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ELLAGIC ACID INHIBITS α-SYNUCLEIN PROPAGATION AND ALLEVIATES THE NEUROTOXICITY BY ENHANCING AUTOPHAGIC FLUX IN ANIMAL MODEL OF PARKINSON'S DISEASE

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College of Medicine and Health Sciences

Department of Biochemistry and Molecular Biology

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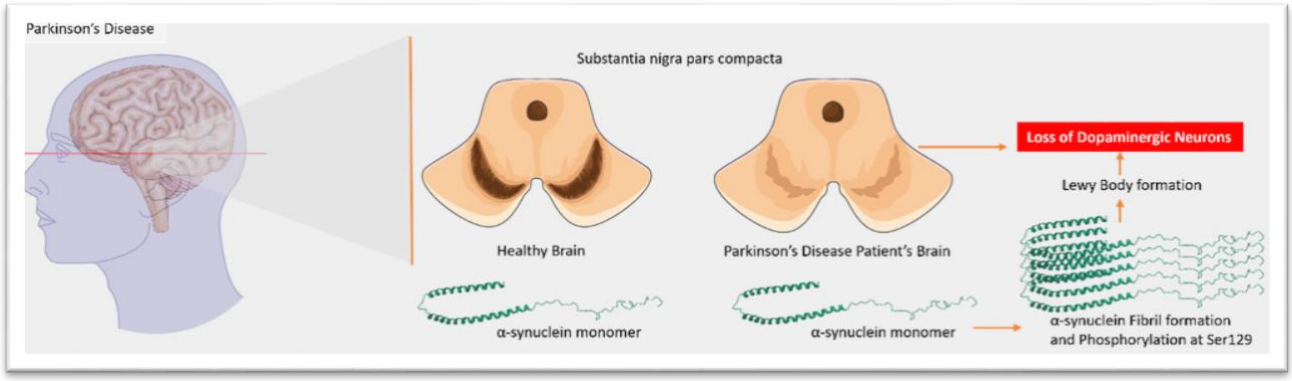

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Cover: The Role of α-synuclein in the progression of Parkinson's Disease

(Photo: By Nada Akram Abdellatif Radwan)

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Declaration of Original Work

I, Nada Akram Abdellatif Radwan, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Ellagic Acid Inhibits α-Synuclein Propagation and Alleviates the Neurotoxicity by Enhancing Autophagic Flux in Animal Model of Parkinson's Disease*", hereby, solemnly declare that this is the original research work done by me under the supervision of Dr. Md Emdadul Haque, in the College of Medicine and Health Sciences at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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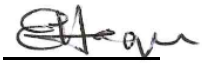
   Signature  Date: April 2, 2024

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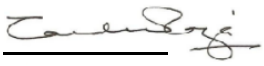
   Signature  Date: April 2, 2024

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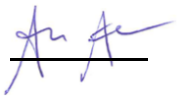
   Signature:  Date: April 2, 2024

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Abstract

Parkinson's Disease (PD) is the second most common neurological disorder pathologically characterized by loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNc) as well as the formation of Lewy bodies composed mainly of α-synuclein (α-syn) aggregates. It has been documented that abnormal aggregation of α-syn is one of the major causes of developing PD. Ellagic Acid (EA) is a widely known dietary supplement found in pomegranates as well as a plethora of berries and nuts. EA has been shown to prevent α-syn aggregates' toxicity in cellular model of PD. In the current study we aim to evaluate whether EA can prevent α-syn spreading and associated toxicity in an animal model of PD.

C57BL/6 male mice were subjected to intrastriatal injections of α-syn Preformed Fibrils (PFF). 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP) (10 mg/kg/b.wt) injections were administered intraperitonially on the 6th week following the stereotaxic surgery for five consecutive days. EA treated animals were injected intraperitonially with EA (10 mg/kg/b.wt) for twelve consecutive days (starting from the day following the stereotaxic surgery). Adjunct EA administration (IP 10 mg/kg/b.wt) with MPTP (EA administration one hour prior to MPTP) continued through the 6th week (seven days). Animals were sacrificed by the end of the 8th week, and brain samples were processed for immunohistochemical analysis. Additionally, an oral route of administration of EA was investigated in this study at a dose of 20 mg/kg/b.wt under the same premises of EA IP administration.

We observed that intraperitoneal administration of EA significantly decreased the spreading of endogenous α-syn from the site of injection (striatum) to the SNc area when compared with the group receiving PFF + MPTP. We also found that EA prevented dopaminergic neuronal loss in the SNc area and nerve terminal density loss in the striatum. We also performed TUNEL assay to evaluate the apoptotic neurons in the SNc which was significantly lower in EA treated group consistent with α-syn spreading and toxicity. Furthermore, the autophagy markers were assessed in TH expressing cells in the SNc and our data suggests that EA enhanced the autophagic flux in animal model of PD. The efficiency of the oral administration of EA retained significance in decreasing the pS129

α-syn spreading but was radically decreased in terms of protection against dopaminergic neuronal loss. This could be attributed to the lower bioavailability of EA upon oral administration. This warrants further study related to oral administration.

The present study suggests the involvement of a restorative mechanism of autophagic flux conferred by EA treatment which prevents toxic α-syn spreading as well as preserves dopaminergic neurons in male mouse PD model. The ameliorative effect of EA in the current PD mouse model was also challenged upon oral administration which needs improvement of oral delivery of EA.

Keywords: α-Synuclein, Autophagy, Ellagic Acid, Parkinson's Disease, PD Mouse Model.

Title and Abstract (in Arabic)

حمض الإيلاجيك يحمي من انتشار بروتين الألفا-سينوكلين ويقلل من سميته عن طريق تحسين آلية الاوتوفاجي في نموذج حيواني (في الفئران) لمرض الباركنسون

الملخص

يحتل مرض الباركنسون المركز الثاني في الامراض العصبية الأكثر شيوعا، ويتصف هذا المرض بانحسار اعداد الاعصاب المسؤولة عن انتاج الدوبامين (Dopamine) في الحيّز الدماغي المسؤول عن التحكم في الحركة الإرادية (الSNc) بالإضافة الى تكون أجسام ليوي المكونة بشكل رئيسي من ألياف الألفا-سينوكلين.

حمض الإيلاجيك (EA) هو مكمل غذائي معروف على نطاق واسع ويتواجد في ثمرة الرمان بالإضافة إلى عدد كبير من التوتيات والمكسرات. لقد أُثبت حمض الإيلاجيك يمنع تراكم ألفا-سينوكلين في النموذج الخلوي لمرض الباركنسون. تهدف هذه الدراسة إلى تقييم قدرة حمض الإيلاجيك في منع انتشار ألفا-سينوكلين والسمية المرتبطة به في نموذج حيواني للمرض.

تم تعريض الفئران (ذكور من نوع C57BL/6) للحقن بألياف الألفا-سينوكلين داخل الأنسجة الدماغية في منطقة الستراياتم او الجسم المخطط الدماغي (striatum) المحتوية على نهايات الاعصاب الدوبامينية من ال SNc مما يشكل بذرة انتشار الياف الألفا-سينوكلين. بعد الجراحة، تم حقن الحيوانات بجرعة واحدة من حمض الإيلاجيك في اليوم داخل الغشاء البريتوني (بجرعة 10 ملغم/كغم وزن الجسم) لمدة 12 يوماً. تم إعطاء جرعة منخفضة من حقن ال1-ميثيل-4-فينيل-1،2،3،6-رباعي هيدروبيريدين (MPTP) (10 ملغم/كغم وزن الجسم) داخل الغشاء البريتوني لمدة 5 ايام في الأسبوع السادس بعد الجراحة. تلقت بعض الفئران عقار حمض الإيلاجيك (بجرعة 10 ملغم/كغم وزن الجسم داخل الغشاء البريتوني قبل ساعة من اعطاء حقن الMPTP) لتقيم مدى قدرة العقار على مكافحة سُمِّية الألفا-سينوكلين. تم التضحية بالحيوانات في الأسبوع الثامن من الجراحة، وتمت معالجة عينات المخ للتحليل الكيميائي المناعي. بالإضافة إلى هذا النموذج، تم إجراء نموذج آخر (مطابق للنموذج السابق) لتقييم فعالية حمض الإيلاجيك عن طريق الفم (جرعة 20 ملغم/كغم وزن الجسم).

لاحظنا أن حمض الإيلاجيك قلل بشكل كبير من انتشار الألفا-سينوكلين الداخلي من موقع الحقن (الجسم المخطط) إلى منطقة SNc بالمقارنة مع المجموعة التي تلقت الياف الألفا -سينوكلين وعقار الMPTP. ووجدنا أيضًا أن حمض الإيلاجيك منع فقدان الخلايا العصبية الدوبامينية في منطقة SNc وفقدان الكثافة العصبية في الجسم المخطط. أجرينا أيضًا اختبار لتقييم الخلايا العصبية المبرمجة للموت في SNc والتي كانت أقل بشكل ملحوظ في المجموعة المعالجة بحمض الإيلاجيك بما يتوافق مع انحسار انتشار الألفا-سينوكلين وسميته. علاوة على ذلك، تم تقييم علامات آلية الاوتوفاجي (أو الالتهام الذاتي) في الخلايا الدوبامينية في الSNc وتشير البيانات الأولية إلى أن حمض الإيلاجيك عزز من آلية الاوتوفاجي في هذا النموذج لمرض الباركنسون. احتفظت كفاءة تناول حمض الإيلاجيك عن طريق الفم

بخاصـية تقليل انتشـار pS129 ألفا-سـينوكلين في ما انخفضـت بشـكل جذري من حيث الحماية ضـد فقدان الخلايا العصبية المنتجة للدوبامين.

تقترح هذه الدراســة تعزيز حمض الإيلاجيك لآلية الالتهام الذاتي والتي تمنع انتشــار الألفا-ســينوكلين الســام وكذلك الخلايا العصبية الدوبامينية في نموذج مرض باركنسون في الفئران. التأثير التحسيني لـــحمض الإيلاجيك في نموذج في الفئران مرض باركنســون لوحِظ بجرعة 10 ملغم/كغم وزن الجســم داخل الغشــاء البريتوني، وعند تناوله عن طريق الفم انحسر هذا التأثير بشكل جذري.

مفاهيم البحث الرئيسية: مرض باركنسون، حمض الإيلاجيك، نموذج مرض باركنسون في الفئران، الالتهام الذاتي، ألفا-سينوكلين.

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This thesis is a culmination of the collective support I have received, and I am truly thankful for the collaborative efforts that have made this achievement possible.

Dedication

To my family, my love and pride.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

6-OHDA	6-Hydroxydopamine
AMPK	AMP-Activated Protein Kinase
ATG	Autophagy Related Genes
BBB	Blood Brain Barrier
CK2	Casein Kinase 2
CMA	Chaperone-Mediated Autophagy
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
COX-2	Cyclooxygenase-2
CYP450	Cytochrome P450
DALYs	Disability Adjusted Life Years
DAT	Dopamine Transporter
DMSO	Dimethyl Sulfoxide
DOPAL	3,4-Dihydroxyphenylacetaldehyde
EA	Ellagic Acid
FIP200	FAK Family-Interacting Protein Of 200 kDa
FPLC	Fast Performance Liquid Chromatography
GRK5	G Protein-Coupled Receptor Kinase
HORMA	Hop1p/ Rev7p/Mad2
HSC70	Heat Shock Cognate Protein
IF	Immunofluorescence
IHC	Immunohistochemistry
iNOS	Inducible Nitric Oxide Synthase
IP	Intraperitoneal

IPGT	Isopropyl B-D-1-Thiogalactopyranoside
LAMP2A	Lysosome-Associated Membrane Protein 2
LB	Lysogeny Broth
LBs	Lewy Bodies
LC3	Microtubule-Associated Protein 1A/1B-Light Chain 3
L-Dopa	Levo-Dihydroxyphenylalanine
MAO	Monoamine Oxidase
MPDP+	1-Methyl-4-Phenyl-2,3-Dihydropyridinium
MPP+	1-Methyl-4-Phenylpyridinium
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
mTOR	Mammalian Target of Rapamycin
NAC	Non-Amyloid-β Component
Nrf2	Nuclear Factor Erythroid 2–Related Factor 2
OD	Optical Density
OMM	Outer Mitochondrial Membrane
p62	P62/Sequestosome 1
PBS	Phosphate Buffered Saline
PD	Parkinson’s Disease
PE	Phosphatidylethanolamine
PFF	α-syn Preformed Fibrils
PI3K	Phosphoinositide 3-Kinase
PINK1	Pten Induced Kinase 1
PK	Proteinase K
PLK2	Polo-Like Kinase 2
pS129 α-syn	α-syn Phosphorylated At Ser129
RNS	Reactive Nitrogen Species

ROS	Reactive Oxygen Species
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SNc	Substantia Nigra pars compacta
TFEB	Transcription Factor EB
TH	Tyrosine Hydroxylase
TUNEL	Terminal Deoxynucleotidyl Transferase Dutp Nick End Labeling
ULK1	Unc-51 Like Autophagy Activating Kinase 1
V	Vehicle
VDAC1	Votage Dependent Anion Channel 1
α-syn	Alpha-Synuclein

Chapter 1: Introduction

1.1 Relevant Literature

1.1.1 Parkinson's Disease (PD) Prevalence

According to the Global Burden of Disease study (Vos et al., 2020), neurological diseases are the second leading cause of disability worldwide. In spite of the decreased incidence in communicable neurological disorders, the absolute number of deaths resulting from neurological disorders had increased by 39% in the span of the past 30 years, which could be attributed to the increased incidence in noncommunicable neurological disorders (James et al., 2018). The Disability Adjusted Life Years (DALYs); an estimate of years lived with disability (Nord, 2014), measured in this study further details a large increase amongst patients suffering from neurological diseases of a degenerative nature (James et al., 2018). The scientific advances aided in the discovery of novel molecular mechanisms and cellular pathways that play a substantial role in disease progression, in many neurodegenerative diseases. However, the progress of therapeutics' development is yet far from effective in light of growing rates of aging populations.

Parkinson's Disease (PD) is the second most common neurodegenerative disorder, and the tenth ranking neurological disorder in the 2019 Global Burden of Disease study (Vos et al., 2020). PD was first reported in 1817 by James Parkinson in the infamous "An Essay on Shaking Palsy" capturing the clinical picture of the disease through his observation of six case studies (Parkinson, 1817), the rare disorder at that time that it escaped nosologists' classification was referred to by Dorsey et al. in 2018 as a "pandemic" (Dorsey et al., 2018). In his publication, Doresy rationalizes this terminology by using some characteristic patterns of pandemics, the first being the area of the afflicted population extending throughout different regions and ethnicities. The second is the migratory trait of PD imposed by the change in the demographics of aging population as well as industrialization. Whether the appropriation of referring to the PD spread as a pandemic remains in question, it is an undoubtedly a widespread disease with detrimental effects.

1.1.2 The Neuropathology of PD

1.1.2.1 Motor vs Non-Motor Symptoms of PD

PD is a neurodegenerative disorder which involves diminished production of dopamine. Dopaminergic neurons in the basal ganglia particularly in the Substantia Nigra pars compacta (SNc) extend to the dorsal striatum, which is referred to collectively as the nigrostriatal dopaminergic pathway, responsible mainly in the facilitation of motor function and coordination. The damage to these dopaminergic neurons is the pathological hallmark of PD and was thought to occur in the early stages of the disease.

