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Sex-based modulation of neuromelanin-linked Parkinson's disease pathology

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PhD Thesis

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3MT. *3-MethoxyTyramine*
5HT. *Serotonin*
5SCD. *5-S-CysteinylDopa*
5SCDA. *5-S-CysteinylDopamine*
6-OHDA. *6-hydroxydopamine*
AAV. *Adeno-Associated Viral vector*
AD. *Alzheimer's disease*
ALP. *Autophagy-Lysosomal Pathways*
BBB. *Blood-Brain Barrier*
CD68. *Cluster of Differentiation 68*
CD8. *Cluster of Differentiation 8*
cDNA. *complementary DNA*
CMV. *CytoMegaloVirus*
COMT. *Catechol-O-MethylTransferase*
DA. *Dopamine or Dopaminergic*
DAT. *Dopamine Transporter*
Dct. *DOPAchrome tautomerase*
DHI. *5,6-DiHydroxyIndole*
DHICA. *5,6-DiHydroxyIndole 2-Carbolic Acid*
DMV. *Dorsal Motor nucleus of the Vagus*
E2. *17β-Estradiol or Estradiol or Oestradiol*
Erα. *Estrogen Receptors α*
Erβ. *Estrogen Receptor β*
GBA. *β-glucocerebrosidase*
GFAP. *Glial Fibrillary Acidic Protein*
GPR30. *G-Protein coupled estrogen Receptor 1 or GPER1*
GWAS. *Genome-Wide Association Studies*
H&E. *Hematoxylin and Eosin*
hTyr. *human Tyrosinase*
i.e.. *id est (That is)*
iLBD. *incidental LB disease*
iPD. *idiopathic Parkinson's disease*
kg. *KiloGram*
LB. *Lewy Body*
LC. *Locus coeruleus*
L-DOPA. *Levodopa*
LID. *L-DOPA Induced Dyskinesias*
LOD. *Limit Of Detection*
LOQ. *Limit Of Quantification*
LP. *LB Pathology*
m. *months*
MAOB. *MonoAmine Oxidase type B*
MB. *Marinesco Bodies*
MDS. *Movement Disorder Society*
mg. *MilliGram*
MHC-I. *Major Histocompatibility Complex class I*
min. *minutes*
MPP+. *1-Methyl-4-PhenylPyridinium*
MPTP. *1-Methyl-4-Phenyl-1,2,3,6-TetrahydroPyridine*
NA. *Noradrenergic or Noradrenaline*
NAT. *NorAdrenaline Transporter*
NBM. *Nucleus Basalis of Meynert*
nM. *nanoMolar*
NM. *Neuromelanin*
NM-MRI. *NM-sensitive Magnetic Resonance Imaging*
NSG. *Normal Goat Serum*
Ob. *Olfactory bulb*
OVX. *Ovariectomized*
Paraquat. *N,N'-dimethyl-4-4-4'-bypiridinium*
PB. *Pale Body*
PD. *Parkinson disease*
PPN. *PedunculoPontine Nucleus*
RBD. *REM sleep Behavior Disorder*
REM. *Rapid Eye Movement*
RN. *Red Nucleus*
ROS. *Reactive Oxygen Species*
RRF. *RetroRubral Field*
SNP. *Single-Nucleotide Polymorphisms*
SNpc. *Substantia Nigra pars compacta*
STR. *Striatum*
TBS. *Tris Buffered Saline*
TFEB. *Transcription Factor EB*
TH. *Tyrosine Hydroxylase*
TYR. *Tyrosinase*
TYRP2. *Tyrosinase-Related Protein-2*
Ub. *Ubiquitin*
UPLC. *Ultra-Performance Liquid Chromatography*
VMAT2. *Vesicular MonoAmine Transporter 2*
VTA. *Ventral Tegmental Area*
y. *years*

ABSTRACT

Parkinson's disease (PD) in males has a higher incidence and prevalence, earlier disease onset, more severe motor symptoms, and more frequent cognitive decline than in females. However, most studies do not consider the influence of sex on PD, and so the molecular mechanisms underlying sex differences in PD remain unknown. Estradiol (E2) modulates dopaminergic pathways and improves PD symptoms in both males and females, and is also able to modulate melanin production in the skin. Age-dependent intracellular neuromelanin (NM) accumulation above a pathogenic threshold triggers PD pathology in rats overexpressing the melanin-producing enzyme tyrosinase (TYR). In this work, I assess whether nigral NM production/accumulation differs by sex, and whether these differences could underlie the influence of sex on PD.

Analysis of healthy human brain tissue showed that intracellular NM levels within nigral dopaminergic neurons are significantly higher in males than in females, with males reaching the pathogenic threshold of NM accumulation earlier, even in the absence of PD. Male AAV-TYR-injected rats exhibited earlier and greater accumulation of NM than females, reaching the pathogenic threshold of intracellular NM accumulation sooner. Remarkably, ovariectomized (OVX) female rats injected with AAV-TYR accumulated NM more rapidly than non-OVX females and reached pathological NM levels at a similar rate to their male counterparts. Treatment of AAV-TYR-injected rats with E2 attenuated motor deficits, and lowered NM levels. Finally, NM-producing cultured neurons treated with E2 showed decreased NM accumulation and increased cell viability.

In conclusion, an increased/accelerated accumulation of NM in males across their life may underlie their higher risk of developing PD, compared to females. E2 treatment may delay or attenuate PD-related pathology if administered early.

INTRODUCTION

1. Parkinson's disease

1.1. Brief history

In 1817, James Parkinson (1775-1824) published his *Essay on the Shaking Palsy* *(Parkinson 1817)*. This article was the first to describe a neurological condition with primary motor symptoms, such as resting tremor and flexed posture, a variable and late onset, and a progressive pathology. Several decades later, in 1872, Charcot expanded upon Parkinson's work by identifying key clinical features such as bradykinesia (slowness of movement) and rigidity *(Charcot 1872)*. It was during this time that the term "Parkinson's disease" (PD) was first introduced, replacing the earlier term "*paralysis agitans*" or "shaky palsy." Charcot recognized that, unlike in Parkinson's original description, patients with PD were not significantly weak, and did not always exhibit tremors, leading to this more precise nomenclature *(Charcot 1872; Goetz 2011; Obeso et al. 2017)*.

In the 1890s, Blocq, Marinesco, and Brissaud were the first to suggest that the Substantia Nigra pars compacta (SNpc) was the primary region affected in PD. This hypothesis arose when they observed unilateral resting tremor in a patient with a tuberculoma on the right-hand side of the brain, opposite to the body's affected side *(Fenelon and Walusinski 2021; Blocq and Marinesco 1893)*. Later on, in the 1920s, Trétiakoff provided the first description of neuropathological changes in the SNpc of patients with PD, noting the loss of neuromelanin (NM)-containing neurons and the presence of cytoplasmic α-synuclein inclusions, which are now known as Lewy bodies (LB) *(Holdorff 2019; Trétiakoff 1919)*. These observations became key neuropathological hallmarks and diagnostic criteria for PD *(Del Rey et al. 2018)*.

Almost forty years later, in 1960, Ehringer and Hornykiewicz observed decreased dopamine (DA) levels in the striatum (STR), – the main synaptic target of SNpc neurons – of patients with PD (*Ehringer & Hornykiewicz, 1960*). Shortly afterwards, the dopamine precursor levodopa (L-DOPA) was first administered intravenously to patients with PD, demonstrating antiakinetic effects *(Birkmayer and Hornykiewicz 1962; Goetz 2011)*. Subsequently, Cotzias and colleagues conducted landmark large-scale trials using oral preparations of L-DOPA, confirming its therapeutic effects and demonstrating long-term benefits in patients with PD *(Cotzias, Papavasiliou, and Gellene 1969)*.

1.2. Epidemiology

PD is considered the most prevalent neurodegenerative motor disorder and the second most frequent neurodegenerative condition after Alzheimer's disease (AD), impacting 2–3% of individuals aged ≥65 years *(Poewe et al. 2017)*. Given its rarity in people under 50 years old, advancing age appears to be the primary risk factor for PD, with incidence rates escalating notably from 60 to 90 years of age *(Poewe et al. 2017)*.

The number of new cases of PD in 2019 was approximately 1.08 million a 160% increase since 1990 and an average annual increase of 0.61% over a 29-year period. In 2019, the global prevalence of PD was 8.5 million, an increase of 156% from 1990. The burden of PD is increasing faster than that of any other neurological condition, surpassing even AD *(Feigin et al. 2019)*. The rising prevalence of PD cases is potentially linked to improved patient survival as well as increasing incidence.

Given the strong correlation of prevalence and incidence with advancing age, and the globally aging population, it is projected that the number of individuals with PD will surpass 12 million by 2040 *(Dorsey and Bloem 2018)*. Numerous investigations are now characterizing this rapid surge in PD cases as resembling a pandemic, necessitating appropriate measures such as risk prevention, specialized healthcare services, and research investment, as well as improved access to therapeutic interventions *(Barker 2020; Dorsey and Bloem 2018; Dorsey et al. 2018)*.

1.3. Pathological hallmarks

PD is a chronic neurodegenerative disorder characterized pathologically by the progressive loss of NM-containing DA neurons in the SNpc *(Lewy 1912; Trétiakoff 1919; Fearnley and Lees 1991; Ma et al. 1997; Dauer and Przedborski 2003; Cheng, Ulane, and Burke 2010; Poewe et al. 2017; Giguère, Burke Nanni, and Trudeau 2018)*. SNpc DA neurons project to the basal ganglia, and their gradual loss leads to worsening DA depletion in their main synaptic target, the STR. This nigrostriatal pathway plays a role in motor control through the release of DA in the caudate-putamen region of the basal ganglia *(Molinoff and Axelrod 1971; Björklund and Dunnett 2007)*, and the loss of SNpc DA neurons explains the motor symptoms of PD, such as resting tremor, rigidity, bradykinesia, and postural instability.

DA neurons possess and accumulate NM throughout the entire human lifespan. NM is a dark-brown pigment formed as a result of the oxidative breakdown of dopamine, which is mainly found in the midbrain and pons *(Carballo-Carbajal et al. 2019)*. NM can also be observed in various brain regions where neurodegeneration has been observed in patients with PD, such as the locus coeruleus (LC), the dorsal motor nucleus of the vagus (DMV), ventral tegmental area (VTA), retrorubral field (RRF), red nucleus (RN), pedunculopontine nucleus (PPN), nucleus basalis of Meynert (NBM), and olfactory bulb (OB) *(Sulzer and Surmeier 2013; Butkovich, Houser, and Tansey 2018; Giguère, Burke Nanni, and Trudeau 2018; Carballo-Carbajal et al. 2019)* (**Figure 1**).

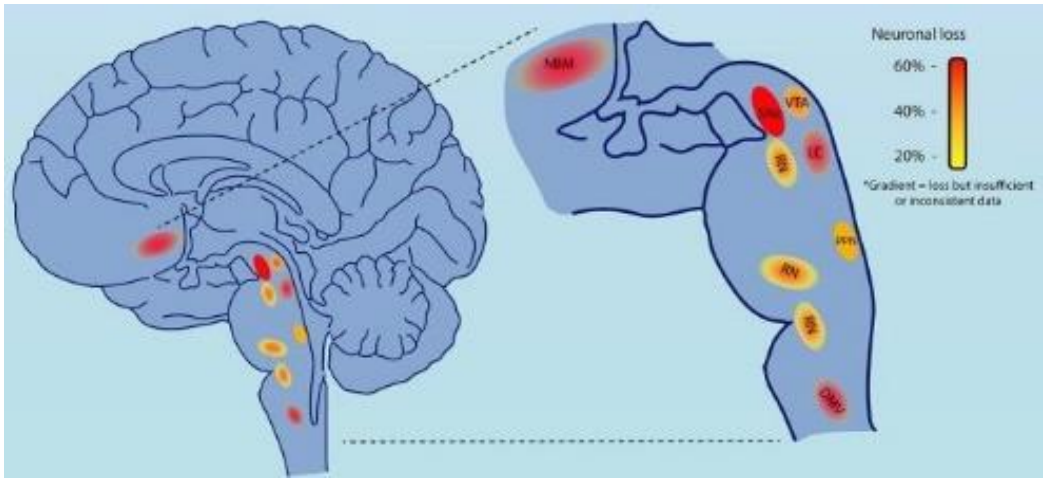

Figure 1 - Cell degeneration in PD. *Schematic representation of brain regions demonstrating cell loss in PD. Color-coded based on the evidence of cell loss: red=60%, orange=40% and yellow=20%. Color gradients indicate uncertainty in the extent of this cell loss. Adapted from (Giguère, Burke Nanni, and Trudeau 2018). VTA: Ventral tegmental area, LC: Locus coeruleus, NBM: Nucleus basalis of Meynert, PPN: Pedunculopontine nucleus, RN: Raphe nuclei, DMV: Dorsal motor nucleus of the vagus*

It is interesting to note that the neurotransmitter DA can be toxic when not encapsulated in synaptic vesicles. DA is oxidized in the cytoplasm and generates toxic quinones, which in turn increase oxidative stress by reacting with proteins such as α-synuclein, tyrosine hydroxylase (TH), DA transporter (DAT) and mitochondrial protein complexes *(Giguère, Burke Nanni, and Trudeau 2018)*. Surprisingly, treatment with L-DOPA, which rises DA levels, does not increase DA cell loss.

Neurodegeneration of DA-producing neurons in SNpc and associated striatal DA denervation not only leads to the motor symptoms of PD but is also associated with non-motor symptoms such as anxiety, depression, and problems with sensory perception *(Schapira, Chaudhuri, and Jenner 2017)*. Patients with PD present with additional non-motor symptoms related to non-DA regions. Such symptoms include autonomic dysfunction (constipation), neuropsychiatric dysfunction (psychosis and dementia), and sensory dysfunction (hyposmia and fatigue) *(Kalia and Lang 2015; Doppler et al. 2017; Obeso et al. 2017)*. Many of these non-motor symptoms appear in the early stages of the disease when patients are not yet diagnosed (**Figure 2**).

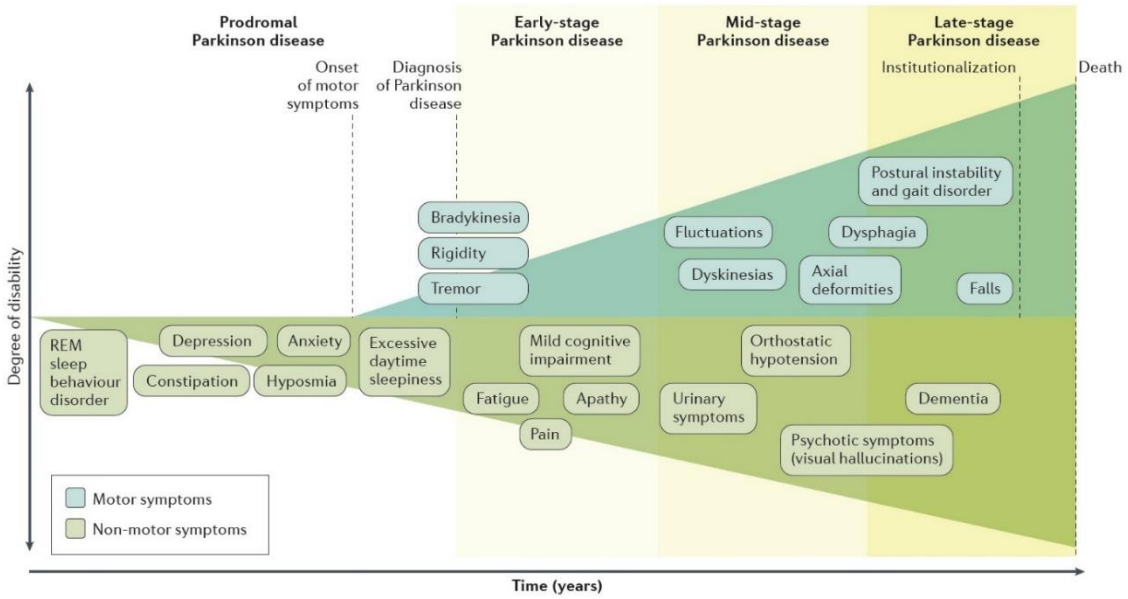

***Figure 2 - Clinical symptoms associated with PD progression**. Schematic representation of the different clinical stages of PD and the appearance of non-motor and motor symptoms. From (Poewe et al., 2017).*

1.4. Disease staging and inclusions

Outside of DA neuronal death, one of the other hallmarks of PD is the presence of cytoplasmic inclusions (LB), which are widely spread aggregations of a hyaline eosinophilic core and a peripheral halo. These aggregates contain several lipids and proteins but the most widely known is α-synuclein *(Spillantini et al. 1997; Hashimoto and Masliah 1999; Wakabayashi et al. 2007)*. They are usually observed after an immunoreactivity for ubiquitin (Ub) in the core and α-synuclein and p62 in the halo. It is interesting to note that α-synuclein aggregates are also found in peripheral organs such as the salivary glands *(Beach et al. 2013)*, the gut *(Borghammer and Berge 2019; Leclair-Visonneau et al. 2020)* and the skin *(Doppler et al. 2017)*.

Another type of inclusion observed in PD patients is Pale bodies (PB). These aggregates, considered to be LB precursors, are pale-stained eosinophilic rounded granular formations that displace NM *(Choong and Mochizuki 2022)*. It has been suggested that α-synuclein-positive structures first appear in perikaryal areas of sparse NM, then p62 and Ub fuse with these, forming PB that progressively dislodge NM before adopting the LB typical aggregates *(Kuusisto, Parkkinen, and Alafuzoff 2003)*.

Braak and colleagues, after observing α-synuclein staining from post-mortem patients with PD, confirmed LB pathology (LP) in many brain areas and noted that the distribution suggested an ascending caudo-rostral course of LP *(H. Braak et al. 2003)*. They proposed that LP would start in the OB and DVC (Stage 1), then progress to the LC (Stage 2) followed by less vulnerable areas such as the SNpc (Stage 3) before, finally, spreading to cortical areas (Stages 4-6) (**Figure 3**). These latter stages are related to motor and dementia symptoms, while motor PD is usually diagnosed at Stage 3, once the SNpc is affected *(H. Braak et al. 2003)*.

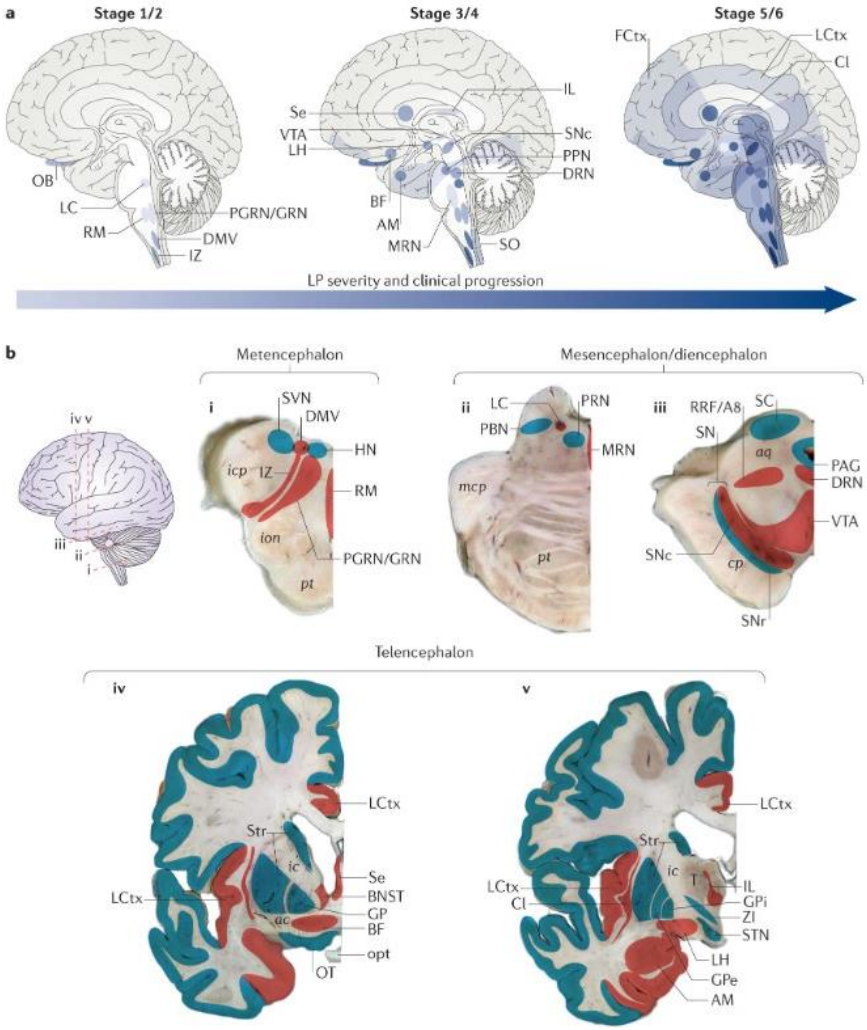

Figure 3 - Staging of Lewy pathology in clinical Parkinson's disease. *a) Schematic representation of the spread of LP within different brain structures. The anatomical progression of disease through the brain increases over time; the darker the color, the more LP is present in each region at a given stage. b) Lateral external surface of a representative brain identifies the levels of each cross-sectional brain slice (i-v). Regions that contain LP at any stage are in red whereas those in blue only rarely or mildly show LP. ac, anterior commissure; aq, aqueduct; AM, amygdala; BF, magnocellular nuclei of the basal forebrain; BNST, bed nucleus of the stria terminalis; Cl, claustrum; cp, cerebral peduncle; DMV, dorsal motor nucleus of the vagus; DRN, dorsal raphe nucleus; FCtx, frontal cortex; GP, globulus pallidus, GPe, GP externa; GPi, GP interna; HN, hypoglossal nucleus; ic, internal capsule; icp, inferior cerebellar peduncle; IL, intralaminar nuclei of the thalamus; ion, inferior olivary nucleus; IZ, intermediate reticular zone; LP, Lewy body pathology; LC, locus coeruleus and subcoeruleus; LCtx, limbic cortex; LH, lateral hypothalamus; mcp, middle cerebellar peduncle; MRN, median raphe nucleus; OB, olfactory bulb; opt, optic tract; OT, olfactory tubercle; PAG, periaqueductal grey; PBN, parabrachial nucleus; PGRN/GRN, paragigantocellular and gigantocellular reticular nucleus; PPN, pedunculopontine nucleus; PRN, pontine reticular nucleus; pt, pyramidal tract; RM, raphe magnus; RRF/A8, retrorubral fields/A8 dopaminergic cell group; SC, superior colliculus; Se, septum; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SO, solitary tract nuclei; STN, subthalamic nucleus; Str, striatum; SVN, spinal vestibular nucleus; T, thalamus; VTA, ventral tegmental area; ZI, zona incerta. From (Surmeier, Obeso, and Halliday 2017).*

Although the LP model seems applicable for most PD cases, a few patients with typical clinical features of PD show no DVC pathology *(Jellinger 2003; Kalaitzakis et al. 2008)* and it does not appear to be suitable for other synucleopathies such as incidental Lewy body diseases (iLBD) or LP with AD *(Dickson et al. 2010; Jellinger 2003)*. Furthermore, LP is not always associated with PD signs and symptoms; it can be observed in clinically healthy subjects such as iLBD cases, which correspond to Stages 4-6 *(Burke, Dauer, and Vonsattel 2008; Jellinger 2003; Parkkinen et al. 2005)*. In addition, LP does not necessarily correlate to cell loss patterns *(Parkkinen et al. 2011; Surmeier, Obeso, and Halliday 2017)*. It is also important to note that some genetic forms of PD cases, such as LRRK2 and Parkin, are also not consistent with LP *(Schneider and Alcalay 2017)*. Thus, while it is apparent that α-synuclein inclusions can be associated with PD, the exact relationship is still unclear.

One last common inclusion in PD is formed in the nucleus of pigmented neurons, in the SNpc and LC. These inclusions are known as Marinesco bodies (MB). MB tend to increase with age and are present in various neurodegenerative diseases, especially AD or dementia with LB *(Beach et al. 2004; Yuen and Baxter 1963)*. It has been reported that, in older brains, DA neuron markers usually decrease while MB frequency is inversely correlated *(Beach et al. 2004)*. While it seems that MB are less frequent in PD, it has been observed that MB prevalence in PD correlates with SNpc neuronal death and pathology span *(Abbott et al. 2017)*.

1.5. Diagnostic window

PD onset remains unclear, as symptoms appear slowly. This means that the initial pathological stages precede the emergence of clinical motor symptoms. Most PD patients are diagnosed only when motor dysfunctions appear (Figure 2), at which point more than 30% of melanized SNpc neurons have already experienced cell death *(Burke and O'Malley 2013)*. Furthermore, PD diagnosis is based on clinical accuracy and post-mortem confirmation, therefore, better diagnostic tests are needed to improve diagnosis and be able to do it at the prodromal phase *(Lau and Breteler 2006)*.

Observations in postmortem brains reveal that patients with PD experience high percentages of neuronal death (30-80%) during the first years of disease pathology *(Kordower et al. 2013)*. However, in all PD stages, loss of TH-positive cells is higher than the loss of pigmented neurons, as these cells first undergo TH downregulation prior to cell death. TH downregulation is the state where pigmented catecholaminergic neurons lower their ability to synthesize catecholamines. TH downregulation has been constantly observed in the SNpc and LC of postmortem PD brains *(Huynh et al. 2021)*, as well as in healthy older brains *(Chu and Kordower 2007; Manaye et al. 1995)* and in stages 1 and 2 of iLBD cases *(Milber et al. 2012)*. Although these neurons have lost a DA phenotype, it is possible that they might recover with neuroprotective therapies as they have not yet undergone cell death.

To be able to diagnose and treat PD earlier, it is necessary to find the mechanisms behind PD onset. It is also crucial to identify people at risk for PD before they develop the disease. Thankfully, the

past few years have seen an increase in epidemiological studies aiming to establish biomarkers and risk factors for PD.

PD diagnosis after the first motor symptoms appear has been considered as early PD, even though it has been established that several non-motor symptoms emerge prior to motor dysfunctions, *(Poewe et al. 2017)* (**Figure 2**). These early non-motor signs and symptoms constitute the prodromal stage of PD. They consist of REM sleep behavior (RBD), olfactory loss, depression, autonomic dysfunction, and emergence of pathological imaging markers of the presynaptic DA system and the cardiac sympathetic system *(Berg et al. 2021)*. It has been reported that most RBD patients later develop a synucleinopathy such as PD or iLBD *(Bloem, Okun, and Klein 2021; Berg et al. 2021)*. In 2015, the Movement Disorder Society (MDS) updated its Clinical Diagnostic Criteria for PD to include the prodromal phase *(Heinzel et al. 2019)*.

1.6. Current PD therapeutic options

L-DOPA therapy was pioneered in the 1960s and remains the primary treatment for PD. L-DOPA restores motor function, but its effect is not permanent: after several years, treatment may lead to dyskinesia and motor response oscillations in a considerable number of patients *(Poewe et al. 2017)*. In recent years, a better understanding of the nigrostriatal pathway has led to new treatments, such as catechol-O-methyltransferase (COMT) or monoamine oxidase type B (MAOB) inhibitors, which inhibit DA degradation and increase DA bioavailability *(Poewe et al. 2017)*, and DA agonists, which counterbalance DA depletion. In some cases, DA agonists are combined with L-DOPA to avoid motor complications *(Connolly and Lang 2014)*, but reportedly this may cause secondary effects such as compulsive behaviors *(Grall-Bronnec et al. 2018)*.

It is important to keep in mind that L-DOPA therapy does not counteract most non-motor PD symptoms. Furthermore, some symptoms appear as a consequence of L-DOPA due to increased DA transmission *(Chaudhuri and Schapira 2009; Del Rey et al. 2018)*. Thus, there is a need for alternative or additional treatments for the non-motor symptoms of PD. DA agonist therapy, tricyclic antidepressants modulating 5HT and NA or DA, cholinesterase inhibitors, blockers of acetylcholine transmission in nerves, and laxatives have all been tried *(Seppi et al. 2011)*. It is clear that a better understanding of PD is necessary to enable the development of treatments that overcome all PD symptoms, and so offer patients a better quality of life.

1.7. Etiology and risk factors

The most important risk factor for PD is age. The world population is aging, especially in Europe, North America, and South America, and as a result an exponential increase in global PD prevalence is being observed *(Kalia and Lang 2015; Ascherio and Schwarzschild 2016)*. In contrast PD prevalence appears to be lower in Asia *(Pringsheim et al. 2014)*. Thus, it appears there are factors other than age to take into account.

Sex is one of the strongest risk factors for PD: males have a 2-fold higher relative risk than females of all ages *(Bourque, Morissette, and Paolo 2019)*. Environmental factors, such as pesticide and metal exposures, viral infections and air pollution, as well as lifestyle factors such as coffee, alcohol and dietary product consumptions, smoking and gut alterations also appear to influence PD risk *(Periñán et al. 2022; Yuan et al. 2022)*.

In the 1990s, α-synuclein was the first gene involved in PD to be identified *(Polymeropoulos et al. 1997)*. Since then, many more genes linked to PD have been determined, including SNCA, PINK1, LRRK2, PARKIN and DJ-1 (**Figure 4**) *(Funayama et al. 2023; Billingsley et al. 2018)*. These genes are mostly associated with either mitochondrial or autophagy-lysosomal pathways (ALP) (Figure 4) *(Kumaran and Cookson 2015; Billingsley et al. 2018)*. Mutations in lysosomal hydrolase β-glucocerebrosidase (GBA) have been reported as the most common PD genetic risk *(Wong, Peng, and Krainc 2019; Billingsley et al. 2018)*. While genetic PD cases only represent 10% of all PD cases, they do allow researchers to gain insights into potential mechanisms underlying idiopathic PD (iPD), as ALP and mitochondrial pathways might be altered in these cases *(Poewe et al. 2017)*. For example, GBA activity is reportedly decreased in iPD *(Gegg et al. 2012)*, and genome-wide association studies (GWAS) have been able to identify more than 90 single-nucleotide polymorphisms (SNP) associated with a higher iPD risk *(Vázquez-Vélez and Zoghbi 2021)*. Moreover, genetic PD risk factors linked to neurotransmission, nucleus and gene regulation, immune system, vesicular trafficking and lipid metabolism might also be impaired in iPD *(Vázquez-Vélez and Zoghbi 2021)*.

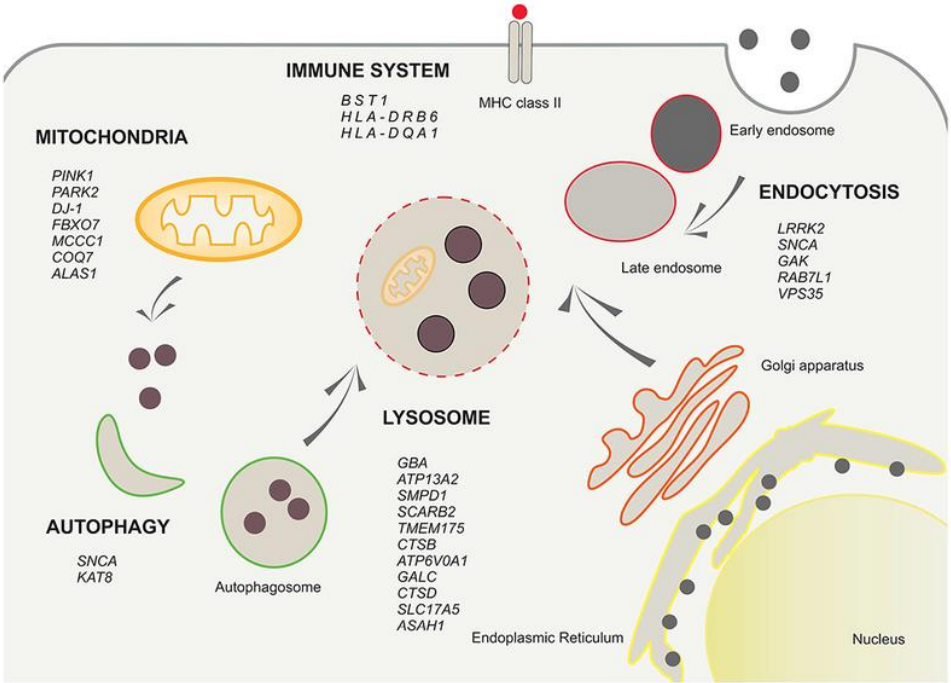

Figure 4 - Overview scheme of PD risk factor genes and the molecular pathways where they are involved. *The genes encode for proteins that mainly participate in mitochondrial turnover, autophagy, endocytosis, and immune system and/or lysosomal function. From (Billingsley et al. 2018). MHC, major histocompatibility complex.*

1.8. Experimental animal models

To better understand the mechanisms underlying PD onset and pathology progression, and to find novel therapeutic targets, using human post-mortem tissue is not enough. Therefore, researchers use experimental animal models to deepen their knowledge of the origin of DA neuronal death and to test potential treatments *(Bezard and Przedborski 2011)*.

