu estágio ativado não foi verificada nos animais salinos.

Apesar dos nossos estudos darem pistas iniciais sobre os efeitos destes miRs na resposta neuroinflamatória, é ainda necessário aprofundar o estudo destes mecanismos, utilizar um maior número de animais e avaliar o seu efeito funcional. Contudo, considerando o potencial efeito anti-inflamatório dos miR-204 e miR-224 na DP, parece óbvio que é de extrema relevância continuar a estudar os processos envolvidos medindo outros aspetos envolvidos na patogénese da DP.

Abstract

Parkinson's disease (PD) is a neurodegenerative disease characterized by the accumulation of α-synuclein protein and, consequently, neuronal degeneration, culminating in motor and non-motor symptoms. Its etiology may come from environmental agents or genetic factors. Currently, there are no effective treatments for PD, so it is important to identify new targets and therapeutic strategies. Experiments carried out in *in vitro* and *in vivo* animal models have been a valuable tool to understand the pathophysiological mechanisms of PD. Animal models allow the study of several characteristics of the PD phenotype and are categorized into genetic models and toxin-induced models, which include the model induced by 6-hydroxydopamine (6-OHDA) used in the present work. 6-OHDA is a neurotoxin that, after being taken up by dopaminergic neurons, through dopamine transporters (DAT), induces the formation of free radicals, mitochondrial dysfunction, and glial activation, culminating in the death of dopaminergic neurons. In different studies, miRNAs (miR) have been identified as attenuating PD due to their potent modulating effect on the regulation of gene expression associated with the pathophysiological mechanisms of PD. Previous studies by our research group showed that miR-124 has a neuroprotective and neurogenic effect in the 6-OHDA model. Other studies report increased levels of miR-204 and miR-224 in the brains of patients with PD and experimental cellular and animal models. Although there is evidence of increased values of these miRs, there are no studies on their cellular and molecular effects in PD models. In this study, we examined the effects of miR-204 and miR-224 on astrocytic and microglial activation, evaluating the protein expression levels of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule-1 (IBA-1) markers by western blot. As expected, we found an increase in GFAP and IBA-1 expression in the SN and SN of mice treated with 6-OHDA toxin compared to saline-treated mice, which indicates astrocytic and microglial activation, respectively. In animals exposed to 6-OHDA in the striatum and miR-204 or miR-224 in the substantia nigra, we observed a decrease in the expression of GFAP and IBA-1 compared to animals treated with 6-OHDA, which suggests an anti-inflammatory and protective effects of these miRs. However, further studies are needed to better understand the effects of these miRs on PD, so that they can be used as therapeutic targets.

Keywords

Parkinson's disease; neuroinflammation; miR-204; miR-224; microglia; astrocytes

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Chapter 1 - Introduction

1.1. Parkinson's Disease overview

Parkinson's disease (PD) is a neurodegenerative disease of the central nervous system that affects around 3% of the population older than sixty-five, with predominance in males. At the cellular level, PD is characterized by the loss of midbrain dopaminergic (DA) neurons in the substantia nigra pars compacta (SN) and their striatal terminals. Pathological features of PD include the presence of Lewy bodies, and alpha-synuclein cytoplasmic inclusions in midbrain dopaminergic neurons. The main symptoms include muscular rigidity, functional deficits, mild cognitive impairment, bradykinesia, tremor, and gait instabilities (Cerri et al., 2019). PD development can be early or delayed with symptoms usually categorized into 5 different categories, as shown in Figure 1: early, primary motor, secondary motor, primary non-motor symptoms, and lastly, late non-motor symptoms (Jamebozorgi et al., 2019).

Parkinson's Disease Symptoms	**Early**	Mild tremors	
		Soft speech	
		Movement problem	
		Depression	
		Fatigue	
		Posture difficulty	
	Motor	*Primary*	Rigidity Bradykinesia Resting Tremor Slow movement
		Secondary	Dystonia Muscle cramps Chewing/ swallowing difficulties Sexual dysfunction
	Non-motor	*Primary*	Dementia Pain Cognitive dysfunction Depression
		Secondary	Emotional changes Urinary problems Sweting Hypotension

Figure 1 - Symptoms of Parkinson's Disease are categorized into three major groups: early, motor, and non-motor, which the last two can also be differentiated into primary and secondary (adapted from (Jamebozorgi et al., 2019)).

Pathologic alterations can precede the motor and non-motor symptoms by two or more decades, which leads to the diagnosis of the disease at a very advanced stage.

Several publications suggest that aging and environmental agents, such as pesticides and drugs of abuse, might stimulate neuropathology. In turn, aging processes may lead to a low-level but chronic inflammation that may impact neuronal function and survival (Beitz, 2014).

PD is usually a sporadic disorder; however, PD genetic traits were also identified in several familial motor disorders. Protein aggregation and gene mutation have an important role in the degeneration of dopaminergic neurons. To date, researchers already identified several monogenic forms of PD and genetic risk factors that increase the possibility of developing PD. The monogenic forms are rare and occur due to a single mutation in a dominant or recessive gene, representing around 3-5% of sporadic cases and about 30% of familiar events

Most gene mutations are associated with mitochondrial dysfunction and result in mitochondrial DNA (mtDNA) damage, reduced mitochondrial membrane potential (MMP), decreased Adenosine 5'-triphosphate (ATP) levels, increased reactive oxygen species (ROS), structural organelle defects, and decreased ATP levels. These flaw phases of mitochondrial dysfunction have already been identified as responsible for PD etiology such as some susceptibility genes that together will allow understanding the neurodegeneration pathogenic mechanisms of this disease (Selvaraj & Piramanayagam, 2019).

Latest discoveries found candidate genes and specific molecular pathways to PD pathogenesis. Around 20 genetic PD loci are already identified. Mutations in nine genes are already confirmed as monogenic PD causes, wherein three have autosomal recessive inheritance (Parkin, PINKI, and DJ-1) and six have autosomal dominant inheritance (SNCA, LRRK2, VPS35, EIF4G1, DNAJC13, and CHCHD2). PARK2 is located on the 6q25.2-27 chromosome and is associated with the development of PD autosomal recessive form, with early initiation. This gene encodes the Parkin protein, cytosolic ubiquitin-E3-ligase, which the main function is mitophagy regulation. Parkin and PINK1, a mitochondrial protein product of another autosomal recessive gene of PD, act in tandem to control the state of mitochondria. What happens is that when the depolarizations of the dysfunctional mitochondria occur, PINK1 is stabilized, Parkin is recruited from the cytosol and will be activated while it's delivered to the mitochondria through PINK1-kinase activity; meanwhile, Park activated form initiates selective autophagy of the damaged organelle. Parkin is currently described as a polyvalent neuroprotective agent of dopaminergic neuron survival with an extremely important role when exposed to neurotoxins(Konovalova et al., 2015).

Epigenetics can also have a massive influence on PD risk. Therefore, genes can be modulated in response to environmental stimuli through processes such as methylation, phosphorylation, acetylation, and generation of microRNAs (miRs). It's already known that epigenetic methylation of the α-synuclein locus upregulates its translation, which leads to reduced levels of methylation in the SN of patients with sporadic PD.

Patient brains have also revealed distinct expression of several miRs, which has been related to mitochondrial dysfunctional development(Mullin & Schapira, 2015).

1.1.1. Pathogenesis features of PD

PD is a chronic and progressive neurodegenerative disorder with several pathological features. The degeneration of dopamine neurons occurs in the SN of the midbrain with consequent loss of their axons projected along the nigrostriatal pathway, which can lead to the loss of the neurotransmitter dopamine that causes the primary motor symptoms.

The two main characteristics of neuropathology in PD are α-synuclein accumulation and dopaminergic neuronal degeneration. The protein α-synuclein is the main component of protein inclusions, also known as Lewy bodies, and therefore, its mutant forms can lead to familial PD. Lewy bodies are present in several brain areas and occur before the loss of dopaminergic neurons (MacMahon Copas et al., 2021). These Lewy bodies are intraneuronal inclusions with immunoreactive aggregates of alpha-synuclein and other neurofilament proteins (as ubiquitin). These aggregates will direct or indirectly spread to other cells leading to increased cell death (Srinivasan et al., 2021). To remove intracellular proteins there are two main procedures, the lysosomal and proteasomal pathways. Intracellular proteins are degraded by 26S proteasome through the ubiquitin system that forms lysine residues by conjugating at least 4 ubiquitin molecules chain shaped. This ubiquitin chain conjugation with substrate proteins arises out of several serial selective enzymatic reactions defined by E3 ligases. The degradation occurs inside 20S proteasome after recognition by ATPase subunits of the 19S proteasome regulatory element of the ubiquitinated substrate protein (Stefanis et al., 2019).

Inflammation and oxidative stress are also important pathophysiologic mechanisms in the process of dopaminergic neuron degeneration (Wei et al., 2018). The innate immune system has pattern recognition receptors (PRRs) that detect general patterns or structures present on the surface of potential pathogens, known as Pathogen-Associated Molecular Patterns (PAMPs), and “danger” signals known as Damage-Associated Molecular Patterns (DAMPs). In general, these receptors stimulate the innate system in response to disease or injury (Kigerl et al., 2014) This is important because PD has already been related to several viral pathogens such as varicella-zoster virus-1 (HSV-1), Ebola virus (EBV), human immunodeficiency virus, *Helicobacter pylori*, and even influenza A. In addition, genes associated with PD risk and peripheral inflammation also represent an essential role in chronic inflammation of PD. This peripheral inflammation involves the innate or adaptative immune system and the release of proinflammatory cytokines. In fact, microbiota exposure, and pathogenic and/or environmental changes may favor α-synuclein protein aggregations with inflammatory responses that will lead to further neurodegeneration. This is why immunomodulatory therapies are being the main focus of PD researchers (Pajares et al., 2020).