Although genetic predisposition plays a role in the development of familial form of PD, sporadic PD cases make up the majority of the PD patient population. Clinical manifestations of PD arise from the decreased dopamine levels which presents the typical parkinsonian symptoms such as tremors, rigidity, muscle stiffness and impairment in coordinated movements. Neuropathological staging based on postpartum PD patients reported by Braak displayed a relatively uniform disease progression pattern (Braak et al., 2003), the initial stages involve alterations in the vagus and olfactory nerve structures followed by worsened lesions in the motor nucleus and Lewy bodies in the locus coeruleus. The following stage marks the damage to the Substantia Nigra highlighting the relative latent involvement of the damage in dopaminergic neurons (the primary cells afflicted in PD patients). Final stages of PD display lesions in the cortex and adjoining temporal neocortical fields.

Non-motor symptoms in the early stages of PD have been reported by PD patients such as depression (van der Hoek et al., 2011), urinary urgency and gastrointestinal disturbances (Sakakibara et al., 2008) which occurs years prior to the development of motor symptoms. The onset of motor symptoms are estimated to manifest at 60-70% loss of SNc neuronal density (Cheng et al., 2010). Although evidence suggest neurodegeneration in PD patients begins in the Dorsal motor nucleus of the vagus and olfactory bulb, the neuropathology of PD has been widely accepted to occur due to the disruption of the nigrostriatal pathway, hence this study will be primarily focused on the dopaminergic cell bodies residing in the SNc and their axonal terminals in the striatum.

1.1.2.2 Lewy Bodies and α-Synuclein

The development of Lewy Bodies (LBs) is the second hallmark of PD. LBs are insoluble inclusions found in various compartments of PD patients' brain and are closely representative of the disease progression (Jellinger, 2009; Spillantini et al., 1997). These inclusions are predominantly composed of alpha-synuclein (α-syn), a 140 amino acid presynaptic protein encoded by SNCA gene, that is hypothesized to function in the regulation of neurotransmitters' release (Bendor et al., 2013). Point mutations of α-syn have been strongly associated with familial PD in an autosomal dominant manner. Despite the fact that these point mutations account for a small fraction of PD cases, implication of α-syn in the neuropathology of sporadic PD cases suggests a causative role of α-syn in PD.

1.1.2.3 α-Syn Protein Structure

While α-syn is innately a soluble protein, insoluble aggregates of α-syn fibrils have been identified as a pathological feature of many neurodegenerative disorders. α-syn primary structure could be divided into 3 sections as represented in Figure 1; the N-terminus domain, Non-Amyloid-β Component (NAC) domain and the C-terminus domain.

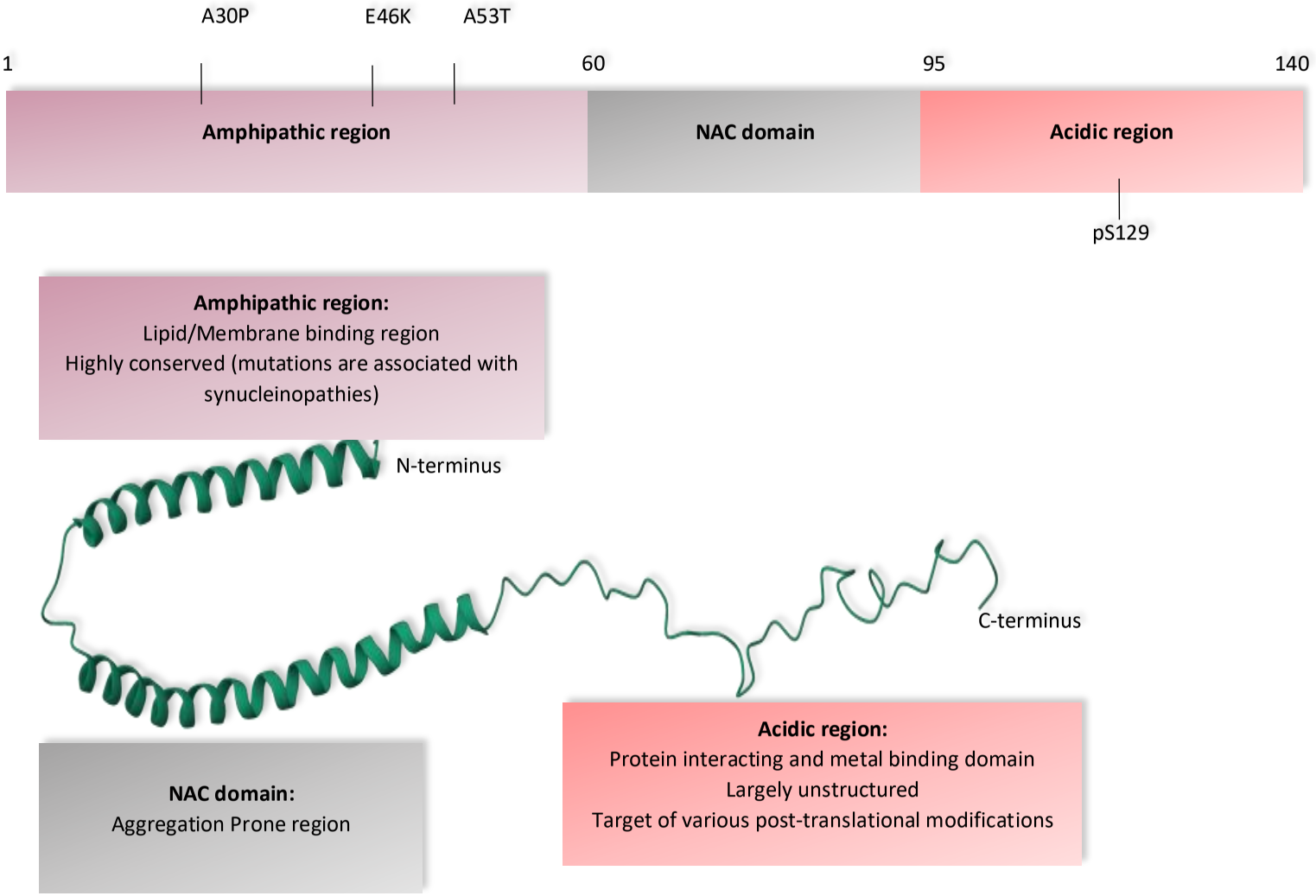

Figure 1: α-Syn Structure

Firstly, the N-terminus domain (also known as the membrane binding domain) is a highly preserved domain across species as well as other isoforms of synuclein protein. This domain (residues 1-60) composes the 2 helices which are separated by a KTEGF motif. The amphipathic nature of this domain aids in the α-syn attachment to cellular phospholipid bilayer structure. It is important to note that major mutations to this domain (A30P, E46K, H50Q, G51D, A53T, and A53E) (Ghosh et al., 2014; Hayakawa et al., 2020; Khalaf et al., 2014; Sakai et al., 2019; Szego et al., 2012) have all been associated to familial PD cases. Furthermore, the N-terminus domain contains a series of threonines preserved at residues 2-5 and 7 that are associated with the attachment of the α-syn protein to the highly curved nature of presynaptic boutons where it is predicted to function in the regulation of neurotransmitter release.

Secondly, the NAC domain is a highly hydrophobic region (residues 61-95) and functions in the α-syn protein aggregation and lipid binding. Posttranslational modifications within this domain includes the phosphorylation at Ser87 which is associated with misfolding, aggregation and fibrillogenesis of α-syn (Sonustun et al., 2022).

Finally, the C-terminus domain (residues 96-140) contains a large percentage of charged residues. The intensely anionic feature of this domain renders it as the least conserved domain amongst synuclein isoforms as well as through different species. This domain is also known as the calcium binding domain and plays a role in the attachment of α-syn to the presynaptic terminal. Phosphorylation occurs at multiple sites of this domain, the most acknowledged being at Ser129 (pS129). pS129 form of α-syn normally occurs at the rate of 4%, but under pathological conditions constitutes up to 90% of aggregated α-syn in the LBs inclusions. The presence of pS129 state amongst α-syn species in neurons is tightly controlled (Waxman & Giasson, 2008). Although the identification of the phosphorylation trigger in pathological context have not been identified, several kinases such as Polo-Like Kinase 2 (PLK2) (Inglis et al., 2009), G protein-coupled Receptor Kinase (GRK5) (Liu et al., 2010), Casein Kinase 2 (CK2) (Castello et al., 2017; Yu et al., 2022) act directly in the development of pS129 α-syn species.

While many researchers debate the protective mechanism pS129 confers over other post-translational modification α-syn induced cytotoxicity (Oueslati, 2016) and its rescuing mechanism of dopamine levels through increased reuptake without alteration of synaptic dopamine transporters (Hara et al., 2013), the implication of pS129 α-syn species in the pathology of many synucleopathies including PD remains undisputed.

1.1.2.4 α-Synuclein Fibrils and Neurotoxicity

Misfolding of α-syn monomers results in the formation of oligomers which further aggregate into α-syn fibrils. α-syn fibrils formation occurs in a nucleation dependent manner which could be roughly divided into 3 stages: lag phase, elongation phase and stationary phase (Villar-Piqué et al., 2016). Figure 2 (Mehra et al., 2021), illustrates the α-syn aggregation pattern.

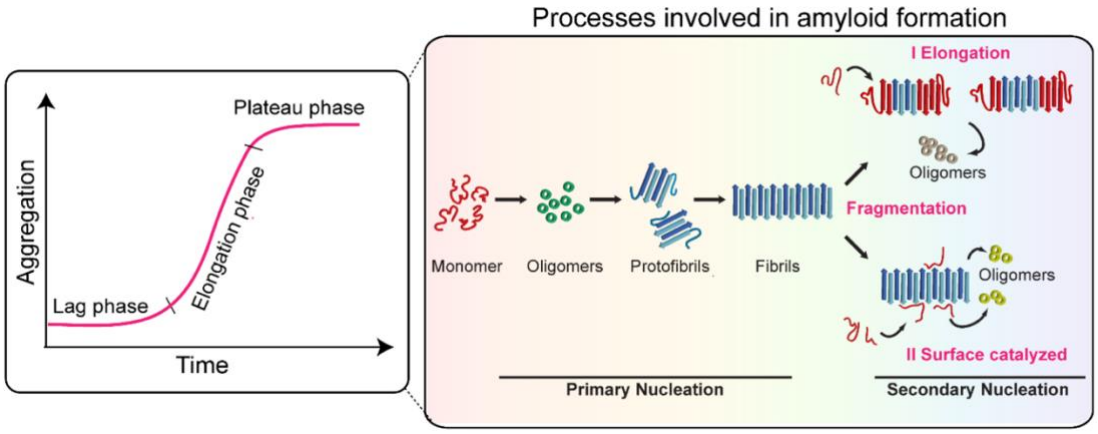

Figure 2: Nucleation Pattern of α-Syn Aggregation

The initial lag phase is the rate limiting step where structural changes in the innate α-syn decrease its presynaptic membrane binding capacity and potentiates monomeric α-syn aggregation to form oligomeric aggregation nuclei. These precursor nuclei enter into an elongation phase where the fibrils undergo exponential growth. Finally, when monomeric α-syn species are depleted, fibrils reach a plateau in the growing rate. Subsequent fractionation of α-syn fibrils result in new aggregations which act as secondary nuclei for further α-syn fibrils formation (Mehra et al., 2021).

Although the development LBs (with the main composite being α-syn fibrils) is the most prominent feature of PD, the toxicity of oligomeric and proto-fibril states of α-syn exceeds that of the fibrils. The toxic nature of α-syn arises from its ability to bind and remodel membranes. Mitochondrial membranes (specifically inner membrane) which are rich in acidic cardiolipin are favorable targets of α-syn (Ghio et al., 2019). The increased levels of α-syn expression have been associated with the increased mitochondrial fragmentation suggesting a disruptive role of α-syn in the mitochondrial membrane and subsequent malfunction in the electron transport chain (Gilmozzi et al., 2020). The rigid form of α-syn fibrils as well as the loss of primary role of α-syn as a neurotransmitter chaperone promotes the loss of synaptic plasticity. Loss of membrane binding capacity of α-syn in addition to the induction of oxidative stress via mitochondrial dysfunction are two promoters of α-syn induced neurotoxicity. Although the SNc is not documented as one of the first α-syn fibrils formation hotspot, the damage witnessed in the dopaminergic neurons of SNc is the greatest in PD pathology. The reason behind this phenomena could be attributed to multiple reasons, first of which is the high content of dopamine which poses oxidative stress of the neurons upon its reuptake and metabolism, secondly, mitochondria in the SNc area had been associated with a larger bioenergetic requirement as well as higher oxidative phosphorylation rate which confers a narrower margin of resistance to neurotoxins in comparison to other neurons (Pacelli et al., 2015).

1.1.3 Autophagy and PD

Autophagy is one of the molecular mechanisms that had gained rapid recognition in the past decades and had been linked to the pathology of many disorders (Ichimiya et al., 2020). Autophagy is a catabolic process where the cell recycles various cellular components by directing them to lysosomal degradation. The catabolic process was discovered in 1963 by dr. Christian de Duve who coined the term “autophagy” literally meaning “self-eating” (De Duve & Wattiaux, 1966). It was not until 1993 that the mechanisms of autophagy were unveiled by dr. Yoshinori Ohsumi, who defined the Autophagy-related Genes (ATG) in the yeast model (Matsuura et al., 1997).

The catabolic role of autophagy is pivotal in the nutrient recycling and elimination of excessive or harmful cellular components. Thus, this process is rendered essential for the

maintenance of cellular homeostasis. This implication of autophagy in the cellular processes have resulted in an explosion of research in the field of autophagy and disclosed the relationship of the catabolic process with the pathogenesis of various diseases like neurodegenerative diseases (where the autophagic process is found significantly dysfunctional) and tumorigenesis (where the autophagic process is exploited to facilitate the tumor malignancy under nutrient-depleted conditions) (Ichimiya et al., 2020).

Autophagy as a process is roughly referred to as the engulfment and delivery of cellular cargo to the lysosomal vacuoles in the cell. This wide definition holds three main mechanisms by which cellular cargo is delivered to the lysosome: Macroautophagy, which involves the gross enclosure of the cellular cargo prior to the fusion to the lysosome, Microautophagy which implicates the direct engulfment of the cellular cargo into the lysosome, and finally Chaperone-Mediated Autophagy (CMA) is directed by the targeting of KFERQ-motifs in proteins by chaperone Heat Shock Cognate protein (HSC70) which are later recognized by Lysosome-Associated Membrane Protein 2 receptor (LAMP2A) and transferred to the lysosome (Parzych & Klionsky, 2014).

Macroautophagy is the most studied form of autophagy and is referred to commonly as autophagy in many studies. The process of macroautophagy depends on the de novo formation of a double membrane (known as phagophore or isolation membrane) around the cellular cargo which proceeds to elongate and enclose the cargo forming the autophagosome. The engulfed cargo (though a cascade of molecular events) fuses with the lysosome and degrades the cargo through the action of lysosomal enzymes, resulting in the removal of toxic substances as well as protein recycling by increasing the amino acid pool that is vital for the formation of new proteins during external nutrient depletion. The formation and processing of the cargo proceeds in the following steps: initiation, nucleation, maturation, fusion, and degradation (Kocaturk et al., 2019).