Cell models, such as human α-synuclein overexpression or cells treated with rotenone or 1-methyl-4-phenylpyridinium ion (MPP+), can be useful but they lack the maturation and the complex interactions and mechanisms of a whole brain *(Falkenburger, Saridaki, and Dinter 2016)*. These models do not allow exploration of the effects of cell-to-cell interactions, pharmacokinetics, or blood-brain barrier (BBB) permeability, nor do they permit examination of behavioral output *(Falkenburger, Saridaki, and Dinter 2016; Dawson, Golde, and Lagier-Tourenne 2018)*. However, cell models can be useful to grasp specific molecular pathways, or as part of patient-specific studies using patient-derived induced pluripotent stem cells *(Le, Sayana, and Jankovic 2014)*.

Over the years, many species have been used as PD animal models, from nematodes and flies to rodents and non-human primates. However, they each fail to reproduce all the aspects of PD *(Hewitt and Whitworth 2017; Ünal and Emekli-Alturfan 2019)*. Nevertheless, the use of animal models in preclinical drug research remains mandatory before moving onto approved human clinical studies *(Singh and Seed 2021)*.

PD animal models can be divided into two categories: neurotoxic, with SNpc cell death and nigrostriatal neurodegeneration induced by specific molecules (**Figure 5**) *(Tieu 2011)*, or genetic. Genetic animal models are based on human genetic studies and present a wide range of phenotypes, but some fail to reproduce the SNpc neurodegeneration of PD.

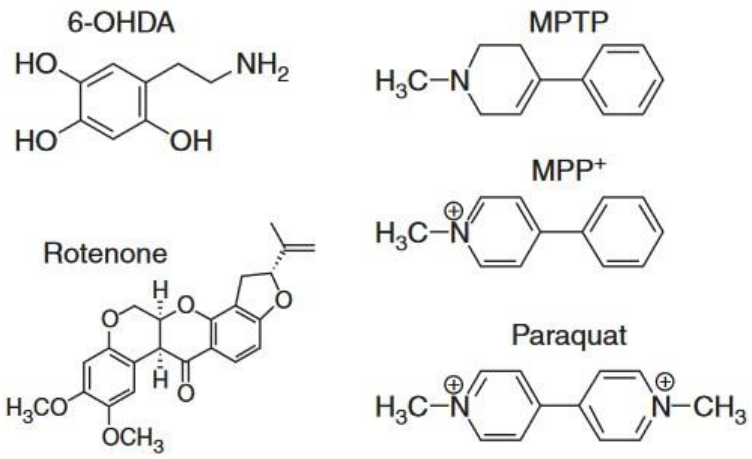

Figure 5 - Structures of neurotoxic molecules used to induced nigrostriatal damage in some common animal models of PD. *From (Tieu 2011).*

1.8.1. Neurotoxic models

1.8.1.1. 6-OHDA

The 6-hydroxydopamine (6-OHDA) model was the first animal model of PD *(Ungerstedt 1968)*. As 6-OHDA cannot cross the BBB, it has to be injected intracerebrally. The molecule is usually injected unilaterally to cause neurodegeneration in only one hemisphere *(Blesa and Przedborski 2014)*. 6-OHDA has a high affinity for DAT and noradrenaline transporter (NAT) which allows it to enter DA cells *(Bové et al. 2005)*. Within neurons, 6-OHDA increases quinone production and reactive oxygen species (ROS) to produce its toxic effect *(Bové et al. 2005)*. However, this model fails to reproduce LP.

1.8.1.2. MPTP

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been used to model PD in animals since the 1980s. It is highly lipophilic, crosses the BBB easily, and therefore is administered systematically. Once MPTP arrives in the brain, it is metabolized by MAO-B astrocytes into its active metabolite, MPP+, and enters neurons via DAT. Once inside neurons, MPP+ inhibits complex I of the mitochondrial electron transport chain, resulting in an increase of ROS and decreased energy production. MPTP induces a PD-like neurotoxic pattern on the nigrostriatal system, such as an increase SNpc DA neuronal death compared to VTA DA cell death and a greater degeneration of DA nerve terminals in the putamen compared to the caudate nucleus *(Blesa and Przedborski 2014)*. However, despite a higher α-synuclein expression, as in the 6-OHDA model, LB-like inclusions are rarely observed in the mouse model *(Vila et al. 2000)*, and are only found in non-human primates treated with MPTP *(Forno et al. 1986; Kowall et al. 2000)*.

1.8.1.3. Herbicides

Paraquat (N,N'-dimethyl-4-4-4'-bypiridinium) has been extensively used in agriculture. Its structure is similar to MPP+ (**Figure 5**) and it is able to cross the BBB and enter DA neurons via DAT. Once there, it activates oxidative stress mechanisms to cause neurotoxicity *(Bastías-Candia, Zolezzi, and Inestrosa 2019)*. Rotenone is not only an herbicide but also an insecticide. Like MPTP, rotenone is highly lipophilic and easily crosses the BBB and cellular membranes. Once inside cells, it inhibits complex I in mitochondria, obstructing oxidative phosphorylation *(Bové et al. 2005)*. While neither 6-OHDA nor MPTP are able to reproduce LP, these two herbicides are able to replicate LB-like inclusions in DA SNpc neurons *(Bové et al. 2005)*.

1.8.1.4. Neurotoxic models summary

While these neurotoxic models enable some recreation of PD within days, or even weeks if chronic administrations are performed, they fail to recreate the multisystemic aspects of the disease. Most importantly, they cannot reproduce the slow, decades-long, progression of PD. Another disadvantage of these models is the toxicity of the administered molecules, which represent a risk for researchers. However, these models have been invaluable to improving our understanding of specific

SNpc neuronal death mechanisms, as well as for testing potential therapies targeting only the motor symptoms of PD.

1.8.2. Genetic animal models

Several genetic models have been developed and extensively characterized, to deepen our knowledge of familial forms of PD. These models replicate identified causative genes and result in a wide range of cellular and molecular alterations within the SNpc DA neurons, such as α-synuclein proteostasis, mitochondrial function, oxidative stress, axonal transport and neuroinflammation (**Figure 6**) *(Poewe et al. 2017)*.

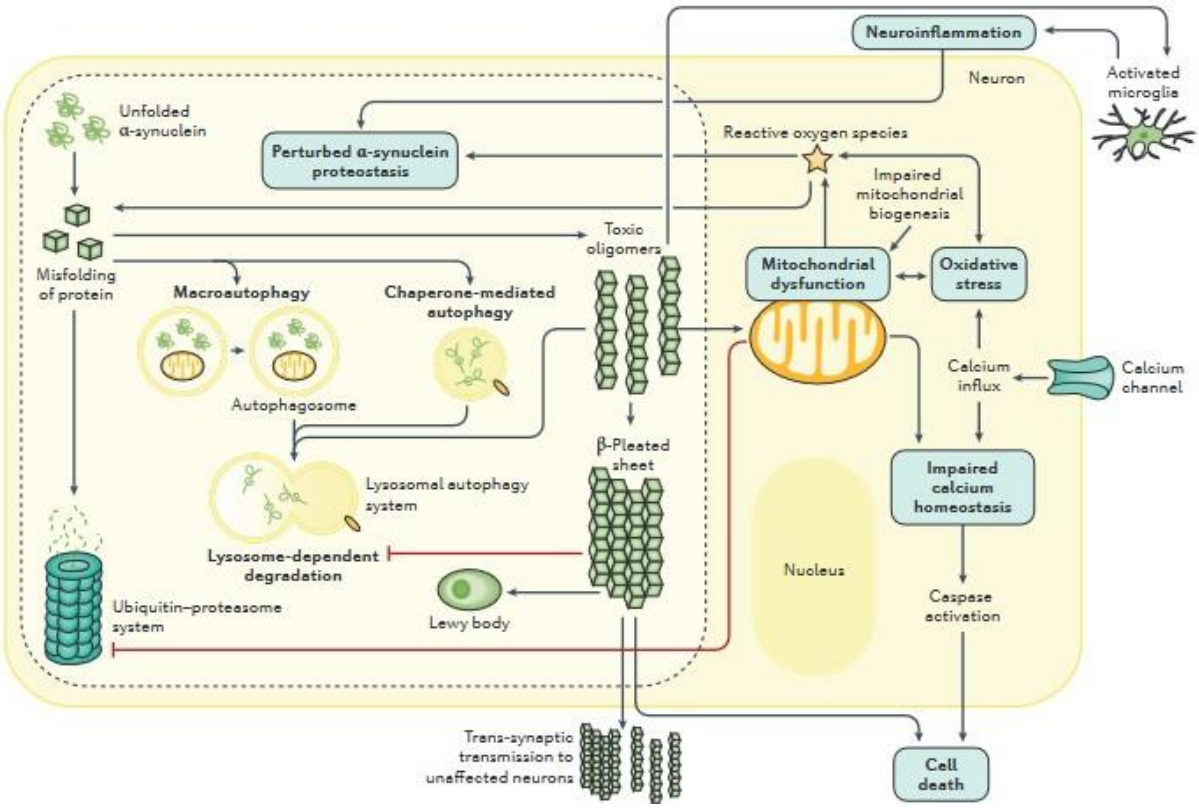

Figure 6 - Molecular mechanisms involved in Parkinson's disease. *Schematic diagram depicting interactions between major molecular pathways that are implicated in the pathogenesis of Parkinson's disease. From (Poewe et al., 2017).*

Genetic animal models of PD are typically transgenic mice modelling point mutations (PARK1/α-synuclein[A53T, A30P, E46K]) or LRRK2 mutations (PARK8/LRRK2 [G2019S, R1441C/G]), or knockout mice modelling loss-of-function mutations (PARK2/Parkin, PARK6/PINK or PARK7/DJ). However, most of these models fail to reproduce neurodegeneration of the nigrostriatal system. The exception is transgenic mice overexpressing α-synuclein (PARK4/α-synuclein duplication/triplication) when a neuronal specific promoter, such as thymocyte differentiation antigen 1 (Thy-1), is used *(Blesa and Przedborski 2014; Konnova and Swanberg 2018)*.

1.8.3. Other models

Other models use different strategies, such as viral vector overexpression of α-synuclein injected directly into the SNpc *(Decressac et al. 2012; Oliveras-Salvá et al. 2013)*, as well as the

injection of α-synuclein aggregations, known as preformed fibrils *(Luk et al. 2012; Paumier et al. 2015)*. In some cases, these two types of injections can be combined to enhance DA neurodegeneration *(Thakur et al. 2017)*.

1.8.4. Animal models summary

Animal models were designed, in part, to test potential treatments for PD patients. Unfortunately, some therapeutic agents that seemed promising in neurotoxic or genetic models failed to demonstrate any efficacy in clinical trials *(Dawson, Golde, and Lagier-Tourenne 2018; C. W. Olanow, Kieburtz, and Katz 2017)*. The lack of good translation between preclinical and clinical phases demonstrates the need for an animal model able to reproduce the multifactorial etiology of PD and its gradual progression *(C. Warren Olanow, Kieburtz, and Schapira 2008)*.

2. Neuromelanin

2.1. Brief history

The first description of a dark pigmented structure in the human SN was made in 1786 by Félix Vicq-d'Azyr *(André Parent 2007; A. Parent 2016; Vicq d'Azyr 1786)*. More than a century later, Blocq and Marinesco proposed a possible link between this pigmented structure and PD *(M. Parent and Parent 2010; Marinescu and Blocq 1892)*. In 1939, Adler described the presence of a dark pigmentation in other animals *(Adler 1939)*. In the late 1950s, this pigment was identified as NM, due to its relationship to peripheral melanin *(Lillie 1955; 1957; Fedorow et al. 2005)*. In 1968, Fenichel and Bazelon analyzed brains from the 34th gestational week to 16 years of age and described an age-dependent increase of NM in the SN, LC and DVC *(Fenichel and Bazelon 1968)*. Finally, in the early 1980s, NM was described in other parts of the human brain, and a correspondence between this pigment and catecholaminergic neurons was observed *(Bogerts 1981; Saper and Petito 1982)*.

2.2. NM in different species

NM is not restricted to humans: it has been found, in lower amounts, in non-human primates, cats, dogs, horses, sheep, giraffes, dolphins, whales and even frogs *(Adler 1939; 1942; Usunoff et al. 2002; Itzev et al. 2002; Sacchini et al. 2018; 2022; Sukhorukova, Alekseeva, and Korzhevsky 2014)*. However, it is uniquely abundant in human brains *(Marsden 1961; Scherer 1939)*. A lack of NM production/accumulation has consistently been described in rodents *(Itzev et al. 2002)*. Rodents represent the majority of PD animal models, therefore the absence of NM in these animals could explain, at least in part, why these models fail to reproduce the multisystemic aspects of the disease. Moreover, the lack of NM in rodents has contributed to a scarcity of *in vivo* NM studies and a relative lack of knowledge of its potential implications in health.

2.3. NM structure and synthesis

NM is structurally similar to peripheral melanin, as it contains both eumelanin and pheomelanin *(Odh et al. 1994)*. In fact, NM granules appear to be a dense pheomelanin core surrounded by eumelanin *(Bush et al. 2006)*. Both peripheral melanin and NM share important spectroscopic and histological aspects, such as basophilia, ultraviolet spectra, and electron paramagnetic resonance spectra *(Usunoff et al. 2002)*. NM granules not only contain pigments but also enclose different lipids and peptides *(Zucca et al. 2014)*. Indeed, lipid droplets entangled with NM have been consistently described within NM granules in the human brain.

Heterogeneous NM organelles are entrapped within either single or double-membranes *(Sulzer et al. 2000; David Sulzer et al. 2008; Zucca et al. 2018)*. As, during autophagy, structures to be degraded are sequestered into double-membrane vesicles (autophagosomes) *(Mari, Tooze, and Reggiori 2011)*, it seems these double-membrane NM organelles might, in fact, be pigmented autophagic vacuoles *(Zucca*

et al. 2018). Therefore, the NM granules could be consumed by the phagophore, engendering autolysosomes containing undegradable material (**Figure 7**) *(Zucca et al. 2018; Sulzer et al. 2018)*.

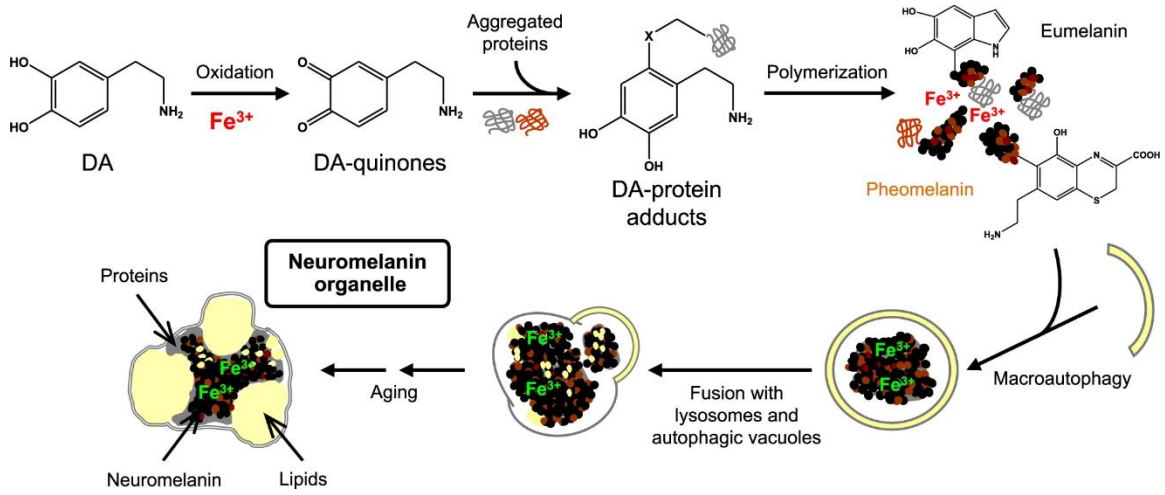

Figure 7 - Mechanisms for biosynthesis of NM pigment and for the formation of NM-containing organelles in human SN. *Excess dopamine in the cytosol of SN neurons can be oxidized to quinones by ferric iron. These highly reactive compounds can bind to aggregated and β-structured proteins that accumulate in the cytosol. An oxidative polymerization initiates formation of the melanin-protein component with eumelanin and pheomelanin moieties that can also bind high amounts of metals, particularly iron. Via macroautophagy, the resulting undegradable material is taken into autophagic vacuoles that fuse with lysosomes and other autophagic vacuoles containing lipid and protein components, thus forming the final NM-containing organelles that contain NM pigment along with metals, abundant lipid bodies, and protein matrix. The process continues during the life of the neuron, so that SN dopamine neurons accumulate high numbers of NM-containing organelles with age. From (Sulzer et al. 2018).* DA: Dopaminergic

The exact NM synthesis pathway is still under debate. However, based on its similarities with peripheral melanin, it is possible to conjecture a pathway based on well-established melanin synthesis mechanisms. In the skin, tyrosine is oxidated into DOPA by the melanogenic enzyme tyrosinase (TYR), and the DOPA into DOPA quinone by tyrosine hydroxylase and DOPA oxidase *(Ito and Wakamatsu 2008; Adhikari et al. 2018)*. For eumelanin formation, DOPA quinone is converted into DOPAchrome by DOPAchrome tautomerase (Dct), also known as tyrosinase-related protein-2 (TYRP2), and is then subdivided into DHICA and DHI by catalytic reaction of DHICA oxidase and DHI oxidase. The final color is due to the ratio of DHICA and DHI. The conversion of DHICA into indole-55,6-quinone carboxylic acid, or the conversion of DHI into indole-5,6-quinone is the final step in the formation of eumelanin *(Ito and Wakamatsu 2008; Adhikari et al. 2018)*. For pheomelanin formation, when cysteine is present, DOPA quinone is converted to cysteinyl-DOPA, forming cysteinilquinones which, in turn, become pheomelanin (**Figure 8**) *(Ito and Wakamatsu 2008; Adhikari et al. 2018)*.

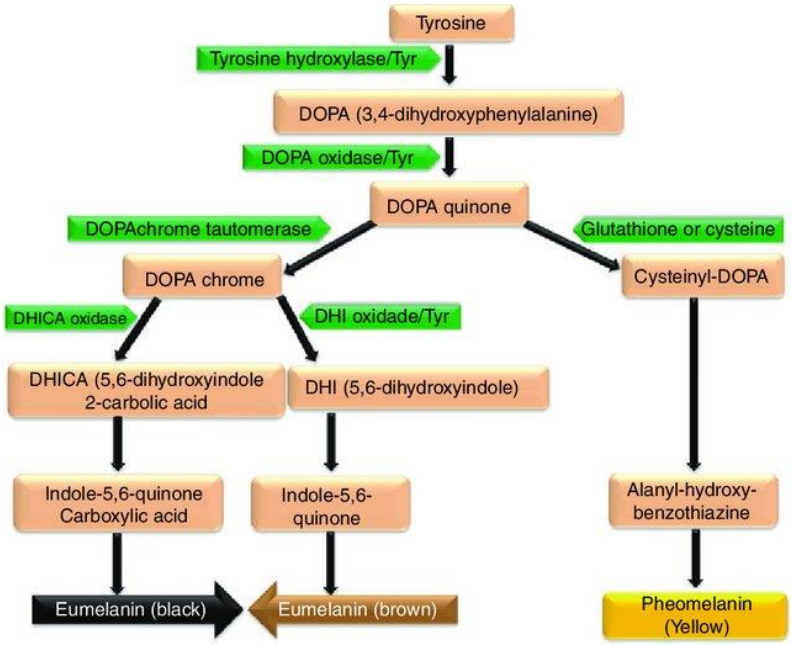

Figure 8 - Melanin biosynthesis pathway. *From (Adhikari et al. 2018).*

NM formation *in vitro* has been reported as being driven by excess cytosolic catecholamines not accumulated by synaptic vesicles *(Sulzer et al. 2000)*. When L-DOPA is added, NM granule formation and accumulation are observed. This accumulation is reduced when an iron chelator and the vesicular monoamine transporter 2 (VMAT2) are overexpressed, due to enhancement of DA uptake by synaptic vesicles. Iron and free cytosolic DA both appear to trigger NM granule formation (**Figure 9**). It is important to note that, in some cases of albinism-associated with a loss of TYR activity, NM is observed in the SNpc *(Fernández et al. 2021; Foley and Banter 1958)*. Therefore, it is widely accepted that NM formation arises from DA autoxidation.

NM appears to be present only in catecholaminergic neurons, but is not in all of them. In addition, as some species completely lack NM despite having DA, it seems unlikely that DA autoxidation is the only explanation for NM synthesis. Moreover, patients with PD receiving enhanced dosages of L-DOPA do not exhibit increased NM levels compared to untreated patients *(David M. A. Mann and Yates 1983; Sulzer and Surmeier 2013)*, while rats receiving chronical administration of L-DOPA do not produce NM *(Murer et al. 1998)*. The accumulation of NM with age suggests an enzyme synthesis origin *(H Fedorow et al. 2006)*. It is important to note that synthetic NM obtained from DA autoxidation presents a different structure of NM granules than that observed in human brains *(Double et al. 2000)*. The enzyme that could be responsible for the origin of NM synthesis is, like in melanocytes, TYR. In support of this, TYR-like activity and expression have been described in both human and mouse brains *(Greggio et al. 2005; Miranda et al. 1984; Tief, Schmidt, and Beermann 1998; Xu et al. 1997)*. In animal models, in the absence of TH, Tyr presents catecholaminergic synthesizing activity. Moreover, PD has been associated with a rare variant of TYR, which seems to suggest that TYR might

play a role *(Lubbe et al. 2016)*. It is possible that other enzymes are involved in NM synthesis, but they have not been identified *(Zucca et al. 2014)*.

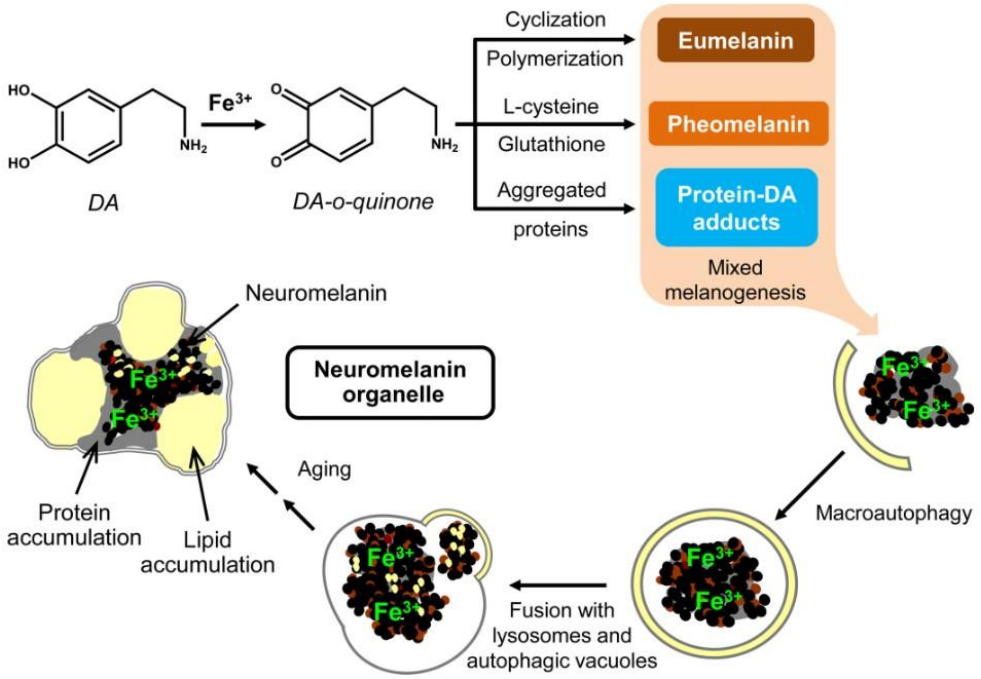

Figure 9 - Possible mechanisms for the synthesis of NM pigment and for the formation of NM-containing organelles. *Excess DA present in the cytosol can be oxidized to DA-o-quinone by ferric iron in a catalytic reaction. In the formation of NM pigment, DA-o-quinone can undergo three different pathways: i) cyclization, further oxidation and polymerization to give eumelanin; ii) reaction with L-cysteine or glutathione to give cysteinyl-DA compounds then oxidized to pheomelanin; and iii) conjugation with protein residues to give DA-protein adducts. The latter two reactions seem to be most rapid and lead to the formation of a protein-pheomelanin core, which is then coated by eumelanin, according to the mixed melanogenesis model. Iron(III) is incorporated into the melanic portion of the forming NM pigment. The resulting undegradable and insoluble pigment is taken into autophagic vacuoles that fuse with lysosomes and other autophagic vacuoles containing lipids, proteins, etc., leading to the formation of NM-containing organelles. These double membrane bounded organelles contain NM pigment along with its components, abundant lipid bodies, and protein matrix. This process continues during the entire neuron life and results in the accumulation of NM-containing organelles with aging. From (Zucca et al. 2017). DA, dopamine; Fe^{3+}, iron (III); NM, neuromelanin.*

2.4. NM function

The exact physiological role of NM is still unknown. NM was initially considered to be a waste product of cellular metabolism. Within the past decade, it has been hypothesized that NM could have a dual function, being either protective or toxic depending on the cellular context *(Zucca et al. 2014)*.

NM synthesis seems to prevent accumulation of toxic catechol derivatives, by integrating them *(L. Zecca et al. 2003)*. NM is able to bind to metals, such as iron, zinc and copper, as well as to toxic chemicals, such as MPTP and paraquat. This means that, under normal physiological conditions, NM would function as an antioxidant. However, when intracellular iron levels are higher than intracellular NM levels – therefore exceeding NM-binding capacity – NM would release iron and have a pro-oxidant effect *(Zucca et al. 2014)*.

It seems that, at first, NM could protect neurons from cellular stress with the formation of autophagic vacuoles which, by macroautophagy, trap NM. However, as NM accumulates, these

vacuoles might interfere with degradative pathways and endocytic/secretory tasks *(Sulzer et al. 2000; David Sulzer et al. 2008)*, which would have a detrimental effect on cell survival. Furthermore, when NM is released in the extracellular space, it activates microglia, becoming a potential cause of chronic inflammation by discharging metals and toxic molecules previously trapped within *(Zucca et al. 2014)*. Thus, it seems that NM accumulates with age to protect the neurons until it occupies all the cytoplasm; this in turn starts to cause neuronal dysfunction, and then cell death. Dying cells are then responsible for the release of NM into the extracellular space, triggering a self-perpetuating cycle of neuroinflammation and neurodegeneration *(Vila 2019)*.

2.5. Intracellular NM levels

Intracellular NM levels are 15% lower in surviving SNpc cells of patients with PD, suggesting that neurons containing higher intracellular NM levels are more prone to cell death *(David M. A. Mann and Yates 1983)*. A positive correlation has been found between cell loss in the midbrain of patients with PD and the percentage of NM-positive neurons usually present in this area~~,~~ in healthy patients *(E. Hirsch, Graybiel, and Agid 1988)*. Moreover, an inverse correlation between intracellular NM levels and the percentage of surviving neurons in PD compared to healthy controls was reported in the 1990s *(Kastner et al. 1992)*. Additionally, in patients with PD, SNpc DA neurons with LP exhibit a decrease in intracellular NM levels whereas morphologically intact DA neurons exhibit an increase of in NM *(Halliday et al. 2005)*. Our own team observed increased intracellular NM levels in patients with PD as well as in patients with iLBD *(Carballo-Carbajal et al. 2019)*. Taken together, these studies seem to confirm that excess NM accumulation above a certain threshold could trigger neuronal death, suggesting a key role for NM in PD progression as a vulnerability factor.

2.6. PD hallmarks and NM

Age is considered to be the main risk factor for PD, and NM accumulation appears to be age-dependent. Indeed, NM-sensitive Magnetic Resonance Imaging (NM-MRI) has shown an age-dependent increase of NM in the SNpc and LC of healthy controls, reaching a plateau at middle age *(Xing et al. 2018; Shibata et al. 2006)*. NM starts to accumulate in the SNpc from 3 years old until it occupies most of the cytoplasm in older humans (**Figure 10**) *(H Fedorow et al. 2006; D. M. A. Mann and Yates 1974; Luigi Zecca et al. 2002)*. NM is also observed in the LC from 3 years old and increases with age *(Fenichel and Bazelon 1968; Manaye et al. 1995)*. NM is higher in the SNpc around middle age and above, but NM levels are higher in the LC in the first decades of a human life *(D. M. A. Mann and Yates 1974; 1979; Luigi Zecca et al. 2004)*. It seems that neurons are unable to degrade or eliminate NM, although it is unclear whether this is caused by inadequate fusion of NM vacuoles with lysosomes or by NM resistance to lysosomal

PD is consistent with neuronal death, mostly in the SNpc and LC, which is also observed in older healthy controls. This supports the idea that NM could be a vulnerability factor for PD, with a pathogenic threshold above which NM accumulation would be toxic and trigger PD progression. It is also interesting to note that extracellular NM, released from dying neurons, is usually seen in aged individuals and is associated with microglial activation *(Beach et al. 2007; Korzhevskii et al. 2021)*. These observations suggest that NM might also have an impact on the healthy aged brain.

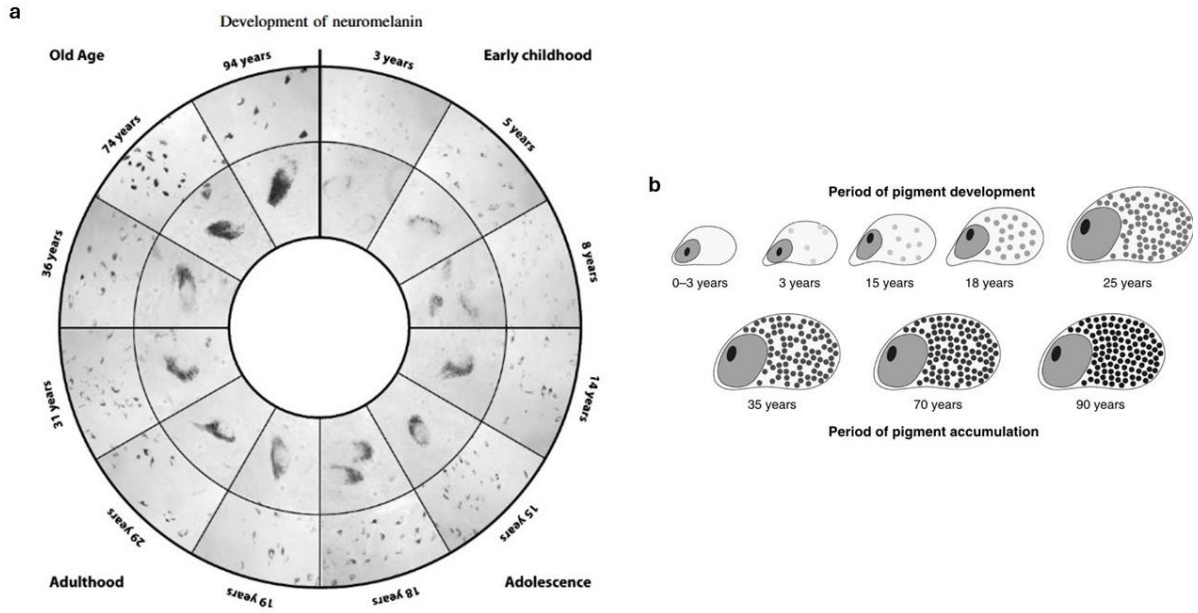

Figure 10 - NM age-dependent accumulation in humans across lifetime. *a) Intermediate and high magnification photomicrographs of representative unstained NM-pigmented neurons from the ventral region of the substantia nigra of humans at various ages. The first appearance of NM pigment is at 3 years of age. The optical density of the NM pigment steadily increases with age, while the average cellular volume occupied by NM pigment increases up to twenty years of age. After twenty years, the average cellular volume occupied by NM does not increase significantly. b) Representation of the three developmental stages of NM. Initiation of pigmentation occurs at around 3 years of age, followed by an increase in cell size and the cellular volume occupied by NM up until the age of ≈20 years. In adulthood, the NM pigment darkens over time. Adapted from (Halliday et al. 2006).* NM, neuromelanin.