In the brain, the activation of astrocytes and microglia, release pro-inflammatory cytokines that result in the degeneration of DA neurons in the SN.

Therefore, tackling neuroinflammation is being proposed as a therapeutic strategy for several neurodegenerative diseases such as PD. Mitochondrial dysfunction can also be observed in PD

animal models. Several studies support that this defect seems a widespread feature in both monogenic and sporadic forms of PD, appearing in an early stage of the disease. The majority of mitochondrial deficiencies associated with PD include modifications in mitochondrial morphology and dynamics, mitochondrial electron transport chain impairments, irregular calcium homeostasis, and mitochondrial DNA mutations which can lead to a decline in energy production and generation of oxygen species with consequent apoptosis (Subramaniam & Chesselet, 2013). Various insults such as spontaneous, genetic, or environmental can cause mitochondrial damage as DNA, lipids, or proteins damage that led to the loss of metabolic functions (such as ATP synthesis), consumption of ATP to generate membrane potential (instead of mitochondria production of ATP) or even more production of ROS by the flawed mitochondria. This damage triggers several cellular responses such as DNA repair, lipases, proteases, responses from mitochondrial unfolded proteins, mitophagy, and even apoptosis (Youle & Van Der Bliek, 2012).Apoptosis is an ATP-dependent process, highly controlled, that depends on caspases activation and consequently, caspases initiators that cleave multiple substrates that precipitate cell death by producing chromatin condensation, DNA fragmentation, membrane blebbing, and cell shrinkage that led to formation of small apoptotic bodies. This overactivation of apoptosis is found in PD patient brains and animal models induced with neurotoxins (Dionísio et al., 2021). Dopaminergic neuron degeneration has been correlated with ROS overproduction, which can occur due to inflammation and/or mitochondrial dysfunctions. In PD, exacerbated ROS production occurs due to neuroinflammation, age, mitochondria dysfunction, dopamine degradation, and increased levels of calcium and iron; and its levels increase in response to pesticides or neurotoxins. SN neurons also accumulate neuromelanin (NM) that accumulates metal ions, iron, and generates ROS. There are two defense systems against ROS production from iron-dopamine chemistry, which are vesicular monoamine transporters 2 (VMAT2) and dopamine transporter (DAT). They can take free dopamine from the synapse into synaptic vesicles, sheltering them from oxidation. With aging, nigral expression of DAT decreases, and alfa-synuclein interacts with VMAT2 in vesicle uptake which leads to impaired dopamine release into synapse and reuptake by the vesicles. Neurodegeneration led to ROS production that can damage lipid membranes and cellular proteins, which causes mitochondrial dysfunction. Deficiencies in Complex I have been associated, in several studies, with neuronal apoptosis which is a pathological feature of PD and associated with specific protein mutations (Teleanu et al., 2022). These processes are represented in a schematic form in the Figure 2.

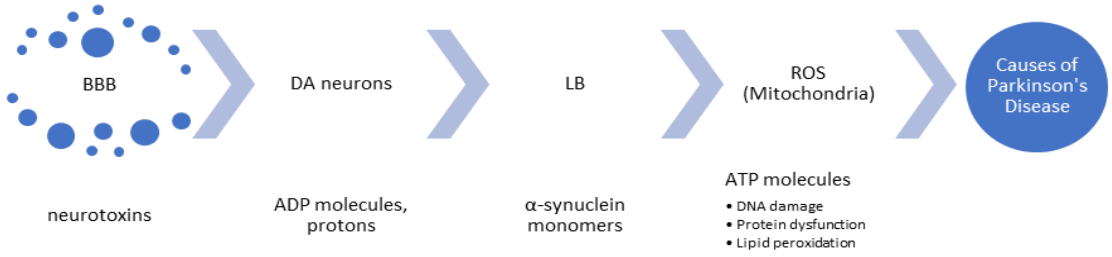

Figure 2 - Parkinson's disease pathological mechanism (adapted from Robea et al., 2020).

1.1.2. Neuroinflammation in PD: focus on astrocytes and microglia cells

Microglia and astrocytes, in physiological circumstances, continuously survey the brain parenchyma aiming to keep central nervous system (CNS) homeostasis by releasing neurotrophic factors, removing synaptic glutamate and other mechanisms. Nonetheless, several factors such as protein aggregates or factors released from injured neurons activate these glial cells resulting in a robust inflammatory reaction. This chronic neuroinflammation is not an early factor in all types of PD but seems to be a cofactor in the evolution of this disease.

Microglia are a type of glial cell with origin in the myeloid cell lineage. They own similar characteristics to macrophages as being involved in immunological surveillance with the release of neurotrophic factors and with phagocytic function by removing external substances and damaged neurons. These mechanisms need to be highly regulated to prevent overactivation which can lead to neurotoxic consequences. Microglia appear with two alternative activation phenotypes, M1 (pro-inflammatory) and M2 (anti-inflammatory) that lead to different types of arrays of cytokines secreted. Pro-inflammatory cytokines such as Tumor Necrosis Factor Alpha (TNF-α), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-12 (IL-12), and other molecules such as ROS, are produced after classical M1 activation, which leads to a magnified pro-inflammatory response and typically adverse effects on neuronal survival. On contrary, M2 microglia have an immunosuppressant role and contraries M1 response, stimulating neuronal survival and repair. Therefore, M2 produces interleukins-4 (IL-4), -13 (IL-13), -10 (IL-10), and Transforming growth factor-beta (TGF-β) anti-inflammatory cytokines. M1 and M2 microglia activation forms are also discerned by gene expression patterns. Several evidence suggest that microglial activation is crucial for the etiology and progression of PD, as studied in (Zhang et al., 2019), where microglia was more reactive in PD model animals and miR overexpression was able to suppress α-synuclein induced microglia cell activation and production of pro-inflammatory cytokines.

Concomitantly, astrocytes also play an important role in PD neuroinflammation. Astrocytes are the most populous glial subtype and provide metabolic and structural support which is crucial for brain function. Recent studies demonstrated that astrocytes express genes important to maintain neuronal survival (e.g., glial-derived neurotrophic factor), the reason why this cell type, when dysregulated, can contribute to PD. They produce pro-inflammatory cytokines, IL-1ß and TNF-α, that allow them to react to stimulus in a similar but slower way as microglia (Q. Wang et al., 2015).

These cells also contribute to the maintenance of the blood-brain barrier, which is disturbed in PD patients. Upon injury, astrocytes surround the lesioned area and create a barrier to prevent toxic signals spread into the adjacent healthy tissue. There are 8 known PD-related genes with an important role in astrocyte biology: PARK7, SNCA, PLA2G6, TP13A2, LRRK2, GBA, PINK1, and PARK2. These PD-related genes have an effect in astrocyte inflammatory response and the metabolism of fatty acids, but also other cellular pathways such as lysosome and mitochondrial functions. The dysfunction of these processes occurs in familial and idiopathic PD. Studies about astrocytes' contribution to PD pathogenesis are less than the ones about neuronal function but its involvement in PD is an emerging field of study with important effects on potential treatments (Booth et al., 2017).

All these factors point to astrocytes and microglia cells as extremely interesting cell populations to understand neuroinflammation and to identify new therapies for PD.

1.1.3. Experimental models for PD

Experimental models are essential to understand PD pathophysiology and identifying novel therapeutic targets and strategies (Raza et al., 2019).

Animal models allow the study of distinct characteristics of PD phenotypes pursuant to potential therapies. None of the present models available so far entirely manifest all the phenotypic traits of PD. These experimental models can be categorized into two categories as toxin-induced and genetic models (Jagmag et al., 2016).

Toxin-based models include 6-hydroxidopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, paraquat, and methamphetamine (Meth) and are used to evaluate nigrostriatal DA neuronal degeneration in a more acute form. Meanwhile, genetic models allow researchers to study the genetic etiology of PD and uncover new diagnoses, therapies and/or drug designs (Shimohama & Hisahara, 2011). Both models' categories are displayed in table 1 above.

Table 1 - Experimental models for PD (adapted from (Shimohama & Hisahara, 2011)).

Experimental models for PD		
Toxin Models	**Genetic Models**	
	Autosomal-dominant (AD)	*Autosomal-Recessive (AR) (KO or knockdown genes)*
6-OHDA	α-synuclein	Parkin
MPTP	Leucine rich repeat kinase 2 (LRRK2)	DJ-1
Rotenone	Ubiquitin carboxyl-terminal esterase L1 (UCHL1)	Phosphatase and tensin homolog- (PTEN-)
Paraquat	High-temperature requirement A2 (HTRA2/Omi)	Induced novel kinase 1 (PINK1)
Methamphetamine		

The toxin-based models, 6-OHDA, MPTP, and rotenone can lead to DA neuronal loss in the SN. Additionally, MPTP and rotenone can lead to higher degeneration of the nerve terminals in the putamen and be systemically administrated while, 6-OHDA can't, and must be directly injected into the brain. Regarding Lewy Body formation, rotenone is the only of the three that can lead to this pathophysiologic feature. MPTP models don't show typical PD behavior and the mechanisms involved aren't fully understood. Regarding rotenone, this model has low reproducibility with rats and the highest mortality. Paraquat can also lead to the loss of SN DA neurons and Lewy Body formation but with higher variability in the nigrostriatal DA system. The last toxin-induced model mentioned, Meth, can result in neurite damage with later neuronal damage with progressive and afterward loss of DA neurons and can increase the effect of other neurotoxins (such as MPTP) when administrated in elevated concentrations. (Jagmag et al., 2016).