Autophagy results from the formation of a phagophore, initiated by the Unc-51 Like Autophagy Activating Kinase 1 (ULK1) complex and the ATG5-ATG12-ATG16 complex. The phagophore encloses and elongates around the cellular cargo to form an autophagosome, which then fuses with a lysosome resulting in the degradation of the

contents. Autophagy is initiated through the stimulation of ULK complex formation. It is important to note that this complex formation is highly regulated by the mammalian Target of Rapamycin (mTOR) signaling pathway, which senses the nutritional status of the cell. mTOR is a core protein of complexes mTORC1 and mTORC2 both of which are involved in the regulation of a plethora of anabolic cellular functions including cell proliferation and protein synthesis (Kim & Guan, 2015). Under nutrient-rich conditions, mTORC1 suppresses autophagy by its attachment to the ULK complex, whereas during nutrient deficiency conditions mTORC1 is inhibited through the action of AMP-activated protein Kinase (AMPK) which senses the ATP depletion in the cell (Gwinn et al., 2008). The inhibition of mTORC1 results in the activation of Phosphoinositide 3-Kinase (PI3K) complex and initiation of the autophagy cascade. The PI3K is responsible for the engulfment or nucleation of the cargo (Kocaturk et al., 2019).

The ULK1 complex is a key regulator in the initiation of the autophagic process. The Serine/threonine-protein kinase activity of the ULK complex mediates upstream signaling of autophagosome precursors (such as PI3K complex) in response to nutrient starvation. The ULK1 complex (homologous to ATG1 in yeast) is localized in the phagophore and is comprised of several proteins including ULK Kinase, FAK family-Interacting Protein of 200 kDa (FIP200) and ATG13, and ATG101. ULK kinase activity stabilizes the ULK complex which is required for the autophagy initiation. ULK kinase is activated by the phosphorylation by AMPK which is itself phosphorylated during cellular starvation (Gwinn et al., 2008).

The action of AMPK, as a metabolic sensor, arises from its direct stimulatory action on autophagy initiator complex (through the phosphorylation of ULK and mTOR complexes) and the indirect transcriptional activation of autophagy thought Transcription Factor EB (TFEB) stimulation (Li & Chen, 2019). The dimerization of ATG13:ATG101 mediated through their Hop1p/ Rev7p/MAD2 (HORMA) domains is important for the function of the initiation complex and is inhibited through phosphorylation by mTOR pathway induction (Rosenberg & Corbett, 2015). Additionally, the HORMA domain plays a significant role in the recruitment of PI3K complex, thus affecting subsequent steps in the phagophore elongation and cargo nucleation (Jao et al., 2013). The phosphorylation of

FIP200 by ULK kinase activity is mediated by ATG13 (Hara & Mizushima, 2009; Jung et al., 2009), the activation of FIP200 is rendered important in various cellular processes such as cell division and migration. However, it is particularly found to colocalize in the phagophore upon cellular starvation along with ULK complex suggesting structural importance in the initiation of autophagy (Hara et al., 2008).

Although the precise origin of the double membrane that forms the phagophore is yet to be determined, many proteins are identified as regulators of the phagophore formation. One of the most prominent proteins identified in the process of phagophore formation is Beclin-1, which highlights the beginning of autophagic flux in the cell. The elongation and maturation of the phagophore are achieved via the action of the ATG family and the Microtubule-Associated Protein 1A/1B-Light Chain 3 (LC3) protein activation. Upon the fusion of the autophagic vesicle with the lysosome, the autolysosome forms where the lysosomal activity takes place and the autophagic cargo is degraded. It is important to note that the LC3 is modified upon the lysosomal fusion from LC3 I to LC3II making it an essential biomarker in the study of the activity of the autophagic process (Tanida et al., 2008). The conjugation of LC3 to Phosphatidylethanolamine (PE) is mediated by the cleaving action by protease ATG4 followed by the sequential activation by ATG7 to result in the formation of the membrane-bound form of LC3 (LC3 II-PE) (Lee & Lee, 2016). Many autophagic proteins are being utilized in the detection of the activity of the autophagic process, the lipidated form of LC3 (LC3II) with respect to the unlipidated form (LC3I) has been actively used by researchers as an essential marker to detect the autophagic rate, the importance this protein had gained is due to its transformation under active lysosomal degradation of cellular cargo to LC3II as opposed to its primary form LC3I which could be used in the estimation of the autophagic activity.

Although the process of macroautophagy is considered the least precise autophagic mechanism as gross engulfment of the cellular cargo within the cytoplasm by the phagophore occurs, multiple selective forms of macroautophagy process specific cellular targets such as mitophagy, pexophagy, lipophagy and ER-phagy. The prominent role of mitochondria in the understanding of the neurodegeneration and neuronal ageing processes have prompted the research directions into the study of mitophagy.

Mitophagy regulation in neurodegenerative diseases is a research area that has gained wide recognition especially in PD research. Mitophagy is a highly regulated process through which the autophagy lysosomal pathway targets the dysfunctional mitochondria. It is proposed that the catalytic process takes place in a 2-step model in a mammalian model, the first step being triggered by the depletion of ATP due to the dysfunctional respiratory chain which further triggers the Reactive Oxygen Species (ROS) to rise as well as AMPK. Elevated ROS and AMPK levels decrease the action of mTOR (a suppressor of the autophagic flux). The second step is via mitochondrial priming where the disintegrated mitochondrion is tagged with Parkin (Ding & Yin, 2012).

Parkin is an essential E3 ubiquitin ligase encoded by PRKN gene, mutations in PRKN gene have been associated with familial PD cases and autosomal recessive juvenile PD (Dawson & Dawson, 2010; Kitada et al., 1998). During compromised mitochondrial efficiency, recruitment and phosphorylation of Parkin is promoted via a serine/threonine protein kinase that localizes in the mitochondria PTEN Induced Kinase 1 (PINK1). Parkin phosphorylation and localization onto the Outer Mitochondrial Membrane (OMM) of dysfunctional mitochondria initiates a cascade of molecular events. Overexpression of Parkin has been associated with reduced α-syn toxicity, although it is estimated that this effect is due to the E3 ligase activity of Parkin, the exact mechanism remains unclear (Wilkaniec et al., 2019).

Parkin and PINK1 interaction controls mitochondrial quality control via inducing defective Voltage-Dependent Anion Channel-1 (VDAC1) by poly or mono- ubiquitination that result in either the rescuing of the compromised mitochondrion by reducing the Calcium efflux (thus putting a stopper in the ROS leakage and preventing further mitochondrial disintegration action) or by promoting autophagic lysosomal pathway, respectively (Ham et al., 2020). The VDAC protein family is associated with a plethora of neuronal functions that arises from its function as gatekeeper for the passage of various metabolites as well as ions (Camara et al., 2017), the role of VDAC1 in triggering Parkin-mediated mitophagy is impartial in retaining mitochondrial efficiency. The poly-Ubiquitinated VDAC1 protein recruits p62/sequestosome1 (SQSTM1) and LC3 to the mitochondria initiating mitophagy of dysfunctional mitochondria (Ham et al., 2020).

Dopaminergic neurons in the SNc, being the primary focus of PD research, display a rather higher rate of neuronal death that is not in concordance with the level of α-syn when compared with the α-syn spreading pattern (Bendor et al., 2013; Braak et al., 2003). The relatively higher levels of ATP consumption (Pacelli et al., 2015) in addition of the high presence of the bioactive dopamine, its toxic metabolite 3,4-Dihydroxyphenylacetaldehyde (DOPAL) (Goldstein et al., 2013) and the progressive buildup of neuromelanin (Zucca et al., 2023) are few factors that potentate the relatively higher susceptibility of SNc neuronal death. The significance of autophagy in the pathophysiology of PD arises from the neutralizing effect that it confers afflicted neurons. Autophagic lysosomal pathways offer a quality control mechanism that is essential in the maintenance of cellular functions.

1.1.4 PD Therapy

The role of striatal dopaminergic loss in the pathology of PD was reported in 1960, roughly a century after the first report of PD cases. This discovery led to the development of Levo-Dihydroxyphenylalanine (L-Dopa). The introduction of L-Dopa presented the first substantial foothold in PD therapeutics. The conversion of the dopamine precursor (L-Dopa) (which is readily available across the Blood Brain Barrier (BBB) unlike Dopamine) stabilized the dopamine deficiency in the nigrostriatal pathway (as illustrated in Figure 3). Later Dopamine decarboxylase inhibitors were added as an adjunct therapy with L-Dopa to counteract peripheral conversion to dopamine allowing increased bioavailability at synaptic terminals. More recent adjunctive additions of Catechol-O-Methyltransferase (COMT) and Monoamine Oxidase (MAO) type B inhibitors have been added in PD management therapy to delay the enzymatic metabolism of the newly formed dopamine hence prolonging the half-life of L-Dopa. Dopamine agonists induce receptor sensitivity to the endogenous dopamine levels. The use of these agents is limited to patients at early PD stages, this is due to the association of higher side effects and lower therapeutic efficacy than L-Dopa. The progressive nature of neurodegeneration in PD establishes a limit to the effectiveness of replacement therapies. Although their use is still reserved for life threatening cases, the neuropathology of PD is still not targeted by established therapies. The search for agents that target α-syn accumulation and alleviate neuronal stress continues as more molecular targets of PD are being discovered.

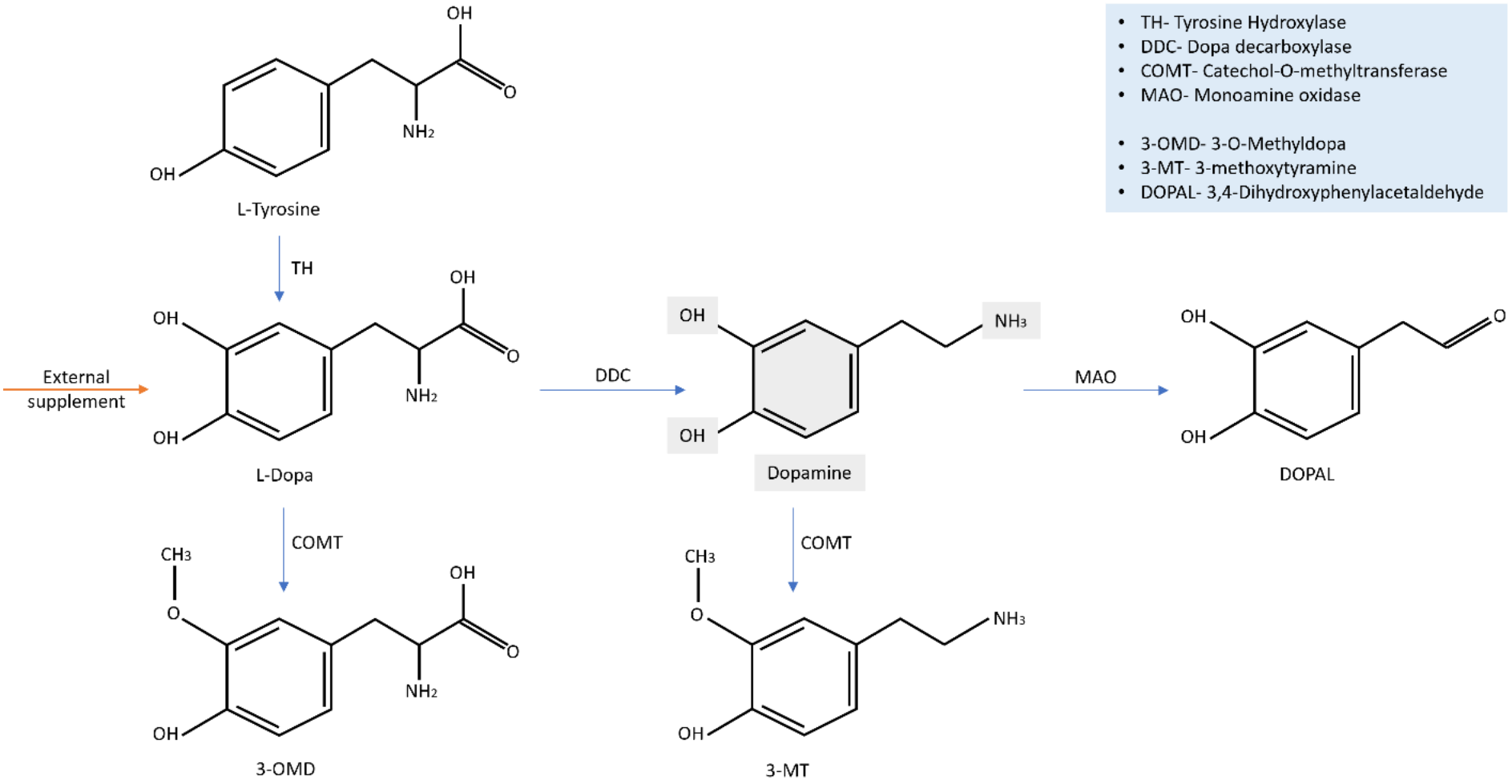

Figure 3: L-Dopa Transformation to Dopamine

1.1.5 Natural Products Prospects in PD Therapy

The use of naturally occurring chemicals in deriving therapeutic agents has been impartial in the development of the pharmacotherapeutic field. Whether by prior investigation or pure serendipity, the discovery of many natural products that symptomatically alleviated PD dyskinesia provided the primary therapeutic regimen prior to L-Dopa discovery. Many early therapies included the use of anticholinergics, (which to this date are effective in certain cases in early stages of PD) (Katzenschlager et al., 2002; Rascol et al., 2021), and alkaloids (Ordenstein, 1868). Furthermore, the consumption of *Mucuna pruriens* as well as other L-Dopa rich leguminous plants in many populations improved parkinsonism (Cilia et al., 2017).

Although the prodigious drug L-Dopa is considered the gold standard for PD therapy, it is not capable of halting the progression of the disease. The interest in chemicals derived from natural products have increased due to the success of many agents in targeting causative neuropathological features of PD such as targeting oxidative stress, stimulating anti-inflammatory markers as well as upregulation of the autophagic flux. Additionally, some natural compounds were associated with the direct inhibition of α-syn aggregation by binding to the protein as well as induction of proteasomal degradation.

Polyphenols are among the most researched naturally occurring chemicals in the field of preventive medicine, their antioxidant capacity had attracted therapeutic potential in many pathologies. One of the most prominent polyphenolic compounds is Ellagic Acid (EA). The polyphenolic compound (displayed in Figure 4) is found in a plethora of dietary sources including walnuts, cashews, cranberries, strawberries, raspberries (Daniel et al., 1989; Landete, 2011) and pomegranates (Sharifi-Rad et al., 2022). In pomegranates, there are several therapeutic compounds but EA is found in the highest concentrations (Viladomiu et al., 2013).