NM is related to LB formation, as LB are present in accumulated NM within the neuron cytoplasm *(Jellinger 2003; Kuusisto, Parkkinen, and Alafuzoff 2003; E. Braak et al. 2001)*. Additionally, a major component of NM granules found only in PD brains, is α-synuclein *(Fasano et al. 2003)*. Microgliosis, astrogliosis, and lymphocyte infiltration are all localized in the SNpc and LC of PD, especially within high NM-positive cells or extracellular NM proximity *(E. C. Hirsch and Hunot 2009; Miklossy et al. 2006; Pey et al. 2014)*.

Major histocompatibility complex class I (MHC-I), which is necessary for antigen presentation, colocalizes with NM granules only in the SNpc and LC *(Cebrián et al. 2014)*. Therefore NM could present as an antigen, meaning that NM-positive neurons could be attacked by T-cells. CD8-positive T-cells have been observed in physical contact with NM-positive neurons as well as with extracellular NM *(Galiano-Landeira et al. 2020)*. Furthermore, extracellular NM is usually surrounded by, or

enclosed by, microglial cells in both patients with PD and healthy elderly patients *(Beach et al. 2007; Langston et al. 1999; McGeer et al. 1988)*. Extracellular NM seems to induce a pro-inflammatory microglial phenotype, both *in vitro* and *in vivo* *(Wilms et al. 2003; Luigi Zecca et al. 2008; Zhang et al. 2011)*. It is interesting to note that increased NM antibodies have been found in PD patients' sera *(Double et al. 2009)*.

It is also important to note that the two most vulnerable brain areas in PD, the SNpc and VTA, also correspond to the most pigmented catecholaminergic areas in the human brain *(Zucca et al. 2014)*. Consistent neurodegeneration involving an increased loss of pigmented cells in these areas has been consistently observed in PD brains *(Giguère, Burke Nanni, and Trudeau 2018; Sasaki et al. 2006)*.

2.7. First *in vivo* NM-producing rodent model

Even though NM seems to have a key role in PD, it has been poorly studied because rodents do not accumulate NM, making its investigation difficult *(Barden and Levine 1983; Sukhorukova, Alekseeva, and Korzhevsky 2014)*. To better understand the potential role of NM in PD, it seems evident that an *in vivo* model able to reproduce age-dependent NM accumulation is needed.

Our team has demonstrated that NM can be synthesized enzymatically by TYR in the SNpc of rats *(Carballo-Carbajal et al. 2019)*. Adeno-associated viral vector (AAV)-driven overexpression of human TYR (AAV-hTyr) in the SNpc of rats was able to reproduce a human-like accumulation of NM (**¡Error! No se encuentra el origen de la referencia.**a-c). Quantification of intracellular NM in these rats confirmed an age-dependent NM accumulation, reaching levels observed in older humans (**¡Error! No se encuentra el origen de la referencia.**d). As in PD patients, this accumulation led to motor symptoms and neuronal dysfunction, with impaired DA release, nigrostriatal neurodegeneration, and intracellular inclusions formation (**¡Error! No se encuentra el origen de la referencia.**e). These inclusions were also observed in the absence of α-synuclein, in α-synuclein knockout mice, which suggests that their formation is a consequence of NM accumulation and, above all, independent of α-synuclein (**¡Error! No se encuentra el origen de la referencia.**f,g). Furthermore, AAV-hTyr rats were co-injected with an AAV expressing a transcription factor EB (TFEB), a master regulator of autophagy which, once activated, induces biogenesis of lysosomes and autophagosomes, promoting lysosomal exocytosis. These rats showed a decrease in inclusion formation, as well as reduced NM accumulation and NM-linked neurodegeneration. Moreover, motor impairments were not apparent in these animals (**¡Error! No se encuentra el origen de la referencia.**a-d) *(Carballo-Carbajal et al. 2019)*.

The above results suggested that age-dependent NM accumulation interferes with the ALP, leading to the alteration of endocytosis which, in turn, creates a cellular traffic jam, prompting LP and PD-linked neuronal dysfunction *(Vila 2019)*.

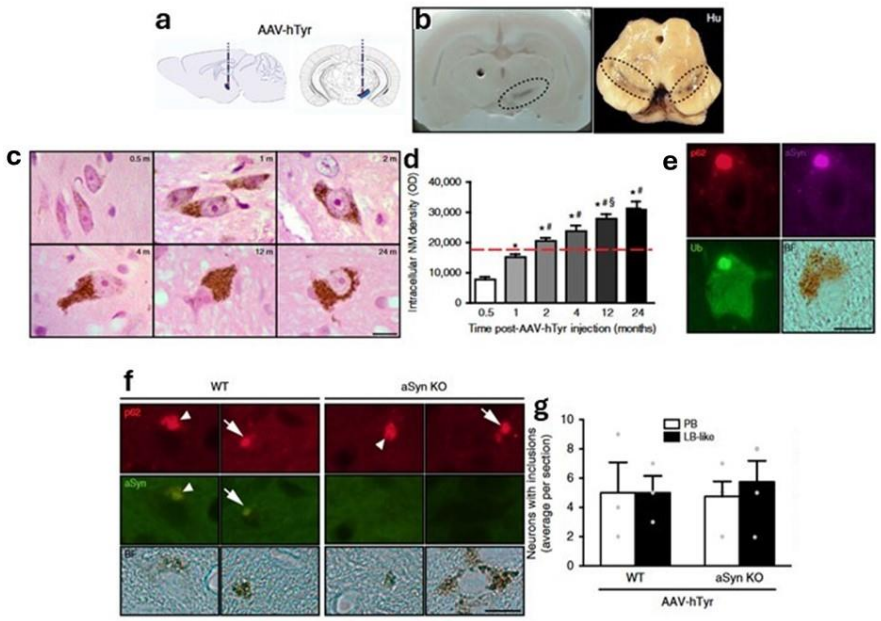

Figure 11 - Age-dependent NM accumulation in SNpc rats prompts PD. *a) Schematic representation of the site of AAV-hTyr unilateral stereotaxic injection above the SNpc of the rat brain. b) Left, representative unstained AAV-hTyr-injected rat brain (2 m post-AAV injection) mounted in a cryostat in which ipsilateral SNpc can be detected macroscopically as a brown, darkened area (dashed outline). A hole was made in the contralateral hemisphere as anatomical reference. Right, representative unstained midbrain from a 62-year-old human control subject (Hu) in which the SNpc can be detected macroscopically (bilateral dashed outlines). c) Hematoxylin-eosin (H&E)-stained brain sections showing progressive intracellular NM accumulation (brown) within ipsilateral SNpc DA neurons from AAV-hTyr-injected rats. Scale bar, 12.5 µm. d) Quantification of intracellular NM optical density in ipsilateral SNpc DA neurons of AAV-hTyr-injected rats. *p < 0.05, compared to 0.5 m; #p < 0.05, compared to 1 m; §p < 0.05, compared to 2&4 m. *p < 0.05, compared to control subjects. e) Ipsilateral SNpc section from an AAV-hTyr-injected rat exhibiting a NM-laden neuron with an intracytoplasmic LB-like inclusion immunopositive for p62 (red), aSyn (purple) and ubiquitin (Ub, green). Scale bar, 12.5 µm. f) Ipsilateral SNpc sections from AAV-hTyr-injected aSyn KO and WT mice exhibiting NM-laden neurons with p62-positive (red) PB (arrowhead) and LB-like inclusions (arrow) at 2 m post-AAV injection. aSyn immunofluorescence is shown in green. Scale bar, 12.5 µm. g) Quantification of NM-laden neurons with p62-positive PB or LB-like inclusions in AAV-hTyr-injected aSyn KO and WT mice at 2 m postAAV injection. p = 0.866 (two-way ANOVA). Adapted from (Carballo-Carbajal et al. 2019).*

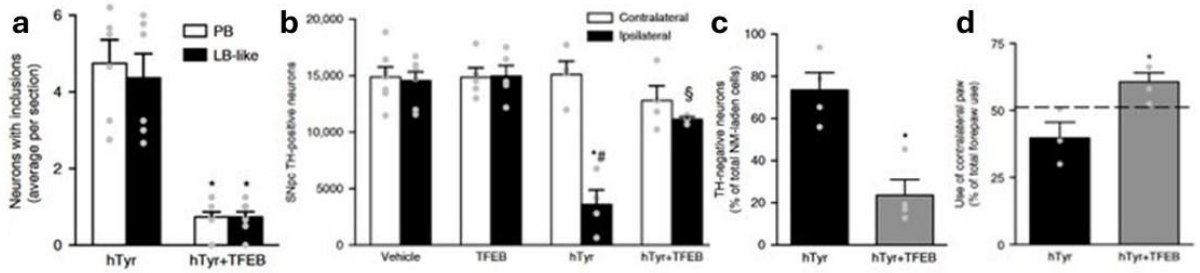

Figure 12 - Therapeutic enhancement of lysosomal proteostasis in NM-producing rats. *a) PB/LB-like inclusions in ipsilateral SNpc from AAV-hTyr- and AAV-hTyr/TFEB-injected rats (2 m post-AAV). *p < 0.05, compared to AAV-hTyr-injected animals (two-way ANOVA, Student–Newman–Keuls post-hoc test). b) SNpc TH-positive neurons at 12 m post-AAV/ vehicle injections. *p < 0.05, compared to respective contralateral side; #p < 0.05, compared to ipsilateral vehicle- and AAV-TFEB-injected animals; §p < 0.05, compared to ipsilateral AAV-hTyr-injected animals (two-way ANOVA; Student–Newman–Keuls post-hoc test). c) TH downregulation within NM-laden neurons (12 m post-AAV). *p < 0.05, compared to AAV-hTyr-injected animals (two-tailed t-test). d) Contralateral forepaw use in AAV-hTyr- and AAV-hTyr/TFEB-injected rats (12 m post-AAV). *p < 0.05, compared to AAV-hTyr (only)-injected animals (two-tailed t-test). Dashed line indicates average contralateral forepaw use in control-injected rats. In all panels, values are mean ± SEM. Adapted from (Carballo-Carbajal et al. 2019).*

3. Sex steroids and Parkinson's disease

3.1. Sex differences in PD

There are several sex differences in the incidence and presentation of PD. Males have a 1.5 to 2-fold higher relative risk for developing PD (Bourque, Morissette, and Paolo 2019; Bourque and Di Paolo 2022; L. Hirsch et al. 2016) and onset is around two years earlier on average than in females *(Haaxma et al. 2007; Twelves, Perkins, and Counsell 2003)*. Males and females typically exhibit differences in motor symptoms: females usually present a tremor-dominant phenotype that is associated with slower PD progression, while males display more rigidity *(Haaxma et al. 2007; Baba et al. 2005; Bourque and Di Paolo 2022)*. Moreover, females may have a higher risk of L-DOPA-induced dyskinesias and L-DOPA-related motor complications *(Colombo et al. 2015; Bjornestad et al. 2016; Bourque and Di Paolo 2022)*. Non-motor symptoms, such as depression, pain, and constipation, appear to be reported more frequently in females (**Figure 13**) *(Meoni, Macerollo, and Moro 2020)*, while *de novo* male patients display a greater degree of brain atrophy and connectivity disruption, indicative of more severe neurodegeneration *(Tremblay et al. 2020)*.

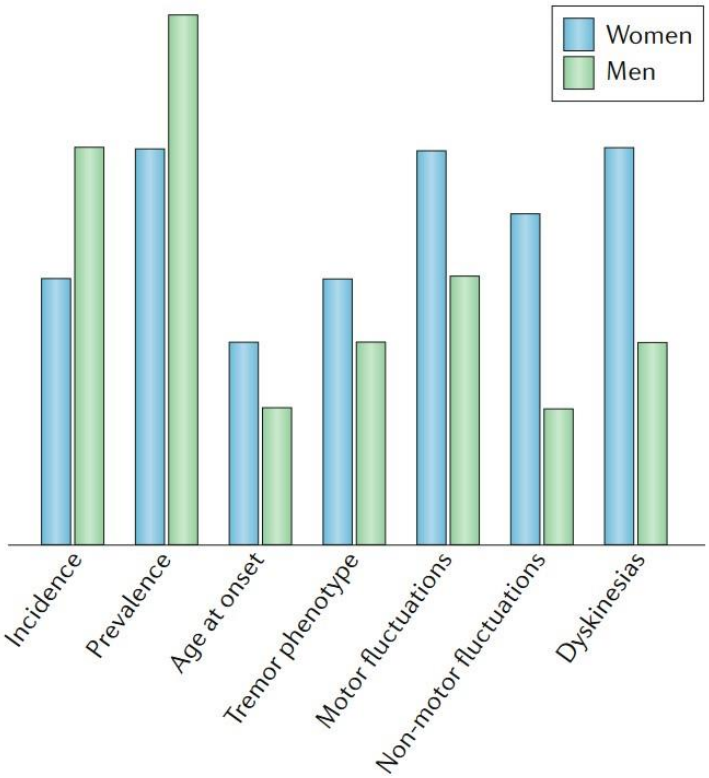

Figure 13 - Sex differences in Parkinson's disease. *Compared to males (green bars), females (blue bars) tend to have: older age at disease onset; lower prevalence and incidence; higher rate of tremor phenotype; and greater likelihoods of dyskinesia, and motor and non-motor fluctuations. From (Meoni, Macerollo, and Moro 2020).*

Risk and protective factors appear to be different between males and females *(Savica et al. 2013)*. DA transporter binding in the caudate nucleus is generally higher in females than in males, both with and without PD *(Kaasinen et al. 2015)*.

The origins of these sex differences in PD have been hypothesized to be genetic, environmental, related to sexual dimorphism, caused by gonadal hormones, or due to some combination of the above. A key protective role for ovarian hormones appears to be the current best hypothesis: 17β-estradiol (E2) and progesterone seem to have a neuroprotective effect on DA neurons in the female brain *(Blauwendraat et al. 2021; Bourque and Di Paolo 2022)*. However, genes carried on the sex chromosomes, especially the Y-chromosome may also be responsible for the higher prevalence of PD in males *(Lee et al. 2019)*.

In summary, sex differences in the onset and presentation of PD may be explained by the neuroprotective effects of sex steroids in females in combination with genes located on the sex chromosomes, a sex-dependent dimorphic brain development allowing changes in its vulnerability to environmental factors, and differences in risk exposure *(Savica et al. 2013)*.

3.2. Impact of ethnicity on sex differences in PD

The reported prevalence of PD is lower in Eastern countries (China, Japan, India, Taiwan, Hong Kong, Singapore) than Western countries (Spain, Italy, France, United Kingdom, Netherlands, United States of America, Australia, Brazil, Argentina, Bulgaria) *(Abbas, Xu, and Tan 2017)*. Furthermore, with a male:female ratio of 1-1.2, sex differences in PD prevalence are less apparent in Asian populations than worldwide *(Abbas, Xu, and Tan 2017)*. Notably, among people living in the United States, Caucasians have a substantially higher PD prevalence and incidence than Asians *(Wright Willis et al. 2010)*. Moreover, males of Japanese ancestry living in the United States have higher incidence and prevalence rates of PD than males resident in Japan *(Abbas, Xu, and Tan 2017)*. It is interesting to note that the prevalence of PD in African Americans is lower than in Caucasians, but higher than in Africans from Nigeria *(Abbas, Xu, and Tan 2017)*.

These differences in PD prevalence across different race groups are interesting to note, because E2 levels in African Americans are 18% higher and in Asian Americans are 22% higher than in Caucasians *(Pinheiro et al. 2005)*. It is possible that higher E2 levels may provide greater protection from PD. Furthermore, a higher prevalence in females with GBA-associated PD has been reported in North America and Europe but not in Asia nor Oceania where the population have higher E2 levels *(Li et al. 2021)*. These data show correlation between E2 and prevalence: the higher incidence in Asian Americans vs Asians, and the less apparent sex differences in Asian populations, both suggest lifestyle factors may be key in modifying PD risk (and may also modify E2 levels, partly explaining the correlation) .

3.3. Impact of age on sex differences in PD

As discussed, age is the main risk factor for PD *(Collier, Kanaan, and Kordower 2017)*. Both PD and aging share several impaired cellular processes, such as mitochondrial dysfunction, inflammation, proteasome/lysosome function, oxidative stress and dopamine metabolism *(Collier, Kanaan, and*

Kordower 2017). These changes reduce the brain's ability to respond to stressful events, making it more vulnerable to damage. For example, in mice and monkeys treated with MPTP, greater degeneration is observed in aged animals compared to younger ones *(Jiang et al. 2014; McCormack et al. 2004)*. As several pathways are impaired in both PD and aging, a drug focusing only on one pathway involved might not be effective against the range of pathological processes leading to dopaminergic degeneration in PD *(Bourque and Di Paolo 2022)*. A therapeutic strategy targeting several mechanisms that contribute to a dysfunctional nigrostriatal dopaminergic system seems to be a more promising lead *(Bourque and Di Paolo 2022)*.

3.4. Neuroactive steroids as potential treatments in PD

Neurosteroids are synthesized by neurons and glia *(Giatti et al. 2019)*. Neuroactive steroids are hormonal steroids that affect the brain; they may be produced by peripheral glands, by the central nervous system, or be synthetic. Steroids synthesized in the periphery are highly lipophilic and can easily cross the BBB by diffusion or via a specific transporter *(Giatti et al. 2019; Grube, Hagen, and Jedlitschky 2018)*. The brain expresses steroidogenic enzymes that regulate the production of neuroactive steroids from cholesterol (**Figure 14**a).

Neuroactive steroids have multiple mechanisms of action *(Giatti et al. 2019; Yilmaz et al. 2019; Bourque, Morissette, and Di Paolo 2024)* and they modulate several functions of the central nervous system (**Figure 14**b) *(R. C. Melcangi, Garcia-Segura, and Mensah-Nyagan 2007; Bourque, Morissette, and Di Paolo 2024; Bourque and Di Paolo 2022)*. As they target various functions impaired in PD, neuroactive steroids make an attractive therapeutic strategy *(Bourque, Morissette, and Di Paolo 2024)*. A better understanding of these sex steroids would help in the identification of tailored therapeutic approaches specific for males and females with PD *(Bourque, Morissette, and Di Paolo 2024)*.

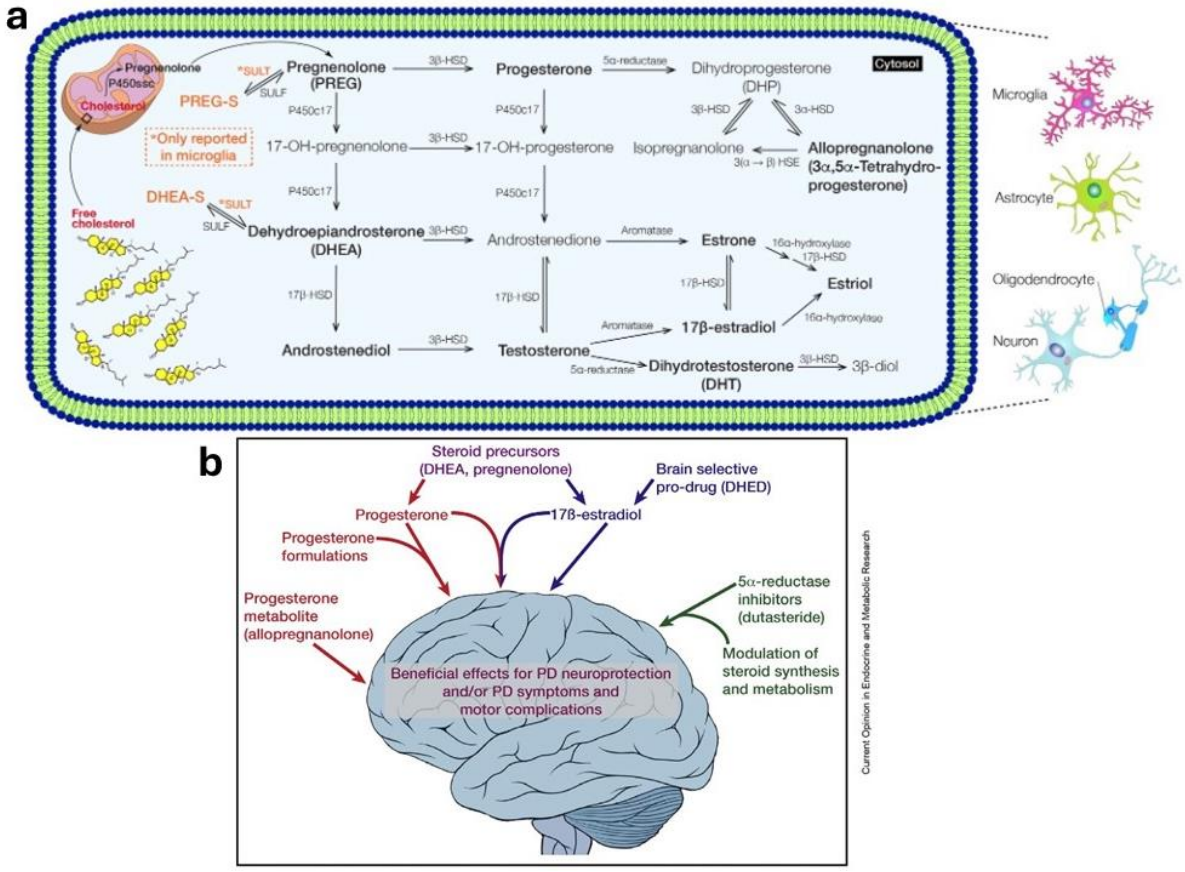

Figure 14 - Neuroactive steroids and their potential beneficial effects in Parkinson's disease. *a) Schematic diagram of steroidogenesis of major neuroactive steroids. Neuron, oligodendrocyte, astrocyte and microglia possess enzymes involved in steroidogenesis. Cholesterol enters the metabolon protein complex where CYP11A1 converts cholesterol to pregnenolone (considered the rate-limiting step in steroidogenesis) (Elustondo et al., 2017). Pregnenolone is then converted to 17-hydroxypregnenolone or to progesterone. Steroidogenesis for progesterone, testosterone and estrogens is shown. Steroids investigated for PD neuroprotection and/or PD symptomatic treatment are shown in bold. Adapted from (Bourque, Morissette, and Di Paolo 2024). b) Summary of neurosteroid treatment options tailored for males and females with PD. 17b-estradiol and progesterone both have neuroprotective and neuromodulation effects in the brain. 17b-estradiol levels can be increased selectively in the brain via a pro-drug (DHED) or via the use of precursors (DHEA, pregnenolone). Brain progesterone levels can be increased with novel formulations, and its effects can also be increased through the activity of its metabolites. Modulation of steroid synthesis and metabolism with 5a-reductase inhibitors such as dutasteride could also be an option for males. From (Bourque and Di Paolo 2022).*

3.5. Androgens and PD

Androgens, such as testosterone and dihydrotestosterone, decrease inflammation, activate signaling pathways and regulate mitochondrial functions in the brain *(Bianchi et al. 2020; Mohajeri et al. 2019)*. Androgen effects are initiated upon binding to classical androgen receptors or to membrane androgen receptors *(McEwan and Brinkmann 2000; Thomas 2019)*.

As males have a higher incidence and prevalence of PD than females, it is important to determine how androgens might be associated with PD *(Bourque, Morissette, and Di Paolo 2024)*.

3.5.1. Human studies

Males experience a slow, gradual decrease in testosterone blood levels from their fourth-fifth decade *(Bourque, Morissette, and Di Paolo 2024)* and, from 50 years of age, healthy males show an age-dependent increase in testosterone deficiency *(Harman et al. 2001)*. In comparison, males with PD appear to have higher testosterone blood levels *(Bourque, Morissette, and Di Paolo 2024)* and lower rates of testosterone deficiency than healthy controls *(M. S. Okun et al. 2004; Michael S. Okun, McDonald, and DeLong 2002)*. Furthermore, testosterone blood levels in males with PD seem to correlate inversely with α-synuclein CSF levels, suggesting that higher testosterone levels correlate with a more pronounced pathology *(Bourque, Morissette, and Di Paolo 2024)*. Conversely, androgen deprivation therapy given to prostate cancer patients is not associated with an increased risk of PD, *(Chung et al. 2016; Young et al. 2017)* and in fact patients receiving androgen deprivation therapy for 5 years present a lower risk of PD than patients who received no hormonal deprivation *(Young et al. 2017)*. This suggests that males with low levels of testosterone may be at reduced risk for PD, and decreasing androgen levels might be beneficial in some at-risk males.

Furthermore, in PD patients with testosterone deficiency, testosterone replacement therapy resulted in some improvement on non-motor symptoms, but motor symptoms were still present. In PD patients with low normal testosterone levels, no beneficial effect of testosterone replacement therapy was observed *(Mitchell, Thomas, and Burnet 2006; Michael S. Okun, McDonald, and DeLong 2002; Michael S. Okun et al. 2002; M. M. S. Okun, Fernandez, and Rodriguez 2006)*. Testosterone deficiency is associated with decreased energy, impaired physical performance, and mobility limitations. Testosterone replacement therapy leads to an improvement of energy but has modest effects on physical function *(Bourque, Morissette, and Di Paolo 2024)*. It is possible that improvements in motor symptoms seen in patients with PD when testosterone levels are restored to normal relate to the treatment of testosterone deficiency rather than to PD *(Bourque, Morissette, and Di Paolo 2024)*.

3.5.2. Androgen treatments in animal models of PD

Neurosteroid levels in the cerebral cortex change after gonadectomy, in both males and females *(Caruso et al. 2010)*. In the 6-OHDA rat model of PD, toxicity was reduced in castrated male rats, with less damage in DA neurons compared to intact males *(Gillies et al. 2004; Murray et al. 2003; Tamás et al. 2006)*. However, in the MPTP mouse model of PD, no significant difference in neuronal damage was observed between castrated and intact males, whether young or aged *(Antzoulatos et al. 2011; Dluzen 1996; Isenbrandt et al. 2021)*. These inconsistent findings could be due to the different species and/or toxin used.

No effect on DA neuron damage was observed in castrated male 6-OHDA rats and MPTP mice treated with testosterone or dihydrotestosterone *(Dluzen 1996; Gillies et al. 2004; Murray et al. 2003)*. Moreover, these hormones had no neuroprotective effect in intact male MPTP mice *(Ekue et al. 2002)*.

Similarly, androgen treatment was not associated with any beneficial effect in either–6-OHDA ovariectomized (OVX) female rats or intact female MPTP mice *(Gillies et al. 2004; Murray et al. 2003; Tomas-Camardiel et al. 2002)*.

To conclude, castration-induced reductions in male sex hormones do not induce additional toxicity in the MPTP mouse model of PD. Furthermore, androgen treatments do not show any beneficial impact in castrated male and female 6-OHDA rats or intact male and female MPTP mice *(Bourque, Morissette, and Di Paolo 2024)*.

3.6. Progesterone and PD

Brain progesterone is metabolized by 5α-reductase into dihydroprogesterone and then into allopregnanolone *(Roberto Cosimo Melcangi and Panzica 2014)*. Progesterone increases cell survival and tropic factors, regulates calcium channels, modulates the inflammatory response, and has anti-apoptotic effects *(Arevalo et al. 2013; Djebaili et al. 2005; Guerra-Araiza et al. 2009; Kaur et al. 2007; Luoma, Kelley, and Mermelstein 2011; O'Connor et al. 2007)*. It also increases the oxidative capacity of mitochondria, while decreasing oxidative stress and enhancing antioxidant enzyme levels *(Aggarwal et al. 2008; Irwin et al. 2008)*. Progesterone and its metabolite, allopregnanolone, also increase autophagy *(Kim, Lee, and Koh 2012)*. Allopregnanolone inhibits mitochondrial permeability transition pore currents as well as mitochondrial cytochrome c release *(Sayeed et al. 2009)*. Allopregnanolone also modulates microglial morphology and phagocytic function *(Jolivel et al. 2021)*.

Progesterone acts by binding to intracellular progesterone receptors A and B, which act as nuclear transcription factors, to putative membrane-associated progesterone receptor component 1, or to one of the five isoforms of the transmembrane progesterone receptor that activates G proteins *(Guennoun 2020)*.

3.6.1. Human studies

The highest levels of dihydroprogesterone and allopregnanolone are found in the SNpc *(Bixo et al. 1997)*. Levels of brain progesterone and its metabolites are higher in fertile females than in post-menopausal females *(Bixo et al. 1997)*, indicating that changes in brain steroid levels occur with menopause.

In post-mortem PD brains, progesterone metabolism is impaired relative to age-matched controls *(di Michele et al. 2003; Luchetti et al. 2010)*. Males with PD have lower levels of progesterone metabolites in CSF and plasma than healthy controls *(di Michele et al. 2003)*. As 5α-reductase type 1 is reduced in the SNpc of both males and females with PD, this could explain the observed decrease in progesterone metabolite levels *(di Michele et al. 2003; Luchetti et al. 2010)*.

While levels of progesterone metabolites – allopregnanolone and 5α-dihydroprogesterone – are reduced in the later stages of PD, allopregnanolone, and its own metabolite, 3α5α20α-

hexahydroprogesterone, are increased in the SNpc of both males and females in the early stages of PD *(Luchetti et al. 2023)*. This increase in progesterone metabolism in early stages could be a protective mechanism; it is then followed by a downregulation of metabolism that may exacerbate pathological changes in the SNpc *(Bourque, Morissette, and Di Paolo 2024)*. Notably, all other steroids, their precursors, and their metabolites show no differences between patients with PD and controls *(Luchetti et al. 2023)*.

Taken together, the above data suggest that progesterone metabolites may provide therapeutic benefits for PD patients. However, it is important to note that an increased PD risk has been reported in females using progestin-only hormone *(Simon et al. 2009)*.Unfortunately, as progesterone has a short half-life and poor bioavailability, most progesterone treatments are in fact synthetic progestins that bind to different steroid receptors, in addition to progesterone receptors *(Stanczyk et al. 2013)*. Post-menopausal females with PD who received conjugated equine estrogens followed by two weeks of medroxyprogesterone acetate treatment showed improvements in L-DOPA induced dyskinesias (LID) but it is unclear whether the improvements were due to the medroxyprogesterone acetate or the conjugated equine estrogens *(Nicoletti et al. 2007)*. When post-menopausal females with PD who were in their 60s (therefore around a decade after menopause) received progesterone for two weeks after E2 treatment, they displayed worsened motor symptoms, while the E2 treatment had no effect *(Strijks, Kremer, and Horstink 1999; Nicoletti et al. 2007)*. As there was a substantial 10-year gap between menopause and hormonal treatments in these females, it would be interesting to know the effect of such interventions in younger patients.

3.6.2. Progesterone treatments in animal models of PD

In females, progesterone levels decrease after short- and long-term OVX, while dihydroprogesterone levels increase after short-term OVX and then decrease after long-term OVX *(Caruso et al. 2010)*. In males, dihydroprogesterone levels similarly show an initial increase after short-term gonadectomy which is followed by a decrease after long-term gonadectomy;~~,~~ allopregnanolone levels also decrease after long-term gonadectomy *(Caruso et al. 2010)*. These results are consistent with the decrease in progesterone and its metabolites seen in human menopausal females *(Bixo et al. 1997)*.

In intact, young or old male MPTP mice, low doses of progesterone appear to protect against MPTP-induced neurotoxicity *(Bourque, Morissette, and Paolo 2015; Callier et al. 2001; Grandbois et al. 2000; Morissette et al. 2008)*. Additionally, male MPTP mice treated with high doses of progesterone show decreased MPTP toxicity on DA neurons The beneficial effect of progesterone is apparent when it is administered 24h after MPTP, but not when it is administered 5 days after MPTP *(Litim, Morissette, and Paolo 2017)*. Allopregnanolone treatment in male MPTP mice leads to improved motor performance and appears to restore both the number of TH-positive cells and the total number of cells in the SNpc *(Adeosun et al. 2012)*.

In male 6-OHDA rats, pregnenolone and dihydroprogesterone levels are decreased in the striatum relative to control animals *(Roberto Cosimo Melcangi et al. 2012)*. Progesterone treatment (4 mg/kg) for 3 days, 7 days after 6-OHDA infusion in the striatum, improves motor symptoms and cognitive deficits compared with untreated animals, and normalizes glutamate and DA release *(Casas et al. 2011; 2013)*. However, progesterone treatment (4 and 8 mg/kg) for 13 days, 24h after 6-OHDA lesion has no protective effect on striatal DA concentration and animals display more severe motor deficits than untreated animals *(Chao et al. 2011)*. Interestingly, allopregnanolone treatment (5 and 20 mg/kg) every other day for 8 weeks, starting 24h after 6-OHDA lesion in male rats results in improved motor symptoms and appears to preserve the density of synaptic proteins *(Nezhadi et al. 2017)*. It is important to note that the effect of progesterone treatment has not been investigated in female rodents, but MPTP female monkeys receiving progesterone treatment display no effect on LID *(Gomez-Mancilla and Bédard 1992)*.