Regarding genetic models, the five genes most regularly targeted for models of PD are α-synuclein, LRRK2, Parkin, DJ-1, Pink1, Nurr1 (NR4A2). It isn't so far clear if the genes encoded in those loci are helpers, carriers, or drivers of PD development but these models can give excellent vehicles to reveal insights about molecular signaling, genetics networks, and screening tests. *C. elegans* and *D. melanogaster* are two of the most known and popular non-mammalian genetic models of PD. The combination of both toxin and genetic models would be of extreme gain (Jagmag et al., 2016). 6-OHDA is a neurotoxin that acts in the periphery and the CNS, but it is unable to cross the blood-brain barrier. Therefore, 6-OHDA toxicity in the CNS only occurs when it's injected directly into the brain by stereotaxic surgery, making it the most widely well-described tool to study PD motor deficits (Simola et al., 2007). Several aspects influence the outset effect of 6-OHDA: dose, molecular vector used, and even the site of administration. Ungerstedt and colleagues showed degeneration of

the nigrostriatal pathway through intracerebral stereotaxic injection of 6-OHDA that can be achieved from 3 different types of injury *in vivo* models: intranigral, intra-striatal, and medial forebrain bundle injection, all with distinct advantages and disadvantages. While injury of the SN and medial forebrain bundle can cause immediate neurotoxic effects, it quickly causes widespread degeneration of the damaged nucleus, which represents an advantage in studying cell death resulting from prolonged oxidative stress damage. Otherwise, injection of this toxin in the ST lead to degeneration of DA neurons of the SN and the reduction of DA neurons in the VTA neurons that innervate the nucleus accumbens and that create the mesolimbic pathway (Hernandez-Baltazar et al., 2018). This toxin is taken into dopamine neurons via the dopamine transporter DAT, causing irreparable death of the dopamine cells by generating reactive free radicals, oxidative stress, mitochondria dysfunction, and microglial activation. 6-OHDA is the model of choice to mimic PD motor dysfunctions and molecular mechanisms. However, has some limitations, such as the absence of Lewy body aggregates, an important pathophysiology feature of PD (Tronci & Francardo, 2018).

This toxin is captured from the extracellular space by NAT or DAT and then stored in the catecholaminergic neurons. Once inside the neurons, 6-OHDA suffers enzymatic degradation through the MAO-A and further autoxidation, which leads to cytotoxic species that will damage the nucleus and intracellular proteins with consequent neuronal damage. Additionally, 6-OHDA can also impair complex I mitochondrial activity and lead to neurotoxicity, as represented in Figure 3 (Simola et al., 2007).

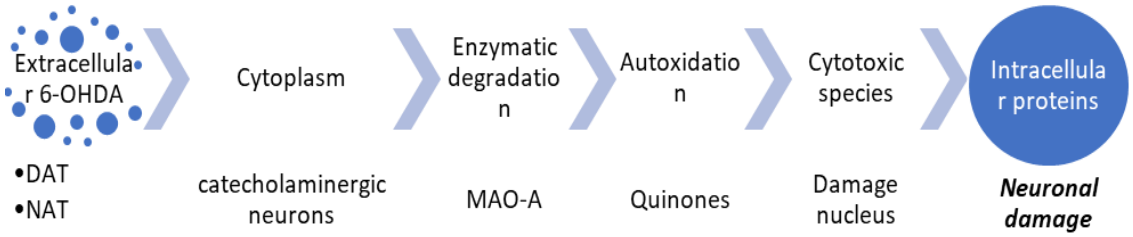

Figure 3 -6-OHDA neurotoxicity-induced mechanisms.

1.1.4. microRNAs (miRs)

microRNAs (miRs) are small (19 to 25 nucleotides), endogenous, and noncoding RNAs with functions on gene expression regulation. This process is possible due to the silencing of specific mRNAs targets through post-transcription mechanisms (Paul et al., 1234).

After pre-miRs production in the nucleus, they are transported to the cytoplasm via Exportin-5 and Ran-GTP for an additional process by Dicer until mature miRs. Then, single miR mature strand uploads to the RISC complex, with Ago-2 and GW182 proteins. In this phase, differing on mRNA target complete or incomplete complementarity, the result is either translation inhibition or degradation of the mRNA target (Figure 4) (Gan et al., 2015).

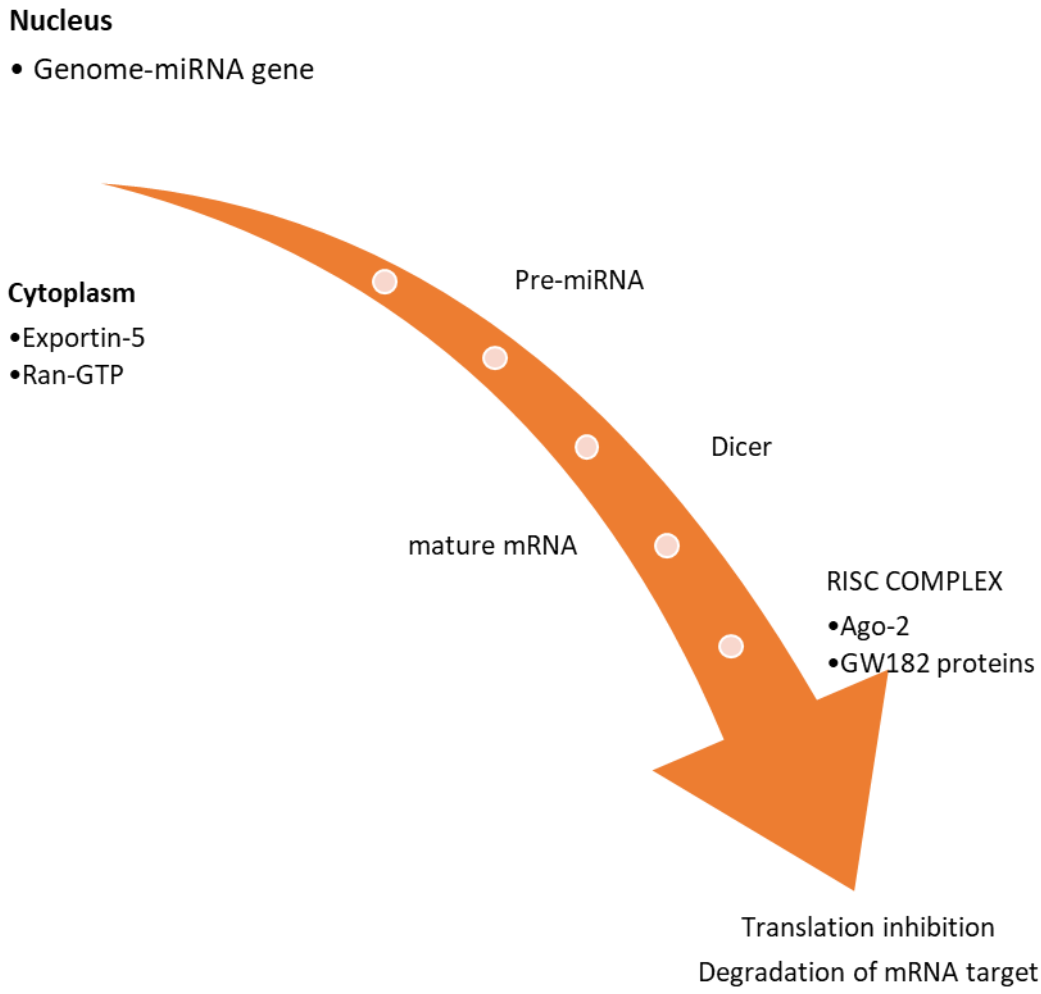

Figure 4 - miR biosynthesis pathway and mechanism of action.

Therefore, miRs have several functions. They are capable of binding to complementary target sequences in mRNA and obstructing translational machinery, which prevents and alters the subsequent protein product manufacture. They can also trigger the recruitment and association of mRNA decay factors that ultimately lead to the destabilization of mRNA and reduced expression levels due to the degradation of these molecules (Bhaskaran & Mohan, 2014).

It has been suggested that miRs engage in essential functions in the development of CNS and disease. In fact, miRs emerge dysregulated in several neurodegenerative diseases. Artificial miRs to inhibit certain transcripts, and administration of modified oligonucleotides mimicking or inhibiting certain miRNAs are some of the mechanisms that are already efficient to counteract neurodegeneration. Several studies performed over the years showed that a number of miRs are dysregulated in PD, making miRs one of the most promising novel therapeutics tools in PD (Hutchison et al., 2009).

1.2. miRs in PD

miRs have been identified as aggravators or mitigators of PD due to their potent modulator effect on gene expression that can regulate signaling pathways. Many studies have shown that dysregulated miRs expression happens in PD and contributes to its pathogenesis. Figure 5 illustrates the major dysfunctional processes that occur in PD and that could be regulated by miRs. Hence, it is important to understand the mechanisms underlying dysregulated miRs in PD (Goh et al., 2019).

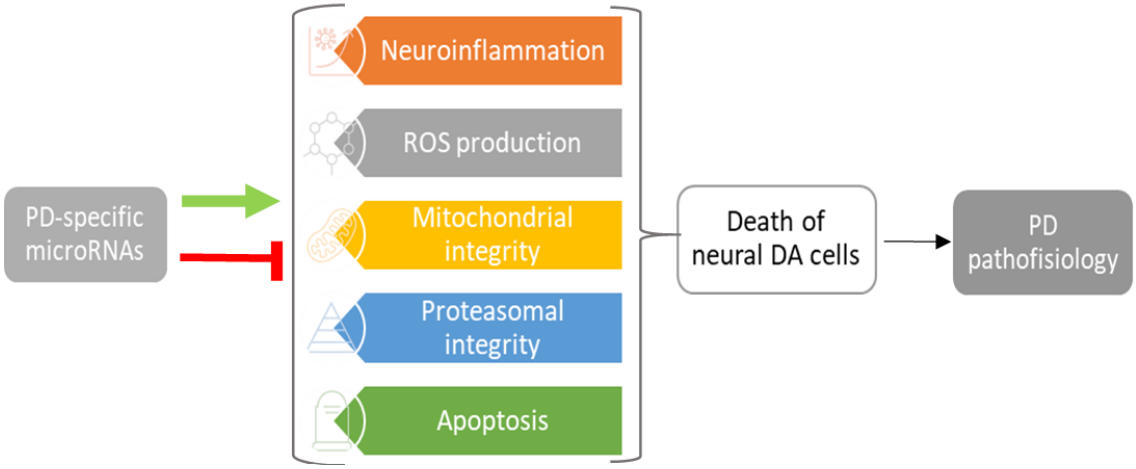

Figure 5 - Major dysfunctional processes in PD regulated by miRs.

miRs have a huge potential as therapeutic molecules due to the application of miRs inhibitors and mimics applied to target pathological down- and upregulated mRNAs (Hussein & Magdy, 2021).