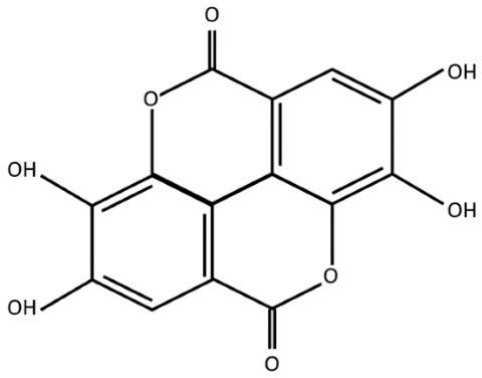

Figure 4: Ellagic Acid Molecular Structure

The polyphenolic compound is a derivative of gallic acid and is often found in the form of ellagitannins. Hydrolysis of precursor ellagitannins consumed from natural sources into EA which is later transformed into urolithins by gut microbiota is often the case in the metabolism of EA. Several factors hinder the activity of ellagic acid molecule, degradation throughout the microbiota as well as the hepatic metabolism by Cytochrome P450 (CYP450) system all of which radically decrease the bioavailability of EA (González-Sarrías et al., 2015). Additionally, the lipophilic nature of the polyphenolic compound as well as the intrinsic interaction of the aqueous OH groups with lipophilic rings (which results in the low water solubility of the compound) presents a challenging circulation of the molecule form the site of administration to the target site. Still, it is the lipophilic nature of this molecule that promotes its passage through the blood brain barrier which is one of the main challenges of administration of Central Nervous System (CNS) targeted pharmaceuticals.

The phenolic groups of EA are good hydrogen doners conferring their antioxidant effect on ROS and well as Reactive Nitrogen Species (RNS) both of which are capable of eliciting harmful oxidative cellular stress. Furthermore, the presence of benzene rings creates a capacity to participate and buffer redox reactions (Alfei et al., 2019).

The high antioxidative properties of EA had attracted a great deal of attention in various chronic diseases many of which share inflammatory etiologies such as cardiovascular disease (Basu & Penugonda, 2009), Inflammatory Bowel Disease (Rosillo et al., 2012), respiratory syndromes (Mansouri et al., 2020) and liver diseases (Aishwarya et al., 2021).

The anti-tumorigenic properties of EA had been extensively studied in many oncologic diseases including pancreatic (Kim et al., 2021), lung (Duan et al., 2020), bladder (Ceci et al., 2016), colorectal (Yousef et al., 2016) and breast cancers (Yousuf et al., 2020), Mitigation of adverse effects of anticancer therapies had been reported in multiple studies (Engelke et al., 2016; Goyal et al., 2022; Yakobov et al., 2023). Furthermore EA plays a role in the induction of Keap1-Nrf2-ARE signaling pathway which is responsible for the regulation of over 200 proteins responsible in the detoxication and the elimination of ROS, RNS as well as the neutralization of electrophiles (Wang et al., 2022).

EA had gained a peculiar scope of interest in PD research, given the antioxidative nature of the molecule, researchers aimed to investigate its potential effectiveness in therapeutic context. Multiple studies discussed the direct physical interaction between EA and α-syn in vitro which prevented the aggregation of monomeric α-syn to oligomeric and filamentous forms of α-syn. Furthermore, Two published studies from Dr. Emdadul Haque's lab documenting the effect of EA in preventing the toxic effect of α-syn aggregates in the PD cell culture model (SH-SY5Y cells) (Ardah et al., 2021) in addition to the capacity of EA in eliciting an antioxidative response in a mouse model of PD (Ardah et al., 2020).

The current study investigates the role of EA in a mouse PD model in retarding the toxic spreading of α-syn aggregates and the induction of autophagic flux, both of which play a significant role in the protection of dopaminergic neurons in the nigrostriatal pathway.

1.1.6 PD Modeling and Research Challenges

Challenges in PD modeling systems include the lack innate ability of rodents or drosophila (which are the most common models of neurological disorders) to develop α-syn fibrils.

Based on the hypothesis of template α-syn toxic aggregation (Yoshida & Hasegawa, 2022), neurons afflicted with α-syn toxic aggregates are capable of inducing aggregation in nearby healthy neurons. α-syn Preformed Fibrils (PFF) had been utilized in this light to initiate α-syn aggregation in a manner similar to that of α-syn aggregation. PFF acts as a seed for α-syn aggregation thus bypassing primary nucleation and stimulating the spreading of α-syn aggregates in a retrograde manner along the nigrostriatal pathway.

Although the extensive growth in publications on α-syn had aided in a better understanding of fibril formation and LB development, the conformational changes in α-syn protein and the role of post translational modifications are two important fields of PD research that remain elusive to some extent.

Additionally, the timeline of mitochondrial damage is harder to model due to the relatively briefed duration of the studies and therefore necessitates the induction of mitochondrial damage. neurotoxins such as 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP), rotenone, 6-Hydroxydopamine (6-OHDA) all of which primarily damage the respiratory chain resulting in the increase oxidative stress which mimics the PD pathology.

Furthermore, the heterogeneity of the disease development confers harder modeling strategies, the combination of genetic predisposition in addition to the environmental factor aid in the development of age-related neurological disorder like PD with a relatively latent onset are all factors that increase the complexity in the development of accurate modeling systems.

Loss of dopaminergic neurons in the SNc and their neuronal terminal ends in the striatum leading to the dysfunction and dopamine deficiency in nigrostriatal pathway is one of the major pathological features of PD. Dopamine Transporters (DAT) in the striatum are important determinants of the presynaptic dysfunction in PD pathogenesis and primarily responsible for the dopamine reuptake in the synaptic cleft. DAT is used as a marker for determining the functional integrity of dopamine in synapses. Tyrosine Hydroxylase (TH) on the other hand is the rate limiting enzyme in dopamine synthesis and is considered an essential marker of dopaminergic neuron soma in the SNc area.

Acute MPTP induced dopaminergic neurodegeneration was shown earlier to be significantly counteracted by EA IP administration in C57BL/6 mice (Ardah et al., 2020). The neurotoxicity of MPTP clinically presented among young abusers as parkinsonian symptoms during early 1980s (Langston et al., 1983). The selective neurotoxicity of MPTP to dopaminergic neurons in the SNc significantly added to the capacity of animal modeling of PD (Meredith & Rademacher, 2011). MPTP is of lipophilic nature which aids in crossing BBB and is converted to 1-Methyl-4-Phenyl-2,3-Dihydropyridinium (MPDP+)

by the enzymatic action of Monoamine Oxidase-B (MAO-B), MPDP+ spontaneously oxidizes into mitochondrial complex I inhibitor 1-Methyl-4-Phenylpyridinium (MPP+) which disrupts both respiratory chain and calcium homeostasis resulting in increased ROS, ATP depletion. The specificity of MPP+ toxicity to the SNc dopaminergic neurons in many PD models is prominently due to the high selectivity of MPP+ to DA uptake sites (Shen et al., 1985). Increased concentration of intracellular MPP+ results in impairment of mitochondrial functions of the high energy demanding neurons and progression of PD (Pissadaki & Bolam, 2013). Previously reported IP administration of a low dose of MPTP (10 mg/kg b.wt.) resulted in the spreading of α-syn aggregation stimulated by template α-syn PFF intrastriatal inoculation (Merghani et al., 2021). The current study utilizes this newly developed model to investigate the neuroprotective role of EA in vivo in male C57BL/6 mice.

Chapter 2: Methodology

2.1 Research Design

Previous data obtained in Dr. Haque's lab have confirmed the physical interaction of ellagic acid on the aggregation of α-syn in vivo. The ameliorative effect of EA on the α-syn aggregate toxicity was furthermore tested in vitro in SH-SY5Y cells, a cellular model of PD where a novel increased autophagic flux was reported (Ardah et al., 2021). Following the proving of EA's efficacy in cell culture PD model, the research design proceeded to animal PD model. These set of experiments evaluated the anti-inflammatory role of EA in vivo via the detection of various inflammatory neuronal features such as activated microglia and astrocytes in addition to amelioration in the levels of inflammatory biomarkers (such as iNOS and COX2). The main aim of the current study was to report the novel autophagic stimulatory effect of EA on dopaminergic neurons in PD mouse model and the subsequent preventive effect of the α-syn toxic spreading to the SNc. Figure 5 illustrates the research design of the current study.

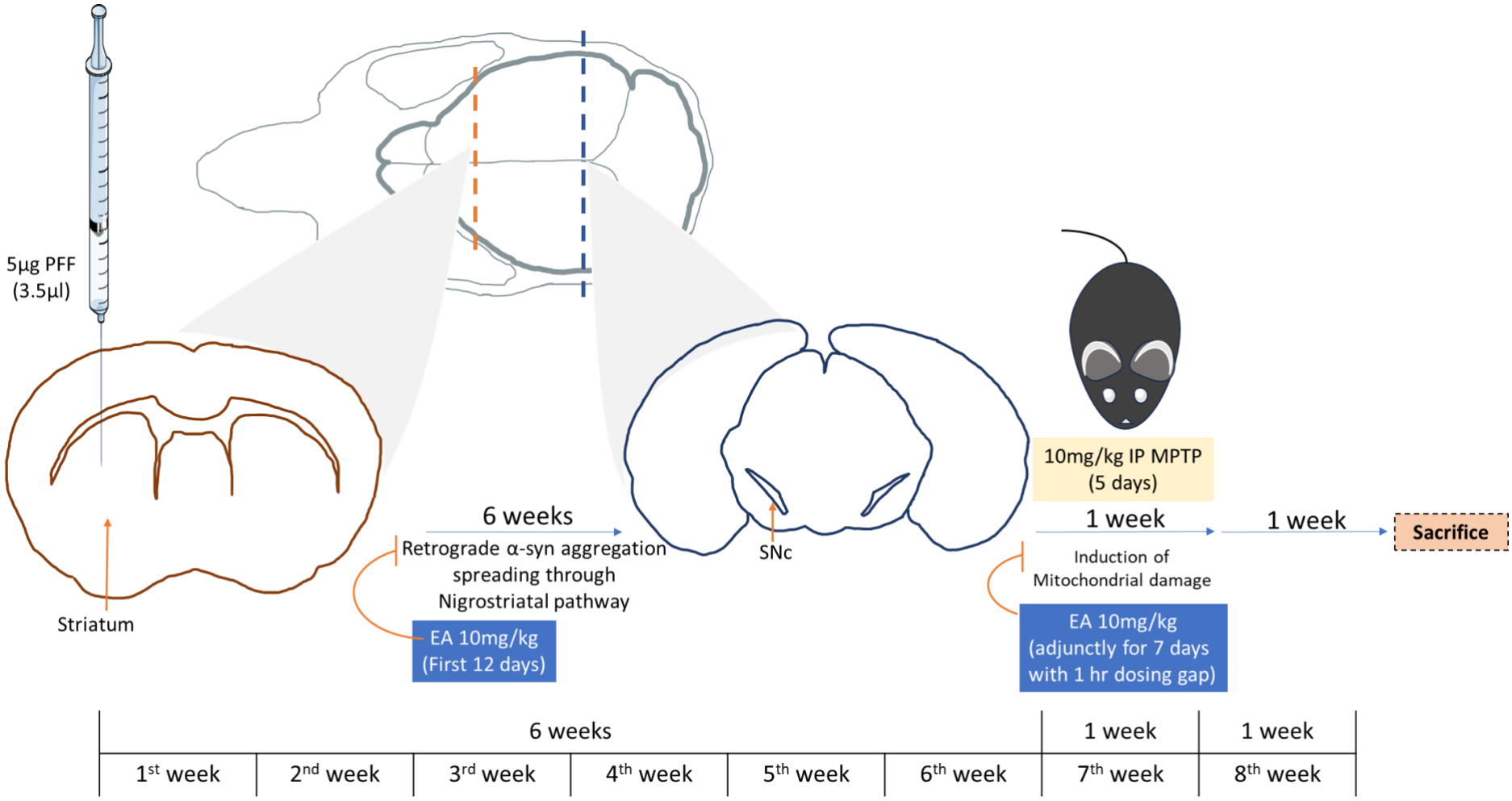

Figure 5: Research Outline

2.2 Methodology

A general outline of the methodology of this study includes the synthesis of the PFF seed which was used in the latter stages of the study as detailed in subsequent sections. The study plan and sample processing was reported as published (Radwan et al., 2024).

2.2.1 PFF Seed Synthesis

PFF seed synthesis proceeded through multiple stages as illustrated in Figure 6. The first step proceeded by the bacterial transformation with pT7-7 Wild Type α-syn vector and plating onto ampicillin selection plates to identify bacterial colonies successfully expressing the alpha-syn vector. The selection of one colony was inoculated in LB overnight at 37°C in the shaker. Expansion of this colony into a larger LB volume and kept overnight, Optical Density (OD) was regularly assessed until an OD of 0.5-0.6 at which the bacterial growth is exponential was achieved. Lac-repressor was inhibited by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPGT) to the bacterial culture to induce the Wild type human α-syn production (2 hours at 37°C in the shaker).

Bacterial culture underwent pelleting and lysis using a detergent-free lysis buffer. The resulting lysate underwent dialysis in a gel filtration buffer (Tris-EDTA) through a dialysis tube with a 7 kDa cutoff molecular weight, which is compatible with subsequent isolation techniques. Following dialysis, the lysate underwent filtration (using 2 um filters) and concentration (approximately 10 kDa cutoff molecular weight). The concentrated lysate was then processed through FPLC. Fractionated protein samples were eluted, and the molecular weight of α-syn was determined relative to the range of eluted fractions on the standard curve (peaks of the chromatogram). Further analysis of the collected fractions was performed using SDS-PAGE to confirm the optimal representation of α-syn elution. The sample's purity was enhanced by subjecting the collected fraction to ion exchange chromatography, leading to the isolation of the α-syn monomer. Monomeric α-syn was transformed into aggregated α-syn fibrils via subjecting to constant shaking (800 rpm/min) at 37°C over a period of 7-8 days. Periodic testing of fibrils formation was achieved using thioflavin-S assay. Furthermore, the visualization of the aggregatory phases of α-syn

fibrils is confirmed using western blotting and electron microscopy. Finally, the aggregated α-syn fibrils were pelleted and washed to remove trace monomeric α-syn.

Application of the α-syn fibrils in pathological context require a more interactive state which is consistent with a smaller length of fibrils, this is achieved by the sonication of the aggregated α-syn fibrils to form α-syn PFF seed. This seed was then aliquoted and stored at -80°C.