3.7. Estrogens and PD

E2 increases mitochondrial function, reduces oxidative stress, impacts the integrity of DA neurons, and activates cell survival signaling pathways and trophic factors. It also modulates inflammatory processes, prevents apoptosis, inhibits the formation of protein aggregates, and decreases the activity of calcium channels *(Arnold, Victor, and Beyer 2012; Bourque, Morissette, and Paolo 2015; Brewer et al. 2009; D'Alessandro et al. 2012; D'Astous et al. 2006; Villa et al. 2016)*.

E2 can bind to estrogen receptors α (Erα) or β (Erβ) or to G-protein coupled estrogen receptor 1 (GPER1 or GPR30), and this binding activates both genomic and non-genomic mechanisms *(Arterburn and Prossnitz 2023; Levin 2015; Nilsson et al. 2001)*. E2 is known to play a role in brain development, notably influencing the DA system *(McCarthy 2009)*. The reported sex differences in PD incidence could be a consequence of the actions of E2 in DA neurons, which might somehow favor PD development in males and/or prevent PD in females *(Villa et al. 2016)*.

3.7.1. Human studies

In males with PD, blood levels of E2 are increased compared to age-matched healthy controls *(Bovenzi et al. 2023)*. Male patients with less motor symptoms or in the early stages of the disease have higher blood levels of E2, which suggests that E2 may have a protective effect on motor dysfunction *(Bovenzi et al. 2023)*.

In females, E2 levels fluctuate across the lifespan: important events impacting E2 levels are menarche, pregnancy, menopause, and the type of menopause. Menopause usually occurs between 45 and 55 years of age and is characterized by a drop in E2 levels *(Davis and Baber 2022)*. It is important to keep in mind that the prodromal phase of PD may occur 20 years prior to the onset of motor symptoms, and therefore could correspond to the time of perimenopause *(Fereshtehnejad et al. 2019)*. It is interesting to note that perimenopause has been proposed to be a period in which females not only

experience hormonal changes, but also alterations in the immune system and metabolic function. These changes might create a vulnerable state favorable for neurodegeneration *(Brinton et al. 2015; Wang, Mishra, and Brinton 2020)*. While females experiencing natural menopause experience a progressive decrease in ovarian hormone levels, females who go through surgical menopause undergo a sudden drop in these hormones. This abrupt decline may lead to an increase risk of PD whereas longer exposure to endogenous ovarian hormones could confer protection against PD *(Bourque, Morissette, and Di Paolo 2024)*.

Events that negatively impact estrogen stimulation, such as a short fertile lifespan, cumulative period of pregnancies over 30 months, or earlier menopause, appear to be associated with an increased risk of PD in females *(Ragonese et al. 2004)*. It is interesting to note that, during pregnancy, estriol is the main estrogen and ovarian hormones are lower. Estriol has a lower affinity for ER than E2, and has both estrogenic and antiestrogenic effects *(Bernstein et al. 1985; Kuiper et al. 1997; Melamed et al. 1997; Morel et al. 2016)*. On the other hand, a longer duration of fertile lifespan is associated with a lower PD risk, as late age of menopause is linked to a decreased risk of PD *(Yoo et al. 2020; Kusters et al. 2021; 2022)*. Indeed, each year of delay in menopause is associated with a 7% decrease in PD risk *(Kusters et al. 2021)*. In accordance with this, females with PD typically have a shorter reproductive lifespan than control females *(Nitkowska, Czyżyk, and Friedman 2014)* and are more likely to have experienced surgical menopause *(Nitkowska, Czyżyk, and Friedman 2014)*. Furthermore, females that undergo bilateral oophorectomy before menopause present a higher PD risk than gonad-intact females *(Benedetti et al. 2001; Canonico et al. 2021; W. A. Rocca et al. 2008; Walter A. Rocca et al. 2022)*. Overall, females who have had more than two pregnancies, experienced menarche either early or late, and gone through early and/or artificial menopause have a more than 2-fold increased PD risk compared to females who have had fewer than two pregnancies experienced menarche at median age, and gone through natural menopause *(Pesce et al. 2023)*. It is important to take into account that females who undergo surgical menopause usually have an underlying condition that could be an indicator of hormonal dysfunction, and they might receive hormonal therapy, which could be confounding factors that influence the results observed *(Unda et al. 2022)*.

Females with a high cumulative estrogen exposure have a significantly lower risk of PD *(Gatto et al. 2014)*. Hormonal therapy given during menopause to treat menopausal symptoms can be given to postmenopausal females to prevent osteoporosis *(Davis and Baber 2022)*. Females who receive hormone replacement therapy during menopause are less at risk of PD than those who do not receive hormone treatment *(Currie et al. 2004; Pesce et al. 2023)*. Nevertheless, it is important to note that, the impact of hormonal therapy on PD risk might depend on the type of menopause. For example, estrogen therapy is linked to an increased PD risk in females with hysterectomy but not in females who experienced natural menopause *(Popat et al. 2005)*. Furthermore, the formulation and duration of hormonal therapy need to be taken into account. The use of esterified estrogens combined with progestin

is associated with an increased risk of PD, while conjugated equine estrogens associated (or not) with progestin are not linked to any higher risk *(Lundin et al. 2014)*. Estrogen-progesterone formulation is associated with a greater PD risk if used for less than five years, but does not appear to alter risk if used for more than five years *(Gatto et al. 2014; Wu et al. 2020; R. Liu et al. 2014)*. In summary, assessments of age, type of menopause, and timing of hormonal therapy are needed to fully elucidate the impact of hormonal therapy on PD risk in females *(Bourque, Morissette, and Di Paolo 2024)*. Overall, it appears necessary to also take into account lifestyle, comorbidities and risk factors, hormonal formulation, and duration and time of initiation *(Bourque, Morissette, and Paolo 2019; Unda et al. 2022)*.

In post-menopausal females with early PD who are not taking L-DOPA, use of hormonal therapy is linked to lower disease severity *(Saunders-Pullman et al. 1999)*. However, E2 therapy in later stages of PD does not seem to have an impact on motor symptoms *(Strijks, Kremer, and Horstink 1999)*. Equine estrogens appear to improve motor scores and fluctuations in post-menopausal females *(Tsang, Ho, and Lo 2000)*, while transdermal E2 treatment reportedly decreases the dose of L-DOPA required to improve motor functions but has no effect on dyskinesia scores *(Blanchet et al. 1999)*. Additionally, treatment with conjugated equine estrogens, associated or not with medroxyprogesterone acetate, improves LID in post-menopausal females *(Nicoletti et al. 2007; Villeneuve, Langlier, and Bédard 1978)*. Long-term hormonal therapy is associated with increased DA transporter density and increased DA activity in post-menopausal females *(Gardiner et al. 2004; Craig et al. 2004)*. Thus, it seems that hormonal therapies stimulate the remaining nigrostriatal DA neurons in female patients with PD, encouraging better reuptake, storage and release of DA. This in turn leads to more stable DA levels, enabling a reduction in L-DOPA dose and leading to an increase in the duration of L-DOPA response, resulting in a decrease in LID *(Bourque, Morissette, and Di Paolo 2024)*.

It is important to note that E2 has been reported to increase melanin production in the skin, by increasing melanogenesis through GPER and tyrosinase activity *(Natale et al. 2016)*. The possible effect of E2 on NM synthesis has not yet been studied, but it could be hypothesized that E2 is involved in the regulation of NM production in the brain.

3.7.2. Estrogen treatments in animal models of PD

It appears that males are more sensitive to toxicity than females in both the MPTP and the 6-OHDA rodent models of PD *(Dluzen 1996; Isenbrandt et al. 2021; Murray et al. 2003; Miller et al. 1998)*. Compared to gonad-intact females, OVX 6-OHDA rodents and OVX MPTP mice are more susceptible to DA neurodegeneration *(Murray et al. 2003; Isenbrandt et al. 2021)*. Aged acyclic rats, which are considered to be in natural menopause, and young OVX females show a similar loss of TH immunoreactivity in the SNpc, which implies that both natural and surgical menopause induces DA neurodegeneration *(Rodriguez-Perez et al. 2012)*. Furthermore, female rats lesioned with 6-OHDA in

diestrus (which corresponds to minimal E2 levels), present more DA loss than females lesioned in proestrus (which corresponds to maximal E2 levels) *(Datla et al. 2003)*.

In gonad-intact female MPTP mice, high doses of E2 induce a partial beneficial effect on some DA markers, while lower doses reduce the toxic effect of MPTP *(Ookubo et al. 2009; Mitra et al. 2016)*. In OVX rodents in which 6-OHDA is injected in the SNpc, E2 treatment displays neuroprotective effects *(Bourque, Morissette, and Di Paolo 2024)*. As E2 treatment also has neuroprotective effects in aged OVX females, this implies the aging brain is responsive to an E2 effect *(Dluzen 1996; Dluzen, Mcdermott, and Anderson 2001; Miller et al. 1998)*. Overall, low doses of E2 provide greater DA protection than high doses *(Cordellini et al. 2011)*. When E2 treatment is started two months after OVX, neuroprotection is observed, but when treatment is delayed until 20 months after OVX, the neuroprotective effect is lost *(Peinado, González, and Leret 2004; Rodriguez-Perez et al. 2015; 2012)*. Similarly, aged acyclic gonad-intact females present a loss of E2 beneficial effect compared to younger gonad-intact animals *(Rodriguez-Perez et al. 2012)*. These results suggest that the decrease in circulating E2 after both types of menopause induces changes in the brain that lead to a loss of E2 responsiveness in females *(Bourque, Morissette, and Di Paolo 2024)*. This is in contrast to male MPTP mice, in which E2 treatment has neuroprotective effects at all ages *(Bourque, Morissette, and Di Paolo 2024)*. As in females, low doses of E2 seem to be more effective than high doses~~,~~ in males *(Ookubo et al. 2009; Cordellini et al. 2011; Ramirez, Liu, and Menniti 2003)*.

6-OHDA mice present a loss of SNpc excitation due to mGluR1 down-regulation on striatal cholinergic interneurons *(Cai et al. 2021)*. When mGluR1 is selectively expressed in these neurons, improvement in motor symptoms is observed *(Cai et al. 2021)*. Membrane ERα, which are present in striatal cholinergic neurons, appear to mediate mGluR1 through direct interaction, therefore the beneficial effect of E2 on motor symptoms could be mediated by mGluR1 *(Dewing et al. 2007; Almey et al. 2012)*. On the other hand, an increase in NR2B is associated with LID, and GPR30-mediated neuroprotection is associated with inhibition of NR2B-containing NMDA receptors. Thus, GPR30 activation could down-regulate NR2B which, in turn, would decrease LID *(S. Liu et al. 2012; Calon et al. 2003)*.

3.8. Conclusions

In conclusion, sex differences should be taken into account in PD research. Furthermore, the hormonal status of male and female PD patients requires study, in order to develop therapeutic approaches that could be specifically adapted. Therefore, studying the impact of sex steroids on NM accumulation appears to be important for improving our understanding of PD.

HYPOTHESIS AND OBJECTIVES

The main goal of this thesis is to determine whether differences between males and females in age-dependent NM production/accumulation could underlie the differential effect of sex on PD.

Main hypothesis: Male sex bias in PD is due to a higher rate of intracellular NM accumulation across life compared to females, thus reaching the pathogenic threshold of intracellular NM accumulation at an earlier time point. Higher estrogen levels in females may slow down age-dependent NM accumulation and thus explain this effect.

To address this hypothesis, I propose to elaborate on the following specific aims:

AIM 1: Assess levels of age-dependent intracellular NM accumulation in human female and male postmortem brains

Hypothesis 1: *Human females exhibit a slower rate of intracellular neuromelanin (NM) accumulation with age compared to males, thereby reaching the pathogenic threshold of NM accumulation at a later stage, resulting in a delayed potential onset of disease and a distinct PD phenotype.*

Previously, our research group established that human postmortem tissue from control subjects exhibited lower levels of intracellular neuromelanin (NM) compared to individuals with idiopathic Parkinson's disease (iPD), the latter being associated with increased degeneration of pigmented neurons. In this Aim, I investigated whether these differences in NM accumulation and neuronal dysfunction/degeneration also displayed sex disparities. To do so, I analyzed human post-mortem samples from the substantia nigra pars compacta (SNpc) of both male and female individuals to identify potential sex-related variations in age-dependent NM accumulation and neuronal dysfunction/degeneration.

This investigation was structured into the following sub-studies:

a) Comparison between male and female age-controlled subjects and iPD patients.
b) Stratification of male and female age-controlled subjects into two age groups: 59-79 years old and over 80 years old.
c) Examination of male and female control subjects spanning from 5 years old to over 90 years old.

AIM 2: Determine NM accumulation and NM-linked PD pathology in female and male AAV-hTyr-injected rats

Hypothesis 2: *Aging AAV-hTyr-injected female rats accumulate intracellular NM more slowly than their male counterparts, thus reaching the pathogenic threshold of intracellular NM accumulation later and exhibiting a delayed PD phenotype/progression.*

In previous research, our team pioneered the development of the first *in vivo* rodent model exhibiting progressive accumulation of human-like NM over time, up to levels reached in aged human brains. This model involves the AAV-mediated overexpression of TYR (AAV-TYR), resulting in unilateral pigmentation of the rodent SNpc. Here, my aim was to investigate sex-related differences in NM accumulation and NM-linked PD pathology in this animal model.

To address this question, I conducted a comprehensive examination of both male and female AAV-hTyr-injected rats over time. My investigations encompassed the following key themes:

a) Assessment of motor asymmetry
b) Analysis of NM accumulation
c) Evaluation of neuronal viability.

AAV-hTyr-injected rats were categorized into three distinct disease stages:

a) Pre-symptomatic
b) Prodromal
c) Established Parkinson's disease (PD).

AIM 3: Contribution of gonadal steroids on NM accumulation and NM-linked PD pathology

Hypothesis 3: *Gonadal steroids, particularly estradiol, reduce the rate of intracellular NM accumulation/production.*

Ovariectomy (OVX):

As discussed in the introduction, menopause has been associated with an elevated risk of PD in females. Hence, I aimed to replicate a "post-menopausal" state in our rodent model. To

achieve this, I examined prodromal ovariectomized (OVX) female rats injected with AAV-hTyr to evaluate:

a) Motor asymmetry
b) NM accumulation
c) Neuronal viability.

These findings were compared to those of non-OVX females and males from our earlier study (Aim 2) to ascertain whether, as hypothesized, estrogen deficiency in OVX (i.e., postmenopausal) rats accelerates NM production/accumulation and PD pathology.

Estradiol (E2) treatment:

TR5TY6 cell line:

As outlined in the introduction, estradiol has the capability to directly influence hTyr activity and melanin production in the skin, as well as to promote melanin extrusion from melanocytes. Consequently, I sought to determine whether estradiol could modulate NM accumulation in a NM-producing TR5TY6 neuroblastoma cell line. I evaluated:

a) Cell viability
b) Intracellular NM levels
c) Extracellular NM levels.

AAV-hTyr-injected rats:

To determine whether estradiol treatment could impact intracellular NM accumulation *in vivo*, I administered two different estradiol dosages to male and female AAV-hTyr-injected rats. Once again, my investigations encompassed the following key themes:

a) Motor asymmetry
b) Neuromelanin accumulation
c) Neuronal viability.

MATERIAL AND METHODS

STUDY DESIGN

In this study, our goal was to explore if the sex differences observed in the incidence and prevalence of Parkinson's disease (PD) are linked to differences in age-dependent neuromelanin (NM) accumulation between males and females. We utilized human postmortem brain tissues and a unique rodent model of NM production that we recently developed. This model involves unilateral overexpression of tyrosinase (TYR) in the substantia nigra pars compacta (SNpc) of rats, using a viral vector approach (Carballo-carbajal et al. 2019)**.**

Alongside NM accumulation, these rats exhibit a progressive PD-like phenotype. This includes motor impairments, formation of Lewy body (LB)-like inclusions, degeneration of the nigrostriatal pathway, release of NM from dying neurons, and neuroinflammation. We used adult male and female Sprague–Dawley rats, randomly assigned into five groups based on different time points after receiving unilateral intranigral injections of AAV-TYR.

We assessed several parameters including motor asymmetry (behavioral), and various histological markers such as intracellular NM levels, LB-like inclusion formation, tyrosine hydroxylase (TH) downregulation, nigrostriatal degeneration, extracellular NM release, and neuroinflammation. From prior data, we selected three experimental groups for detailed evaluation: (i) at the initial stage of NM accumulation, before NM-linked neuronal dysfunction (1 month post-AAV injection), representing pre-symptomatic stages; (ii) once NM-linked neuronal dysfunction begins (i.e. TH downregulation) but before evident neurodegeneration (between 2 and 4 months post-AAV), corresponding to prodromal PD stages; and (iii) once full nigrostriatal neurodegeneration has developed (from 6 to 20 months post-AAV), representing established PD stages.

All analyses throughout this thesis were performed blind to the experimental groups.

HUMAN TISSUE

HUMAN POST-MORTEM BRAIN TISSUE

Paraffin-embedded midbrain sections (5 µm) were obtained from idiopathic Parkinson's disease patients (iPD; n = 30; 13 female and 17 male) and age-matched control individuals (n = 32; 19 female and 13 male). These samples were provided by various institutions: the Neurological Tissue BioBank at IDIBAPS-Hospital Clinic in Barcelona, the Tissue Biobank at IMIB in Murcia, the Tissue Biobank at the CIEN Foundation in Madrid, and the Vall d'Hebron Tissue BioBank. All procedures followed the guidelines of the BPC (CPMP/ICH/135/95) and Spanish regulation (223/2004), and received approval from the Vall d'Hebron Research Institute (VHIR) Ethical Clinical Investigation Committee [PR(AG)370/2014].

For the quantification of intracellular neuromelanin (NM) in human brain samples, two groups were evaluated: control subjects (1008 cells analyzed across 32 cases) and PD patients (646 cells analyzed across 30 cases). Standard hematoxylin and eosin (H&E) staining was conducted on 5-µm-thick sections from the substantia nigra pars compacta (SNpc) of each subject. Dopamine (DA) neurons were identified by the presence of the distinctive, unstained NM pigment in H&E-stained sections. All NM-positive neurons in each section were examined under an objective magnification of ×20. Intracellular NM optical density was measured using ImageJ software by an investigator who was blinded to the group identities (Carballo-carbajal et al. 2019).

For TH-positive cell count in human brain samples, two groups were evaluated: control subjects (1008 cells analyzed across 32 cases) and PD patients (646 cells analyzed across 30 cases). Tyrosine Hydroxylase (TH) histochemistry was conducted on 5-µm-thick sections from the substantia nigra pars compacta (SNpc) of each subject. TH-immunostained 5-µm-thick paraffin-embedded slides were scanned with an Olympus Slideview VS200 slide scanner, and the resulting images were obtained and quantified with the QuPath software. Assessment of the total number of TH-positive neurons, the number of NM-laden neurons (with or without TH), and extracellular NM aggregates in the SNpc was performed an objective magnification of ×20 by an investigator who was blinded to the group identities.

ANIMALS

ANIMAL HANDLING

Male and female adult Sprague–Dawley rats from Charles River, weighing 225–250 g at the time of surgery, were kept in groups of two to three per cage. They had unrestricted access to food and water and were maintained on a 12-hour light/dark cycle. All experimental and surgical procedures adhered to the European Directive 2010/63/EU and Spanish regulations (Real Decreto 53/2013; Generalitat de Catalunya Decret 214/97) regarding the protection of animals used for experimental and other scientific purposes. The procedures were also approved by the Ethical Experimentation Committee at Vall d'Hebron Research Institute (VHIR).

STEREOTAXIC INFUSION OF VIRAL VECTOR

Recombinant AAV vectors serotype 2/1 (batch #846a) and serotype 9 (batch #1946b) expressing human tyrosinase cDNA under the control of the CMV promoter (AAV-hTyr) were produced at the Viral Vector Production Unit of the Autonomous University of Barcelona (UPV-UAB, Spain). These vectors were from batches #846a, with a titer of 2.49×10^{12} gc/mL, and #1946b, with a titer of 9.11×10^{12} gc/mL, respectively.

All surgical interventions on rats were conducted using a stereotaxic frame under general anesthesia administered with isoflurane (5% for induction and 2% for maintenance), supplied by Baxter. The vector solutions were injected using a 10 µL Hamilton syringe equipped with a glass capillary (Hamilton model Cat#701). Each animal received a 2 µL unilateral injection of AAV-hTyr. The infusion was carried out at a rate of 0.4 µL/min, after which the needle was left in place for an additional 4 minutes before being slowly withdrawn. The injections were performed unilaterally on the right side of the brain at specified coordinates above the SNpc. The coordinates, set in the flat skull position and calculated relative to bregma according to the stereotaxic atlas by Paxinos and Watson (2007), were: anteroposterior: −5.29 mm; medio-lateral: −2 mm; dorso-ventral: −7.6 mm below dural surface.

ESTRADIOL SUBCUTANEOUS PELLET INSERTION

Subcutaneous pellet containing releasing 17β-estradiol during 21 days (Innovative Research of America (IRA) (#E-121) were implanted subcutaneously between the skin and

muscle tissues in the lateral side of the neck between the ear and the shoulder. Both male and female rats received either 0.025mg or 0.5mg 17β-estradiol, two-weeks after stereotaxic infusion of viral vector (batch #1946b).

CYLINDER BEHAVIORAL TEST

Rats underwent assessments of left and right forepaw use employing the cylinder test one week before surgery and again the day prior to their sacrifice, scheduled at: (i) 1, 2, 4, 6, 12, and 20 months post-injection (batch #846a) and (ii) once a week for 20 days post-injection (batch #1946b). For the cylinder test, rats were first acclimatized to the experimental room for at least one hour before testing. Subsequently, they were placed in a transparent glass cylinder, and the total number of contacts made with the left and right forepaws was recorded over a five-minute period. Results are expressed as the percentage of contralateral paw usage.

To maintain test integrity, the behavioral equipment was sanitized with 70% ethanol following each session to eliminate olfactory cues. All behavioral assessments were conducted during the daylight portion of the light-dark cycle by an investigator who was blinded to the group allocations of the animals.

BRAIN PROCESSING FOR HISTOLOGICAL ANALYSIS

INTRACARDIAC PERFUSION

Wash and fixative solutions were prepared prior to perfusion. The wash solution was prepared immediately before use by adding 3.75mL 1% $NaNO_2$ and 0.11mL heparin (25000 IU/mL) to 300mL of 0.9% NaCL irrigated solution. The fixative solution consisted of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4).

Prior to perfusion, rats were anesthetized by intraperitoneal overdose with 5% sodium pentobarbital solution (2500 mg/50mL) according to the animal's weight (50mg/kg). After around 10 minutes, rats were checked for complete anesthetic state by loss of the pedal pain reflex to ensure the absence of paw movement. If rats still reacted with pedal pain reflex after 15-20 minutes, additional anesthetic (same amount as first injection) was administered.

Under a fume hood, the animals were placed in supine position, with the animals' limbs pinned to facilitate exposure of the peritoneal cavity. An incision of the skin reveal the outer abdominal wall which is cut laterally and through the ribs, parallel to the lungs to create a chest

“flap”. The flap is folded back towards the head, to clamp the aorta. A venous catheter is inserted in the left ventricle while an incision in made in the right atrium for drainage.

The heart is rinsed for 5 minutes with wash solution applied with a peristatic pump (9mL/min) to remove blood prior to fixation. Wash solution is replaced by ice-cold (4ºC) paraformaldehyde fixative solution (9mL/min) for 15 minutes.

MICROTOMY

Once perfusion was completed, the animals were guillotined, and the brains removed and post-fixed for 24 hours in the same fixative. Paraffin embedding was performed at the VHIR’s Drug Delivery and Targeting facility.

Each brain was sectioned with a sliding microtome (Leica, Germany) at 5µm-tickness to collect 200 slides of the SNpc and 50 slides of the striatum (10 slides per section).

H&E, IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

HEMATOXYLIN-EOSIN STAINING

Standard hematoxylin-eosin (H&E) staining was performed in 5µm-thick paraffin-embedded SNpc section for each animal. In these sections, SNpc DA neurons were identified by the visualization of unstained NM pigment.

IMMUNOHISTOCHEMISTRY

SNpc and striatum sections were stained with an antibody against tyrosine hydroxylase (TH), which is the rate limiting enzyme involved in DA biosynthesis. Briefly, endogenous peroxidase activity was inhibited by incubating tissue sections with 3% H202 and 10% methanol in Tris Buffered Saline 1x (TBS) (pH=7.4) for 10 min at room temperature (RT). After 3 TBS washes, tissue sections were incubated for 1h in blocking buffer containing 5% normal goat serum (NSG) in TBS.

Next, sections were incubated in TBS with 2% NSG and the corresponding primary antibody at 4ºC for 24h or 48h respectively. Sections were washed in TBS and then incubated with the corresponding secondary biotinylated antibody (Vector Laboratories) in blocking buffer for 1h at RT. For antibody detection, SNpc sections were incubated in an avidin-biotin complex (ABC Peroxidase Standard Staining Kit, ThermoFisher, 32020) that reacted with Vector® SG Peroxidase HRP Substrate Kit (Vector Laboratories, SK-4700) and striatum sections were visualized by DAB Peroxidase Substrate kit (Vector laboratories).

Slides were then subjected to a dehydration process following the next protocol: submerge slides in 70% ethanol for 1 minute; rinse slides quickly for 10 seconds in 95% ethanol; immerse slides for 1 minute in 100% ethanol; leave slides in xylene for 1 minute or more until mounting with the coverslip. Finally, slides were cover slipped using DPX mounting medium (Sigma Aldrich, 06522).

IMMUNOFLUORESCENCE

A similar protocol was used without the quenching step. Preincubation was performed with 5% (vol/vol) NGS and 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich) in phosphate buffered saline (PBS) solution. Corresponding primary antibodies were incubated together overnight at 4 °C in 2% (vol/vol) serum and adequate Alexa 488 and 594-conjugated secondary antibodies (1:1000, ThermoFisher Scientific) were incubated simultaneously for 1 h at RT in 2% (vol/vol) serum. Nuclei were stained with Hoechst 33342 (1:10000, ThermoFisher Scientific) in 1× PBS for 10 min. Sections were cover slipped using the Dako Cytomation Fluorescent Mounting Medium (Dako).

Primary Antibody	Manufacturer	Dilution
Anti-TH	Calbiochem (657012)	1:500 (IHC for human SNpc) 1:40000 (IHC for rat SNpc) 1:3500 (IHC for rat Striatum) 1:1000 (IF for rat SNpc)
Anti-Iba1	Wako #019-19741	1:1000 (IHC) 1:500 (IF)
Anti-CD68	Serotec #MCA341R	1:100 (IHC)
Anti-ERα	ThermoFisher Scientific #MA1-310	1:100 (IF)
Anti-ERβ	ThermoFisher Scientific #PA1-310B	1:100 (IF)
GPER (GPR30)	ThermoFisher Scientific #PA528647	1:100 (IF)

Table 1 - Primary antibodies used in histochemistry (IHQ) and immunofluorescence (IF). *IHQ was performed on paraffin sections for optical density analysis, stereological cell count and neuroinflammation parameters (TH, Iba1, CD68) . IF was performed on paraffin sections for estrogens receptors (ERα, ERβ, GPR30 and TH).*

Secondary Antibody	Manufacturer	Dilution
Anti-mouse (goat)	Vector Laboratories #BA-9200	1:1000 (IHC)
Anti-rabbit (goat)	Vector Laboratories #BA-1000	1:1000 (IHC)
Anti-mouse Alexa 488 (goat)	ThermoFisher Scientific #A11001	1:1000 (IF)
Anti-mouse Alexa 594 (goat)	ThermoFisher Scientific #A11005	1:1000 (IF)
Anti-rabbit Alexa 488 (goat)	ThermoFisher Scientific #A11008	1:1000 (IF)
Anti-rabbit Alexa 594 (goat)	ThermoFisher Scientific #A11012	1:1000 (IF)
Hoescht 33342	ThermoFisher Scientific #H3570	1:10000 (IF)

Table 2 - Secondary antibodies used in histochemistry (IHQ) and immunofluorescence (IF). *IHQ was performed on paraffin sections for optical density analysis, stereological cell count and neuroinflammation parameters (TH, Iba1, CD68). IF was performed on paraffin sections for estrogens receptors (ERα, ERβ, GPR30 and TH).*

INTRACELLULAR NM QUANTIFICATION

Intracellular NM levels were quantified in AAV-TYR-injected animals in 5-µm-thick paraffin-embedded SNpc sections (12 slides for each animal, every 17th section, to cover the whole extent of the SNpc). In these sections, SNpc dopaminergic neurons were identified by the visualization of unstained NM brown pigment. Midbrain sections were scanned using the Panoramic Midi II FL, HQ SCIENTIFIC 60× and section images were acquired with CaseViewer software at an objective magnification of 63×. For illustration purposes, high resolution micrographs were acquired with an Olympus Slideview VS200 slide scanner and the QuPath software. All NM-positive neurons in a representative SNpc middle section exhibiting high numbers of NM-containing neurons were analyzed by means of optical densitometry using ImageJ software (RRID:SCR_003070; https://imagej.net/) to quantify the intracellular density of NM pigment, as described in published protocol *(Guillard-Sirieix et al., 2024*). The pixel brightness values for all individual NM-positive cells (excluding the nucleus) in all acquired images were measured and corrected for non-specific background staining by subtracting values obtained from the neuropil in the same images. For the AAV-hTyr groups, animals were analyzed at different time-points after AAV-hTyr injection.

For rats injected with AAV-hTyr 846a: pre-symptomatic group 1 m (Females n = 6; Males n = 7), prodromal group 2 m (Females n = 6; Males n = 7) and 4 m (Females n = 6; Males n = 7), and established PD 6 m (Females n = 6; Males n = 6), 12 m (Females n = 5; Males n = 7) and 20 m (Females n = 5; Males n =5).

For rats injected with AAV-hTyr 1946b: group without estradiol treatment (Females n = 6; Males n = 6), group receiving 0.025mg of estradiol (Females n = 6; Males n = 6), and group receiving 0.5mg of estradiol (Females n = 6; Males n = 6). All quantifications were performed in blind.

STRIATAL OPTICAL DENSITOMETRY

The density of TH-positive fibers in the striatum was measured by densitometry in serial coronal sections covering the whole region (5 sections/animal) (**Figure 15**). TH-immunostained 5-µm-thick paraffin-embedded sections were scanned with an Olympus Slideview VS200 slide scanner, and the resulting images were obtained with the QuPath software and quantified using ImageJ software (RRID:SCR_003070; https://imagej.net/). Striatal densitometry values were corrected for non-specific background staining by subtracting densitometric values obtained from the cortex. Data are expressed as the percentage of the densitometric value of the equivalent anatomical area from the non-injected contralateral side of the same animal. For the AAV-hTyr groups, animals were analyzed at different time-points after AAV-hTyr injection.

For rats injected with AAV-hTyr 846a: pre-symptomatic group 1 m (Females n = 6; Males n = 7), prodromal group 2 m (Females n = 6; Males n = 7) and 4 m (Females n = 6; Males n = 7), and established PD 6 m (Females n = 6; Males n = 6), 12 m (Females n = 5; Males n = 7) and 20 m (Females n = 5; Males n =5).

For rats injected with AAV-hTyr 1946b: group without estradiol treatment (Females n = 6; Males n = 6), group receiving 0.025mg of estradiol (Females n = 6; Males n = 6), and group receiving 0.5mg of estradiol (Females n = 6; Males n = 6). All quantifications were performed in blind.

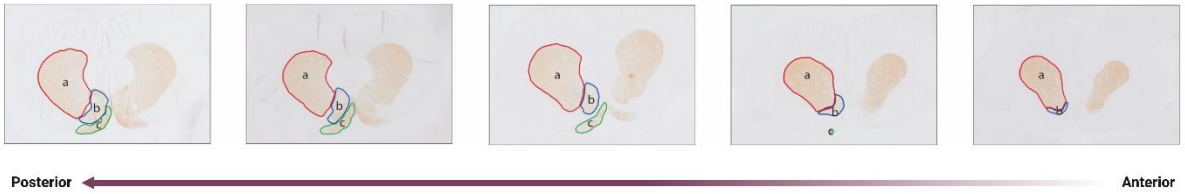

Figure 15 - Regions of interest for the optical densitometry in the rat striatum of one animal. *a) Caudoputamen in red; b) Nucleus accumbens in blue; c) Olfactory tubercle in green. Five 5µm-thick paraffin sections covering the rat Str were Immunostained for TH.*

STEREOLOGICAL CELL COUNTING

For slide visualization a Zeiss Imager.D1 microscope coupled to an AxioCam MRc camera (Zeiss, Germany) was employed. Assessment of the total number of TH-positive neurons, the number of NM-laden neurons (with or without TH), and extracellular NM aggregates in the SNpc was performed according to the fractionator principle, using the MBF Bioscience StereoInvestigator 11 (64 bits) Software (Micro Brightfield).