There are several miRs described as therapeutical targets used for some deregulated pathways, such as for α-synuclein therapies described in (Elkurd et al., 2018). In our research group, we showed that the intraventricular administration of miR-124 coupled to polymeric nanoparticles induced neurogenesis and were able to counteract motor deficits in a 6-OHDA model *in vivo* (Saraiva et al., 2018). Moreover, using exosomes (small extracellular vesicles) as delivery agents for miR-124-3p also had a therapeutic effect in the same *in vivo* PD model by boosting a neuroprotective effect (Esteves et al., 2022).

Taking this evidence into account, we aimed to test other putative miRs, the miR-204 and miR-224, on a selected pathological mechanism of PD such as neuroinflammation.

The following table 2 shows some studies developed in the past few years detailing their effects on PD.

Table 2 – Effects of miRs on PD experimental models.

miRs	Experimental model	Administration method	Effects	References
miR-224	Cell cultures of human SH-SY5Y neuroblastoma and human SK-MEL28 cell lines	Transfection using HiPerfect Transfection reagent	miR-224 lead to increased α-synuclein accumulation which contributes to LB formation and PD pathogenesis, suggesting that silencing of this miR is a suitable target for PD	(Alvarez-Erviti et al., 2013)
miR-204	PC12 cells treated with MPP+	In Silico methods	miR-204 levels upregulated in PC12 cells in response to MPP+, suggesting that its deregulation contributes to the neurotoxicity mechanisms of PD	(Talepoor Ardakani et al., 2019)
miR-124	SVZ NSPCs cultures *in vitro*; healthy and 6-OHDA lesioned mice *in vivo*	*In vitro*: polymeric nanoparticles *In vivo*: intracerebroventricular injection	Increased neurogenesis and counteracted motor deficits	(Saraiva et al., 2016)
	N27 cell line *in vitro;* healthy and 6-OHDA lesioned mice *in vivo*	*In vitro*: Transfection of exosomes with Exo-Fect *In vivo*: intracerebroventricular injection	Increased neuroprotection and improved motor function	(Esteves et al., 2020)
miR-137	Healthy and MPTP lesioned mice *in vivo*	Intraperitoneal injection with serum-derived exosomes	OXR1 upregulation through miR-137 down-regulation and inhibition of oxidative stress injury in PD neurons	(Jiang et al., 2019)

Chapter 2 – Objectives

The main aim of this work was to evaluate the role of miR-204 and miR-224 on glial activity in a 6-OHDA *in vivo* model of PD. To achieve that we evaluated:

- The expression of the astrocytic marker GFAP and the microglial marker IBA-1 in the ST and SN by WB.
- The levels of GFAP, DAT, and IBA-1 by immunohistochemistry.

Chapter 3 – Materials and Methods

3.1. *In vivo* Studies

All procedures were performed in agreement with the protocols approved by the national ethical requirements for animal research and according to European Community guidelines (2010/63/EU). The animals used for *in vivo* experiments were wild-type C57BL/6J adult male mice with ages between 8 and 10-week-old. The stereotaxic administration of 6-OHDA in the ST and the miRs in the SN was performed previously by other team members. Briefly, mice were kept in the same room and similar cages with controlled conditions of food, water, and 12h light/dark cycle and a 22ºC temperature room. They were submitted to stereotaxic surgery with previous anesthesia with an intraperitoneal injection of ketamine and xylazine (90 mg/kg and 10mg/kg of each mouse weight, respectively), before 6-OHDA injection. To perform this procedure, the animals were placed in the digital stereotaxic frame (51900 Stoelting, Dubin, Ireland) and sanitized the scalp with Betadine®. To expose the skull an incision was performed with a scalpel along the midline to set the zero at the bregma point. Stereotaxic injection of 7 ug 6-OHDA at a rate of 0.2 uL/min was performed using a Hamilton syringe in the right ST with coordinates anteroposterior=3, mediolateral=1.4, dorsoventral=4.2. Then, the incision was sutured, and the mice were kept until recovery at 37ºC.

3.1.1. Brain Slices Preparation

Brain slice preparations were also performed by other group members. Brains were frozen and quickly immersed and preserved in a cryoprotective solution at 4ºC. Then, the brains were cut into 40 µm sections on a freezing cryostat-microtome (Leica CM 3050S, Leica Microsystems). Then, ST and SN sections of each animal were selectively collected into six wells of 24-well plates and preserved in an anti-freeze solution (30% ethylene glycol, 30% glycerol, 30% water, 10% phosphate buffer solution) until further processing for immunohistochemistry.

3.2. Western Blot

Western Blot analyses were performed to evaluate GFAP and IBA-1 expression. Total concentration of proteins was determined using the Pierce Bicinchoninic Acid Protein Assay. This is a copper-based colorimetric assay whose principle is that proteins decrease Cu^{2+} to Cu^{+} in an alkaline solution (biuret reaction) resulting in the formation of a purple color by bicinchoninic acid (Pierce, 2020). Subsequently, samples were denatured at 95ºC for 5 minutes with Loading Buffer, at 6x concentration (350 mM Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.06% bromophenol blue).

The macromolecules were separated into 10, 12,5 or 15% bisacrylamide gels, according to the molecular weights. The samples were loaded with a total of 40 µg of protein into the SDS polyacrylamide gels and the proteins were separated by SDS-PAGE electrophoresis in a running buffer solution (25mM Tris, 190 mM glycine, 10% SDS and pH 8.3) at RT and a power of 120V.

Then, proteins were transferred into a polyvinylidene difluoride membrane (Millipore), through a semi-dry transfer for 1 hour and 30 minutes using the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 0.75 A and Room Temperature (RT). Post-transfer, membranes were blocked in Tris buffer saline solution-Tween 20 0.1% (20 mM Tris, 137 mM NaCl solution, and 0.1% Tween 20) with 5% BSA for 1 hour at RT.

After that, membranes were incubated with the primary antibodies mouse anti-GFAP (1:1000, 52 kDa, BD Biosciences), rabbit anti-GAPDH (1:1000, 37 kDa, Millipore), and mouse anti-IBA-1 (1:100, 17 kDa, Santa Cruz) followed by the incubation with the secondary antibody anti-mouse and anti-rabbit antibody conjugated with horseradish peroxidase (1:5000).

Then, membranes were incubated with Pierce™ ECL Western Blotting Substrate (Thermo Scientific) for 2 minutes in the dark. Protein lanes were detected using the ChemiDoc™ MP Imaging System (Bio-Rad) and quantified using the Image Lab 5.1 software (Bio-Rad Laboratories).

3.3. Immunostainings and Imaging

3.3.1. Immunohistochemistry

Immunohistochemistry was performed in free-floating sections for the detection of DAT, GFAP, and IBA-1 in the SN and ST.

All protocol steps were performed on a rotating platform with gentle agitation. First, slices were washed to remove the cryopreservation solution, followed by an antigen retrieval procedure to increase the efficacy of protein detection. Slices were incubated with sodium citrate 10 mM pH=6, at 80 ºC for 30 minutes, cooled down at RT, placed in water for 5 minutes, and then washed one time with 0.1% PBS+Tween-20 (0.1% PBS-T) for 5 minutes. The next step was permeabilization with FBS serum blocking solution (10% FBS serum and 0.1% Triton X-100 in PBS) for 1 hour at RT. After 2 washes of 10 minutes with PBS-T, endogenous peroxidase activity was inhibited using a 3% hydrogen peroxidase (H_2O_2) solution for 10 minutes. Then, slices were washed 2 times for 10 min with 0.1% PBS-T and incubated for 2 overnights at 4ºC with primary antibodies (mentioned in Table 3) diluted in 5% FBS and PBS. Slices were then incubated with IgG (H+L) (anti-DAT) for 1h at RT, diluted at 1:200 in 1% FBS in PBS. After washing 3 times for 10 min with 0.1% PBS-T, slices were incubated with the Avidin/Biotin (AB) solution to improve sensitivity and decrease background. Samples were then washed 3 times for 10 minutes with PBS-T and 1 time with PBS. Finally, the DAB solution was used as a substrate, for color development for 5 minutes. The reaction was stopped by washing 2 times with 0.1% PBS-T for 10 minutes, and one last wash with PBS 1x for 10 minutes.

Sections were then mounted in *Super Frost* blades and let dry for 2 overnights. After drying, the tissue was dehydrated, starting with distilled water, passing through increasing concentrations of ethanol solutions, and finally across xylene. After that, they were mounted with *Entellan* mounting medium and let dry for 2 overnights until microscope analysis.

Table 3 - Primary and secondary antibodies used for immunostainings.