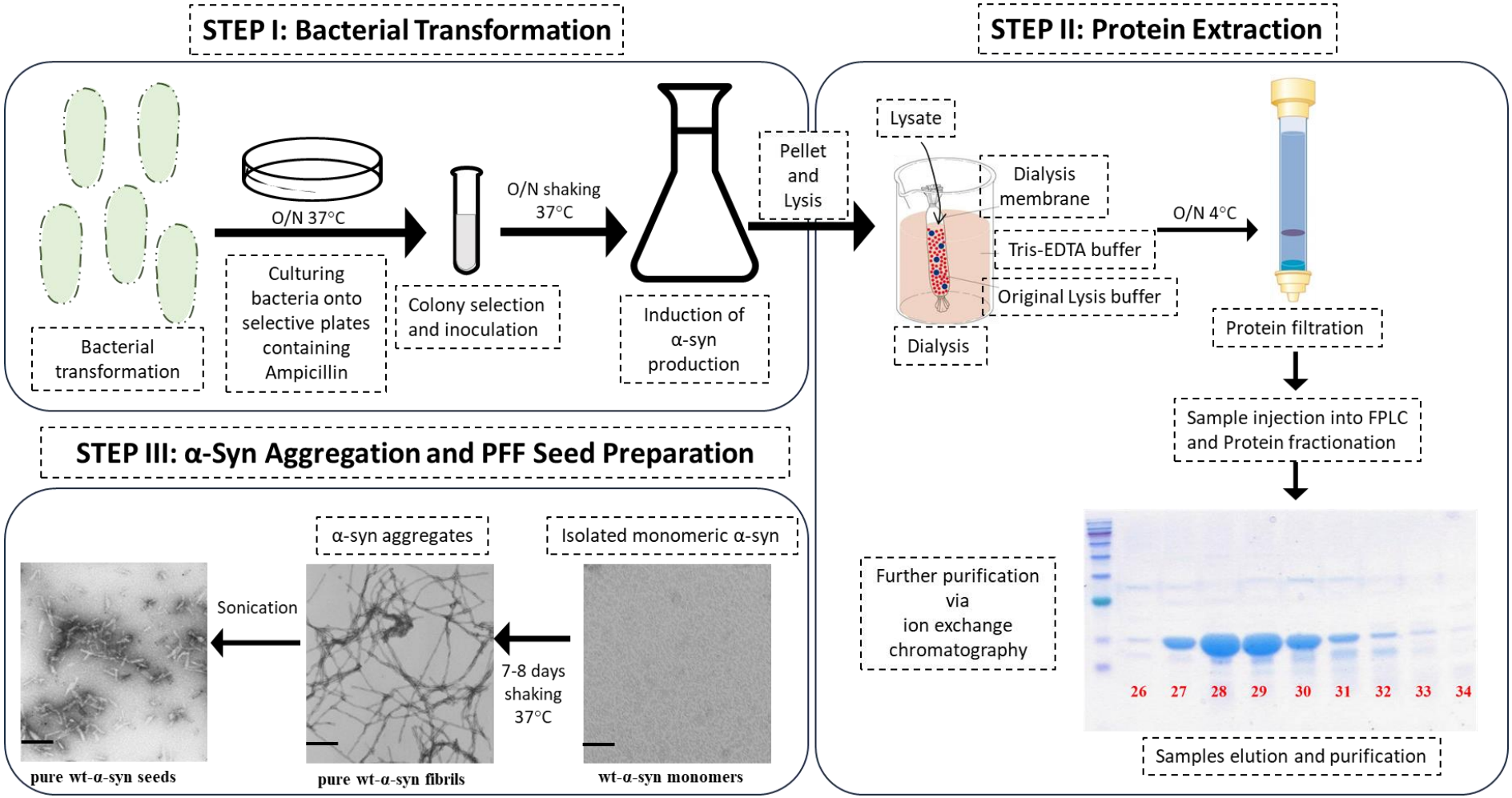

Figure 6: Human α-Syn PFF Seed Synthesis

2.2.2 Animals

C57BL/6 mice were obtained from UAE University animal house. Male mice of ages 2-2.5 months which weighed 20-26 grams were included in the experiments. Mice were housed in a 12hr light/dark cycle with access to food and water throughout the experiment duration. All experiments were performed in accordance with UAE University Animal Ethics guidelines (Approval number: ERA_2021_8408).

2.2.3 Study Plan

Male C57BL/6 mice were acclimated to the polystyrene cages for a week, animal sedation was conducted using Ketamine HCL (Ketamil) and Xylazine HCL (Xylazil-20) solutions diluted in normal saline (at ratios 1:1:10 respectively) and dosed accordingly (1ml/kg).

Following sedation, the head was fixed onto the stereotaxic surgery apparatus (WPI) and a surgical scalpel blade (No. 11) was used to perform a cut (approximately from the sagittal suture to lambda) fully exposing the bregma.

Identification of the bregma as a locus was used to identify the Caudate Putamen Striatal area, the following coordinates (Anterior-Posterior: +0.5 mm, Medial-Lateral: -2.2 mm, Dorsal-Ventral: -3.4 mm) have been used in the intrastriatal stereotaxic surgeries with respect of the inoculation of α-syn PFF seed (Merghani et al., 2021; Paxinos & Franklin, 2013).

The PFF used in the stereotaxic surgery was stored in -80°C, transferred in Liquid nitrogen to the surgery room in the animal house facility and thawed on ice prior to the start of the surgery. Intrastriatal injection (using Hamilton 10 µl syringe) was conducted at the following infusion rate: 0.5 µl/minute using a syringe pump (Micro4 ^{TM} MicroSyringe Pump Controller, WPI, USA), PFF infusion of 2.5 µl (a total of 5 µg of PFF seed) was delivered at a constant rate and an extra minute was kept allowing remanent PFF seed in the syringe to diffuse properly.

Following the stereotaxic surgery, wound was sealed by application of a tissue adhesive (3M Vetbond$^{TM/MC}$, Japan) onto the wound edges that have been clamped together using a forceps.

Intraperitoneal administration of the Drug of interest (Ellagic acid-E2250-Sigma, USA) proceeded for the following 12 days. EA was prepared in a 20 mg/ml DMSO stock then aliquoted into 200 µl aliquots and stored in -80°C, upon dose administration prepared EA aliquots were mixed in saline solution (in a 1:20 ratio to achieve final dose of 10 mg/kg to test group). A 4-week gap was given before proceeding with the neurotoxin (MPTP-M08896-Sigma, USA) intraperitoneal administration at 10 mg/kg for 5 days (a dose optimized based on previous project (Merghani et al., 2021)). A conjunctive administration of EA was given to test groups with a 1-hour dosage gap (EA prior to MPTP) and alternate site of injection to avoid Drug-Drug interactions. EA administration continued for 2 extra days, 1 week gap was given before the sacrifice. Controls were dosed with the Vehicle (V) (1:20 - DMSO: saline ratio). The three groups assessed in this study were:

1. PFF+MPTP+EA.
2. PFF+MPTP+V.
3. PBS+V.

Oral administration of EA followed the same study plan with only a modification in the final dose of EA (20 mg/kg b.wt), homogenization of the suspension was performed prior to each dose.

Animal sacrifice was performed at the end of the 8th week of the experiment. Animals were dosed with sedative (Ketamine: Xylazine: normal saline at ratios 1:1:10 respectively) and dosed accordingly (1 ml/kg). Following sedation, the animal was pinned onto a dissection pan on its back, and a cut made by gently holding the skin above the thoracic cavity using forceps then cutting an incision extending to the abdominal area. Exposing the heart by making bilateral cuts through the ribs was operated. Next a blunt forceps was used to stabilize the insertion of the perfusion needle into the apex of the left ventricle, followed by a cut in the right atrium as illustrated in Figure 7 (Ghosh & Higgins, 2018). The perfusion was conducted using 10 ml of normal saline followed by 10 ml of 4% Paraformaldehyde solution for tissue fixation (animals specified for the following tissue processing through western blotting were not perfused with the fixation solution and were

directly placed in 1.5 ml tubes, subjected to flash freeze in liquid nitrogen during the collection and stored in -80°C).

The collected brains are kept in 4% Paraformaldehyde solution for 24 hours, for the following 3 days this solution is changed twice a day with 10% sucrose in 0.1 M PB, 0.02% sodium azide. Finally, the brains were carefully dried and frozen at -80°C. Cryosectioning of the brain samples were performed at -15°C, 40 µm sections, striatal and midbrain (SNc region) sections were saved in a serial manner in PBS 0.02% sodium azide (free floating).

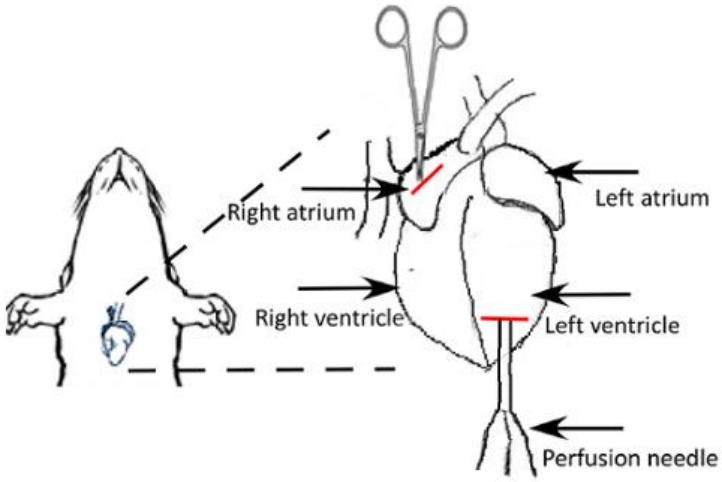

Figure 7: Heart Perfusion Protocol

2.2.4 Immunohistochemistry

The assessment of the pathological features of the PD model with and without EA inferred in this study was evaluated via Immunohistochemistry (IHC). The following sections will further illustrate the methodologies implemented. Table 1 highlights the antibodies used in the IHC assays in this study with their respective dilutions. Figure 8 illustrates the tissue sampling process.

Table 1: IHC Antibodies

Antibodies	Host Species/ Cat. No.	Dilution	Assay
Primary Antibodies			
DAT	Rat/ TEMECULA MAB369	1:1000	DAB
TH	Mouse/ Immuno star 22941	1:1000	DAB/IF
pS129 α-syn	Rabbit/ ab59264	1:1000	IF
Conformational α-syn	Rabbit/ ab209538	1:1000	IF
LC3A/B	Rabbit/ CST 12741	1:500	IF
p62	Mouse/ ab56416	1:500	IF
Secondary Antibodies			
Biotin-sp-conjugated	Donkey anti-Rat/ Jackson Immuno Research 712-065-153	1:1000	DAB
Biotin-sp-conjugated	Donkey anti-mouse/ Jackson Immuno Research 715-065-150	1:1000	DAB
Alexa Fluor 594	Goat anti-mouse/ Invitrogen; A11032	1:1000	IF
Alexa Fluor 488	Goat anti-rabbit/ Invitrogen; A11034	1:1000	IF
Tertiary Antibody			
Streptavidin-horseradish Peroxidase conjugate	AntiDonkey/Amersham™ ;RPN1231-2ML	1:200	DAB

2.2.4.1 DAB (3,3'-Diaminobenzidine) Stain

DAB stain was performed to the collected striatal sections (to assess the Dopaminergic nerve terminals -DAT-loss), collection of the striatal sections (area from -1.54 to -0.22 mm of bregma) of the free-floating samples. These samples were washed in Phosphate Buffered Saline (PBS), pH 7.4 in a 5-minute incubations 3 times. Following these washes, sectioned were blocked to minimize background signal with 10% normal goat serum in PBS for 1 hour at room temperature. PBS washes (5 minutes incubations 3 times) was performed followed by incubation with the Primary antibodies overnight at -4°C. The sections were kept at room temperature for at least 20 minutes before further processing to avoid tissue damage, primary antibody was washed with standard PBS washes (5 minutes incubations 3 times) then incubated with secondary antibody (Biotin conjugated) for 1 hour at room temperature to increase the signal specificity as well as intensity. PBS washes were performed prior to incubation with tertiary antibody (Streptavidin-HRP

Conjugate) for 1 hour at room temperature followed again by PBS washes. Signal acquisition was performed by DAB reaction (by incubation in DAB solution detailed in Table 2 for 2-10 minutes) which is stopped by 0.1 M sodium acetate solution.

Table 2: DAB Solution

DAB Solution Components	mL
0.2 M sodium acetate	2.5
8% Nickel-Ammonium Sulfate	1.56
1% Ammonium chloride	0.2
4% D-Glucose	0.25
DAB (10 mg/mL)	0.125
H2O	0.365
Glucose Oxidase Dehydrogenase	0.075

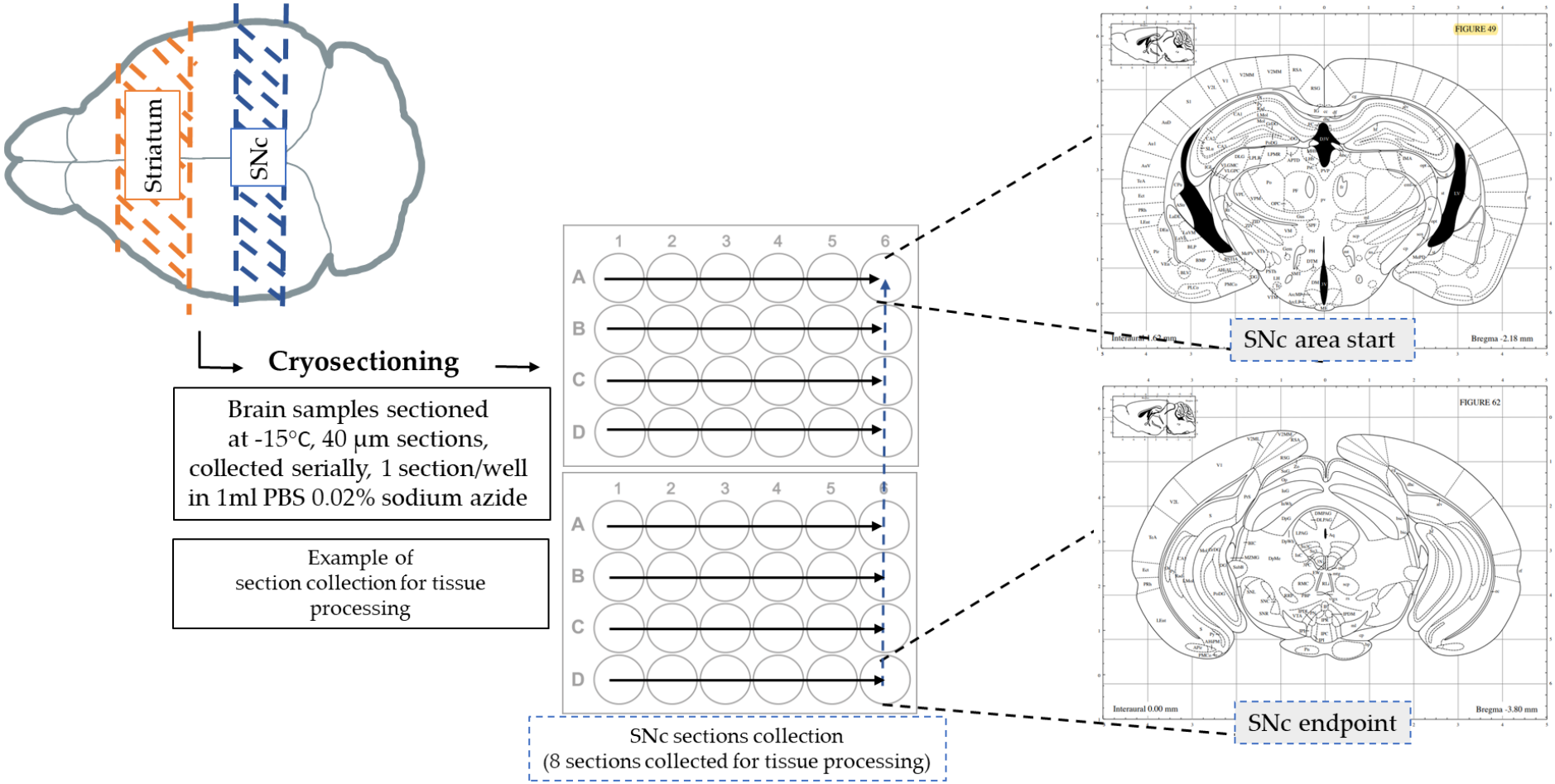

Figure 8: Cryosectioning and Section Collection of C57BL/6 Mouse Brain. Solid arrows indicate serial section collection following brain sectioning, dotted arrows indicate tissue collection for further sample processing.