Serial 5 µm-thick paraffin sections covering the entire SNpc were included in the counting procedure (every 17th section for a total of 10-12 sections analyzed/animal) (**Figure 16**). The following sampling parameters were used: (i) a fixed counting frame with a width and length of 50 µm; (ii) a sampling grid size of 100 x 75 µm and (iii) a multiplication factor of 51. The counting frames were placed randomly by the software at the intersections of the grid within the outlined structure of interest. Objects in both brain hemispheres were independently counted following the unbiased sampling rule using a 100x lens and included in the measurement when they came into focus within the dissector. A coefficient of error of <0.10 was accepted.

The total number of SNpc DA neurons was calculated by considering all TH+NM+, TH-NM+ and TH+NM- neurons. The percentage of TH downregulation was calculated by considering the total number of TH+NM+ and the total number of TH-NM+ with respect to the total number of neurons containing NM. All quantifications were performed by an investigator blinded to the experimental groups.

For rats injected with AAV-hTyr 846a: pre-symptomatic group 1 m (Females n = 6; Males n = 7), prodromal group 2 m (Females n = 6; Males n = 7) and 4 m (Females n = 6; Males n = 7), and established PD 6 m (Females n = 6; Males n = 6), 12 m (Females n = 5; Males n = 7) and 20 m (Females n = 5; Males n =5).

For rats injected with AAV-hTyr 1946b: group without estradiol treatment (Females n = 6; Males n = 6), group receiving 0.025mg of estradiol (Females n = 6; Males n = 6), and group receiving 0.5mg of estradiol (Females n = 6; Males n = 6). All quantifications were performed in blind.

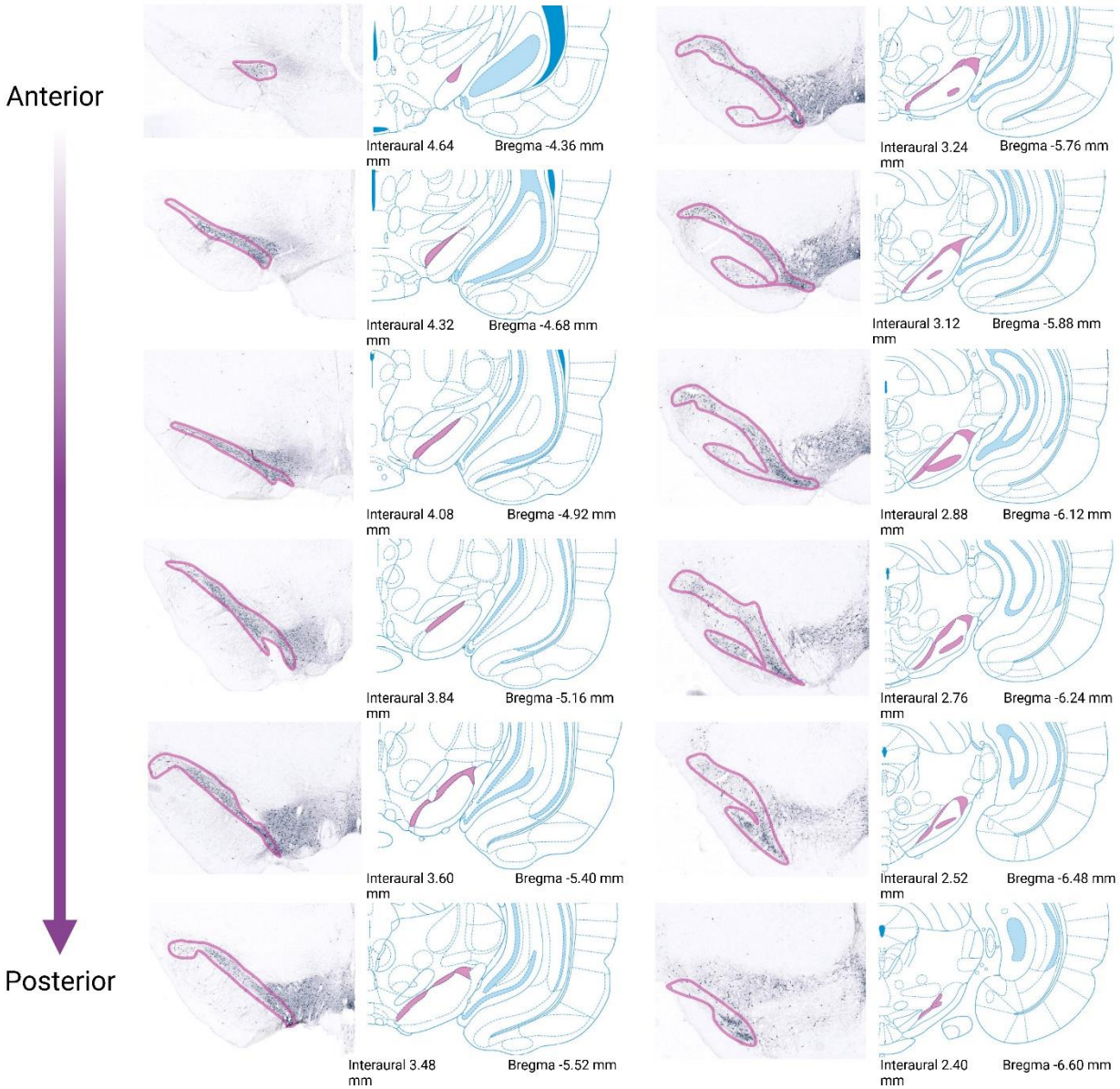

Figure 16 – Anatomical levels of rat SNpc used for quantifications. *Twelve serial 5µm-thick paraffin sections covering the entire rat SNpc (one every 17th section) were Immunostained for TH and matched with the corresponding anatomical level from the rat brain atlas. Pink color indicates the borders of the SNpc in both the atlas and TH-immunostained sections. Figure was created using BioRender.com and adapted from Compte J. (2022). In vivo modulation of neuromelanin levels: therapeutic approaches via VMAT2-overexpression and application of transcranial focused ultrasound in neuromelanin-producing Parkinson's disease model. [PhD thesis, Universitat Autònoma de Barcelona].*

QUANTIFICATION OF NEUROINFLAMMATION PARAMETERS

Quantification of Iba-1 and CD68-positive cells was performed in SNpc sections adjacent to those used for stereological cell counts. Slides of 5-µm thick paraffin sections covering were scanned using the Panoramic Midi II FL, HQ SCIENTIFIC 60x scanner and section images were acquired with CaseViewer software (RRID:SCR_017654; https://www. 3dhistech.com/caseviewer). For quantification of Iba-1 and CD68-positive cells, specific AI-based algorithms were implemented using the Aiforia 5.3 platform (RRID:SCR_022739, https://www. aiforia.com). Iba-1-positive cells were counted separately in two different groups according to their activation state: non-reactive (branched) and reactive (amoeboid). CD68-positive cells were counted individually. Data are presented as the number of positive cells per quantified area (in mm^2). All quantifications were performed by an investigator blinded to the experimental groups. For illustration purposes, high resolution micrographs were acquired with the Olympus Slideview VS200 slide scanner and the Olyvia 3.3 software.

ELISA FOR ESTRADIOL LEVELS

SERUM EXTRACTION

Animals were anesthetized with isoflurane 5%. After opening the thoracic cavity, a direct intracardiac puncture was performed for blood collection. Approximately 500µL of blood were collected into the clotting activator tubes (Micro sample tube Serum Gel, 1.1 mL, #41.1500.005, Sarstedt) and kept at room temperature for 30 minutes. After coagulation, tubes were centrifuged at 1500g for 10 minutes and rapidly stored at -80ºC.

BRAIN COLLECTION

Animals were euthanized and the brains removed and snap-frozen for 20 seconds on dry ice cooled with 2-methylbutane (isopentane) and stored at -80ºC. The tissues were minced and homogenized with PBS (1:9) with a glass homogenizer on ice. An ultrasonic cell disrupter was used to obtained a homogenized solution which was centrifuged for 5 minutes at 10000g and the supernatant was collected and stored at -80ºC. Protein samples were quantified using BCA method (pierce BCA Protein Assay Kit, Thermo Scientific #23227).

Determination of brain and serum estradiol levels was performed using Rat Estradiol ELISA Kit (Biorbyt #orb782508) following the manufacturer's instructions. Absorbance was read at λ=405nm in a Varioskan LUX multimode microplate reader using the SkanIt Software 6.1.

CELL LINE

TR5TY6 NEUROBLASTOMA CELL LINE

A stable inducible SH-SY5Y cell line, TR5TY6, which expresses human tyrosinase under the control of the T-Rex TM Tet-On system (Invitrogen), was provided by Dr. T. Hasegawa from the Department of Neurology at Tohoku University in Sendai, Japan. This cell line was verified as mycoplasma-free through routine PCR analysis. The cells were cultured in low glucose (1 g/L) Dulbecco's Modified Eagle's Medium (DMEM) from Gibco, supplemented with penicillin/streptomycin and a combination of antibiotics (7 µg/mL blasticidin and 300 µg/mL Zeocin from Life Technologies). The medium also included tetracycline-free fetal bovine serum (FBS) from Clontech to prevent unintentional transgene expression.

For various assays, TR5TY6 cells were plated at densities of 2.5×10^5 or 10^6 cells/plate in 24-or 6-well plates respectively. For neuromelanin (NM) quantification, cells were cultured on 12-mm slides coated with 50 µg/ml poly-D-lysine (Sigma-Aldrich). Typically, 24 hours after seeding, the cells were differentiated using 10 µM retinoic acid (RA) from Sigma-Aldrich for three days, then treated with 80 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) for an additional three days, before induction of tyrosinase expression with 2 µg/ml doxycycline for up to six days.

NEUROMELANIN QUANTIFICATION

Quantification of intracellular and extracellular nNM in the differentiated and induced TR5TY6 cells was carried out as follows: (i) optical densitometry was performed on fixed cells viewed under transmitted light at an objective magnification of ×40; slides were divided into a grid, with images captured from 25 fields covering 50% of each slide, and analyzed as previously described for rat sections; (ii) tyrosinase activity was performed on the cell medium to measure extracellular NM.

OPTICAL MICROSCOPY FOR INTRACELLULAR NEUROMELANIN

Cells fixed with 4% paraformaldehyde for 30 min at 4 °C were blocked with 3% NGS (Atom) and 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich) in PBS solution. Hoechst 33342 (1:10,000, Thermo Fisher Scientific) was used for nuclei counterstain for 1 h at RT in 2% (vol/vol) NGS. Cells were coverslipped using Dako Cytomation Fluorescent Mounting Medium (Dako) and images acquired using an Zeiss LSM 980 confocal microscope and Airyscan 2 visualization software.

TYROSINASE ENZYME ACTIVITY FOR EXTRACELLULAR NEUROMELANIN

Cell medium was incubated at 37ºC for 30 minutes. L-DOPA [0,5mM] was added before the real-time change of absorbance for 3 hours, every minute for 20 minutes then every 5 minutes for 2 hours and 40 minutes. Tyrosinase from mushroom ([0,5mg/mL], Sigma-Aldrich #T3824-25100) was used as a positive control and 4-Butylresorcinol [10mM] was added to the tyrosinase from mushroom as a negative control. Absorbance was read at λ=484nm in a Varioskan LUX multimode microplate reader using the SkanIt Software 6.1.

UPLC-MS/MS ANALYSIS

Samples were performed using our previously validated method (*Gonzalez-Sepulveda et al., 2020*). An Acquity UPLC system coupled with a Xevo TQ-S triple quadrupole mass spectrometer with electrospray ionization interface was used. Instrument control, data acquisition, and analysis were performed using MassLynx V4.1. The chromatographic separation of cell samples was performed on a Acquity HSS T3 column (1.8µm; 2.1 × 100mm) couple to an Acquity HSS T3 VanGuard pre-column (100 Å, 1.8µm, 2.1mm × 5mm). The column temperature was set at 40ºC, and samples were maintained at 6ºC in the thermostatic autosampler. The mobile phase consisted of solvent A (methanol 100%) and solvent B (265nM FA in water) at a flow of 0.4mL/min with the following gradient profile: 0.5% B maintained for 0.5min, 8% B at 2.6min, 55%B at 2.9min, 60% B at 3.3min, 80% B at 4.3min, 90% B at 4.4min and maintained for 0.5min, 0.5% B at 5min followed by 1min of equilibration.

The mass spectrometer detector operated under: source temperature 150ºC, desolvation temperature 450ºC, cone gas flow 50L/h, desolvation gas flow 1100L/h, and collision gas flow 0.15mL/min. Argon was used as the collision gas. The capillary voltage was set at 0.5kV for MIX1 and MIX3, and at 2kV for MIX2 detection. The electrospray ionization source was operated in both positive and negative modes, depending on the analyte. Multiple reaction monitoring acquisition settings for the targeted metabolites are summarized in Table 3.

Each sample was injected three times into the UPLC-MS/MS system to analyze different sets of compounds (i.e., MIX1, MIX2 and MIX3). Samples with a concentration between limit of detection (LOD) and limit of quantification (LOQ) or bigger than LOQ were considered acceptable; samples with a concentration lower than LOD were considered as the LOD value. Data were normalized to the protein concentration and presented as the ratio.

Analyte	MRM transition (m/z)	MIX	ES	Retention time (min)	Cone Voltage (V)	Collision energy (eV)	Capillary Voltage (kV)
3MT	150,7 > 90,96	1	+	3,09	35	20	0,5
AC	149,61 > 121,91	1	+	3,36	25	25	0,5
NE	151,75 > 106,94	1	+	0,69	15	20	0,5
DA	153,93 > 90,57	1	+	1,46	10	20	0,5
L-DOPA	198,1 > 152,1	1	+	1,48	15	15	0,5
DOPAC	166,99 > 122,82	2	-	3,72	18	22	2
DOMA	182,86 > 136,85	2	-	1,62	20	14	2
5SCDA	273,1 > 166,9	3	+	1,73	20	20	0,5
5SCD	317 > 154,86	3	+	2,01	24	30	0,5

Table 3 - MRM acquisition settings for the targeted metabolites and the internal standard.

STATISTICAL ANALYSIS

Statistical analysis were performed using GraphPad Prism software (v9.5.1, GraphPad Software Inc, USA) using the appropriate statistical tests, as indicated in each figure legend. No statistical methods were used to pre-determine sample size but our sample sizes are equivalent to those reported in previous similar publications. Non-parametric tests that do not rely on assumptions about the parameters of the underlying population distribution were performed. Accordingly, differences among means were analyzed either by Kruskal-Wallis ANOVA on ranks, Mann-Whitney test or multiple Mann-Whitney test, as appropriate. In all analysis, the null hypothesis was rejected at the 0,05 level. When above 0,05, the specific p-value is reported.

RESULTS

1. CHAPTER 1: Sex-specific NM accumulation and PD pathology development in human females and males across lifespan.

1.1. Intracellular NM levels in female and male controls and PD subjects:

Human SNpc dopaminergic (DA) neurons accumulate NM with age throughout life. Analyzing SNpc from HE-stained postmortem idiopathic Parkinson's disease (iPD) and age-matched control brains, I found that intracellular NM levels within SNpc DA neurons were significantly higher in PD patients than in age-matched controls (**Figure 17**a,b). Additionally, I observed an increase in the percentage of cell area occupied by NM in PD patients compared to age-matched controls (**Figure 17**c).

To determine whether sex impacts on NM accumulation, I re-analyzed the post-mortem cases of PD patients and age-matched controls classified according to sex. I found that intracellular NM levels within SNpc DA neurons from elderly control subjects were significantly higher in males than in females (**Figure 17**d,e). Males reached the previously established pathogenic threshold of cell dysfunction/degeneration earlier than females *(Carballo-Carbajal et al. 2019)*, even in the absence of overt PD, suggesting that NM-containing nigral neurons may already be dysfunctional in apparently healthy elderly males (**Figure 17**e). The percentage of cell area occupied by NM was also higher in males compared to females in both groups (**Figure 17**f).

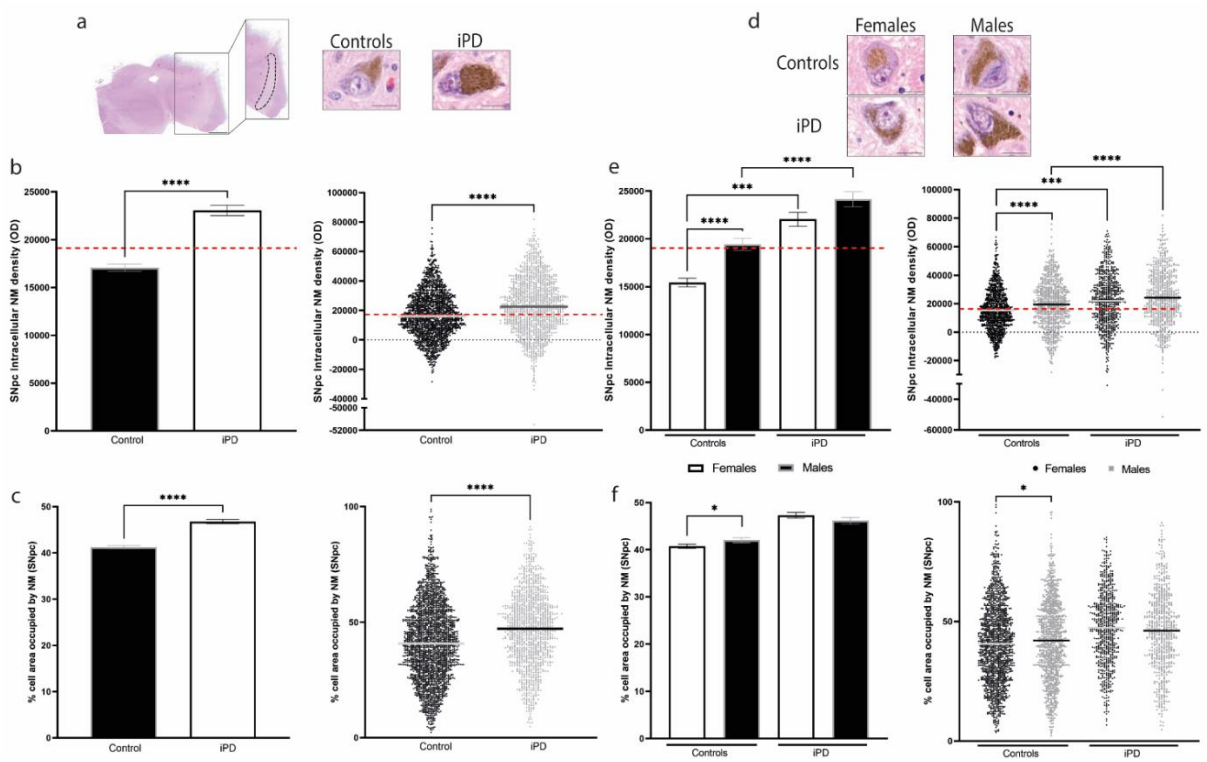

Figure 17 – Intracellular NM levels in SNpc of male and female control and iPD brains. *a) Representative images of SNpc NM-containing neurons in HE-stained human postmortem control and iPD brains. Values are mean ± SEM. b) Quantification of intracellular NM optical density in post-mortem SNpc sections from elderly human (average age 76.25) control subjects and age-matched idiopathic PD (iPD) patients without classification by sex. ***p<0.0001 (Mann Whitney). c) Quantification of percentage of cytosolic area occupied by NM in post-mortem SNpc sections from elderly human (average age 76.25) control subjects and age-matched iPD patients without classification by sex.***p<0.0001 (Mann Whitney U test). d) Representative images of SNpc NM-containing neurons in HE-stained human postmortem control and iPD brains from females and males. e) Quantification of intracellular NM optical density in post-mortem SNpc sections from elderly human control and iPD cases classified by sex. p<0.0001 Control data, p=0.0670 PD data; p<0.001 PD females and p<0.0001 PD males compared to respective control. f) Quantification of percentage of cytosolic area occupied by NM in post-mortem SNpc sections from elderly human control and iPD cases classified by sex. Data comparisons were made by Mann Whitney U test; the null hypothesis was rejected at the 0.05 level; p=0.0245 Control data and p=0.1903 PD data. Controls: n=1008 neurons (19 females), n=699 neurons (13 males); PD patients: n=646 neurons (13 females), n=588 neurons (17 males). In c and d, dotted red line indicates the previously established threshold of NM-linked neuronal dysfunction/degeneration (Carballo-Carbajal et al., 2019).*

To determine if increased NM accumulation above the pathogenic NM threshold in apparently healthy (non-PD) males is already associated with alterations in cell function/viability, I performed stereological cell counts of SNpc TH-positive neurons in these cases, classifying the results by sex (**Figure 18**a). While the numbers of TH-positive and total DA nigral neurons were reduced in PD cases compared to controls, no significant differences between males and females were observed in either group (**Figure 18**b,d). However, control and PD males exhibited higher numbers of extracellular NM debris compared to females, indicating enhanced ongoing degeneration in males, even in the absence of overt PD (**Figure 18**e). No differences were found in either group concerning the percentage of dysfunctional melanized neurons exhibiting a downregulation of the TH phenotype (**Figure 18**c).

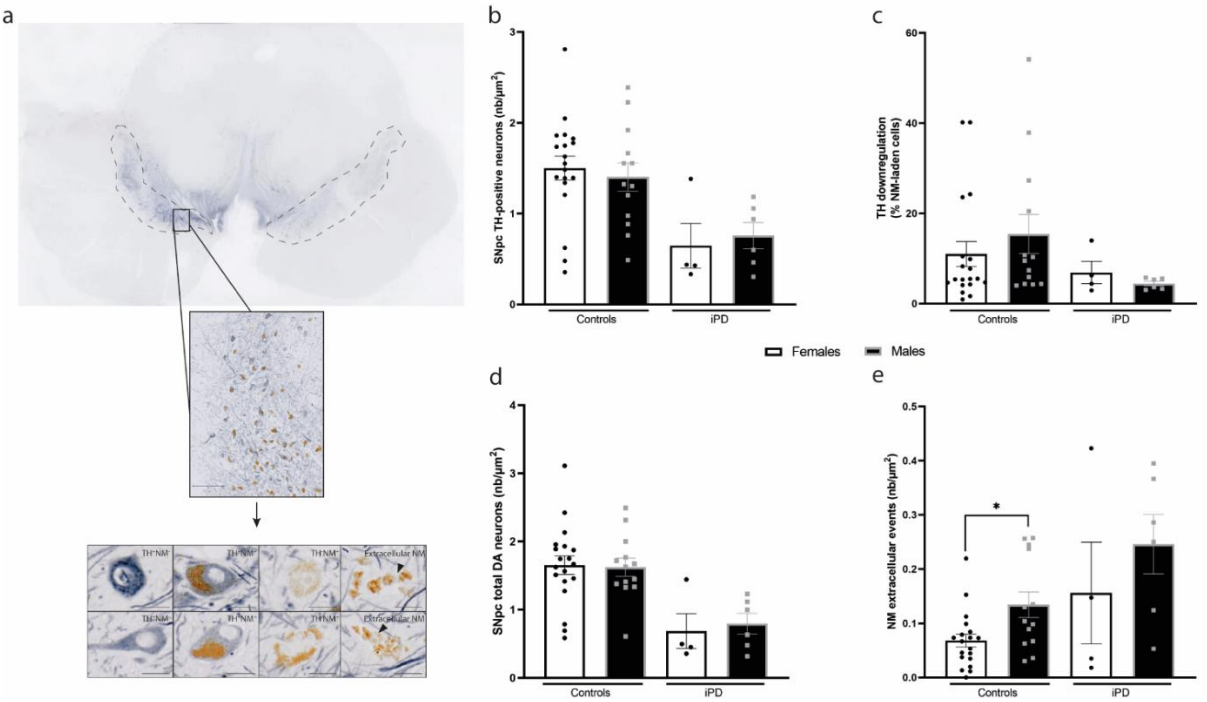

Figure 18 – SNpc cell viability in male and female control and iPD brains. *a) Representative images of TH-immunostained SNpc from elderly human brain tissue. b) Stereological cell counts of SNpc TH-positive neurons in control and iPD cases classified by sex. c) Stereological assessment of TH downregulation within melanized neurons (TH–NM+ neurons vs total NM+ neurons. d) Stereological cell counts of total DA SNpc neurons (including TH-immunopositive and TH-immunonegative melanized neurons). e) Stereological cell counts of extracellular NM events. Controls: n=19 females, n=13 males; iPD: n=4 females, n=6 males.*

1.2. Intracellular NM levels in elderly female and male human controls:

To determine whether the differences in intracellular NM levels between female and male control brains observed above could be due to age-dependent differences, I classified these cases not only by sex but also by age, using two age group intervals: (1) from 59 to 79 years (y) of age, and (2) >80y (**Figure 19**a). I found that intracellular NM levels within SNpc DA neurons were significantly higher in males than in females for both age groups, with males reaching the pathogenic NM threshold as early as 59-79 years of age (**Figure 19**b). In contrast, the percentage of cell area occupied by NM was increased in males compared to females only in the >80y age group (**Figure 19**c).

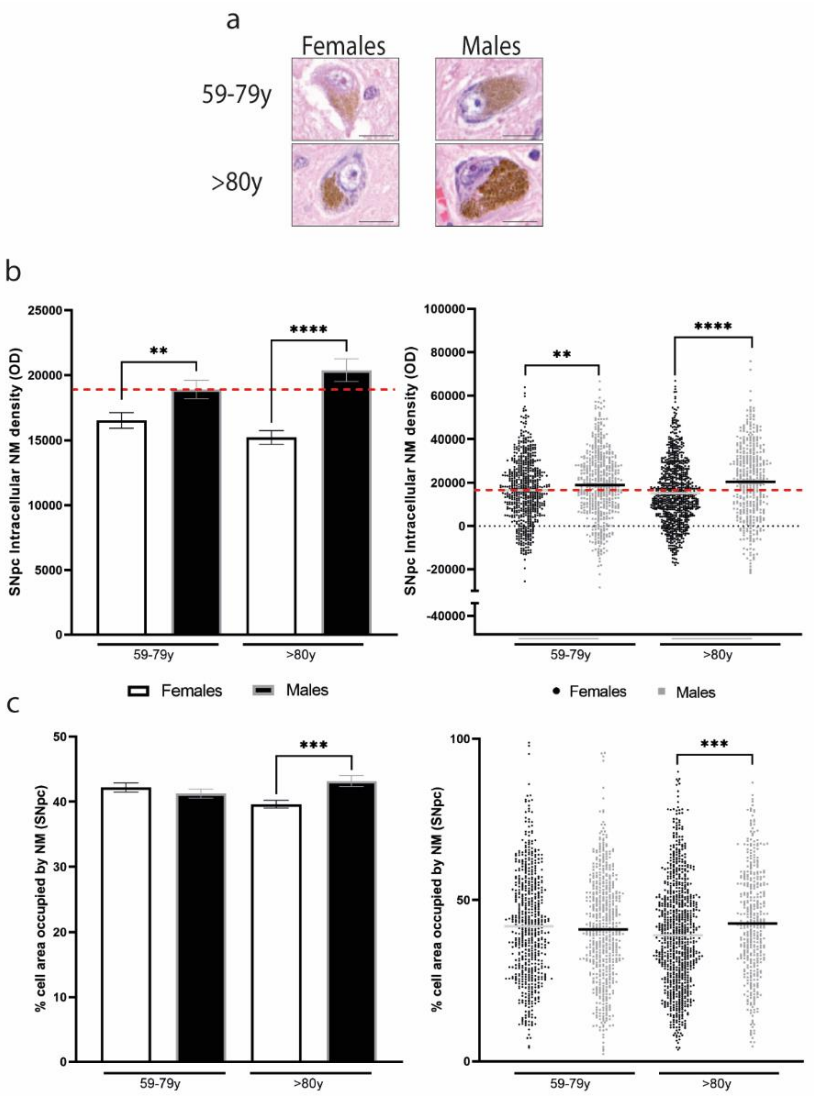

Figure 19 - Age-related intracellular NM levels in female and male control brains. *a) Representative images of SNpc NM-containing neurons in HE-stained postmortem human control brains from females and males, classified by age and sex. b) Quantification of intracellular NM accumulation optical density (OD) in SNpc in control males and females classified into two age groups: 59-79 years (59-79y) and >80 years (>80y). **p=0.0087 and ***p<0.0001 (Mann-Whitney U test). Dotted red line indicates the previously reported threshold of NM-linked neuronal dysfunction/degeneration. c) Quantification of percentage of cytosolic area occupied by NM in post-mortem SNpc sections from the same human samples. ***p=0.0003 >80y (Mann-Whitney U test). 59-79y: n=13 females, n=9 males; >80y: n=6 females, n=4 males.*

I next assessed whether NM-containing nigral neurons reaching the pathogenic threshold in apparently healthy elderly males may already exhibit functional/degenerative changes, even in the absence of overt PD (**Figure 20**). By performing stereological cell counts of SNpc TH-positive neurons, I observed an increased SNpc DA cell loss and a tendency to a greater TH downregulation in males compared to females in controls >80y (**Figure 20**b-d). Furthermore, in this older control group, there was a tendency towards an increased number of extracellular NM events in males compared to females (**Figure 20**e). These results suggest that apparently healthy aged control males may already exhibit incipient neuronal dysfunction/degeneration in melanized SNpc DA neurons.

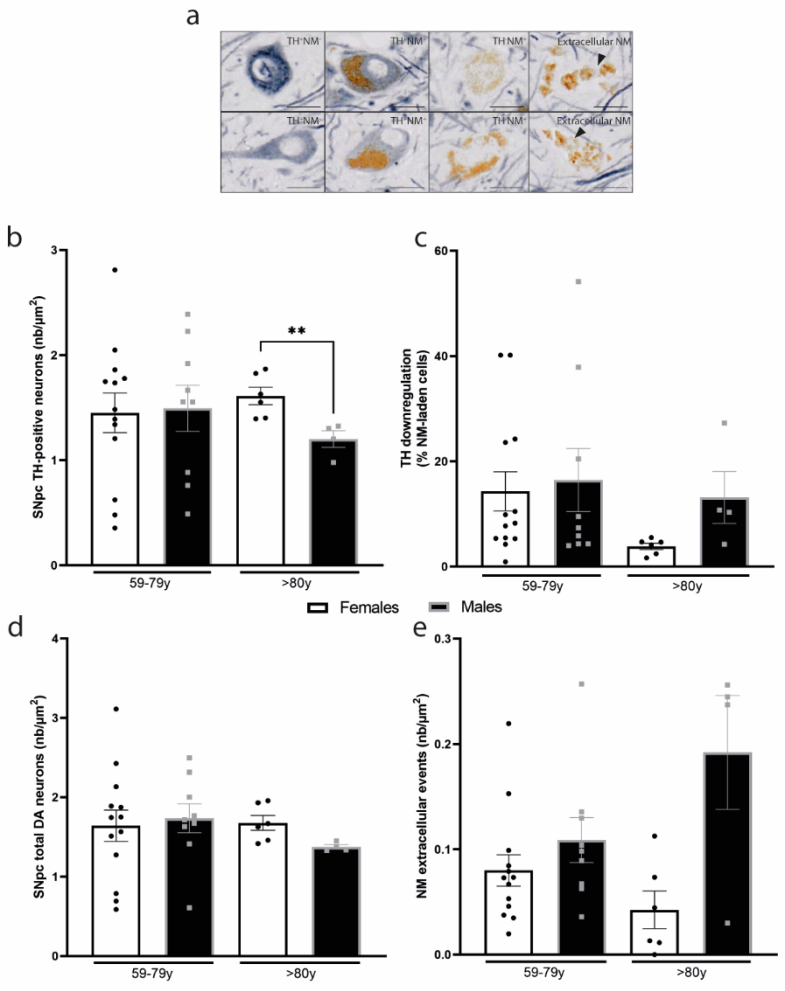

Figure 20 - SNpc cell viability in aged male and female control brains. *a) Representative images of TH-immunostained SN from elderly human cases. b) Stereological cell counts of SNpc TH-positive neurons in male and female controls classified by age. ** p= 0.0096 (Mann-Whitney U test; Two-way ANOVA N.S.). c) Stereological assessment of TH downregulation within melanized neurons (TH−NM+ neurons vs total NM + neurons). d) Stereological cell counts of total SNpc DA neurons (including TH-immunopositive and TH-immunonegative melanized neurons). e) Stereological cell counts of extracellular NM events. Data comparisons were made by Mann-Whitney U test. n=22, 59-79y (n=13 females and n=9 males) and n=10, >80y (n=6 females and n=4 males).*

1.3. NM production in human female and male control brains across lifespan:

To determine at what age female and male SNpc DA neurons start exhibiting differences in intracellular NM levels, I analyzed human postmortem SNpc tissues from male and female control subjects aged from 5 to 90y. These cases were classified into five age groups: 0-5y, 15-20y, 31-50y, 51-70y, and 71-90y. Within each group, cases were also classified by sex. We found that males presented an increase in NM accumulation compared to females as early as 15-20y (**Figure 21**a). While NM levels in females remained constant from age 31y onwards (**Figure 21**a), males exhibited continuously increasing intracellular NM levels with age, until reaching the pathogenic NM threshold by 51-70y (**Figure 21**a). Similarly, the percentage of cell area occupied by NM was also increased in males compared to females by 15-20y onwards (**Figure 21**b).