Primary Antibodies				
Reactivity	**Species**	**Company**	**Reference**	**IHC Dilution**
DAT	Rabbit	Abcam	Ab184451	1:500
GFAP	Mouse	Cell Signaling	3670	1:2500
IBA-1	Rabbit	Fujifilm Wako	019-19741	1:2500
Secondary Antibodies				
Reactivity	**Species**	**Manufacture**	**Reference**	**IHC Dilution**
Anti-Rabbit*	Goat	Vector Labs	BA-1000	1:200
Anti-Mouse*	Heparan Sulfate	Vector Labs	BA-2001	1:200

*Biotinylated Goat Anti-Rabbit IgG (H+L).

3.4. Statistical analysis

Statistical significances were determined with Student's t-test or one-way analysis of variance proceeded of Dunnett's Comparison Test, with relevant p values <0.05. The data were demonstrated with the mean ± standard error of mean (SEM) and the software used for statistical analysis was GraphPad Prism 6.1 (GraphPad Software, San Diego, CA). The ZEN 3.5 software was used to obtain, analyze, and treat microscopy images in the ZEN 3.5 blue edition (ZEISS Group).

Chapter 4 – Results

4.1. Effects of miRs in GFAP expression in the ST

GFAP protein is an astrocytic marker used to assess glial reactivity. We performed WB against GFAP in ST and SN samples dissected from mice that received intracerebral injections with saline, 6-OHDA, 6-OHDA+miR-204, and 6-OHDA+ miR-224 and collected 1- and 5-days post-surgery.

In Figure 5 A and B we showed that exposure to 6-OHDA seems to induce an increase in the expression levels of GFAP compared to saline animals both at 1- and 5-days post-surgery. This effect is not statistically significant due to the low number of mice tested (n=2 and n=3 respectively). One day post-surgery, we observed that miR-204 conferred a protective effect, reducing the expression levels of GFAP as compared to 6-OHDA-treated animals (Figure 5 A, $mean_{saline}$ = 100±16,4, n=2; $mean_{6\text{-}OHDA}$=143,9±63,8, n=4; $mean_{6\text{-}OHDA+miR\text{-}204}$=131,6±20,3, n=2; $mean_{6\text{-}OHDA+miR\text{-}224}$=68,3±11,4, n=3), while at 5 days both miR-204 and miR-224 reduced substantially GFAP expression levels (Figure 6 B, $mean_{saline}$=99,6±50,1, n=3; $mean_{6\text{-}OHDA}$=225,3±31,6, n=4; $mean_{6\text{-}OHDA+miR\text{-}204}$=109±2,4, n=3; $mean_{6\text{-}OHDA+miR\text{-}224}$=119,7±31,6, n=3).

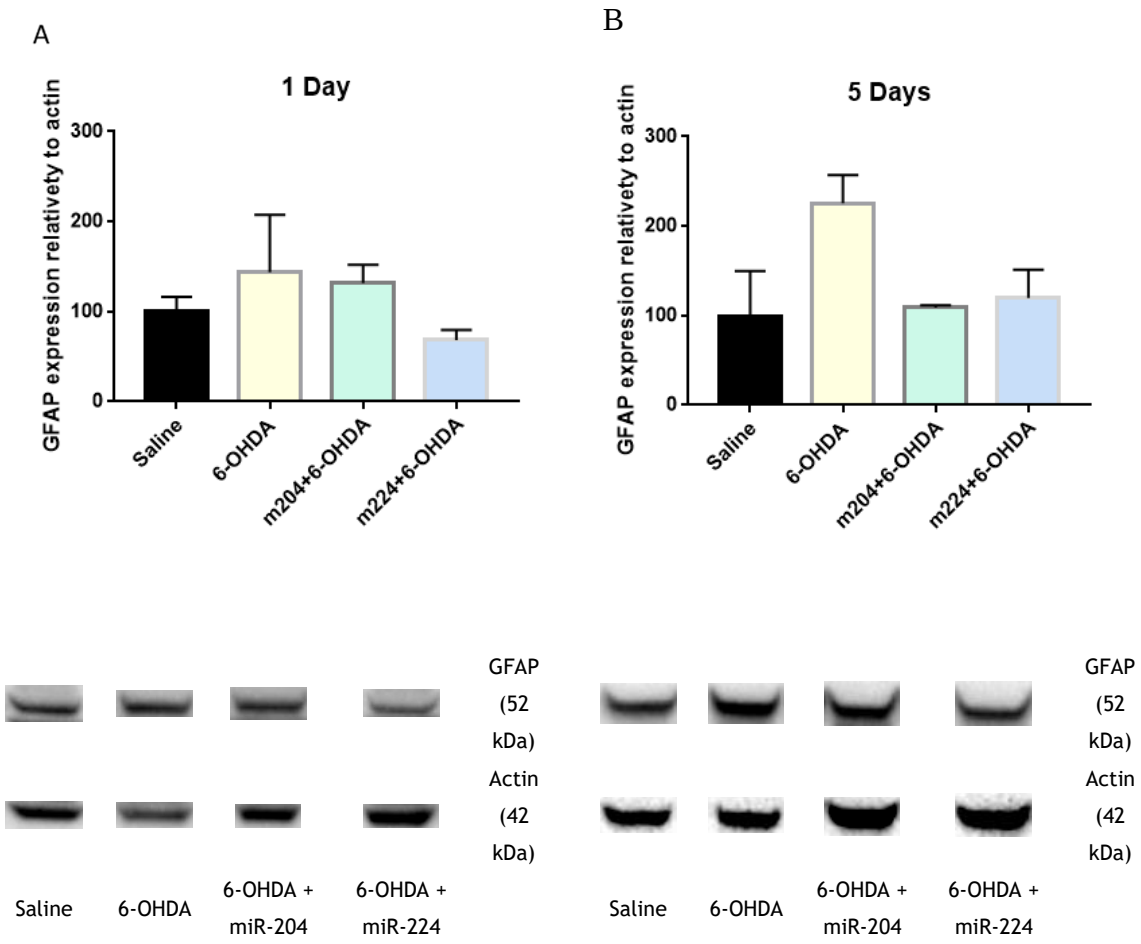

Figure 6 - GFAP protein expression levels in the ST at 1 (A) and 5 (B) days post-administration with saline, 6-OHDA, 6-OHDA+miR-204, and 6-OHDA+miR-224. Protein expression was normalized to actin. Statistics are present as a percentage of mean ± SEM (n=3-4 mice). The expression of GFAP levels in saline animals was normalized to 100%. A representative WB of GFAP and actin is present below each graph.

Then, we evaluated GFAP expression at two weeks post-injections, through immunohistochemistry, to sustain a qualitative analysis of the results observed in WB. It is notorious that 6-OHDA induced a more reactive and higher intensity of GFAP staining than the other experimental conditions (saline, 6-OHDA+miR-204, and 6-OHDA+miR-224). This qualitative analysis by immuhistochemistry sustains WB results since GFAP expression was higher in 6-OHDA samples than in other conditions. Together, these results suggest that miR-204 and miR-224 act as anti-inflammatory molecules in a 6-OHDA *in vivo* model. The results described are shown in Figure 7.

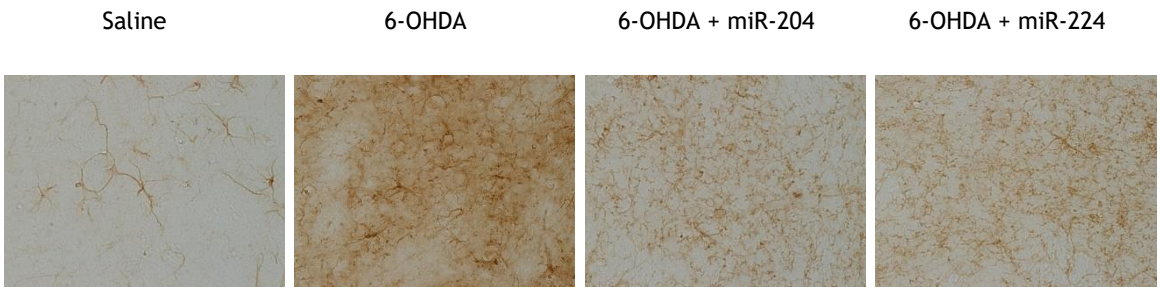

Figure 7 - GFAP protein expression detected by immunohistochemistry in the ST at two weeks after surgery.

4.2. Effects of miRs in GFAP expression in the SN

Following the previous analysis in the ST, we then evaluated the expression levels of GFAP by WB in the SN, in the same experimental groups as indicated previously.