Sections were mount on the slides and dehydrated by exposure to serial dilutions of ethanol. Followed by sealing using Shandon Synthetic mounting media (Ref 6769007) mounting media. Optical density of DAT was measured using ImageJ.

DAB stain was also performed to the collected SNc sections (neuronal cell body loss using TH) by collection 8th sample of the SNc sections (area from -2.18 to -3.80 mm of bregma). The protocol proceeded in the same manner as DAT stain with the compatible secondary and tertiary antibodies to TH antibody as detailed in Table 1.

2.2.4.2 Immunofluorescence (IF)

Immunofluorescent double labeling of the collected brain samples was performed to assess the following aspects in our study; (1) α-syn species spreading to the dopaminergic neurons in the SNc in phosphorylated form at Ser129 (pS129) and filamentous Proteinase K (PK) resistant form. (2) Expression of autophagic markers (LC3, p62) in dopaminergic neurons in the SNc.

Sample collection of SNc sections (TH expressing dopaminergic neurons) by collection 8th sample of the SNc sections (area from -2.18 to -3.80 mm of bregma). TH-pS129, TH-LC3 and TH-p62 co-stains were conducted in a similar manner, respectively. The collected sections were blocked and washed as detailed in the DAT staining. Primary antibodies were diluted in PBS following the dilutions mentioned in Table 1 overnight at -4°C. The sections were kept at room temperature for at least 20 minutes before further processing to avoid tissue damage, primary antibody was washed with standard PBS washes then incubated with florescent secondary antibody (TH was tagged with correspondent alexa594 while pS129, LC3& p62 were tagged with correspondent alexa488, respectively) for 1 hour at room temperature and covered with aluminum foil to avoid photobleaching. Following 3 PBS washes, sections were mount onto the slides and sealed using Fluoroshield (F6182, Sigma). TH-conformational α-syn co-stain required exposure to Proteinase K (PK) 5 µg/ml for 30 minutes at 25°C prior to the blockade and proceeded as other stains but required additional care when handling the tissues as they were more fragile due to PK digestion.

2.2.4.3 TUNEL Assay (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling)

In Situ cell death detection kit (Roche Cat. No. 11 684 817 910) was used in this assay, PK 5 µg/ml for 30 minutes at 25°C prior to the blockade and proceeded as per manufacturer recommendations. Nucleus was stained using TO-PRO™-3 Iodide (642/661, Invitrogen), TOPRO (originally blue) was pseudo colored to magenta for better visualization.

2.2.5 Microscopy

Confocal microscope Nikon EZC1 was used in the image acquisition of colocalizing fluorescent markers (TH-pS129, TH-conformational α-syn, TH-LC3 and TH-p62, respectively). Leica DM4000 B LED Microscope (Leica Microsystems, Wetzlar, Germany).

Stereology of dopaminergic neuronal bodies (TH expressing cells in the SNc are visualized by DAB reaction) was performed using an optical fractionator of automated stereoinvestigator as previously described in (Ardah et al., 2020).

2.2.6 Western Blot Analysis

Sectioned samples of SNc (approximately from area -2.18 to -3.80 mm of bregma) were collected (in a serial manner similar to the IHC and IF protocol) in lysis buffer to assess the levels of the endogenous α-syn, pS129 as well as autophagy markers LC3II/I and p62. 0.1% Triton solution with 1x Protease Inhibitor cocktail was used in the lysis procedure, collected samples were placed in Eppendorf tubes, respectively, and sealed with parafilm and placed on a rotator (25 rpm) overnight at 4°C.

LC3II/I and p62 0.1% Triton solution with 1x Protease Inhibitor cocktail was used in the lysis procedure, collected samples were placed in Eppendorf tubes, respectively, and sealed with parafilm and placed on a rotator (25 rpm) overnight at 4°C.

Samples were homogenized by sonication (sonication was performed on ice with 5 seconds on/off cycles to avoid sample overheating). Sample was pelleted by centrifugation at 14,000 rpm 4°C for 15 minutes and supernatant was collected in chilled labeled tubes.

Samples were processed in 15% SDS-Poly acrylamide gel. Protein transfer was performed using wet transfer method onto methanol activated PVDF membrane at 100 V for 1.5 hours. Membrane was later blocked using 5% milk in PBS-T (or 5% bovine serum albumin in case of detection of phosphorus containing protein). Membranes were tagged with the primary antibody in dilutions detailed in Table 3 overnight at 4°C on the shaker. PBS-T washes for 30 minutes preceded 1hour incubation with the correspondent secondary antibody. Bands were visualized using Sapphire (1.3.0219.0) azure biosystems.

Table 3: Western Blot Antibodies

Antibodies	Host Species/ Cat. No.	Dilution
Primary Antibodies		
TH	Mouse/ Immuno star 22941	1:1000
pS129 α-syn	Rabbit/ ab59264	1:1000
LC3A/B	Rabbit/ CST 12741	1:1000
p62	Mouse/ ab56416	1:1000
GAPDH	Rabbit/ CST 2118	1:1000
Secondary Antibodies		
Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson Immuno Research (115-035-166)	1:10,000
Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L)	Jackson Immuno Research (111-035-144)	1:10,000

2.2.7 Data Acquisition and Statistical Analysis

ImageJ software was used in the analysis of IF colocalization studies as well as DAB signal intensity measurement and the western blotting band intensity study. Graphpad prism 5.0 software was used for statistical analysis (One-way ANOVA and Bonferroni post hoc test). Leica DM4000 B LED Microscope (Leica Microsystems, Wetzlar, Germany). Optical density of DAT was measured using NIH Image J (version: Image J 1.54d).

Chapter 3: Results and Discussions

3.1 Results

3.1.1 EA Inhibits MPTP-PFF Induced α-Syn Spreading and Aggregation

EA was reported previously to play a direct role in the prevention of α-syn aggregates mediated toxicity in cell culture model (Ardah et al., 2021). Building upon prior reports indicating EA's direct role in hindering α-syn toxicity in a cell culture environment and induction of antioxidative property in vivo (Ardah et al., 2020). This study was carried out to validate this effect by in vivo investigation of α-syn spread, aggregations and toxicity within the SNc of mice. The Intrastriatal injection of α-syn PFF seed in the recently developed mouse model (Merghani et al., 2021) induced the secondary nucleation of the α-syn in the neurons thereby skipping past the rate limiting step of the α-syn aggregation reaction which is the formation of the primary fibrillar structures. The spreading of α-syn aggregates from the site of the stereotaxic surgery (striatum) to the site of pathology (SNc) in a retrograde manner across the nigrostriatal pathway in addition to the induction of mitochondrial damage specific to the SNc using low dose of MPTP (10 mg/kg b.wt.) were the two factors that aid in the simulation of the PD pathogenesis in the in vivo model used in this study (Merghani et al., 2021). The evaluation of α-syn spreading and aggregation in C57BL/6 male mouse brain was conducted by two ways, the first was through the detection of the pS219 α-syn which is highly associated with the LB pathology in PD patients, and the second was the detection of PK resistant form of α-syn commonly referred to as conformational or filamentous α-syn which represents digestion resistant form of α-syn, a more pathogenic form of α-syn aggregates. Both α-syn markers in the dopaminergic neurons in the SNc significantly decreased upon EA treatment as displayed in the immunofluorescence staining and presented in Figures 9-A and 10-A. Additionally, the number of synuclein that colocalizes with TH neurons has also been counted and presented in graphs 9-B and 10-B. This result suggests that EA significantly reduced the synuclein level in the TH neurons. These results were further substantiated by the western blot assay measuring the total expression of endogenous α-syn (Figures 9-E and 9-F) which decreased upon EA treatment. Similarly, the pS129 α-syn level (Figures 9-C and 9-D) was significantly lower in EA treated group (+PFF+MPTP) than the vehicle control

group (+PFF+MPTP). Similar to in vitro data, these results suggest the inhibitory effect of EA on α-syn spreading, as well as α-syn aggregates formation in vivo.

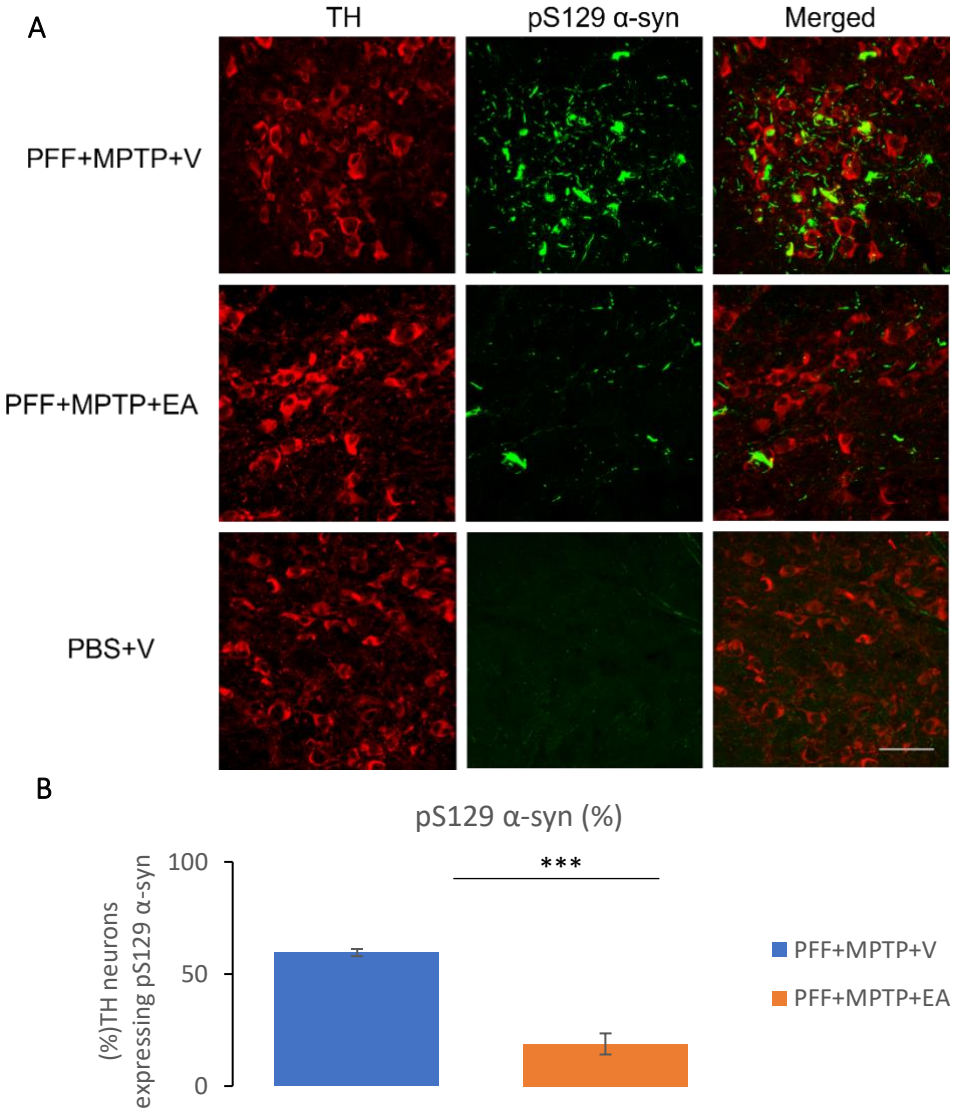

Figure 9: Assessment of α-Syn Spreading from Site of Injection (Striatum) to TH Neurons in the SNc. (A) Representative results showing the mouse pS129 α-syn spreading in the SNc in both PFF+MPTP treated groups in comparison with a control group, EA confers significant protection against synuclein spreading (B) detected in this experiment as the phosphorylated form of α-syn a primary component found in Lewy bodies. Detection of different forms of α-syn protein using SDS-PAGE: (C&D) pS129 α-syn and (E&F) endogenous α-syn. Data represented as percentage ± standard error of the mean (n = 3-4). Scale bar 50 µm (A). (***p < 0.0001, one-way ANOVA and Bonferroni post hoc test). pS129 α-syn: Phosphorylated α-synuclein; TH: Tyrosine hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic acid; V: Vehicle

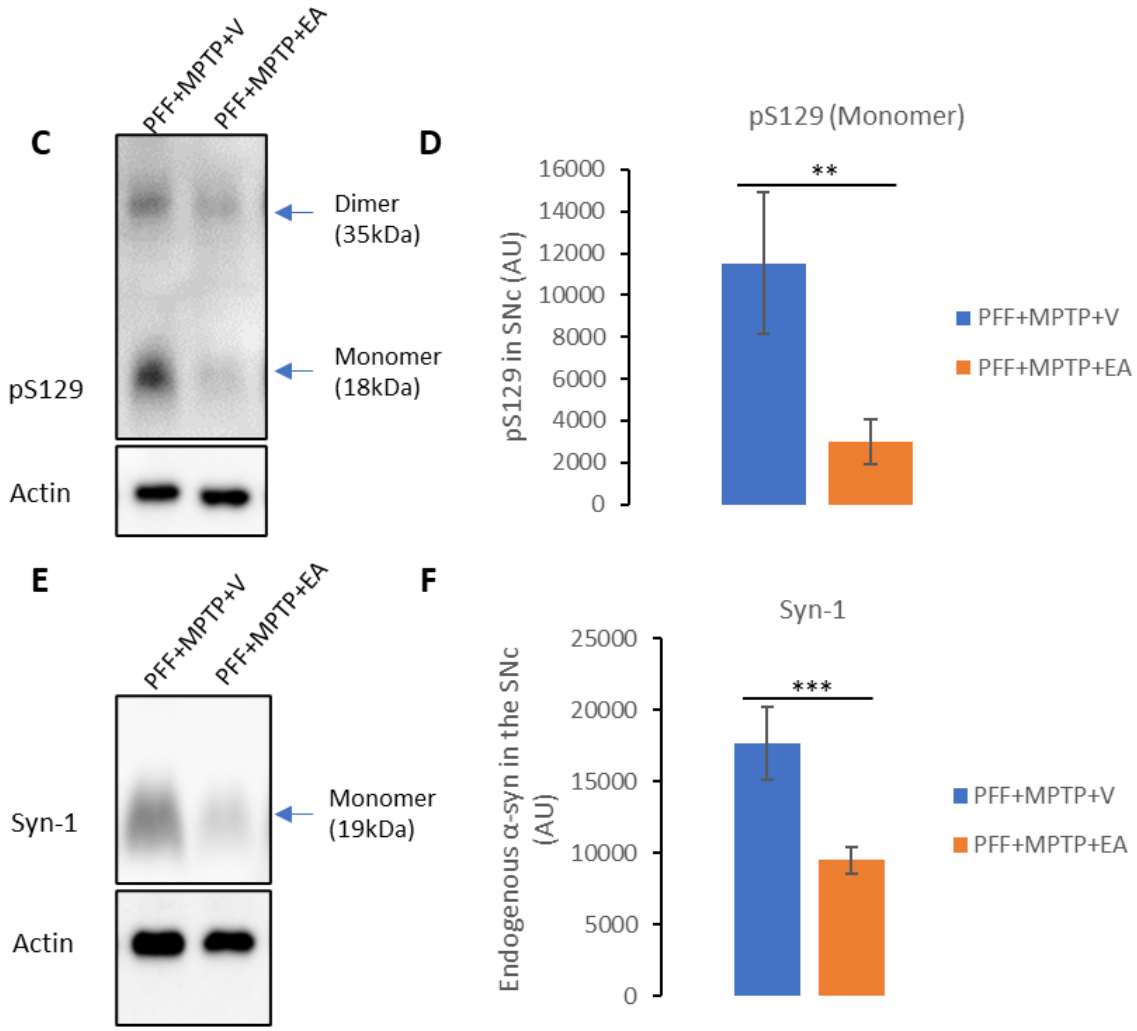

Figure 9: Assessment of α-Syn Spreading from Site of Injection (Striatum) to TH Neurons in the SNc. (A) Representative results showing the mouse pS129 α-syn spreading in the SNc in both PFF+MPTP treated groups in comparison with a control group, EA confers significant protection against synuclein spreading (B) detected in this experiment as the phosphorylated form of α-syn a primary component found in Lewy bodies. Detection of different forms of α-syn protein using SDS-PAGE: (C&D) pS129 α-syn and (E&F) endogenous α-syn. Data represented as percentage ± standard error of the mean (n = 3-4). Scale bar 50 µm (A). (***p < 0.0001 **p < 0.001, one-way ANOVA and Bonferroni post hoc test) (AU: Arbitrary Unit). pS129 α-syn: Phosphorylated α-synuclein; TH: Tyrosine Hydroxylase; PFF: α-synuclein Preformed Fibrils; SNc: Substantia Nigra pars compacta; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle (Continued)