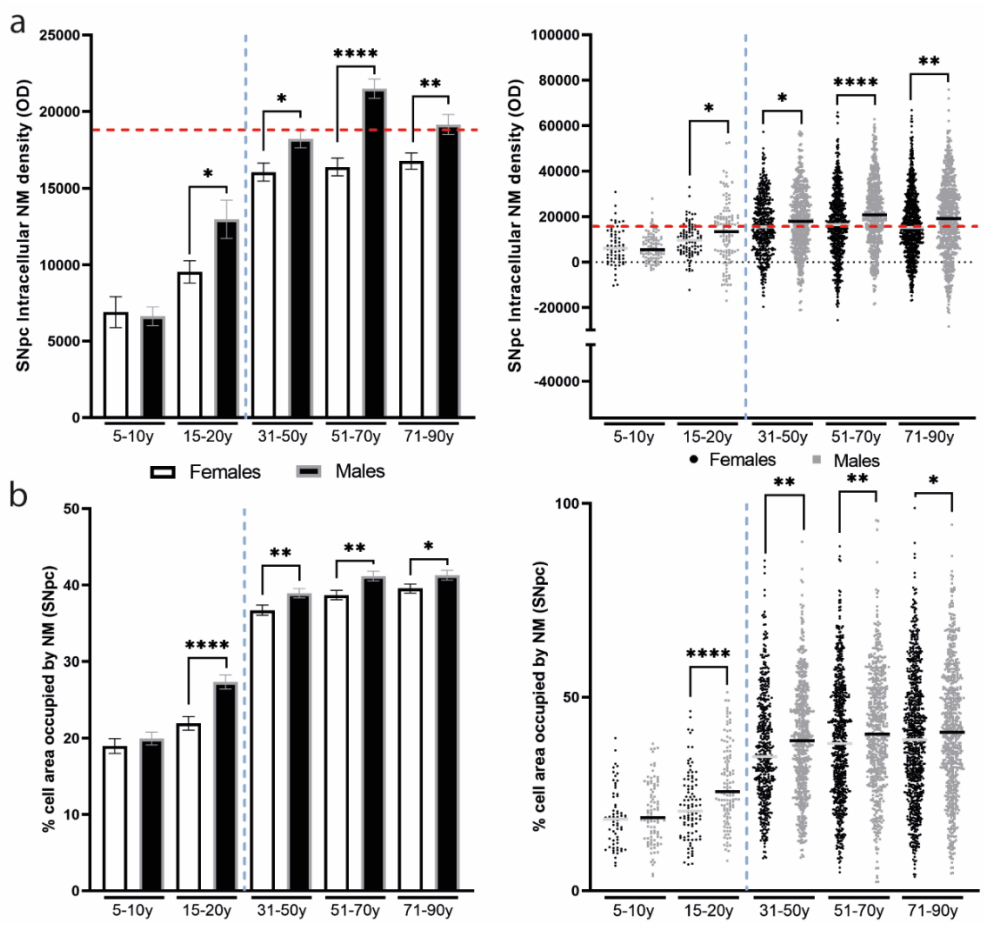

Figure 21 - Intracellular NM levels in male and female control brains across lifespan. *a) Quantification of intracellular NM optical density in post-mortem SNpc sections from 5-90y control female and male subjects. Dotted red line indicates the previously reported threshold of NM-linked neuronal dysfunction/degeneration (Carballo-Carbajal et al., 2019). Data comparisons were made by Mann-Whitney test: 5-10y pair p=0.9362; 15-20y pair p=0.0240; 31-50y pair p=0.0143; 51-70y pair p<0.0001 and 71-90y pair p=0.0011. b) Quantification of the percentage of cytosolic area occupied by NM in post-mortem SNpc sections from 5-90y control female and male subjects. Data comparisons were made by Mann-Whitney U test: 5-10y pair p=0.3951; 15-20y pair p<0.0001; 31-50y pair p=0.0064; 51-70y pair p=0.0023 and 71-90y pair p=0.0380. 59-79y: n=13 females, n=9 males; >80y: n=6 females, n=4 males.*

I next assessed at what age SNpc DA neurons potentially start exhibiting age-related functional/degenerative changes in females and males. By performing stereological cell counts of SNpc TH-positive neurons, I surprisingly found that young (5-10y) males exhibited higher numbers of TH-positive and total DA neurons compared to females, which decreased to female-equivalent levels by puberty (**Figure 22**a,c). From 31y onwards, the number of SNpc TH-positive neurons and total number of DA neurons (including TH-immunopositive and TH-immunonegative melanized neurons) did not differ significantly between males and females (**Figure 22**a,d). However, NM-laden neurons from 31- to 50-year-old males exhibited a significantly higher TH downregulation compared to age-matched females, suggestive of early stages of dysfunction/degeneration (**Figure 22**b). Consistent with this, an increased number of NM extracellular events was observed in males compared to females at later ages (71-90y)

compared to age-matched females (**Figure 22**d). Similarly, Lewy body cytoplasmic inclusions, identified by HE, were also increased in aged males, in the absence of overt PD (**Figure 22**e).

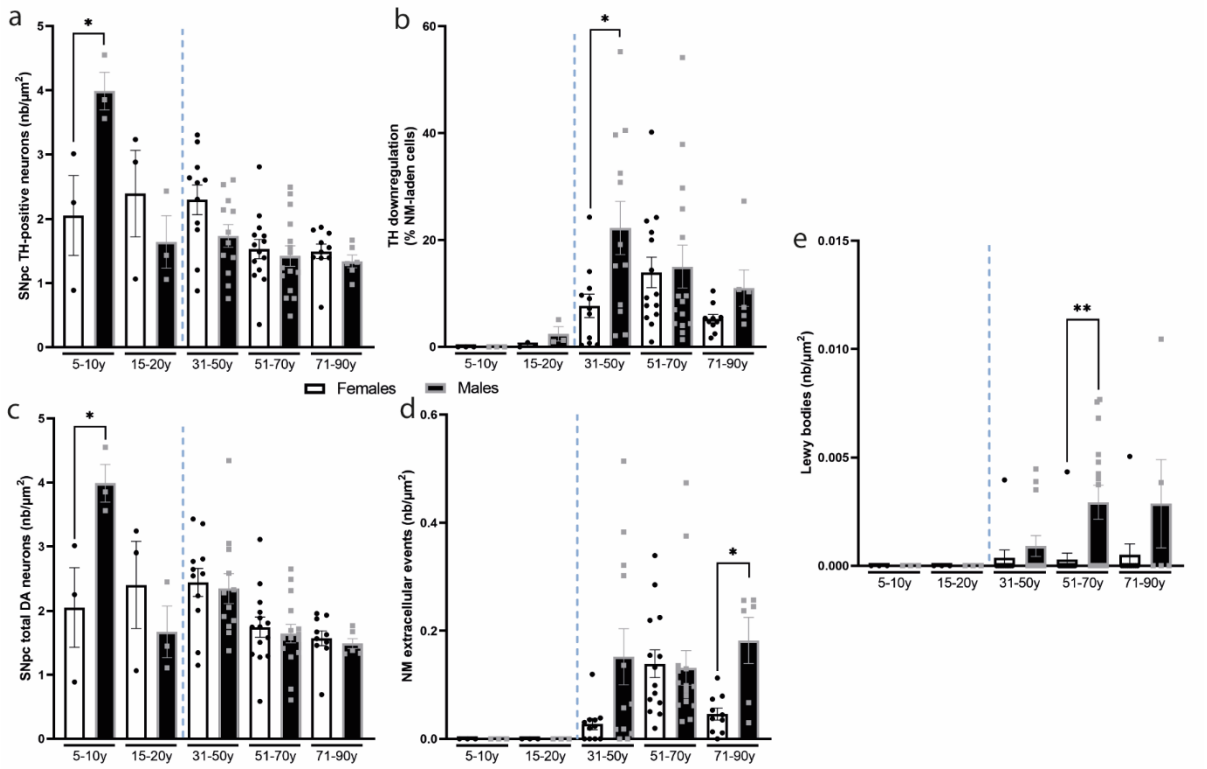

Figure 22 – Cell viability in healthy female and male controls across lifespan. *a) Stereological cell counts of SNpc TH-positive neurons. Data comparisons for 5-10y to 51-70y were made by Student's t-test: 5-10y pair p=0.0479; 15-20y pair p=0.3931; 31-50y pair p=0.0644; 51-70y pair p=0.6257. 71-90y pair compared by Mann-Whitney U test, p= 0.1806. b) Stereological assessment of TH downregulation within melanized neurons (TH−NM+ neurons vs total NM + neurons. 15-20y and 31-50y pairs compared by Student's t-test: 15-20y pair p=0.1969 and 31-50y pair p=0.0168. 51-70y and 71-90y pairs compared by Mann-Whitney U test: 51-70y pair p= 0.7800 and 71-90y pair p=0.0559. c) Stereological cell counts of total SNpc DA neurons (including TH-immunopositive and TH-immunonegative melanized neurons). Pairs compared by Student's t-test: 5-10y pair p= 0.0479; 15-20y pair p=0.4046; 31-50y pair p=0.7652; 51-70y pair p=0.6422; 71-90y pair p=0.6243. d) Stereological cell counts of extracellular NM events. Data comparisons were made by Mann-Whitney U test, 31-50y pair p=0.1265; 51-70y pair p=0.5907 and 71-90y pair p=0.0312. e) Lewy body cytoplasmic inclusions identified by HE. Data comparisons were made by Mann-Whitney U test, 31-50y pair p=0.5223; 51-70y pair p=0.0067 and 71-90y pair p=0.4322. 5-10y and 15-20y: n=3 females and n=3 males in each age group; 31-50y group: n=11 females and n=12 males; 51-70y group: n=14 females and n=15 males; 71-50y group: n=10 females and n=6 males.*

2. CHAPTER 2: NM production and PD pathology in female and male NM-producing rats.

2.1. Intracellular NM levels in AAV-hTyr-injected female and male rats:

While rodent SNpc DA neurons do not naturally accumulate NM, our laboratory established the first NM-producing/accumulating rodent model *(Carballo-Carbajal et al. 2019)*. In the original report of this model, only male animals were used *(Carballo-Carbajal et al. 2019)*. To assess whether this model could also reproduce male sex bias differences observed in humans, male and female adult rats received a single unilateral stereotaxic injection of AAV-hTyr above the right SNpc (**Figure 23**a). Non-injected contralateral brain hemispheres served as internal controls. Analyses were performed at 1, 2, 4, 6, 12, and 20 months post-AAV injections, each time-point corresponding to a different PD-like disease stage, as previously defined by our team in AAV-hTyr-injected male rats *(Carballo-Carbajal et al. 2019)*: pre-symptomatic stage (1m), prodromal/early PD stage (2 & 4m), and established PD stage (≥6m) (**Figure 23**b).

Animals were assessed for contralateral paw akinesia using the cylinder test. At the "established PD" stage, male and female rats exhibited significant motor asymmetry compared to their baseline pre-AAV-hTyr injection performance (**Figure 23**c). At this stage, males tended to exhibit greater motor dysfunction than females, although the difference between sexes was not statistically significant (**Figure 23**c).

To determine whether the sex-related differences in NM levels observed in humans were also present in our rat model, I quantified intracellular NM accumulation in HE-stained SNpc sections from these animals (**Figure 23**d). At the pre-symptomatic and prodromal/early PD stages, male animals exhibited significantly higher intracellular NM levels compared to females (**Figure 23**e), reaching the previously established pathogenic NM threshold as early as at the pre-symptomatic stage. In contrast, females reached the pathogenic NM threshold only at the late, established PD stage (**Figure 23**e). Once male and females reached the final established PD stage, intracellular NM levels became similar in both sexes, exceeding the pathogenic threshold both in females and males (**Figure 23**e). Similarly, male rats also exhibited a higher percentage of cytosolic area occupied by NM compared to females, starting at the pre-symptomatic stage (**Figure 23**f). Once males and females reached the final established PD stage, this difference between sexes was no longer evident (**Figure 23**f).

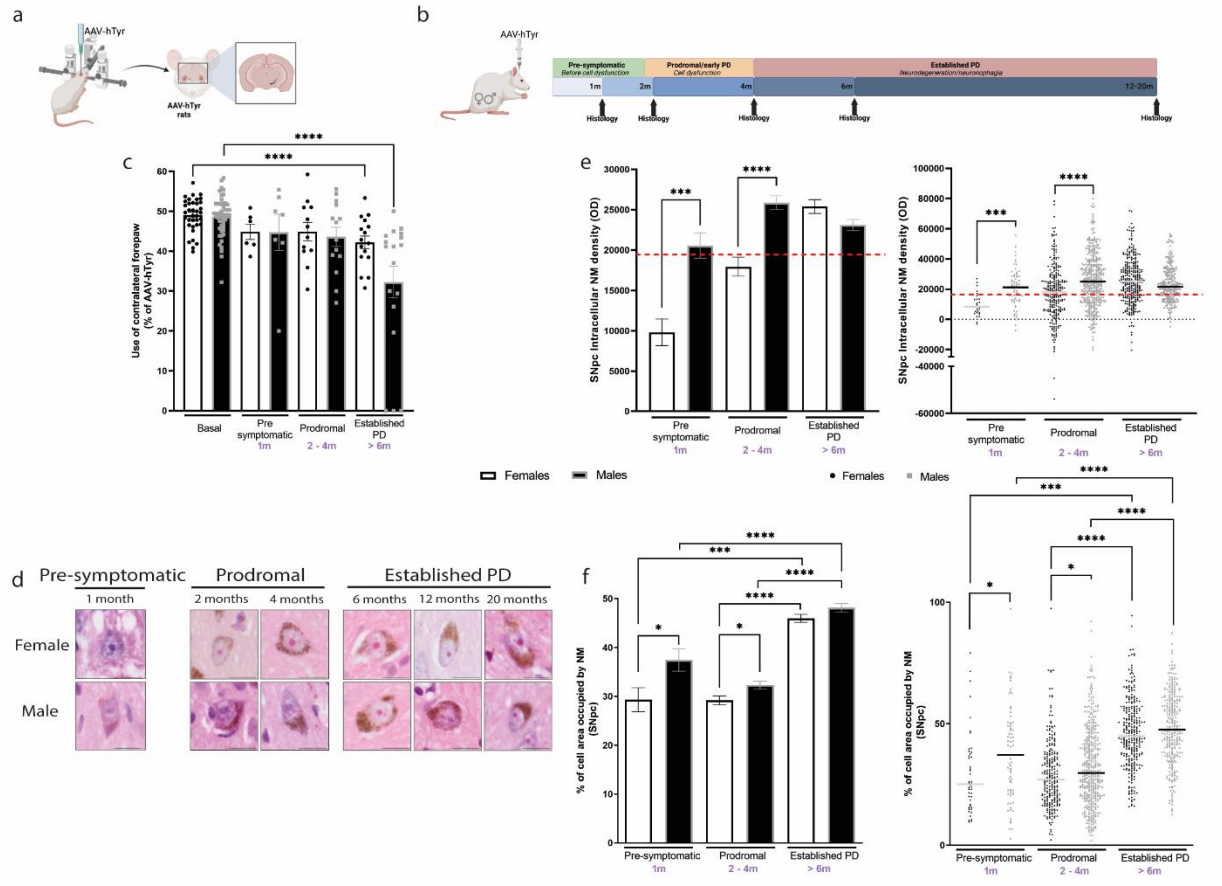

Figure 23 – Intracellular NM levels in AAV-hTyr-injected male and female rats. *a) Schematic representation of the AAV-hTyr unilateral stereotaxic injection above the SNpc of the rat brain. b) Schematic representation of the experimental time-course post-AAV injection with the distribution of the different groups/stages based on the previously described timeline of PD-like pathology occurring in male rats (Carballo-Carbajal et al., 2019). c) Contralateral forepaw akinesia was assessed with the cylinder asymmetry test at different time points post-AAV injection. ***p<0.0001 (Mann-Whitney U test). d) Representative images of NM-containing SNpc neurons in HE-stained postmortem rat brain tissues, shown based on sex and time post-AAV-hTyr injection. e) Quantification of intracellular NM optical density in the ipsilateral SNpc of AAV-hTyr-injected male and female rats at different stages post-AAV treatment. ***p=0.0002, ****p<0.0001, p=0.0332 (Mann-Whitney U test). Dotted red line indicates the previously described threshold of NM-linked neuronal dysfunction/degeneration in male rats (Carballo-Carbajal et al., 2019). f) Quantification of percentage of cytosolic area occupied by NM in the ipsilateral SNpc of AAV-hTyr-injected male and female rats at different stages post-AAV treatment. Data comparisons were made by Student's t-test: p=0.0207 pre-symptomatic; p=0.0114 prodromal/early PD; p=0.0672 established PD. p<0.0001 pre-symptomatic vs established PD females; p<0.0001 pre-symptomatic vs established PD males. Pre-symptomatic: n=6 females, n=7 males; Prodromal: n=12 females, n=14 males; Established PD: n=18 females, n= 18 males.*

2.2. Nigrostriatal degeneration in AAV-hTyr-injected female and male rats:

I next investigated whether the differences in time-dependent NM accumulation observed between female and male AAV-hTyr-injected rats influenced NM-linked nigrostriatal neurodegeneration in these animals. By performing stereological cell counts of SNpc TH-positive neurons, I observed that loss of SNpc TH-positive neurons began earlier in male compared to female animals (**Figure 24**b). Indeed, males tended to exhibit more nigral cell loss than females from as early as the pre-symptomatic stage, although this difference did not reach statistical significance until the prodromal/early PD stages (**Figure 24**b). At the established PD stage, where neurodegeneration was fully developed in both sexes, differences in cell loss were

no longer evident between males and females, as was similarly the case for NM accumulation (**Figure 23**). AAV-hTyr-injected male animals also exhibited an earlier and more sustained TH downregulation compared to their female counterparts (**Figure 24**c). In addition, compared to female animals, males also showed an earlier and greater loss of nigrostriatal DA TH-positive neurons across all stages (**Figure 24**d).

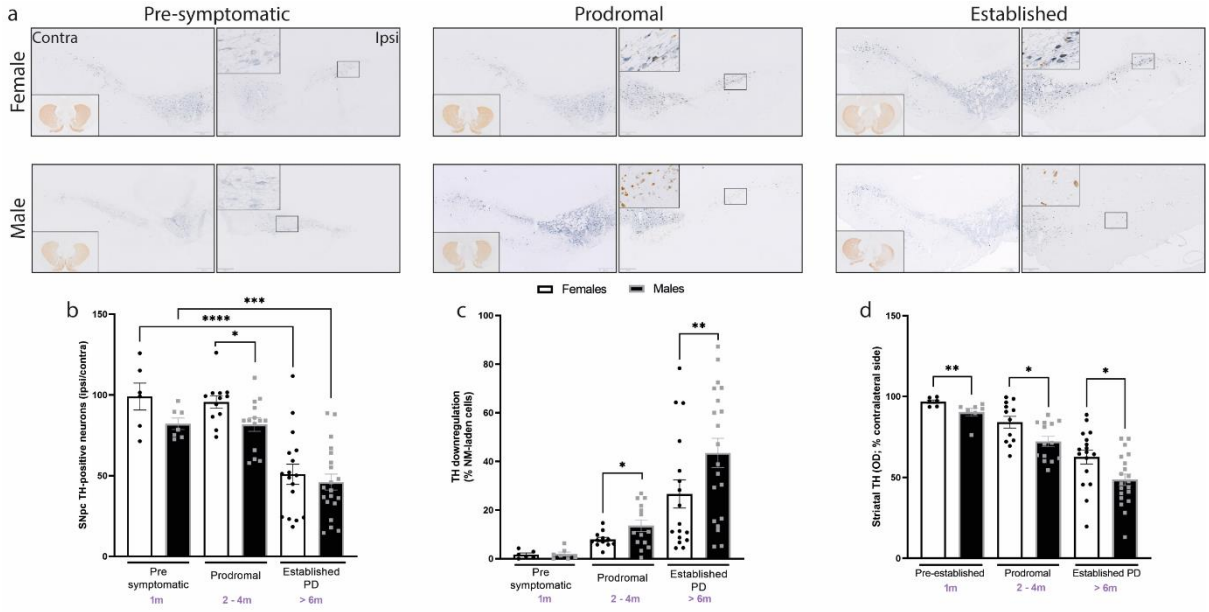

Figure 24 – Nigrostriatal neurodegeneration in AAV-hTyr-injected male and female rats. *a) Representative images of TH-immunostained SNpc and striatum (inset) from male and female AAV-hTyr-injected rats at different PD-like disease stages post-AAV treatment. b) Stereological cell counts of SNpc TH-positive neurons in AAV-hTyr-injected male and female rats. Data comparisons were made by Mann-Whitney U test. Pre-symptomatic: p=0.1375; Prodromal/early PD: p=0.0148; Established PD: p= 0.4785. Pre-symptomatic vs Established PD: p<0.0001 females and p=0.0003 males. c) Stereological assessment of TH downregulation within melanized neurons (TH−NM+ neurons vs total NM + neurons) in AAV-hTyr-injected male and female rats. Pre-symptomatic: p=0.8831. Prodromal/early PD: p=0.0463. Established PD compared with a Mann-Whitney U test, p= 0.0039. d) Optical densitometry of striatal TH-positive fibers in AAV-hTyr-injected male and female rats: Pre-symptomatic data compared with a Mann-Whitney U test, p=0.0047. Prodromal/early PD data compared with a Student's t-test, p=0.0200. Established PD data compared with a Student's t-test, p= 0.0157. Pre-symptomatic: n=6 females, n=7 males; Prodromal: n=12 females, n=14 males; Established PD: n=18 females, n= 18 males.*

These results indicate that, similar to results for human brain tissue, intracellular NM accumulation is accelerated in AAV-hTyr-injected male rats compared to female rats, thus reaching the pathogenic NM threshold earlier to trigger PD-like pathology.

3. CHAPTER 3: Effect of sex-based manipulations on NM production and PD pathology in AAV-hTyr-injected rats.

3.1. OVX rats, a rodent model of menopause:

3.1.1. Intracellular NM levels in AAV-hTyr-injected OVX rats:

OVX female rats serve as an animal model of menopause as the removal of ovaries in these animals mimics the abrupt decrease in estrogen levels observed in human menopause. To investigate whether sex differences in the rodent PD model could be attributed, as hypothesized, to gonadal hormones, OVX female rats received a single unilateral stereotaxic injection of an AAV-hTyr into the right SNpc (**Figure 25**a). The non-injected contralateral brain hemispheres were used as internal controls. Analyses were conducted at 2 and 4 months post-AAV injections, corresponding to the prodromal/early PD stage (**Figure 25**a). OVX females and gonad-intact females and males underwent the same tests and analyses, and the results were compared across these groups.

Animals were evaluated for contralateral paw akinesia using the cylinder test to assess motor dysfunction. At the prodromal stage, OVX females, gonad-intact females, and males exhibited slight motor deficits compared to baseline pre-AAV-hTyr injection levels, but no significant differences were noted (**Figure 25**b). To determine if the removal of ovaries and the subsequent decrease in estrogen levels impacted on NM accumulation in AAV-Tyr-injected female rats, I quantified intracellular NM levels in HE-stained SNpc from these animals (**Figure 25**c). As noted earlier (**Figure 23**e), AAV-hTyr-injected males exhibited significantly higher intracellular NM levels compared to females at the prodromal/early PD stage (**Figure 25**d). Remarkably, AAV-hTyr-injected OVX females also displayed higher intracellular NM levels than gonad-intact females, at levels comparable to those observed in male animals (**Figure 25**d). As for male rats, intracellular NM levels in OVX female rats reached the pathogenic threshold earlier than gonad-intact female rats (**Figure 25**d). The percentage of cytosolic area occupied by NM was also similarly increased both in OVX females and males compared to gonad-intact females (**Figure 25**e).

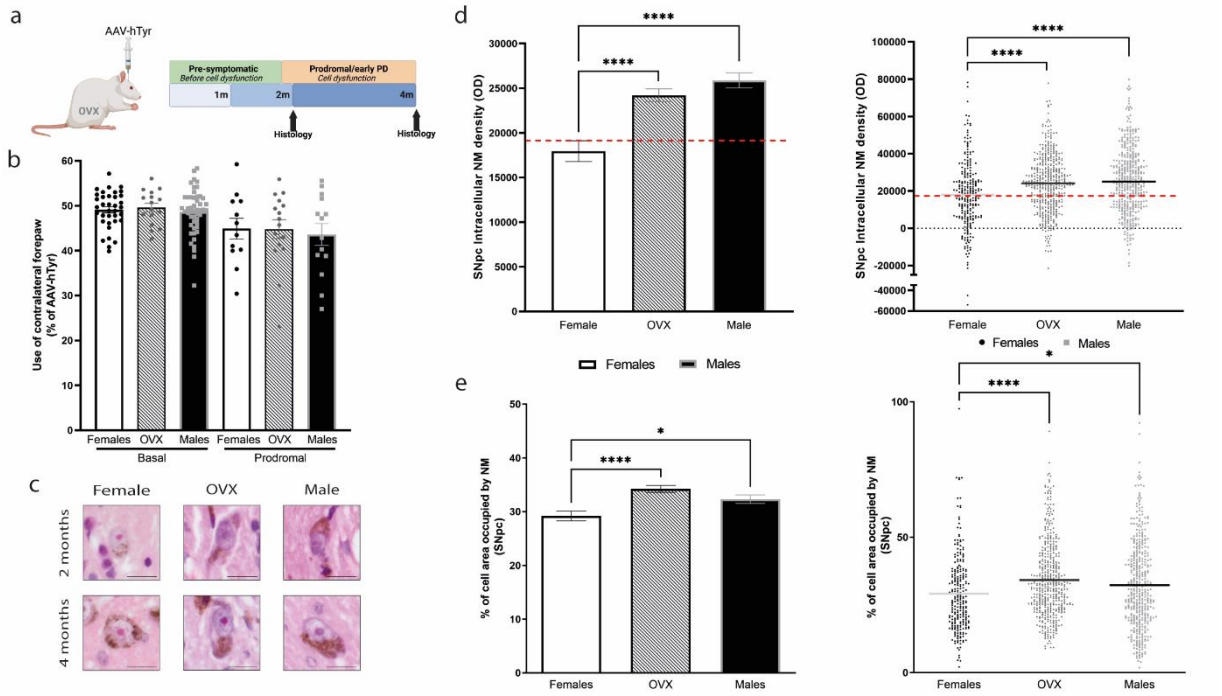

Figure 25 – Intracellular NM levels in AAV-hTyr-injected OVX female rats*. a) Schematic representation of the experimental plan for the OVX female rats. b) Contralateral forepaw use was assessed with the cylinder asymmetry test before (baseline) and after AAV injection. No statistically significant changes were observed between groups (one-way ANOVA). c) Representative images of NM-containing SNpc neurons from HE-stained postmortem rat brain tissues from AAV-hTyr-injected females, OVX females, and males, shown in terms of age and sex. d) Quantification of intracellular NM optical density in the ipsilateral SNpc from AAV-hTyr-injected rats. ****p<0.0001 (Kruskal-Wallis). Dotted red line indicates the previously published threshold of NM-linked neuronal dysfunction/degeneration (Carballo-Carbajal et al., 2019). e) Quantification of the percentage of cytosolic area occupied by NM in ipsilateral SNpc sections from AAV-hTyr-injected rats. ****p<0.0001 and *p<0.05 (one-way ANOVA). N=12 females, n=16 OVX females, and n=14 males.*

3.1.2. Nigrostriatal neurodegeneration in AAV-hTyr-injected OVX rats:

To investigate whether the increase in intracellular NM levels observed in OVX female rats was accompanied by neurodegeneration, I performed stereological cell counts of SNpc TH-positive neurons (**Figure 26**a). As expected, AAV-hTyr-injected male rats exhibited significant loss of SNpc TH-positive neurons compared to gonad-intact female animals (**Figure 26**b). In contrast, the number of SNpc TH-positive neurons in OVX females, while slightly decreased compared to gonad-intact female animals, did not differ significantly from gonad-intact female or male animals, indicating intermediate TH-positive neuron numbers between these two groups (**Figure 26**b). Additionally, as was the case in males, there was a greater TH downregulation in OVX females compared to gonad-intact females, although this did not reach statistical significance (**Figure 26**c). Compared to gonad-intact females, loss of SNpc TH-positive neurons (as measured by optical densitometry) in AAV-hTyr male rats was accompanied by a reduction of striatal DA TH-positive fibers (**Figure 26**d). Again, the levels of striatal TH optical density in OVX were intermediate between gonad-intact females and males, without reaching statistical significance in either group (**Figure 26**d).

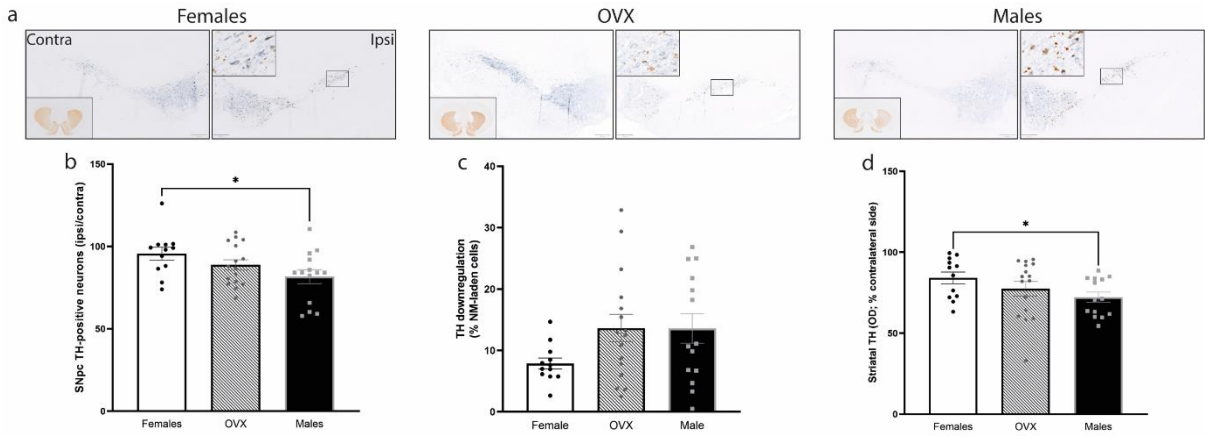

Figure 26 – Nigrostriatal neurodegeneration in AAV-hTyr-injected OVX female rats. *a) Representative images of TH-immunostained SNpc and striatum (bottom inset) from AAV-hTyr-injected OVX female rats, non-OVX female rats, and male rats at the prodromal/early PD stage. b) Stereological cell counts of SNpc TH-positive neurons in AAV-hTyr-injected rats. One-way ANOVA analysis: p= 0.4140 for non-OVX females versus OVX; p=0.3539 for OVX females versus males, and p=0.0394 for non-OVX females versus males. c) Stereological assessment of TH downregulation within melanized neurons (TH−NM+ neurons vs total NM + neurons) in AAV-hTyr-injected rats. No statistically significant changes were observed between groups (one-way ANOVA). d) Optical densitometry of striatal TH-positive fibers in AAV-hTyr-injected rats. Kruskal-Wallis analysis: *p=0.0334, p=0.5325 for non-OVX versus OVX females, and p=0.4738 for OVX females versus males. N=12 females, n=16 OVX females, and n=14 males.*

These results confirm that the loss of estrogen activity in OVX females leads to higher NM accumulation, reaching the pathogenic NM threshold earlier, and a trend to greater nigrostriatal degeneration compared to gonad-intact female animals not subjected to estrogen deprivation. The loss of estrogens in females appears to result in a faster development of PD pathology.