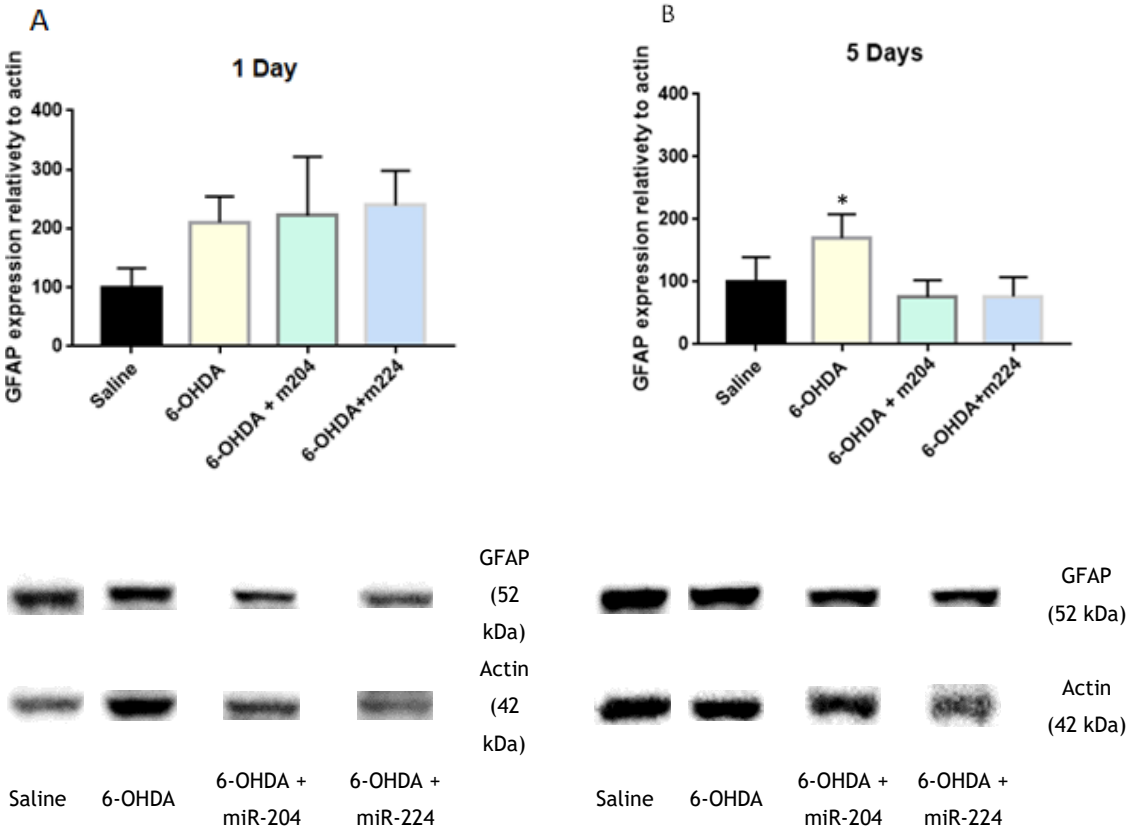

Figure 8 - GFAP protein expression levels in the SN at 1 (A) and 5 (B) days post-administration with saline, 6-OHDA, 6-OHDA+miR-204, and 6-OHDA+miR-224. Protein expression was normalized to actin. Statistics are present as a percentage of mean ± SEM (n=3-4). The expression of GFAP levels in saline animals were normalized to 100%. A representative WB of GFAP and actin is present below each graph.

Figure 8 A and B show GFAP expression levels in SN samples at 1- and 5-days post-surgery, respectively. At day 1, GFAP expression was found increased in animals treated with 6-OHDA, 6-OHDA+ miR-204 and 6-OHDA+miR-224 as compared with saline animals (Figure 8 A, $mean_{saline}$=100±32,5, n=2; $mean_{6\text{-}OHDA}$=210,3±44,1, n=4; $mean_{6\text{-}OHDA+miR\text{-}204}$=221,8±99,7, n=3; $mean_{6\text{-}OHDA}$+miR-224=239±58,9, n=3). At 5 days after injection, we found a significant increase of GFAP expression in 6-OHDA-treated animals as compared with saline. Interestingly, both miRs were able to counteract this increased GFAP expression induced by 6-OHDA (Figure 8 B, $mean_{saline}$=100,1±39,5, n=3; $mean_{6\text{-}OHDA}$=170±38,27, n=3; $mean_{6\text{-}OHDA+miR\text{-}204}$=75,37±27,02, n=3; $mean_{6\text{-}OHDA+miR\text{-}224}$=76,73±30,84, n=2; $p<0.05$).

Then, as mentioned for ST, GFAP spatial expression was evaluated in the SN through immunohistochemistry in samples collected two weeks after injection. We show that 6-OHDA induced a more reactive GFAP morphology with higher staining intensity than saline animals. Moreover, it seems that both miR-204 and miR-224 counteract GFAP reactivity, seeming miR-204 more effective. The results described are shown in Figure 9.

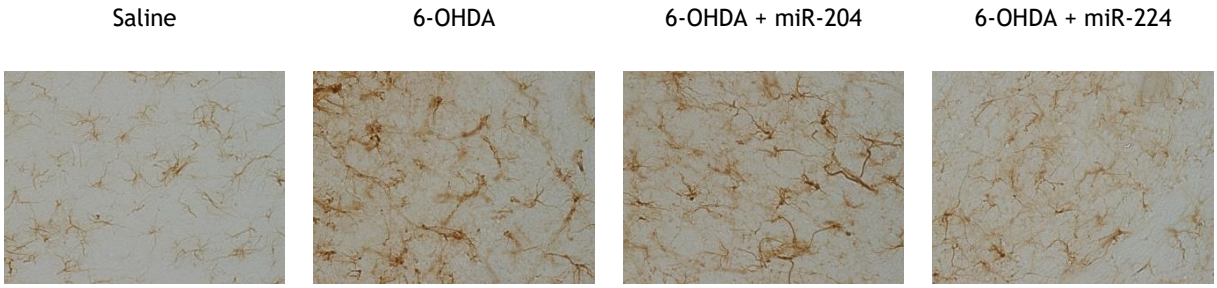

Figure 9 - GFAP protein expression detected by immunohistochemistry in SN samples at two weeks after surgery.

4.3. Effects of miRs in IBA-1 expression in the ST and SN

In the second stage of the study, IBA-1 expression was analyzed in ST and SN samples 2 weeks after surgery by immunohistochemistry. The results obtained show visible differences between experimental conditions (Figure 10). IBA-1 expression was also measured through WB analysis, but no reliable and consistent results were obtained, therefore, these results are not presented.

In the ST, we can identify a ramified IBA-1 staining in saline samples as compared with a more ameboid morphology found in 6-OHDA samples. It seems that the morphology of microglia found in 6-OHDA+miR-204 and 6-OHDA+miR-224 samples was similar to the one found in the saline animals.

Since IBA-1 is a microglia marker, if we compare these results with the ones previously observed for GFAP (astrocyte marker), we see a common pattern, suggesting anti-inflammatory effects of both miRs.

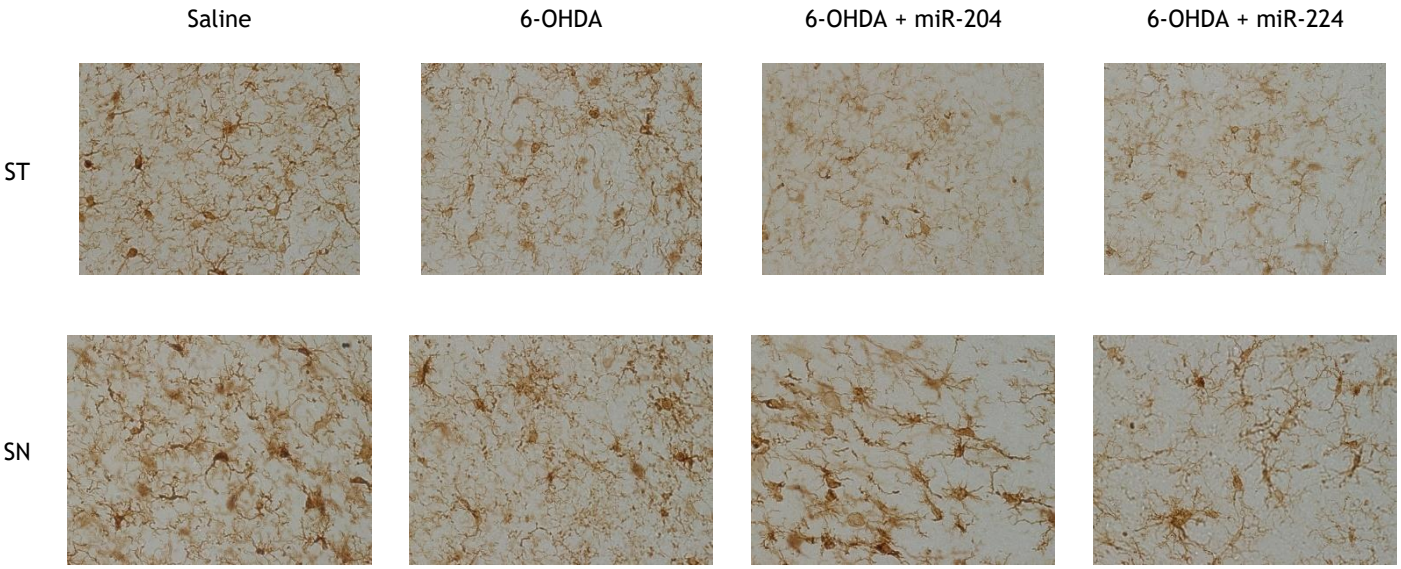

Figure 10 - IBA-1 protein expression detected by immunohistochemistry in ST and SN samples.

4.4. Optimization of DAT expression detection in the ST

Last, we also detected by immunohistochemistry the DAT protein, which is a dopaminergic-specific marker, and therefore, could provide cues to analyze presynaptic dopaminergic terminal loss. After protocol optimization and several procedure experiments, we were not able to detect a reliable signal. The results obtained are displayed below in Figure 11. Samples of SN aren't shown because no visible results were obtained.

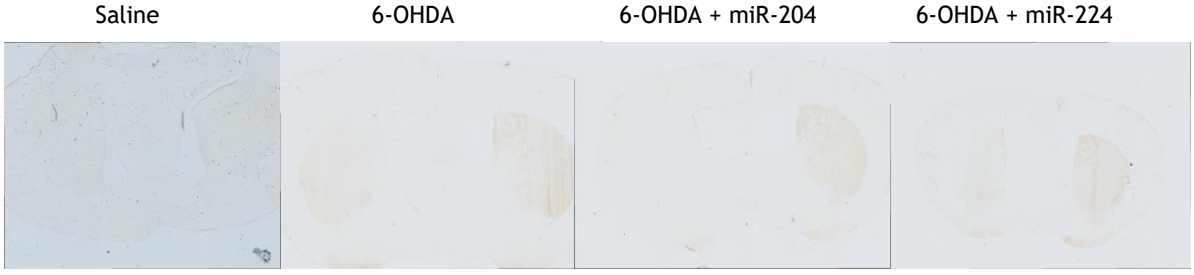

Figure 11 - Representative images of DAT staining obtained by immunohistochemistry in the ST two weeks after stereotaxic injection.