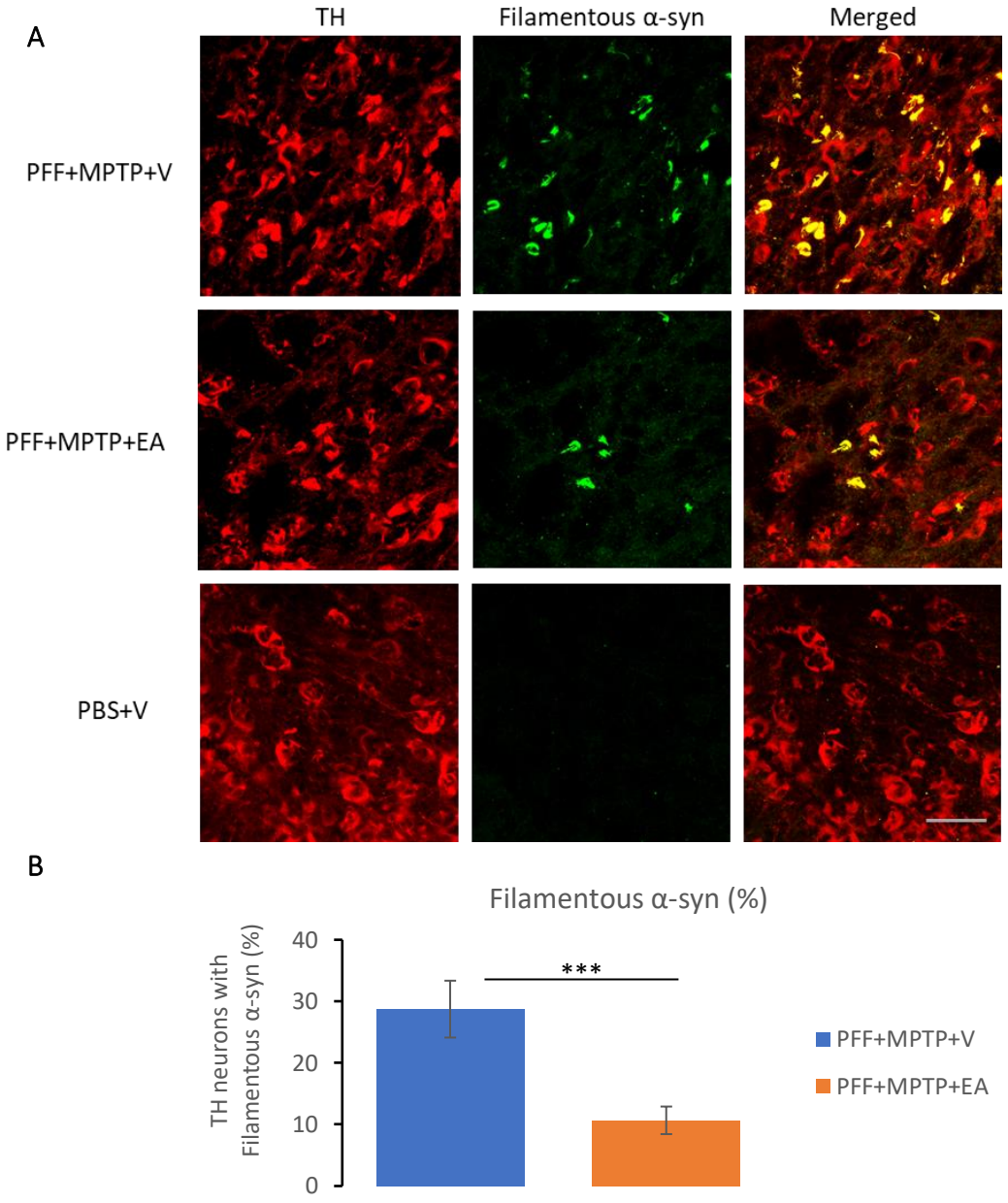

Figure 10: Assessment of α-Syn Spreading from Site of Injection (Striatum) to TH Neurons in the SNc (II). (A) Representative results showing the spreading via detection of Filamentous α-syn in the SNc in both PFF+MPTP treated groups in comparison with a control group, EA confers significant protection against synuclein spreading (B) detected in this experiment as the PK digestion resistant form of α-syn. Data represented as percentage ± standard error of the mean (n = 3-4). Scale bar 50 µm (A). ***p < 0.0001, **p < 0.001 (B) compared with the control group (one-way ANOVA and Bonferroni post hoc test). PK: Protease K; TH: Tyrosine Hydroxylase; SNc: Substantia Nigra pars compacta; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle

3.1.2 EA Inhibits Dopaminergic Neuronal Cell Loss and Preserves DAT Terminals

Loss of dopaminergic neurons in the nigrostriatal pathway is one of the hallmarks of PD. In the current study, PFF+MPTP+V group successfully displayed a significant loss of dopaminergic neurons compared to the PBS+V group, thereby mimicking PD pathology. Assessment of integrity of the dopaminergic neurons was performed via the evaluation of neuronal cell body number (Figure 11-B) and nerve terminals (Figure 11-D). TH expression in dopaminergic neuronal soma in the SNc was quantified via the counting of TH expressing neurons taking 7 serial sections of the SNc using unbiased stereology software as previously described (Ardah et al., 2020).

Result of intraperitoneal administration of EA in the current model showed neuroprotective properties at the dose of 10 mg/kg b.wt which was administered for 12 days (post stereotaxic surgery) as well one hour before each dose of MPTP administration for further seven days (mentioned in methodology section). This indicates consistency with previous findings regarding the neuroprotective effects of EA in the context of the studied model (Figure 11-A).

Since DA neurons project their nerve terminal to striatum, any loss of DA neurons will affect DAT (dopamine transporter) that expresses in the nerve terminal. Thus, the assessment of this nerve terminal has been achieved by staining DAT to measure the intensity of the nerve terminals. Similar to TH neurons, EA also preserved nerve terminal integrity when compared with vehicle treated group (Figure 11-C).

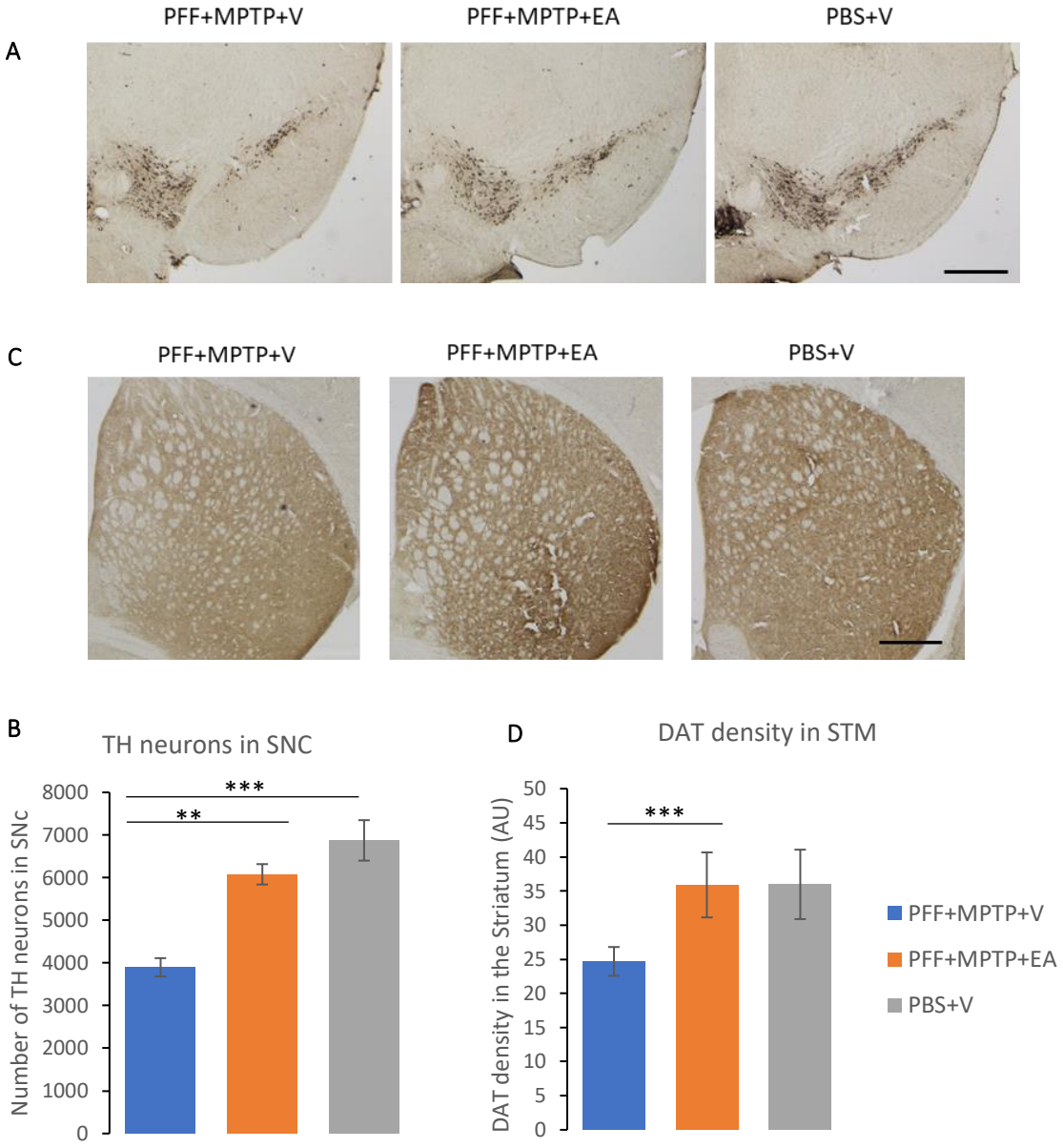

Figure 11: Assessment of Dopaminergic Cell Loss in the Nigrostriatal Pathway. Evaluation of neuronal cell body in both the SNc (A) as well as DAT intensity in the striatum (C) to evaluate the neuronal cell loss. Significant protection against dopaminergic cell loss was conferred by EA treatment (+PFF+MPTP) as compared to the counterpart vehicle treated group (+PFF+MPTP). Quantification dopaminergic neurons as the total number of TH neurons in the SNC using unbiased stereoinvestigator software represented in (B), in addition to measurement of DAT density in the STM (D). Data represented as averages ± standard error of the mean (n = 3-4). Scale bar 500 µm (A & C). ***p < 0.0001, **p < 0.001 (B & D) compared with the control group (one-way ANOVA and Bonferroni post hoc test). STM: Striatum; SNc: Substantia Nigra pars compacta; DAT: Dopamine Transporter; TH: Tyrosine Hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle

3.1.3 EA Decreases the SNc Neuronal Apoptosis in Mouse PD Model

In this study, SNc neurons were also assessed for apoptosis via the detection of DNA fragmentation using TUNEL assay which specifically labels fragmented DNA (a hallmark of apoptosis). The results indicate that the EA-treated group displayed a significantly lower presence of DNA fragmentation, as depicted in Figures 12-A and 12-B. This finding is consistent with previous data, reinforcing the conclusion of the neuroprotective effect of EA treatment against neurotoxicity resulting from the spread of α-synuclein from the striatum to the SNc in the current study.

3.1.4 EA Induces Autophagic Degradation of α-Syn

The autophagic lysosomal pathway is one of the main mechanisms responsible for the degradation of α-syn, therefore we conducted a colocalization study between the TH neurons and the LC3 protein. EA treated samples expressed significantly higher levels of colocalization of the LC3 protein in the TH neurons consistent with displayed inhibition of α-syn spreading and aggregation as well as preserved structural integrity of dopaminergic neurons. Figures 13-A and 13-B display the increased LC3 marker in TH neurons in the SNc of EA treatment. This data was further confirmed by the assessment of the autophagy flux via the quantification of the LC3 II/I ratio by SDS-PAGE assay as illustrated in Figures 13-C and 13-D.