3.1.3. Neuroinflammation in AAV-hTyr-injected OVX rats:

To investigate whether the loss of SNpc DA TH-positive neurons was associated with neuroinflammatory changes, I quantified CD68-positive macrophages and Iba1-positive non-reactive and reactive microglia in AAV-hTyr-injected OVX females, gonad-intact females, and male rats (**Figure 27**a). In all AAV-hTyr-injected groups, there was a marked increase of CD68-positive cells in melanized ipsilateral SNpc compared to non-melanized contralateral SNpc (**Figure 27**a,b), consistent with the role of CD68-positive tissue-resident or blood-borne macrophages at phagocytosing extracellular NM released from dying neurons, as previously reported *(Carballo-Carbajal et al. 2019)*. Males and OVX females tended to exhibit more CD68-positive macrophages than gonad-intact females, although this did not reach statistical significance (**Figure 27**b). Microglial cells are the main players in the recognition, engulfment and clearance of extracellular NM. In agreement with this, the number of microglial cells with non-reactive (ramified) and phagocytic/reactive (large amoeboid de-ramified) morphology was markedly increased in melanized ipsilateral SNpc from AAV-hTyr-injected males. Notably, the

number of microglial cells was increased in OVX females compared to their non-melanized contralateral SNpc, and to the ipsilateral SNpc from gonad-intact females (**Figure 27**c,d).

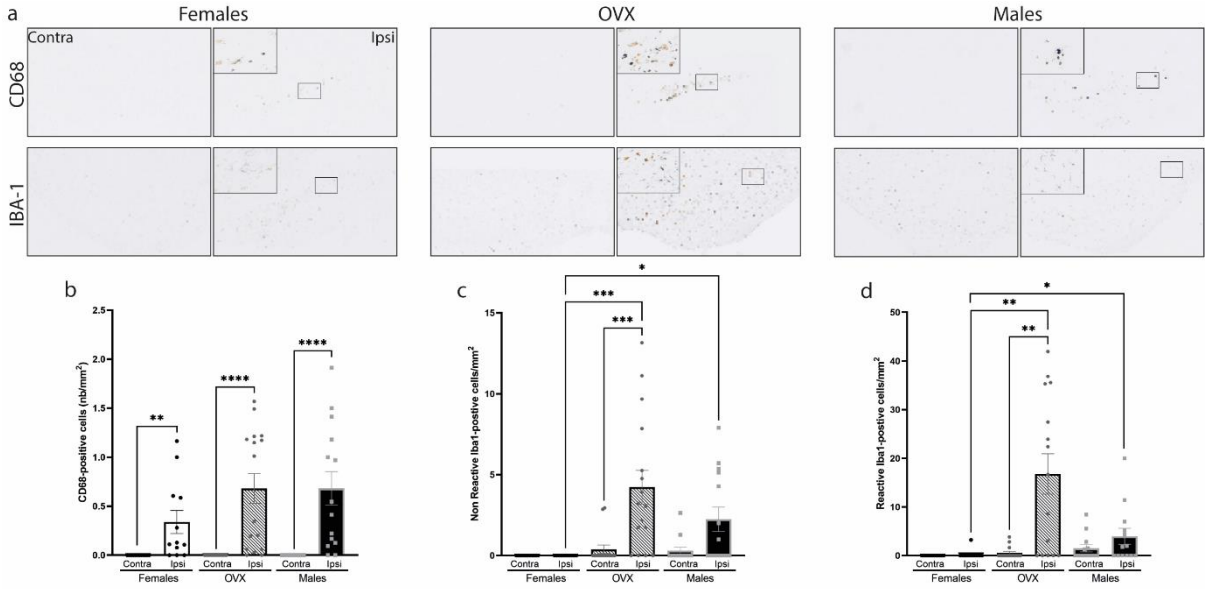

Figure 27 – Neuroinflammation in AAV-hTyr-injected OVX female rats. *a) Representative images of SNpc sections immunostained for CD68 and Iba1 (in blue; unstained NM in brown). b) Quantification of SNpc CD68-positive cells in AAV-hTyr-injected rats. **p=0.0090, ***p<0.0001 (Kruskal-Wallis). c) Quantification of SNpc non-reactive Iba1-positive microglia in AAV-hTyr-injected rats: p<0.0005 for OVX ipsi vs contra; p=0.0001 non-OVX females versus OVX ipsi; p=0.0377 females versus males ipsi (Kruskal-Wallis). d) Quantification of SNpc reactive Iba1-positive microglia in AAV-hTyr-injected rats: p=0.0030 for OVX ipsi vs contra; p=0.0017 non-OVX females versus OVX. N=12 females, n=16 OVX females, and n=14 males. ipsi: ipsilateral; contra: contralateral*

Overall, the loss of estrogens in AAV-hTyr-treated OVX females resulted in accelerated NM accumulation, which was associated with increased SNpc DA TH-positive cell loss and heightened neuroinflammation, similar to findings in male animals.

3.2. Estradiol (E2) treatment:

3.2.1. E2 treatment decreases intracellular NM levels *in vitro*:

3.2.1.1. E2 treatment decreases intracellular NM in NM-producing TR5TY6 cells:

Given that the loss of estrogens was associated with accelerated/increased NM accumulation in rats, and considering that estradiol can influence melanin production and extrusion as outlined in the introduction, I investigated whether estradiol treatment could reduce intracellular NM levels. I first evaluated this possibility in an *in vitro* setting, using an NM-producing human catecholaminergic neuroblastoma SH-SY5Y cell line (TR5TY6) inducible for hTyr expression in response to doxycycline (Tet-On) and differentiated to a neuronal phenotype before hTyr induction *(Carballo-Carbajal et al. 2019)*. Differentiated TR5TY6 cells were treated with two doses of estradiol (E2): 25 nM and 100 nM (**Figure 28**a). Similar to AAV-hTyr-injected rats, induction of hTyr expression in these cells resulted in progressive intracellular NM accumulation, with most of the cytoplasm occupied by NM six

days post-induction, at which time-point cells started to degenerate (**Figure 28**a) *(Carballo-Carbajal et al. 2019)*.

E2 treatment of TR5TY6 cells appeared to influence intracellular NM levels (**Figure 28**b,c). Cells treated with 25 nM E2 accumulated more NM than untreated cells, while cells treated with 100 nM E2 exhibited decreased intracellular NM levels compared to untreated cells and to cells treated with 25 nM E2 (**Figure 28**b,c). These changes in intracellular NM levels were associated with alterations in extracellular NM levels measured in the medium (**Figure 28**d). Indeed, following E2 treatment, I observed a dose-dependent increase of tyrosinase activity in the cell medium, which reflects extracellular NM released from dying cells into the medium (**Figure 28**d). Consequently, cells treated with 100 nM E2 had lower intracellular NM levels and higher tyrosinase activity in the medium compared to untreated cells (**Figure 28**c,d). This was associated with significant changes in the distribution of cell types in cells treated with 100 nM E2 compared to untreated cells and those treated with 25 nM E2 (**Figure 28**e-g). Specifically, cells treated with 100 nM E2 had a lower percentage of collapsed (i.e. dead/dying) cells, with higher NM levels compared to untreated cells (**Figure 28**e). This was, in turn, associated with a higher percentage of cells exhibiting moderate and low levels of NM in the 100 nM E2-treated cell group compared to untreated cells or those treated with 25 nM E2 (**Figure 28**f,g).

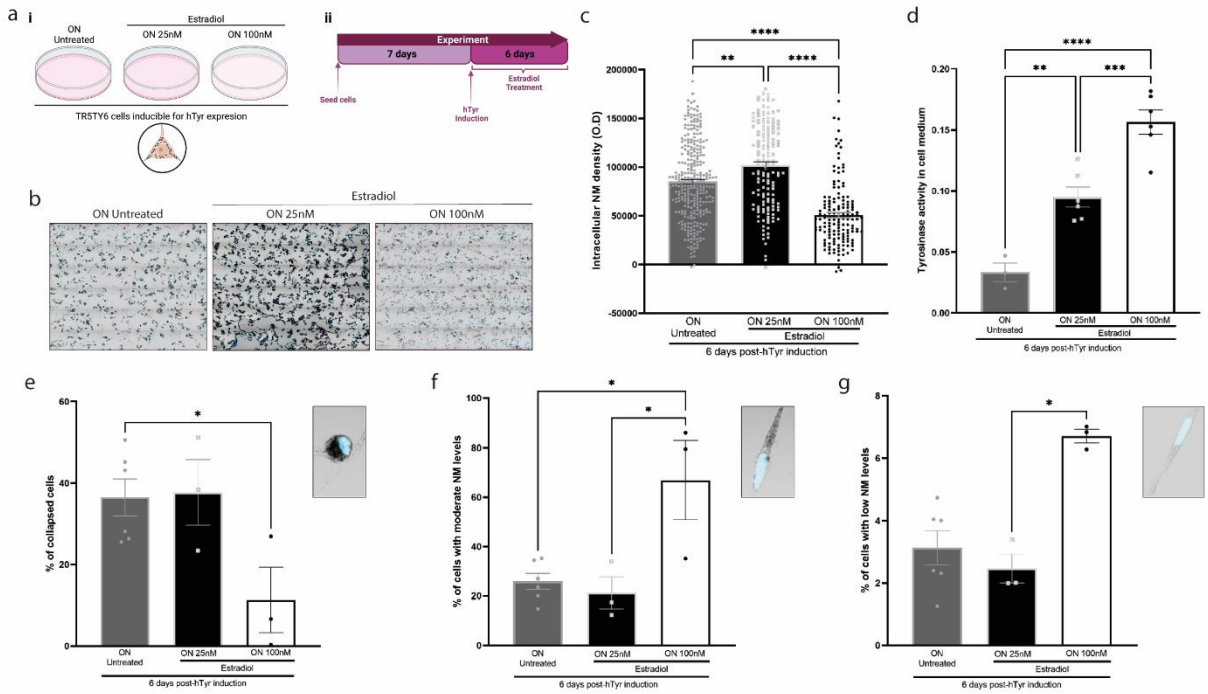

Figure 28 – E2 treatment of NM-producing TR5TY6 cells. *a) Schematic representation of the experimental design for E2 treatment of TR5TY6 cells. b) Representative bright-field photomicrographs with superimposed nuclear Hoechst fluorescent staining (blue) in NM-producing TR5TY6 cells after hTyr induction (ON). c) Quantification of intracellular NM optical density in TR5TY6 cells. **p=0.0012, treated (25 nM) versus untreated cells; ****p<0.0001, treated (100 nM) versus untreated and 25nM-treated cells (Kruskal-Wallis). d) Quantification of absorbance representing tyrosinase activity in the cell medium.*

***p=0.0030, treated (25 nM) versus untreated cells; ****p<0.0001, treated (100 nM) versus untreated cells, and ***p=0.0004 100 nM-treated versus 25nM-treated cells (one-way ANOVA). e) Quantification of collapsed cells with high NM levels. p=0.9897, treated (25 nM) versus untreated cells; *p=0.0441, treated (100 nM) versus untreated cells; p=0.0667 100nM-treated versus 25nM-treated (one-way ANOVA). f) Quantification of cells with moderate NM levels. p=0.9064, treated (25 nM) versus untreated cells; *p=0.0107 treated (100 nM) versus untreated cells; *p=0.0132, 100 nM-treated versus 25 nM-treated cells (one-way ANOVA). g) Quantification of cells with low NM levels. p>0.9999, treated (25 nM) versus untreated; p=0.1093 treated (100 nM) versus untreated cells; *p=0.0382, 100 nM-treated versus 25 nM-treated cells. N=300 cells untreated, and n=150 cells for each treatment condition.*

Overall, these results indicate that E2 can decrease intracellular NM levels *in vitro* by, at least in part, promoting NM extrusion from cells.

3.2.1.2. E2 treatment decreases levels of oxidized DA species that act as NM precursors:

Because estrogens possess intrinsic free radical-scavenging properties *(Prokai et al. 2003)*, I next assessed if E2 could decrease the levels of oxidized catecholamine species that act as NM precursors. This was done by performing UPLC MS/MS analyses of catecholamine metabolites and oxidized species in E2-treated differentiated TR5TY6 cells (**Figure 29**a). hTyr is responsible for the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), the latter being subsequently converted to DA by the enzyme DOPA decarboxylase (**Figure 29**a). Consistent with this, both L-DOPA and DA levels were increased in TR5TY6 cells following hTyr induction (**Figure 29**b). Cytosolic L-DOPA and DA can subsequently be oxidized by hTyr to produce o-quinones. These, in turn, generate aminochrome and 5SCDA/5SCD, which act as precursors of the eumelanin and pheomelanin components of NM, respectively (**Figure 29**a). While aminochrome is too unstable to be reliably measured, both 5SCDA and 5SCD are increased in TR5TY6 cells following hTyr induction, consistent with the formation of NM in these cells (**Figure 29**c). Treatment of cells with 100 nm E2 decreased the oxidation of DA to the NM precursors 5SCDA and 5SCD (**Figure 29**c) and promoted the alternative degradation of DA into 3-methoxytyramine (3MT) (**Figure 29**d).

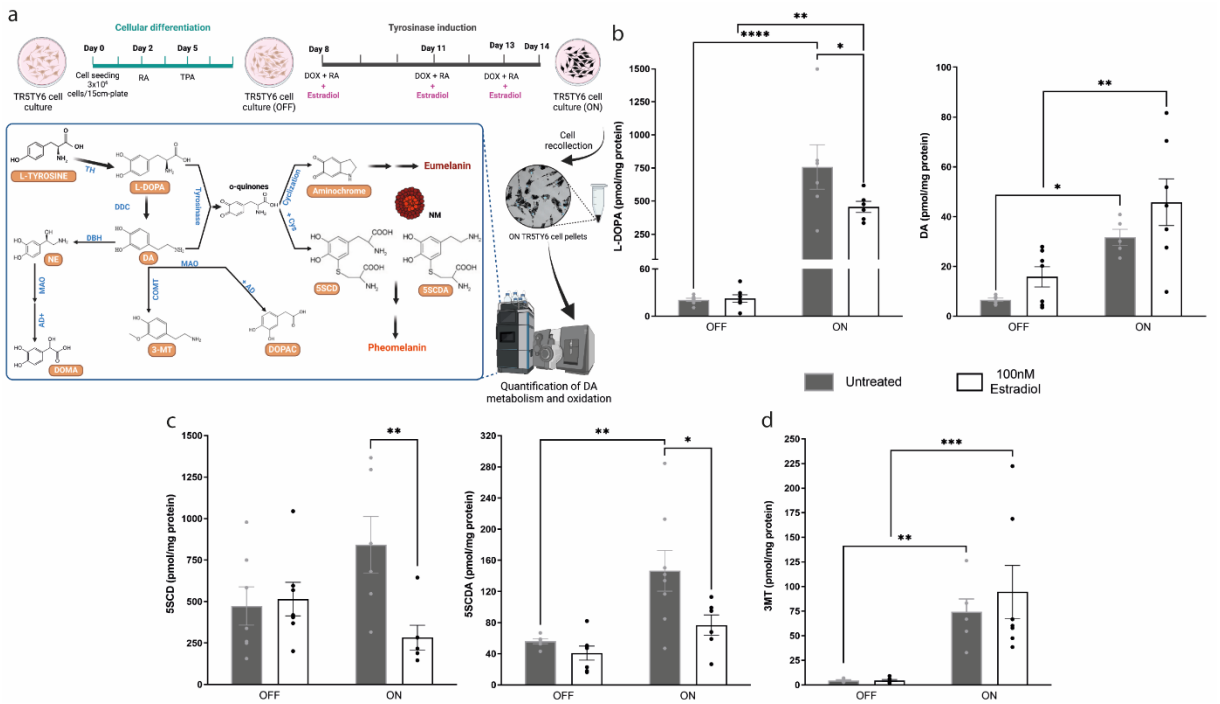

Figure 29 – Effects of E2 treatment on catecholamine metabolism in TR5TY6 cells. *a) Schematic representation of the experimental design for the analysis of catecholamine metabolites by UPLC-MS/MS in E2-treated TR5TY6 cells. Schematic representation of DA metabolism and oxidation pathways. DA is synthesized from L-DOPA. DA can be degraded by MAO to produce 3-MT or by MAO followed by AD to produce DOPAC. DA and L-DOPA can both be oxidized either spontaneously or by tyrosinase to produce o-quinones. These generated AD, 5SCDA and 5SCD, which act as a precursors of the eumelanin and pheomelanin, these being components of NM. 5SCD, 5-S-cysteinyldopa; 5SCDA, 5-S-cysteinyldopamine, AC aminochrome; AD, aldehyde dehydrogenase; COMT, catechol-O-methyltransferase; DA, dopamine; DDC, dopa decarboxylase; DOPAC, 3,4-dihydroxyphenylacetic acid; L-DOPA, 3,4-dihydroxyphenylalanine; MAO, monoamine oxidase; 3-MT, 3-methoxytyramine; NM, neuromelanin. UPLC MS/MS quantification of: b) L-DOPA and DA levels. c) 5SCD and 5SCDA levels. d) 3-MT levels. *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001 (two-way ANOVA). N=7 for each OFF condition and n=8 for each ON condition.*

Overall, these results indicate that the antioxidant properties of E2 may contribute to its effects at reducing intracellular NM levels *in vitro* by decreasing catecholamine oxidation into NM.

3.3.Effects of E2 treatment of AAV-hTyr-injected rats:

4.3.1. E2 treatment decreases intracellular NM levels in AAV-hTyr-injected rats:

To evaluate whether E2 treatment could modulate intracellular NM levels *in vivo*, male and female adult rats received a single unilateral stereotaxic injection of AAV-hTyr into the right SNpc. Two weeks post-AAV injection, rats were implanted with a subcutaneous pellet releasing E2 (at total doses of either 0.025 mg or 0.5 mg) for an additional 21 days (**Figure 30**a).

Motor dysfunction was assessed using the cylinder test to evaluate contralateral paw akinesia at baseline levels (before AAV-hTyr injection), at two weeks post-AAV injection (before E2 pellet insertion), and after the 21-day E2 treatment (**Figure 30**a). Prior to pellet insertion, no significant differences in motor dysfunction were observed between the groups (untreated, 0.025 mg E2, and 0.5 mg E2) or between sexes within these groups (**Figure 30**b). At the end of E2 treatment, untreated female and male animals exhibited enhanced motor dysfunction compared to those receiving either 0.025 mg or 0.5 mg E2 (**Figure 30**c-e). Within the 0.025 mg E2-treated group, the beneficial effect of E2 treatment was more prominent in females than in males (**Figure 30**c-e). To determine whether the observed improvement in motor function in E2-treated animals was associated with reduced NM accumulation, I quantified intracellular NM levels in HE-stained SNpc sections from these animals (**Figure 30**f). A significant, dose-dependent decrease in intracellular NM levels was observed both in males and females treated with E2 (**Figure 30**f), although males consistently showed higher intracellular NM levels than females within each treatment group (**Figure 30**g). The observed sex differences were maintained in relation to the percentage of cytosolic area occupied by NM among E2-treated animals (**Figure 30**h).

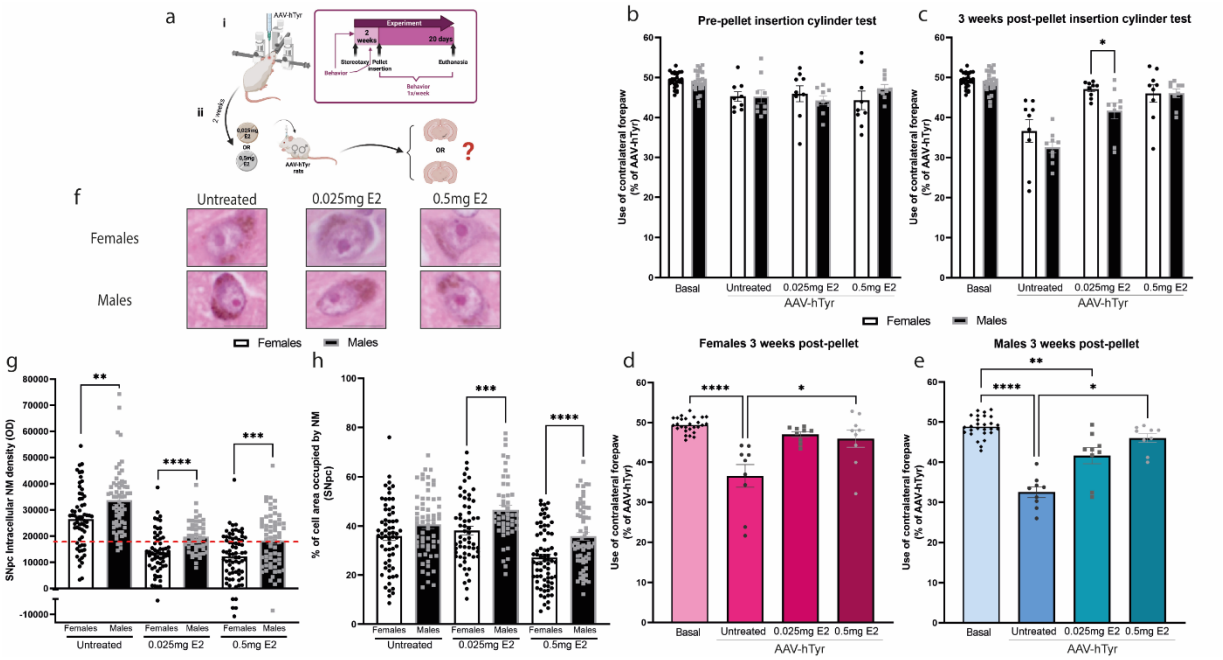

Figure 30 – Effects of E2 treatment on motor asymmetry and intracellular NM density in AAV-hTyr-injected rats. *a) Schematic representation of the experimental design for the in vivo E2 treatment with subcutaneous pellets in AAV-hTyr-injected rats. b) Contralateral forepaw use was assessed with the cylinder asymmetry test under baseline (pre-AAV injection) conditions in female and male rats. No statistically significant differences were observed (two-way ANOVA). c) Contralateral forepaw use assessed with the cylinder asymmetry test after three weeks of E2 treatment in AAV-hTyr-injected female and male rats. Values show mean ± SEM. *p=0.0278 for 0.025mg E2 (two-way ANOVA). d) Contralateral forepaw use assessed with the cylinder asymmetry test after three weeks of E2 treatment in AAV-hTyr-injected female rats. Values show mean ± SEM. ****p<0.0001 for baseline versus untreated rats; *p=0.0470 for untreated versus 0.5 mg E2-treated rats (Kruskal-Wallis). e) Contralateral forepaw use assessed with the cylinder asymmetry test after three weeks of E2 treatment in AAV-hTyr-injected male rats. Values show mean ± SEM. ****p<0.0001 for baseline versus untreated rats; **p=0.0071 for baseline versus 0.025 mg E2-treated rats; *p=0.0278 for untreated versus 0.5 mg E2-treated rats. g) Representative images of NM-containing neurons in HE-stained postmortem rat brain tissues from female and male animals treated or not with E2. g) Quantification of intracellular NM optical density in the ipsilateral SNpc of AAV-hTyr-injected rats, treated or not with E2. ***p=0.0015 (Mann-Whitney U test); ****p<0.0001; ***p=0.0004 (Student's t-test). Dotted red line indicates the previously published threshold of NM-linked neuronal dysfunction/degeneration (Carballo-Carbajal et al., 2019). h) Quantification of percentage of cytosolic area occupied by NM in SNpc sections from AAV-hTyr-injected rats, treated or not with E2. p=0.0501 for untreated animals ***p=0.0015 (Student's t-test); ***p=0.0001 (Mann-Whitney U test). N=8 animals of each sex within each group for the cylinder asymmetry test, and n=6 animals of each sex within each group for histological analysis.*

To investigate whether the decrease in intracellular NM levels observed in E2-treated rats was associated with reduced neurodegeneration, I performed stereological cell counts of SNpc TH-positive neurons for the different treatment groups (**Figure 31**a). In rats treated with E2, I observed a dose-dependent, non-significant trend towards increased DA cell survival compared to untreated rats, both in males and females (**Figure 31**b). Untreated AAV-hTyr-injected rats exhibited greater loss of SNpc TH-positive cells compared to previous experiments (*CHAPTER 2*: NM production and PD pathology in female and male NM-producing rats.), in which a different batch of AAV-hTyr was used. The extent of cell death induced by the new AAV-hTyr vector used in this particular experiment may preclude the observation of a significant protection by E2 treatment. In any case, NM-containing neurons from both males and females treated with 0.5 mg E2 exhibited less TH downregulation compared to their respective untreated controls, suggesting that remaining neurons from E2-treated animals might be better preserved functionally (**Figure 31**c). Consistent with the lack of significant neuroprotection in the SNpc of E2-treated AAV-hTyr-injected animals, the loss of striatal DA TH-positive fibers, as measured by optical densitometry, was similar between all experimental groups (**Figure 31**d). No significant sex differences were observed in relation to nigrostriatal degeneration among untreated or E2-treated animals (**Figure 31**b-d).

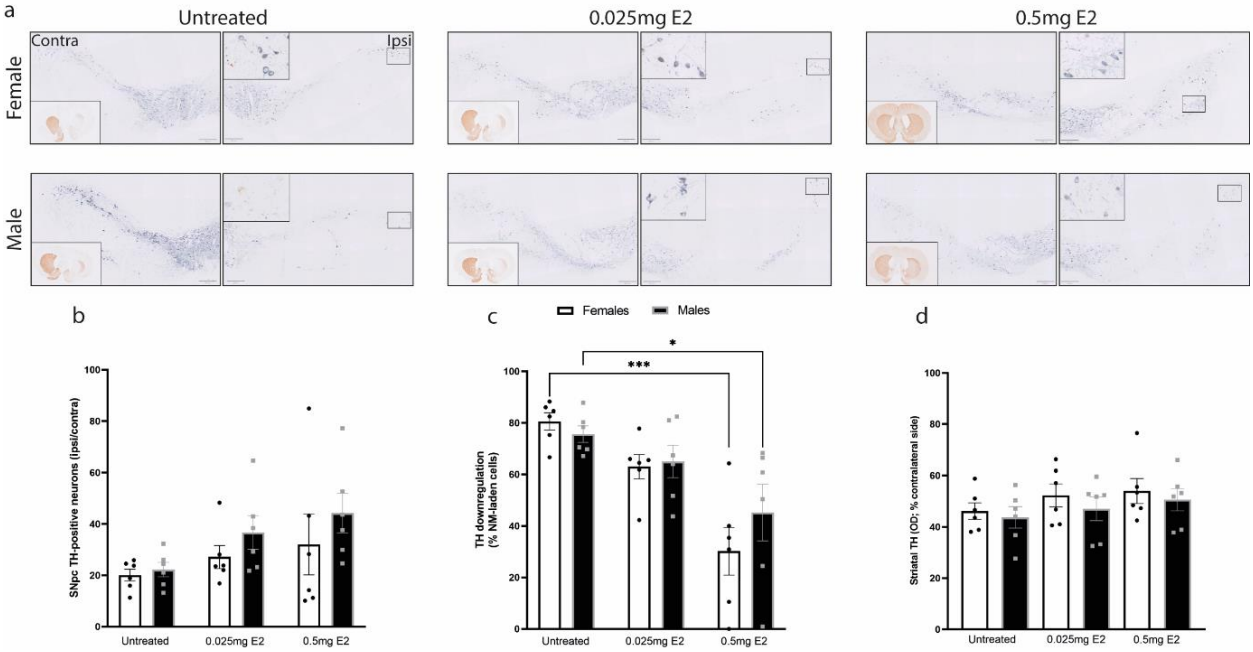

Figure 31 – Effects of E2 treatment on nigrostriatal neurodegeneration in AAV-hTyr-injected male and female rats. *a) Representative images of TH-immunostained SNpc and striatum (bottom inset) from male and female AAV-hTyr-injected rats treated or not with E2. b) Stereological cell counts of SNpc TH-positive neurons in AAV-hTyr-injected rats treated or not with E2. No statistically significant differences were observed (two-way ANOVA). c) Stereological assessment of TH downregulation within melanized neurons (TH−NM+ neurons vs total NM + neurons) in AAV-hTyr-injected male and female rats treated or not with E2. ***p=0.0002; *p=0.0254 (two-way ANOVA). d) Optical densitometry of striatal TH-positive fibers in AAV-hTyr-injected rats treated or not with E2. No statistically significant differences were observed (two-way ANOVA.) N=6 animals of each sex in each group.*

These results suggest that E2 treatment may help slow the progression of PD-linked neuronal dysfunction and degeneration when administered at early disease stages. In contrast, if neurodegeneration is too advanced, the hormonal treatment may not be sufficient to significantly mitigate the nigrostriatal damage.

1.1.1. E2 treatment is associated with a reduction in extracellular NM debris in AAV-hTyr-injected rats:

To assess whether the effects of E2 treatment on neurodegeneration were reflected in reduced extracellular NM released from dying neurons and on subsequent neuroinflammation, I quantified numbers of extracellular NM events and CD68-positive macrophages in these animals (**Figure 32**a,c). AAV-hTyr-injected male and female rats treated with 0.5 mg E2 showed a significant reduction in extracellular NM events compared to untreated animals and animals treated with 0.025 mg E2 (**Figure 32**b). In addition, male and female animals tended to show a similar dose-dependent decrease in the number of CD68-positive macrophages, although these changes were not statistically significant (**Figure 32**d).

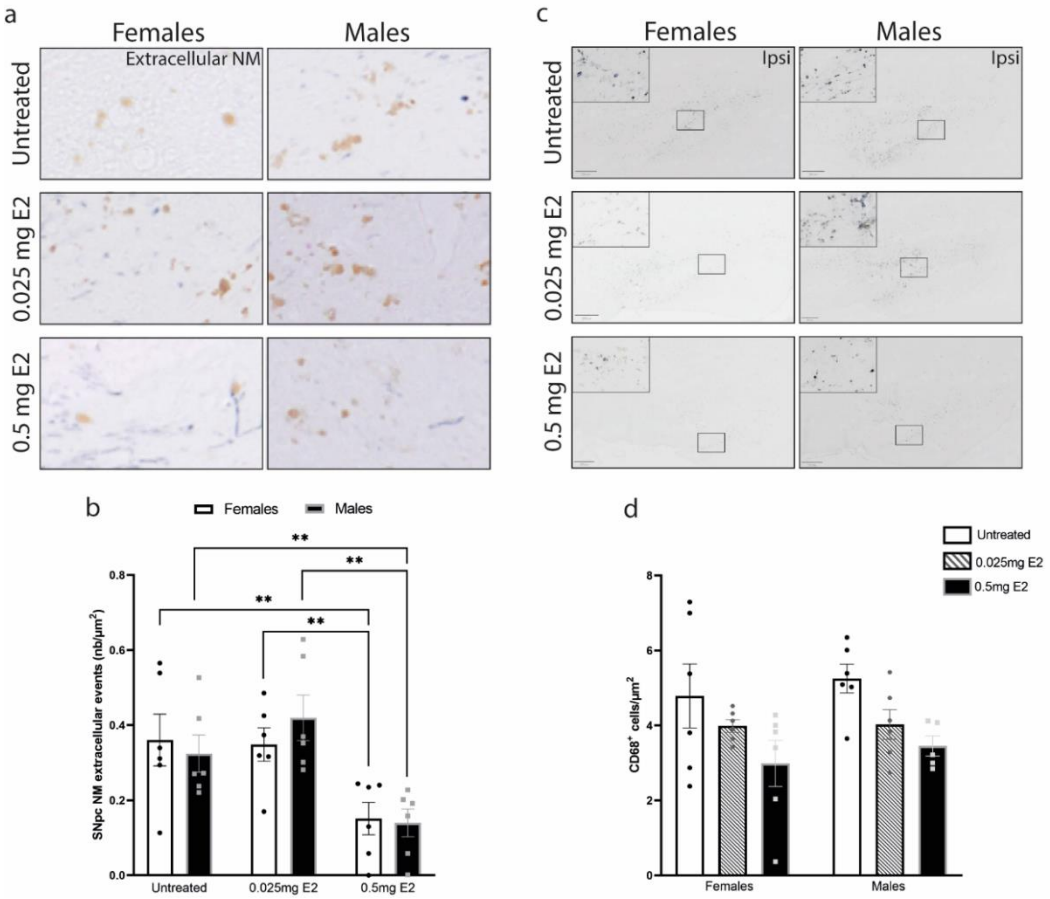

Figure 32 – Effects of E2 treatment on extracellular NM debris in the SNpc of AAV-hTyr-injected rats. *a) Representative images of SNpc sections immunostained for TH (in blue; unstained NM in brown). b) Quantification of SNpc extracellular NM debris in AAV-hTyr-injected rats, treated or not with E2. **p=0.0064. c) Representative images of SNpc sections immunostained for CD68 (in blue; unstained NM in brown). d) Quantification of SNpc CD68-positive cells in AAV-hTyr-injected rats, treated or not with E2. No statistically significant differences were observed (two-way ANOVA). N=6 animals of each sex in each group.*

Overall, E2 treatment in AAV-hTyr rats led to a reversal of motor deficits, reduced intracellular NM accumulation, decreased TH downregulation, increased survival of SNpc DA TH-positive neurons, and decreased extracellular NM debris. E2 treatment appears thus to delay/attenuate PD-related pathology when administered early in the disease course. However, its effectiveness may be limited once the pathology is already well-established.