Chapter 5 – Discussion/ Future Perspectives

PD is characterized by several pathogenic and biochemical mechanisms caused by environmental factors or genetic sources. The two main characteristics of neuropathology in PD are alpha-synuclein accumulation and dopaminergic neuronal degeneration. α-synuclein levels and configuration are essential in PD pathogenesis. To remove intracellular proteins there are two main procedures, the lysosomal and proteasomal pathways. Other mechanisms seek to protect against dopaminergic neuronal degeneration such as antiinflammation strategies.

To induce and mimic PD, we injected the neurotoxin 6-OHDA in the ST which we previously showed that induce significant dopaminergic neuronal death and motor functional deficits(Ambrosi et al., 2017; Esteves et al., 2020). 6-OHDA-induced neurodegeneration is accompanied by gradual microglia repolarization from anti-inflammatory M2 to the pro-inflammatory M1 phenotype (Grotemeyer et al., 2022). In PD, neuroinflammation is one of the most important processes involved in pathogenesis due to the chronic release of pro-inflammatory cytokines by activated microglia and astrocytes (Hernandez-Baltazar et al., 2018; Q. Wang et al., 2015). This explains the need to study in detail glial activity in PD and the role of miRs as therapeutic targets.

Therefore, we performed WB to detect the expression of several proteins involved in neuroinflammation as GFAP and IBA-1. GFAP expression in ST and SN samples 5 days after surgeries was increased in 6-OHDA-treated animals as compared with saline, 6-OHDA+miR-204 and 6-OHDA+miR-224. In accordance, cell morphology looks more reactive in 6-OHDA-treated animals. In (Gomez-Isla et al., 2003), a human α-synuclein A30P transgenic mice with high expression of this protein lead to progressive gliosis with no specific anatomical or biochemical disruption of the nigrostriatal dopaminergic system. In another hand, astrocyte-specific overexpression of the miR-145 reduced astrocytic cell density, size, and number of related cell processes as cell proliferation, migration and ultimately gliosis decrease (C. Y. Wang et al., 2015). These results agree with our findings and exalt the necessity to continue studying this subject.

Then, to assess microglial activation, the detection of IBA-1 protein was performed in brain sections of mice treated with the same conditions as the ones used for GFAP detection. A qualitative observation based on microscopy suggests that microglia seem activated in ST and SN samples of 6-OHDA treated mice as compared with the other conditions. These findings are in agreement with the one reported by (Zhang et al., 2019) showing that microglia cells were found more reactive in a PD model, and that miR-137 overexpression was able to suppress α-synuclein induced microglia cell activation and production of pro-inflammatory cytokines.

Even if without conclusive results due to insufficient time for protocol optimization, we also performed immunohistochemistry against DAT protein because it is the most important mechanism for extracellular reuptake of dopamine into presynaptic terminals. DAT regulates synaptic dopamine in the striatum and DAT modulation can affect locomotor activity. So, in the future, it would also be valuable to study animals' behavior and dopaminergic survival for example by apomorphine test and immunohistochemistry for tyrosine hydroxylase (TH), respectively, to support the results obtained.

Our results didn't conduce to potential breakthroughs, but they allowed us to draw some conclusions. These poor outcomes can be explained because of the small number of animals studied in each condition. This reduced number of samples can be explained because there were no conditions to perform these experiences from the beginning (inexperience *in vivo*). Therefore, we took advantage of the animals ceded from previous experiences performed by other group members, thus reducing the number of animals used herein. Nonetheless, further studies with a larger number of animals and with the corrections already detected are promissory research in this field.

In addition to what was done by immunohistochemistry and WB to study neuroinflammation we could also perform ELISA assays. On the other hand, to compare with these techniques, we could also evaluate *in vivo*, from cerebrospinal fluid and/or blood of animals from every condition, to compare biochemical values such as cytokines (CSF) and C-reactive protein (blood) that allow comparing systemic and peripheral inflammation, respectively.

Even if in the distant future, the direct administration of miR as we performed isn't suitable for clinical trials given its invasiveness. So, it is necessary to unveil other types of administration, like miRs combined with delivery platforms such as exosomes, or nasal administration can be also used. These platforms have already been described, for example, by Guo and collaborators in PD studies where intranasal administration of ExoPTEN led to several functional improvements with reduced neuroinflammation and gliosis(Guo et al., 2019). In parallel, Esteves and collaborators showed cell-derived extracellular vesicles able to deliver miRs-124-3p in a PD mouse model that led to neuronal differentiation and protection against neurotoxin 6-OHDA toxicity(Esteves et al., 2022). Combined, we can verify promising therapeutic approaches from these vesicles as delivery agents in the PD context.

Chapter 6 – Conclusions

Our results suggest that miR-204 and miR-224 can modulate microglia and astrocytes activation which means that they may have an important role in PD pathogenesis and are potential therapeutic targets.

Considering the potential starring role of miR-204 and miR-224 in PD regulation, it seems obvious that is of extreme relevance to continue to study the processes involved by measuring other aspects of PD pathogenesis through other types of techniques.

However, it is essential to notice that the number of animals per group is very low and needs to be increased in further studies to corroborate these conclusions.

Chapter 7 – References

Alvarez-Erviti, L., Seow, Y., Schapira, A. H. V., Rodriguez-Oroz, M. C., Obeso, J. A., & Cooper, J. M. (2013). Influence of microRNA deregulation on chaperone-mediated autophagy and α-synuclein pathology in parkinson's disease. *Cell Death and Disease*, *4*(3), e545-8. https://doi.org/10.1038/cddis.2013.73

Ambrosi, G., Kustrimovic, N., Siani, F., Rasini, E., Cerri, S., Ghezzi, C., Dicorato, G., Caputo, S., Marino, F., Cosentino, M., & Blandini, F. (2017). Complex Changes in the Innate and Adaptive Immunity Accompany Progressive Degeneration of the Nigrostriatal Pathway Induced by Intrastriatal Injection of 6-Hydroxydopamine in the Rat. *Neurotoxicity Research*, *32*(1), 71–81. https://doi.org/10.1007/s12640-017-9712-2

Beitz, J. M. (2014). Parkinson's disease: a review. School of Nursing-Camden, Rutgers University, 311 N. 5. *Frontiers in Bioscience*, *6*(3), 65–74. Doi: 10.02741/s415

Bhaskaran, M., & Mohan, M. (2014). MicroRNAs: History, Biogenesis, and Their Evolving Role in Animal Development and Disease. In *Veterinary Pathology* (Vol. 51, Issue 4, pp. 759–774). https://doi.org/10.1177/0300985813502820

Booth, H. D. E., Hirst, W. D., & Wade-Martins, R. (2017). The Role of Astrocyte Dysfunction in Parkinson's Disease Pathogenesis. In *Trends in Neurosciences* (Vol. 40, Issue 6, pp. 358–370). https://doi.org/10.1016/j.tins.2017.04.001

Cerri, S., Mus, L., & Blandini, F. (2019). Parkinson's Disease in Women and Men: What's the Difference? *Journal of Parkinson's Disease*, *9*(3), 501–515. https://doi.org/10.3233/JPD-191683

Dionísio, P. A., Amaral, J. D., & Rodrigues, C. M. P. (2021). Oxidative stress and regulated cell death in Parkinson's disease. In *Ageing Research Reviews* (Vol. 67). https://doi.org/10.1016/j.arr.2021.101263

Elkurd, B. M. T., Torres-yaghi, Y., Bahroo, L. B., & Pagan, F. L. (2018). *Anti-alpha-Synuclein Therapies for Parkinson ' s Disease*. *September*, 59–61. doi: 10.3389/fneur.2022.852003

Esteves, M., Abreu, R., Fernandes, H., Serra-Almeida, C., Martins, P. A. T., Barão, M., Cristóvão, A. C., Saraiva, C., Ferreira, R., Ferreira, L., & Bernardino, L. (2022). MicroRNA-124-3p-enriched small extracellular vesicles as a therapeutic approach for Parkinson's disease. In *Molecular Therapy*. https://doi.org/10.1016/j.ymthe.2022.06.003

Esteves, M., Serra-Almeida, C., Saraiva, C., & Bernardino, L. (2020). New insights into the regulatory roles of microRNAs in adult neurogenesis. *Current Opinion in Pharmacology*, *50*, 38–45. https://doi.org/10.1016/j.coph.2019.11.003

Gan, J., Qu, Y., Li, J., Zhao, F., & Mu, D. (2015). An evaluation of the links between microRNA, autophagy, and epilepsy. *Reviews in the Neurosciences*, *26*(2), 225–237. https://doi.org/10.1515/revneuro-2014-0062

Goh, S. Y., Chao, Y. X., Dheen, S. T., Tan, E. K., & Tay, S. S. W. (2019). Role of microRNAs in parkinson's disease. In *International Journal of Molecular Sciences* (Vol. 20, Issue 22). https://doi.org/10.3390/ijms20225649

Gomez-Isla, T., Irizarry, M. C., Mariash, A., Cheung, B., Soto, O., Schrump, S., Sondel, J., Kotilinek, L., Day, J., Schwarzschild, M. A., Cha, J. H. J., Newell, K., Miller, D. W., Uéda, K., Young, A. B., Hyman, B. T., & Ashe, K. H. (2003). Motor dysfunction and gliosis with preserved dopaminergic markers in human α-synuclein A30P transgenic mice. *Neurobiology of Aging*, *24*(2), 245–258. https://doi.org/10.1016/S0197-4580(02)00091-X

Grotemeyer, A., McFleder, R. L., Wu, J., Wischhusen, J., & Ip, C. W. (2022). Neuroinflammation in Parkinson's Disease – Putative Pathomechanisms and Targets for Disease-Modification. *Frontiers in Immunology*, *13*(May), 1–18. https://doi.org/10.3389/fimmu.2022.878771

Guo, S., Perets, N., Betzer, O., Ben-Shaul, S., Sheinin, A., Michaelevski, I., Popovtzer, R., Offen, D., & Levenberg, S. (2019). Intranasal Delivery of Mesenchymal Stem Cell Derived Exosomes Loaded with Phosphatase and Tensin Homolog siRNA Repairs Complete Spinal Cord Injury. *ACS Nano*, *13*(9), 10015–10028. https://doi.org/10.1021/acsnano.9b01892

Hernandez-Baltazar, D., Nadella, R., Rovirosa-Hernandez, M. de J., Zavala-Flores, L. M., & Jarquin, C. de J. R. (2018). Animal Model of Parkinson Disease: Neuroinflammation and Apoptosis in the 6-Hydroxydopamine-Induced Model. *Experimental Animal Models of Human Diseases - An Effective Therapeutic Strategy*. https://doi.org/10.5772/intechopen.71271

Hussein, M., & Magdy, R. (2021). MicroRNAs in central nervous system disorders: current advances in pathogenesis and treatment. *Egyptian Journal of Neurology, Psychiatry and Neurosurgery*, *57*(1), 1–11. https://doi.org/10.1186/s41983-021-00289-1

Hutchison, E. R., Okun, E., & Mattson, M. P. (2009). The therapeutic potential of microRNAs in nervous system damage, degeneration, and repair. *Neuromolecular Medicine*, *11*(3), 153–161. https://doi.org/10.1007/s12017-009-8086-x

Jagmag, S. A., Tripathi, N., Shukla, S. D., Maiti, S., & Khurana, S. (2016). Evaluation of models of Parkinson's disease. *Frontiers in Neuroscience*, *9*(JAN). https://doi.org/10.3389/fnins.2015.00503

Jamebozorgi, K., Taghizadeh, E., Rostami, D., Pormasoumi, H., & Barreto, G. E. (2019). *Cellular and Molecular Aspects of Parkinson Treatment : Future Therapeutic Perspectives*. 4799–4811. doi: 10.1007/s12035-018-1419-8.

Jiang, Y., Liu, J., Chen, L., Jin, Y., Zhang, G., Lin, Z., Du, S., Fu, Z., Chen, T., Qin, Y., & Sun, X. (2019). Serum secreted miR-137-containing exosomes affects oxidative stress of neurons by regulating OXR1 in Parkinson's disease. *Brain Research*, *1722*(27). https://doi.org/10.1016/j.brainres.2019.146331

Kigerl, K. A., de Rivero Vaccari, J. P., Dietrich, W. D., Popovich, P. G., & Keane, R. W.

(2014). Pattern recognition receptors and central nervous system repair. In *Experimental Neurology* (Vol. 258, pp. 5–16). https://doi.org/10.1016/j.expneurol.2014.01.001

Konovalova, E. V., Lopacheva, O. M., Grivennikov, I. A., Lebedeva, O. S., Dashinimaev, E. B., Khaspekov, L. G., Fedotova, E. Y., & Illarioshkin, S. N. (2015). Mutations in Parkinson's disease-associated PARK2 gene are accompanied by imbalance in programmed cell death systems. *Acta Naturae*, *7*(4), 146–151. https://doi.org/10.32607/20758251-2015-7-4-146-149

MacMahon Copas, A. N., McComish, S. F., Fletcher, J. M., & Caldwell, M. A. (2021). The Pathogenesis of Parkinson's Disease: A Complex Interplay Between Astrocytes, Microglia, and T Lymphocytes? *Frontiers in Neurology*, *12*(May). https://doi.org/10.3389/fneur.2021.666737

Mullin, S., & Schapira, A. H. V. (2015). Pathogenic Mechanisms of Neurodegeneration in Parkinson Disease. *Neurologic Clinics*, *33*(1), 1–17. https://doi.org/10.1016/j.ncl.2014.09.010

Pajares, M., I Rojo, A., Manda, G., Boscá, L., & Cuadrado, A. (2020). Inflammation in Parkinson's Disease: Mechanisms and Therapeutic Implications. *Cells*, *9*(7), 1–32. https://doi.org/10.3390/cells9071687

Paul, S., Reyes, P. R., Betsabé, ·, Garza, S., & Sharma, A. (1234). MicroRNAs and Child Neuropsychiatric Disorders: A Brief Review. *Neurochemical Research*, *45*, 232–240. https://doi.org/10.1007/s11064-019-02917-y

Pierce. (2020). *BCA Protein Assay Kit 23225*. *2161296*, 0–3.

Raza, C., Anjum, R., & Shakeel, N. ul A. (2019). Parkinson's disease: Mechanisms, translational models and management strategies. *Life Sciences*, *226*(March), 77–90. https://doi.org/10.1016/j.lfs.2019.03.057

Saraiva, C., Ferreira, L., & Bernardino, L. (2016). *Traceable microRNA-124 loaded nanoparticles as a new promising therapeutic tool for Parkinson's disease*. https://doi.org/10.1080/23262133.2016.1256855

Saraiva, C., Talhada, D., Rai, A., Ferreira, R., Ferreira, L., Bernardino, L., & Ruscher, K. (2018). MicroRNA-124-loaded nanoparticles increase survival and neuronal differentiation of neural stem cells in vitro but do not contribute to stroke outcome in vivo. In *PLoS ONE* (Vol. 13, Issue 3). https://doi.org/10.1371/journal.pone.0193609

Selvaraj, S., & Piramanayagam, S. (2019). Impact of gene mutation in the development of Parkinson's disease. *Genes and Diseases*, *6*(2), 120–128. https://doi.org/10.1016/j.gendis.2019.01.004

Shimohama, S., & Hisahara, S. (2011). Toxin-induced and genetic animal models of Parkinson's disease. *Parkinson's Disease*. https://doi.org/10.4061/2011/951709

Simola, N., Morelli, M., & Carta, A. R. (2007). The 6-hydroxydopamine model of Parkinson's disease. *Neurotoxicity Research*, *11*(3–4), 151–167. https://doi.org/10.1007/BF03033565

Srinivasan, E., Chandrasekhar, G., Chandrasekar, P., Anbarasu, K., Vickram, A. S.,

Karunakaran, R., Rajasekaran, R., & Srikumar, P. S. (2021). Alpha-Synuclein Aggregation in Parkinson's Disease. *Frontiers in Medicine*, *8*(October). https://doi.org/10.3389/fmed.2021.736978

Stefanis, L., Emmanouilidou, E., Pantazopoulou, M., Kirik, D., Vekrellis, K., & Tofaris, G. K. (2019). How is alpha-synuclein cleared from the cell? In *Journal of Neurochemistry* (Vol. 150, Issue 5, pp. 577–590). https://doi.org/10.1111/jnc.14704

Subramaniam, S. R., & Chesselet, M. F. (2013). Mitochondrial dysfunction and oxidative stress in Parkinson's disease. *Progress in Neurobiology*, *106–107*, 17–32. https://doi.org/10.1016/j.pneurobio.2013.04.004

Talepoor Ardakani, M., Rostamian Delavar, M., Baghi, M., Nasr-Esfahani, M. H., Kiani-Esfahani, A., & Ghaedi, K. (2019). Upregulation of miR-200a and miR-204 in MPP + -treated differentiated PC12 cells as a model of Parkinson's disease. *Molecular Genetics and Genomic Medicine*, *7*(3), 1–9. https://doi.org/10.1002/mgg3.548

Teleanu, D. M., Niculescu, A.-G., Lungu, I. I., Radu, C. I., Vladâcenco, O., Roza, E., Costăchescu, B., Grumezescu, A. M., & Teleanu, R. I. (2022). An Overview of Oxidative Stress, Neuroinflammation, and Neurodegenerative Diseases. *International Journal of Molecular Sciences*, *23*(11), 5938. https://doi.org/10.3390/ijms23115938

Tronci, E., & Francardo, V. (2018). Animal models of l-DOPA-induced dyskinesia: the 6-OHDA-lesioned rat and mouse. *Journal of Neural Transmission*, *125*(8), 1137–1144. https://doi.org/10.1007/s00702-017-1825-5

Wang, C. Y., Yang, S. H., & Tzeng, S. F. (2015). MicroRNA-145 as one negative regulator of astrogliosis. *Glia*, *63*(2), 194–205. https://doi.org/10.1002/glia.22743

Wang, Q., Liu, Y., & Zhou, J. (2015). Neuroinflammation in Parkinson's disease and its potential as therapeutic target. In *Translational Neurodegeneration* (Vol. 4, Issue 1). https://doi.org/10.1186/s40035-015-0042-0

Wei, Z., Li, X., Li, X., Liu, Q., & Cheng, Y. (2018). Oxidative Stress in Parkinson's Disease: A Systematic Review and Meta-Analysis. In *Frontiers in Molecular Neuroscience* (Vol. 11). https://doi.org/10.3389/fnmol.2018.00236

Youle, R. J., & Van Der Bliek, A. M. (2012). Mitochondrial Fission, Fusion, and Stress HHS Public Access. *Science*, *337*(6098), 1062–1065. https://doi.org/10.1126/science.1219855

Zhang, J., Dongwei Zhou, Zhang, Z., Xinhui Qu, Kunwang Bao, Guohui Lu, & Jian Duan. (2019). miR-let-7a suppresses α-Synuclein-induced microglia inflammation through targeting STAT3 in Parkinson's disease. *Biochemical and Biophysical Research Communications*, *519*(4), 740–746. https://doi.org/10.1016/j.bbrc.2019.08.140