DISCUSSION

While sex differences were previously described in PD, it was unclear whether these differences could be observed in NM accumulation. This present work present results suggesting that intracellular NM levels are sex-dependent and supports the need to separate human cases according to sex for future NM analysis. It also demonstrates that our experimental *in vivo* model of age-dependent intracellular human-like accumulation not only reproduces the progression of PD pathology but also reproduces the sex differences observed in humans. Furthermore, E2 treatment in these animals not only improves motor symptoms, as observed in humans, but it also appears to lower NM accumulation indicating a promising therapeutic approach to not only alleviate symptoms but potentially protecting DA cells from neuronal dysfunction if given at an early stage of the disease.

1. Sex-specific NM accumulation and PD pathology development in human females and males across lifespan.

This analysis of human data revealed several differences between males and females. In particular, that males have higher levels of intracellular NM than females, independently of their diagnostic status. Surprisingly, NM levels in healthy control males were already above the pathogenic threshold that we had previously defined for the initiation of dopaminergic cell dysfunction *(Carballo-Carbajal et al. 2019)*. This observation led to the hypothesis that males produce and accumulate more NM than females across life, which could be underlying the higher incidence of iPD reported in males. While the number of human cases would need to be increased, especially for younger age groups, the results presented in this work demonstrate that, indeed, males accumulate more NM than females as early as 15 years old. The mechanisms by which NM is produced in higher amounts in men still need to be clarified, i.e. higher brain tyrosinase expression, higher production of toxic DA metabolites, higher iron accumulation. Moreover, it has been reported that more than 52% of the population above 85 years present parkinsonian symptoms *(Bennett et al. 1996)*. Our results might have broader implications for the general population since the percentage of males considered healthy but with parkinsonian signs could be much higher than 52%, and/or the age at which healthy males present parkinsonian signs might be much earlier than previously defined.

Both sexes showed a significant loss of cells and a significant increase in TH downregulation in aged controls and especially in the iPD condition, as expected, but, while the cell death was significant in aged controls there were no significative difference between sexes in the iPD group. Interestingly, control males also showed higher number of LB in the SNpc compared to control females. These results favor the hypothesis that males tend to accumulate more NM and start presenting neuropathological events characteristic of the disease earlier than females. Therefore, these results suggest that PD develops in a different manner and has a different time-course in males than in females.

Overall, our results confirm that sex differences can play a very important role in the onset and progression of neurodegenerative pathologies like PD and strengthen the importance of carrying research in both males and females cohorts separately, instead of analyzing both sexes as a mixed group in translational biomedical research. Moreover, our results highlight the possibility that PD progression and the response to certain treatments in diagnosed females might differ from that of diagnosed males. However, as mentioned above, this work has been done using a limited number of cases, especially in younger age groups, so it would be necessary to repeat the analysis with more cases in order to further confirm the results. Furthermore, it would be compelling to also analyze data from patients having a genetic form of PD, such as LRRK2 or GBA mutations, which are the main causal mutation and the most common genetic risk factor for PD, respectively. Additionally, it could also be interesting to analyze preparkinsonian cases, such as idiopathic REM sleep behavior disorder (iRBD) patients, who represent a prodromal form of PD, and subjects with incidental Lewy bodies disease (iLBD), who are clinically healthy but display LB at neuropathological examination. For all these additional cases it would be compelling to compare the phenotype between both sexes to know if these particular subgroups also show differences between males and females, as we have shown for iPD.

Finally, as it appears that sex differences arise from difference in sex steroid levels bot endogenous and exogenous, it would be interesting to know whether these cases, especially female cases but not only, have received hormonal therapy during their life, as well as know the age of their menarche and menopause, to be able to analyze these cases accordingly and determine if these event also affect NM levels. Furthermore, as PD prevalence and incidence differ depending not only on sex but also on ethnicity and/or geography, it would be interesting to analyze post-mortem tissues of cases from different ethnicities within the same country, as well as from different countries. Indeed, same sex cases can have different sex steroid levels, notably E2, depending on their ethnicity and/or country of residence.

Moreover, females and males present differences in microbiota and, while PD females display differences in microbiota composition compared to healthy females, PD males and control males do not show significances in microbiota composition *(Cerri, Mus, and Blandini 2019)*. Sex hormones have been shown to affect gut microbiota composition, and gut microbiota regulates E2 levels *(Shin et al. 2019)*. There is a clear correlation described between E2 levels and gut microbiota richness and diversity, where the higher the E2 levels are, the higher richness *(Shin et al. 2019)*. As age and ethnicity appear to influence gut diversity and, as gut microbiota regulate E2 levels, it is possible to wonder if a combination of sex, age, ethnicity could influence gut microbiota composition which would, in turn impact NM accumulation.

Additionally, this work studied brain tissues from cisgender individuals but in the future, it would also be interesting to study transgender people in order to gain more knowledge about how both

endogenous sex levels and gender-affirming hormonal therapy could impact NM accumulation in both transwomen and transmen, in order to better design a potential hormonal therapeutic approach specific to each patient and/or person at risk for PD. Furthermore, as transgender people experience higher rates of depression and anxiety, which are both non-motor symptoms appearing in the prodromal stage of PD *(Poewe et al. 2017)*, and as it seems crucial to be able to diagnose PD as early as possible. Unfortunately, people from different ethnicities and LGBTQ+, especially transgender, patients, are still at risk for discrimination making their medical care more complex, and research lacking crucial information *("Parkinson's Disease in the LGBTQ+ Community: Three Things to Know" 2023; Scorza et al. 2023)*.

2. NM production and PD pathology in female and male NM-producing rats.

The conclusions from the human data analysis results were supported by the conclusions from our animal model. Indeed, even if our rat PD model did not display sex differences in behavioral motor alteration, an intracellular NM accumulation is observed in both sexes and, importantly, male rats presented an increase in intracellular NM accumulation compared to females reaching the pathogenic NM threshold as early as the pre-symptomatic stage while females reach it only in the established PD stage, which is analogous to the increased density of NM observed in human males. As in humans, once males and females reached the final established PD stage, the sex differences are lost.

Similarly, as in humans, a higher loss of striatal TH-positive terminals and of SNpc TH-positive cells, as well as an increased TH downregulation was observed within the injected side in males. This results confirm that our model reproduces the age-dependent PD progression but it also reproduces the sex differences described in humans, confirming it as a solid PD model as it reproduces PD features, such as slow progression, which are not found in other PD models. It also confirm the importance of using both males and females in all studies not to generate false conclusions, and also because studies in females might help to understand the physiological and molecular events underlying sex differences in human pathologies, despite the existing differences between the reproductive and hormonal systems of females rodents and humans.

However, the AAV-hTyr used in this work is different than the one in the original report of this model *(Carballo-Carbajal et al. 2019)* and animals appear to accumulate intracellular NM more slowly than in our original publication. As more sex differences are observed in the prodromal/early PD group but are lost in the established PD group, it would be interesting to establish more time-points in between in order to better study these differences across time. It could also be intriguing to repeat the experiment with, not only closer and more numerous time-points between the pre-symptomatic and the beginning of the established PD stage, but also with a different AAV-hTyr which would act slightly faster in order to better apprehend these sex differences.

3. Effect of sex-based manipulations on NM production and PD pathology in AAV-hTyr-injected rats.

3.1. OVX rats, a rodent model of menopause

The sex-related differences in NM levels observed in our prodromal/early PD rats appeared to be linked to gonadal hormones since OVX rats accumulate as much NM as their male counterparts and reach earlier the pathogenic NM threshold, compared to gonad-intact females. OVX AAV-hTyr rats at this stage also experience more neuroinflammation compared to the gonad-intact AAV-hTyr females. These observations suggest that surgical menopause in rats result in a more rapid progression of PD-associated pathology, as described in humans confirming, once more, that our rat model trusty option to study PD development and sex differences influencing it. However, these results present the same limitations described in the chapter, as the OVX were injected with the same AAV-hTyr as their gonad-intact female and male counterparts. Therefore, to study in more depth the slow progression and differences, it would be wise to reproduce the experiment with a vector inducing a faster NM accumulation with time-points closer to each other in the earlier stages.

3.2. Estradiol (E2) treatment

3.2.1. E2 treatment decreases intracellular NM levels *in vitro*

The results from NM-producing human catecholaminergic neuroblastoma SH-SY5Y cells inducible for hTyr expression, treated with E2, especially 100nM, show a decrease in intracellular NM levels, an increase of NM release into the medium as well as a reduced cell death. This suggest that, while E2 stimulates melanin production in the skin *(Natale et al. 2016)*, in neuronal cells, low doses of E2 appear to also increase NM production but high doses of E2 present drastic opposite results. It would be interesting to explore further if these observation are only dose-dependent or if they are also time-dependent. Indeed, it is possible to wonder if, even with high doses, cells do not, first experiment an increase in NM levels followed by an increase in NM release. This would imply that cells receiving a lower dose of E2 might need more to time to activate the NM release, therefore the neuroprotective effect on NM levels of E2 would be only visible with a higher dose. A time-lapse cell experiment could be an interesting option in order to determine if E2 might only activate NM production in low doses and stimulate NM release in high doses, or if E2 always triggers both but depending on the dosage, the time needed is different. E2 treatment in those cells also display a decrease in DA oxidation into NM precursors which suggest antioxidant properties in these cells. Overall, it could also be interesting to repeat the experiments with different doses to determine if it is indeed dose-dependent, and if so, which stimulates NM release, as well as decreases DA oxidation the most. As estrogen receptors, ERα, ERβ and GPR30, are present in these cells (**Figure 33**), it seems that E2 has a direct effect on these cells. It could also be interested to develop a time-lapse experiment observing these receptors activation in response to E2 treatment and determine if they are all needed for E2 neuroprotection or if they have different roles.

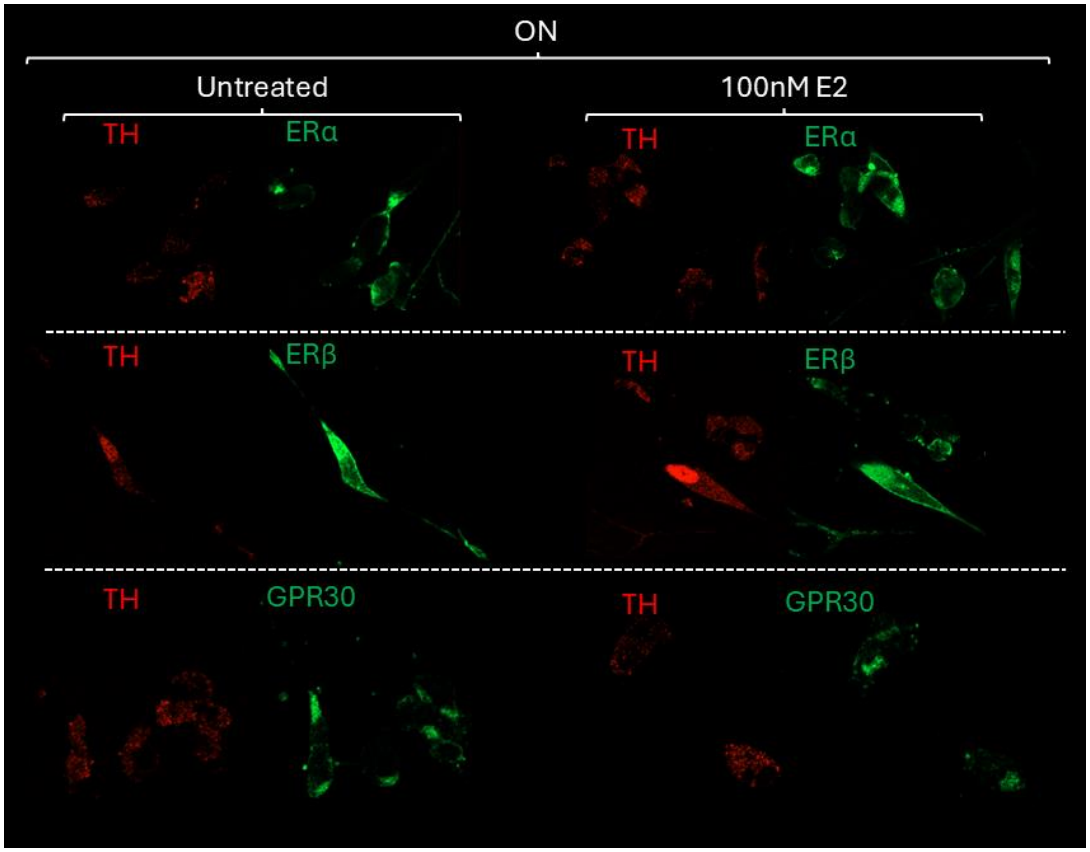

Figure 33 -– Estrogen receptors in NM-producing human catecholaminergic neuroblastoma SH-SY5Y cells inducible for hTyr expression. *Red = Tyrosine hydroxylase (TH)-positive cells and green = estrogen receptors, Erα : estrogen receptor α; Erβ : estrogen receptor β; GPR30: G-protein coupled estrogen receptor 1 also known as GPER1.*

3.2.2. E2 treatment decreases intracellular NM levels *in vivo*

The E2 treatment in our NM-producing AAV-hTyr-injected rats showed improved motor symptoms, as described in humans. The E2 treatment also reduced intracellular NM levels in a dose-dependent manner and attenuated NM-linked neuronal dysfunction (i.e. TH downregulation, motor asymmetry). While a dose-dependent attenuation of neurodegeneration was observed in E2-treated males, it is nonsignificant. As animals were injected with, another AAV-hTyr, different from both the one used in Chapter 2 of this work or in our previous publication *(Carballo-Carbajal et al. 2019)*, and as both males and females display a high cellular death, it appears that this vector induces a very rapid NM accumulation leading to a precocious neurodegeneration. Therefore, it would be compelling to repeat the experiment with yet another AAV-hTyr in order to induce a NM accumulation slightly faster than the one observed in Chapter 2 of this work but slower that the one studied. Also, the experiment were set in one time-point but it could intriguing to repeat them with different time-point within the pre-symptomatic and prodromal/early PD stages. It would also be interesting to determine the best moment to start E2 treatment, testing different times from before AAV-hTyr injection to early established PD stage. A late beginning in treatment could allow to establish if E2 could still help manage some PD-

associated symptoms in an established PD stage. E2 treatment in OVX AAV-hTyr injected rats appears crucial to determine if E2 has a different impact on those animals compared to gonad-intact females.

Preliminary results of E2 levels in the E2 treated AAV-hTyr injected rats, surprisingly suggest a dose-dependent decrease in serum E2 levels in both males and females (**Figure 34**a). Furthermore, AAV-hTyr males display higher E2 serum levels than AAV-hTyr females except in the higher dose of E2 treatment group, which also corresponds to the lower intracellular NM levels observed in these rats, where this sex difference is lost. These counterintuitive results are exploratory and the measurement of E2 levels needs to be repeated before confirming or affirming these observation, especially because the ELISA kit used was able to detect brain E2 levels in females but not in males as they were too low (**Figure 34**b). However, if these first observations are later confirmed, it would be possible to hypothesize that in a possible adaptative response to high NM accumulation, there is an increased dose-dependent estradiol mobilization to stimulate NM release. This is highly speculative and E2 levels in these animals need to be measured with more certainty.

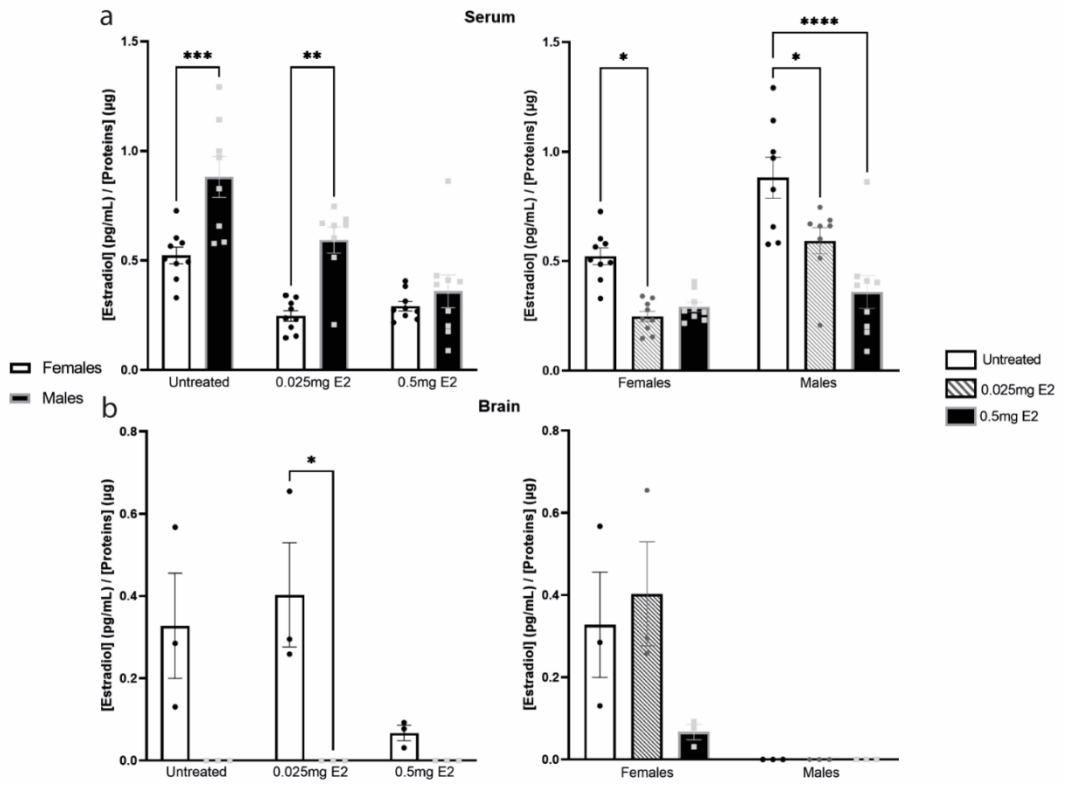

Figure 34 -– E2 levels decrease in a dose-dependent manner in AAV-hTyr injected rats. *a) E2 levels in serum. b) E2 levels in brain.*

As in the *in vitro* experiment, immunofluorescence of Erα, Erβ and GPR30 was done confirming the presence of these receptors in SNpc NM-containing TH-positive cells of these E2 treated AAV-hTyr males and females (**Figure 35**). These observations suggest that E2 could have a direct effect on these cells *in vivo*. It could be interesting to quantify the number and the optical density of each receptors to determine if there are differences between sexes and/or between E2 treated and untreated animals. It

would also be compelling to perform the immunofluorescence in human SNpc, in order to confirm the presence of these receptors, and complete similar quantify to know if potential differences observed in our rats are also potentially present in humans.

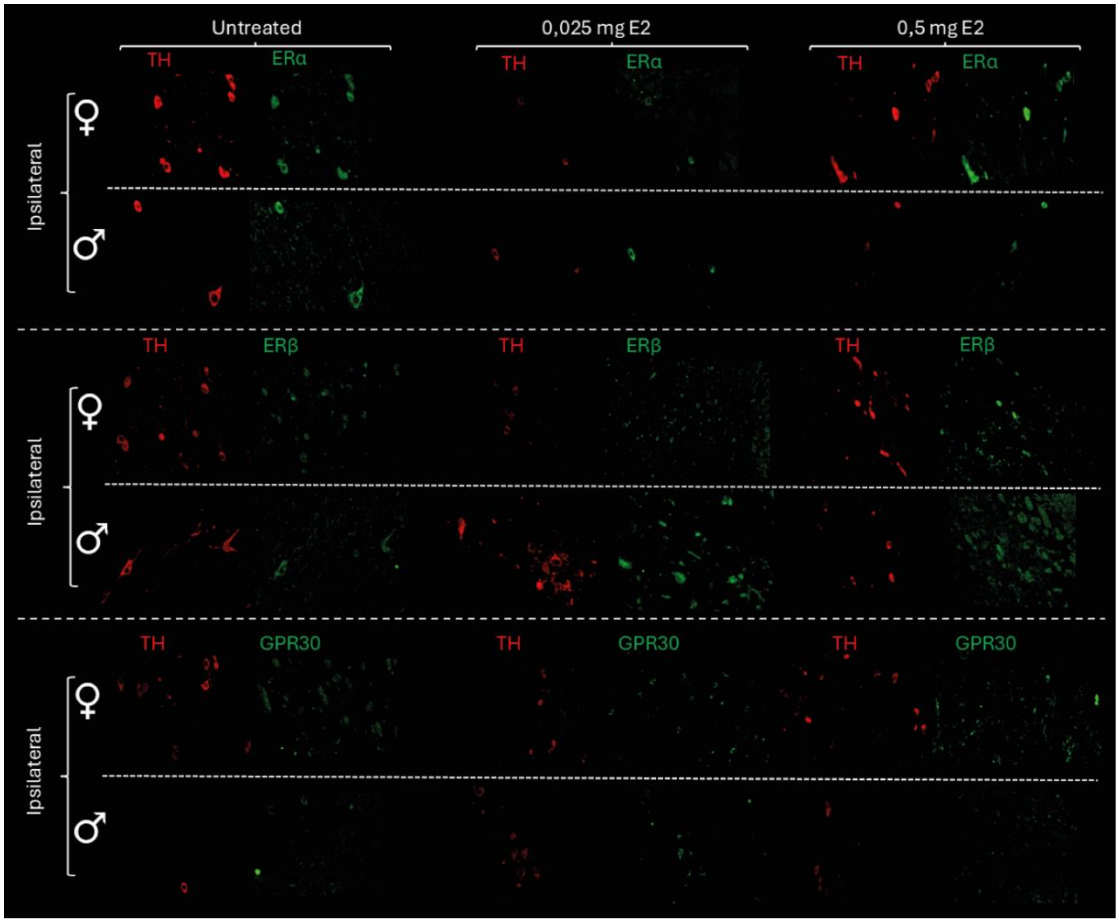

Figure 35 - Estrogen receptors in AAV-hTyr injected rats. *Red = Tyrosine hydroxylase (TH)-positive cells and green = estrogen receptors, Erα : estrogen receptor α; Erβ : estrogen receptor β; GPR30: G-protein coupled estrogen receptor 1 also known as GPER1.*

On the other hand, DHED (10β,17β-dihydroxyestra-1,4-dien-3-one), a small-molecule bio precursor prodrug that converts to E2 in the brain after systemic administration while remaining inert in the rest of the body, has been shown to provide neuroprotection in a rat stroke model, AD model and an αSyn-based transgenic mouse model of PD, as well as in neurological and psychiatric conditions developing from estrogen deficiency *(Katalin Prókai-Tátrai et al. 2019; Molly M Rajsombath et al. 2019; Tschiffely et al. 2018; Kapic et al. 2024; Prokai et al. 2015).* As DHED is brain selective, it does not have peripheral adverse side-effects of estradiol therapy. In addition, DHED is described as more potent to deliver E2 into the brain than direct administration of the hormone and has better physicochemical, pharmacological, and biopharmaceutical properties *(Prokai et al. 2015)* it could be interesting to repeat the experiments with this precursor. However, as, DHED is not found in the periphery, a reliable brain E2 level measurement technique is necessary to make sure that it is possible to verify the potential effects observed would be due to the treatment.

CONCLUSIONS

1. Chapter 1 – Aim 1

1. PD patients accumulate more intracellular NM than age-matched controls, they also display a higher percentage of cell area occupied by NM.
2. In both groups, controls and PD, males present a higher intracellular NM accumulation than females.
3. In healthy controls, males accumulate more NM with age than females and reach earlier the pathogenic threshold of NM accumulation even in the absence of PD. They also have an increased percentage of cell area occupied NM, and present more extracellular NM events.
4. NM-filled nigral neurons from apparently healthy elderly males exhibit early signs of dysfunction/degeneration.
5. Healthy males start to accumulate more intracellular NM, as well as have a higher percentage of cell area occupied by NM as early as 15 years old, compared to females.
6. From 31 years old, healthy females present constant intracellular NM levels while males present an age-dependent increase, followed by a decrease from 71 years old.
7. At 31-50 years old, healthy males experience more TH downregulation than females.

2. Chapter 2 – Aim 2

1. Our AAV-hTyr rat model reproduces the age-dependent NM accumulation, as well as the motor symptoms and the neuronal dysfunction associated with PD.
2. In AAV-hTyr-injected rats, males accumulate more NM over time, particularly at early disease stages (i.e. pre-symptomatic/prodromal), and reach earlier the NM pathogenic threshold compared to females, reproducing the sex differences observed in humans. They also have a greater percentage of cell area occupied by NM, compared to females.
3. In the prodromal stage, AAV-hTyr males experience more SNpc TH-positive cell death and TH downregulation, as well as a greater loss of nigrostriatal DA TH-positive neurons.

3. Chapter 3 – Aim 3

1. Sex-related differences in NM levels may be linked to sex hormones since ovariectomized female rats accumulate as much NM as their male counterparts and reach earlier the pathogenic NM threshold, compared to female animals. They also display a higher percentage of cell area occupied by NM.
2. OVX AAV-hTyr rats also experience more neuroinflammation compared to the gonad-intact AAV-hTyr females.
3. In NM-producing human catecholaminergic neuroblastoma SH-SY5Y cells inducible for hTyr expression, treatment with E2, especially 100nM, decreases intracellular NM levels and reduces cell death.

4. E2 appears to decrease the oxidation of DA into NM precursors and therefore has antioxidant properties in these cells.
5. In NM-producing AAV-hTyr-injected rats, treatment with E2 improved motor symptoms.
6. In these animals, E2 treatment reduced intracellular NM levels in a dose-dependent manner and attenuated NM-linked neuronal dysfunction (i.e. TH downregulation, motor asymmetry).
7. A nonsignificant dose-dependent attenuation of neurodegeneration was observed in E2-treated AAV-hTyr-injected NM-producing rats, especially in male animals.
8. E2 treatment also led to the decrease of extracellular NM debris.
9. Overall, in these animals, E2 treatment appear to delay/attenuate PD-related pathology when administered early in the disease progression.

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ANNEX

Cases	Sex	Age (y)	Group	Clinical diagnostic	Brain region
Control-1	Male	5	5-10y	Control (Hemorrhage)	SNpc
Control-2	Male	5		Control (Ischemia)	SNpc
Control-3	Female	5		Control (Hemorrhage)	SNpc
Control-4	Male	6		Control (Diphtheria)	SNpc
Control-5	Female	9		Control (Toxoplasmosis)	SNpc
Control-6	Female	10		Control	SNpc
Control-7	Female	15	15-20y	Control	SNpc
Control-8	Female	16		Control (abscess, hemorrhage)	SNpc
Control-9	Male	17		Control	SNpc
Control-10	Male	18		Control (Lymphoma B)	SNpc
Control-11	Male	18		Control	SNpc
Control-12	Female	19		Control	SNpc
Control-13	Male	31	31-50y	Control	SNpc
Control-14	Female	35		Control	SNpc
Control-15	Male	35		Control	SNpc
Control-16	Male	36		Control	SNpc
Control-17	Female	37		Control	SNpc
Control-18	Male	37		Control	SNpc
Control-19	Male	38		Control	SNpc
Control-20	Female	38		Control	SNpc
Control-21	Female	39		Control	SNpc
Control-22	Male	40		Control	SNpc
Control-23	Male	41		Control	SNpc
Control-24	Female	42		Control	SNpc
Control-25	Female	43		Control	SNpc
Control-26	Male	43		Control	SNpc
Control-27	Male	43		Control (Tau en LC)	SNpc
Control-28	Male	43		Control	SNpc
Control-29	Male	44		Control	SNpc
Control-30	Female	44		Control	SNpc
Control-31	Male	47		Control	SNpc
Control-32	Female	47		Control	SNpc
Control-33	Female	48		Control	SNpc
Control-34	Female	49		Control	SNpc
Control-35	Male	50		Control	SNpc
Control-36	Female	50		Control	SNpc
Control-37	Female	52	51-70y	Control	SNpc
Control-38	Male	52		Control (SN hypopigmented)	SNpc
Control-39	Female	52		Control	SNpc
Control-40	Male	53		Control	SNpc
Control-41	Male	53		Control	SNpc
Control-42	Female	54		Control	SNpc
Control-43	Female	54		Control	SNpc
Control-44	Male	54		Control	SNpc
Control-45	Female	56		Control	SNpc

Control-46	Male	56		Control	SNpc
Control-47	Male	57		Control (Ischemia)	SNpc
Control-48	Male	57		Control	SNpc
Control-49	Female	58		Control	SNpc
Control-50	Female	58		Control	SNpc
Control-51	Male	58		Control	SNpc
Control-52	Female	59		Control	SNpc
Control-53	Male	59		Control	SNpc
Control-54	Male	59		Control	SNpc
Control-55	Male	60		Control	SNpc
Control-56	Female	60		Control	SNpc
Control-57	Female	61		Control	SNpc
Control-58	Female	62		Control	SNpc
Control-59	Male	63		Control	SNpc
Control-60	Male	63		Control	SNpc
Control-61	Female	65		Control	SNpc
Control-62	Male	65		Control	SNpc
Control-63	Female	66		Control	SNpc
Control-64	Male	69		Control	SNpc
Control-65	Female	70		Control	SNpc
Control-66	Female	70		Control	SNpc
Control-67	Female	71	71-90y	Control	SNpc
Control-68	Female	73		Control	SNpc
Control-69	Female	74		Control	SNpc
Control-70	Male	76		Control	SNpc
Control-71	Male	78		Control	SNpc
Control-72	Female	79		Control	SNpc
Control-73	Female	79		Control	SNpc
Control-74	Male	81		Control	SNpc
Control-75	Male	83		Control	SNpc
Control-76	Male	84		Control	SNpc
Control-77	Female	83		Control	SNpc
Control-78	Female	83		Control	SNpc
Control-79	Female	86		Control (Ictus)	SNpc
Control-80	Female	90		Control (Hemorrhage)	SNpc
Control-81	Female	91		Control	SNpc
Control-82	Male	91		Control	SNpc
Control-83	Female	94		Control	SNpc

Table 4 - Controls post-mortem sample information (Chapter 1).

Cases	Sex	Age (y)	Clinical diagnostic	Brain regions
PD-1	Male	76	iPD Braak 5 LBD	SNpc
PD-2	Male	77	iPD Braak 5 LBD	SNpc
PD-3	Male	77	iPD Braak 4/5	SNpc
PD-4	Male	80	iPD Braak 6	SNpc
PD-5	Male	81	iPD Braak 5/6 LBD	SNpc
PD-6	Male	83	iPD Braak 5	SNpc
PD-7	Male	72	iPD Braak 5 LBD	SNpc
PD-8	Male	78	iPD Braak 4/5 LBD	SNpc
PD-9	Male	76	iPD Braak 4/5 LBD	SNpc
PD-10	Male	73	iPD Braak 4/5	SNpc
PD-11	Male	70	iPD Braak 4 LBD	SNpc
PD-12	Male	68	iPD Braak 4	SNpc
PD-13	Male	74	iPD Braak 5	SNpc
PD-14	Male	81	iPD Braak 4/5	SNpc
PD-15	Male	92	iPD Braak 4/5	SNpc
PD-16	Male	83	iPD Braak 4	SNpc
PD-17	Male	65	iPD Braak 4	SNpc
PD-18	Female	77	iPD Braak 5	SNpc
PD-19	Female	81	iPD Braak 5	SNpc
PD-20	Female	88	iPD Braak 4	SNpc
PD-21	Female	85	iPD Braak 5 LBD	SNpc
PD-22	Female	88	iPD Braak 5 LBD	SNpc
PD-23	Female	84	iPD Braak 4	SNpc
PD-24	Female	83	iPD Braak 4	SNpc
PD-25	Female	81	iPD Braak 5 LBD	SNpc
PD-26	Female	81	iPD Braak 4	SNpc
PD-27	Female	81	iPD Braak 4	SNpc
PD-28	Female	79	iPD Braak 4	SNpc
PD-29	Female	69	iPD Braak 4	SNpc
PD-30	Female	69	iPD Braak 4	SNpc

Table 5 - PD post-mortem sample information (Chapter 1).