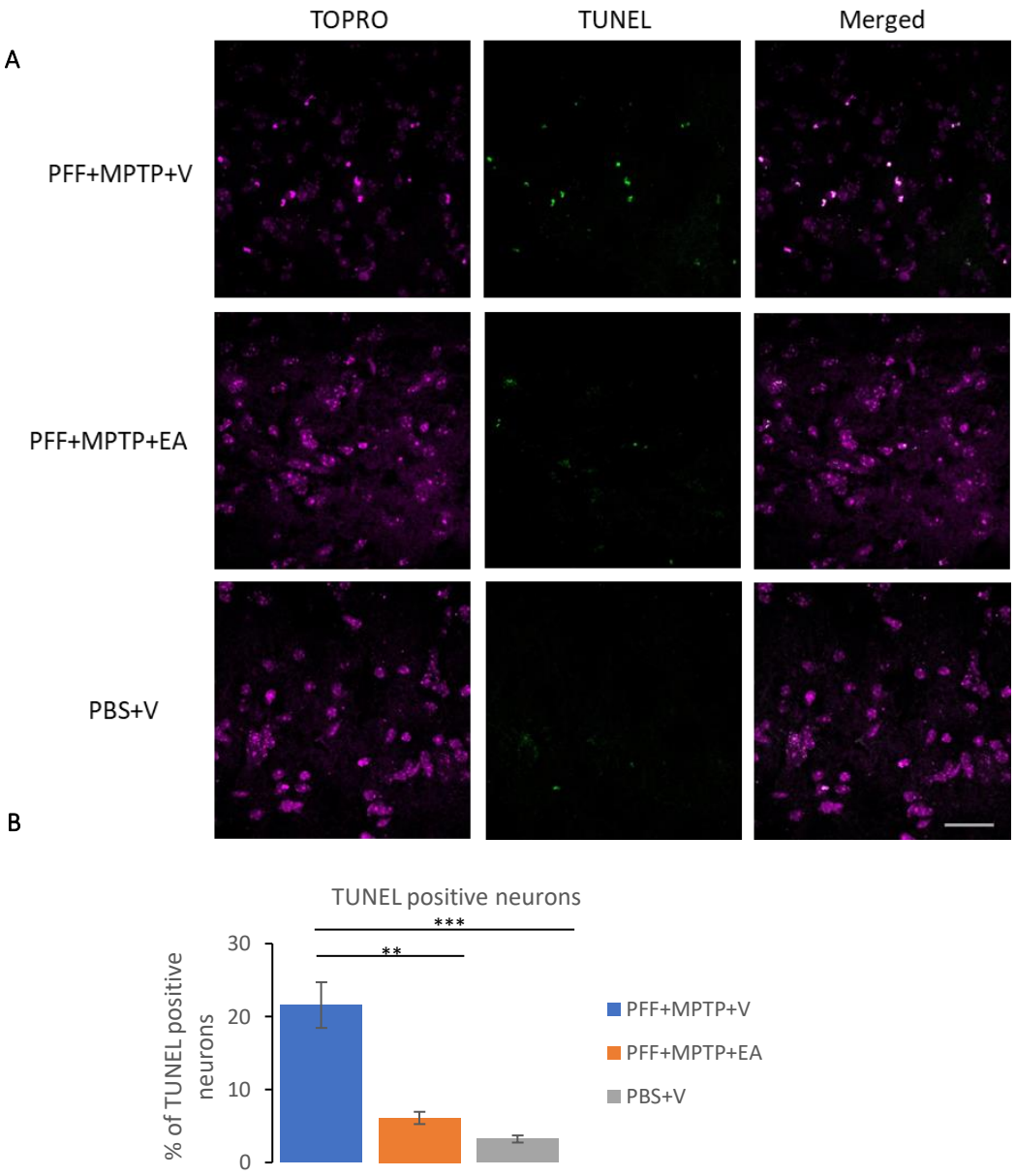

Figure 12: Detection of Apoptotic Neurons by TUNEL Signal. (A) Representative TUNEL positive neurons in the midbrain sections in the SNc region. EA confers significant against apoptosis in presence of PFF+MPTP treatment as represented in (B). TO-PRO (originally blue) was pseudo colored to magenta for better visualization. Data represented as percentage ± standard error of the mean (n = 3-4). Scale bar 25 µm (A). ***p <0.0001, **p < 0.001 (B) compared with the control group (one-way ANOVA and Bonferroni post hoc test). TUNEL: Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle

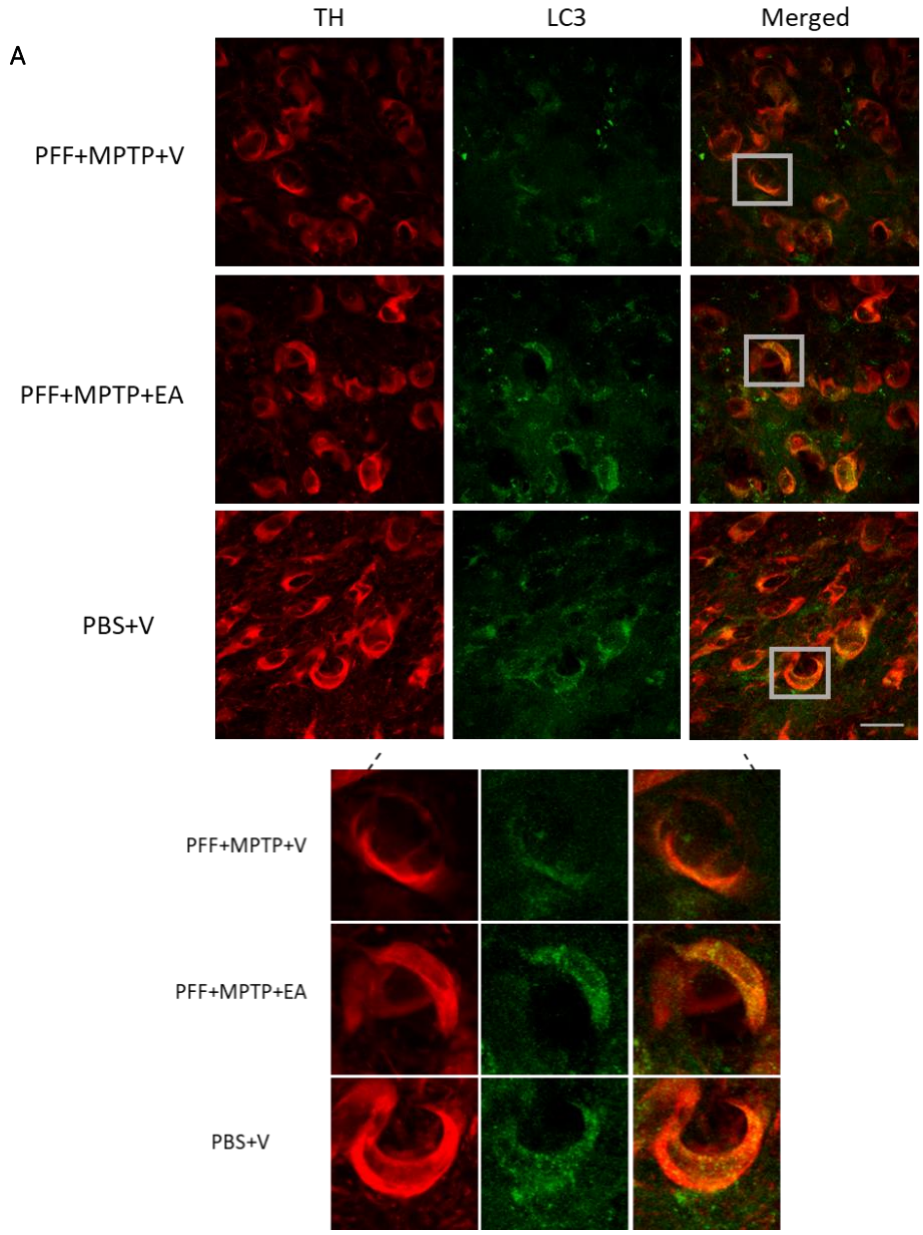

Figure 13: Assessment of Autophagy in TH Neurons in the SNc (I). (A) Representative results showing the LC3 in the SNc in both PFF+MPTP treated groups in comparison with a control group, EA significantly increases LC3 puncta colocalized in the TH neurons of the SNc as compared with vehicle only treated group (+PFF+MPTP) (B). Detection of autophagy marker LC3 in both its isoforms LC3II and LC3I (C) revealed an increase in the LC3II/I ratio in the EA treated group as opposed to control group (D). Data represented as colocalization coefficient ± standard error of the mean (n = 3-4). Scale bar 25 µm (A). (**p < 0.001, *p < 0.05, ns: Not significant, one-way ANOVA and Bonferroni post hoc test). LC3: Microtubule-associated protein 1A/1B-light chain 3; TH: Tyrosine hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic acid; V: Vehicle

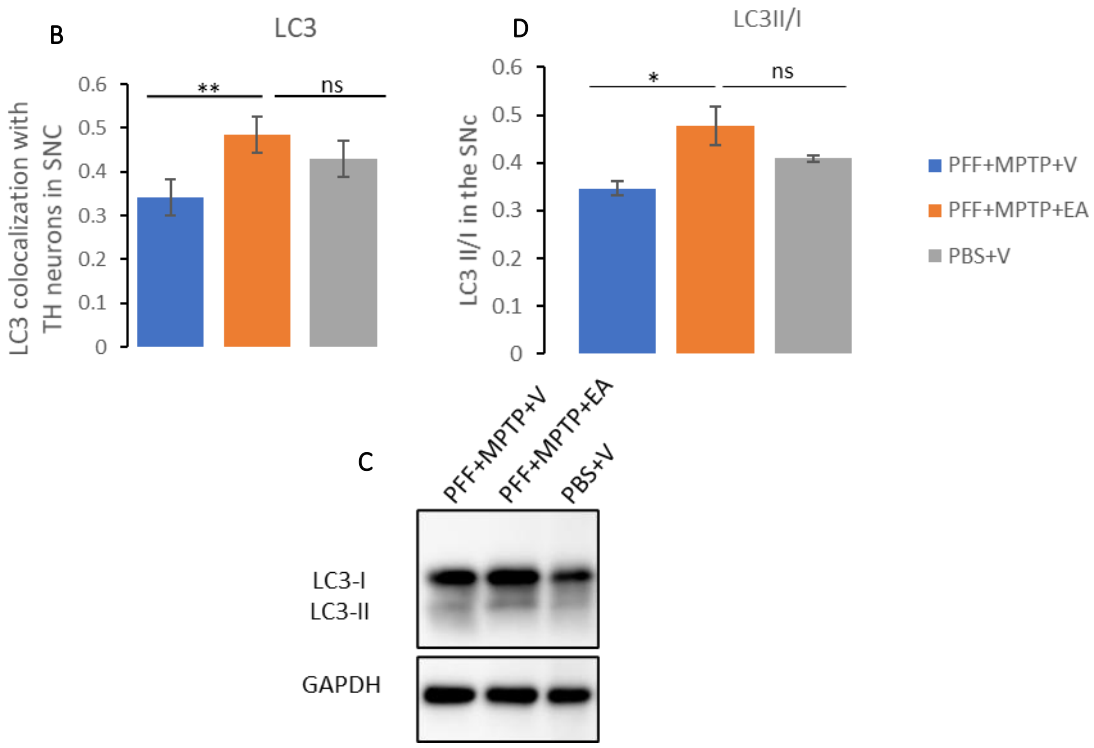

Figure 13: Assessment of Autophagy in TH Neurons in the SNc (I). (A) Representative results showing the LC3 in the SNc in both PFF+MPTP treated groups in comparison with a control group, EA significantly increases LC3 puncta colocalized in the TH neurons of the SNc as compared with vehicle only treated group (+PFF+MPTP) (B). Detection of autophagy marker LC3 in both its isoforms LC3II and LC3I (C) revealed an increase in the LC3II/I ratio in the EA treated group as opposed to control group (D). Data represented as colocalization coefficient ± standard error of the mean (n = 3-4). Scale bar 25 µm (A). (**p < 0.001, *p < 0.05, ns: Not significant, one-way ANOVA and Bonferroni post hoc test). LC3: Microtubule-associated protein 1A/1B-light chain 3; TH: Tyrosine hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic acid; V: Vehicle (Continued)

The molecular chaperone, p62 is essential in the recruitment of autophagic biomarkers (autophagosome) and cellular cargo, thus aiding in the facilitation of the catabolic process. p62 is a substrate of the autophagic flux, in other words, its accumulation is a sign of dysfunctional autophagy. Decreased p62 inclusions colocalized with TH neurons in the SNc in the EA treated groups in comparison with control group (Figures 14-A and 14-B) confirmed the capacity of EA to restore normal autophagy in the current PD mouse model. The western blot analysis of p62 also confirmed the immunostaining data related to p62 (Figures 14-C and 14-D).

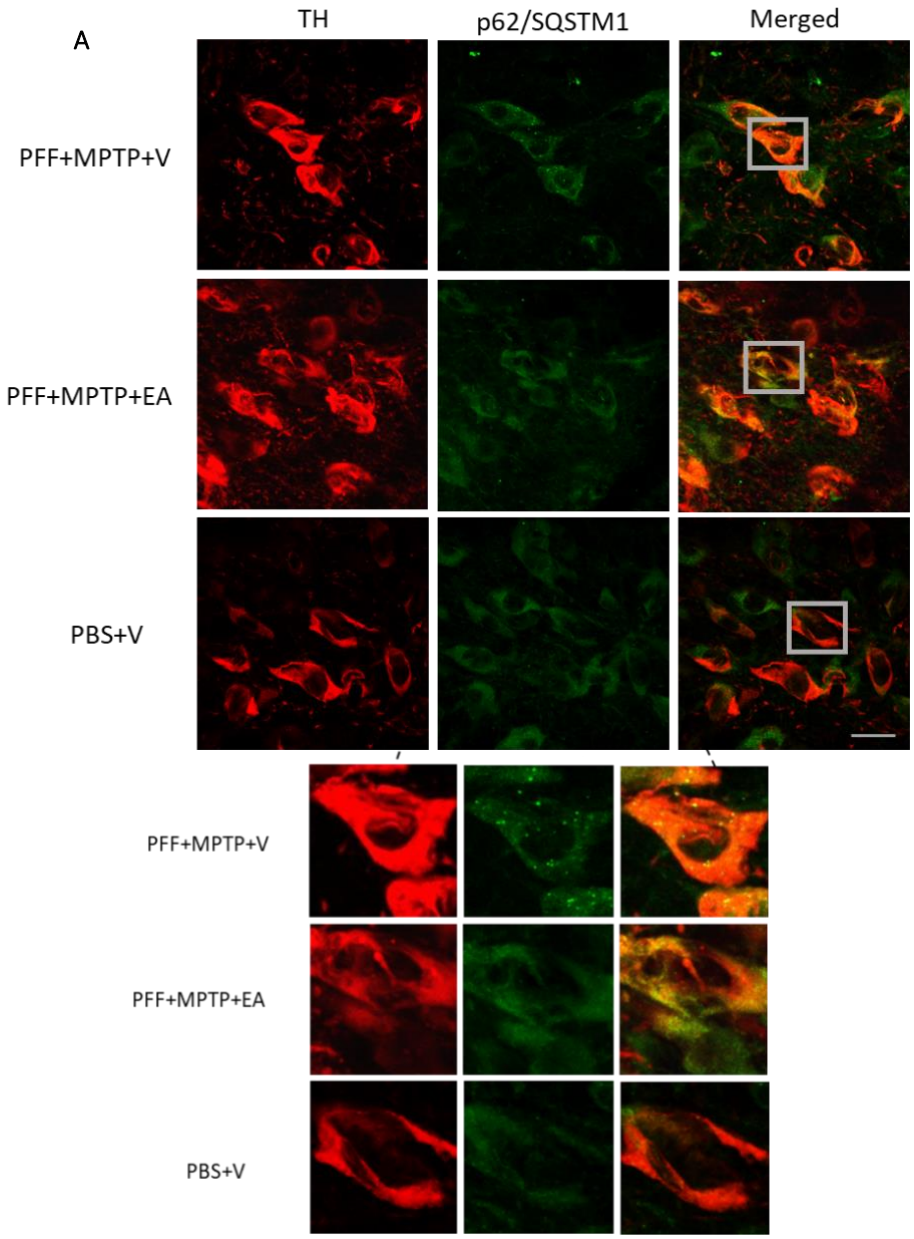

Figure 14: Assessment of Autophagy in TH Neurons in the SNc (II). (A) Representative results showing the p62 in the SNc in both PFF+MPTP treated groups in comparison with a control group, EA significantly decreases p62 puncta colocalized in the TH neurons of the SNc as compared with vehicle only treated group (+PFF+MPTP) (B). (C) Detection of lower levels of p62 in the EA treated group is indicative of a functional autophagy as opposed to control group as represented in (D). Data represented as p-value ± standard error of the mean (n = 3-4). Scale bar 25 µm (A). (*$p < 0.05$, one-way ANOVA and Bonferroni post hoc test). p62/SQSTM1: Sequestosome 1; TH: Tyrosine Hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle

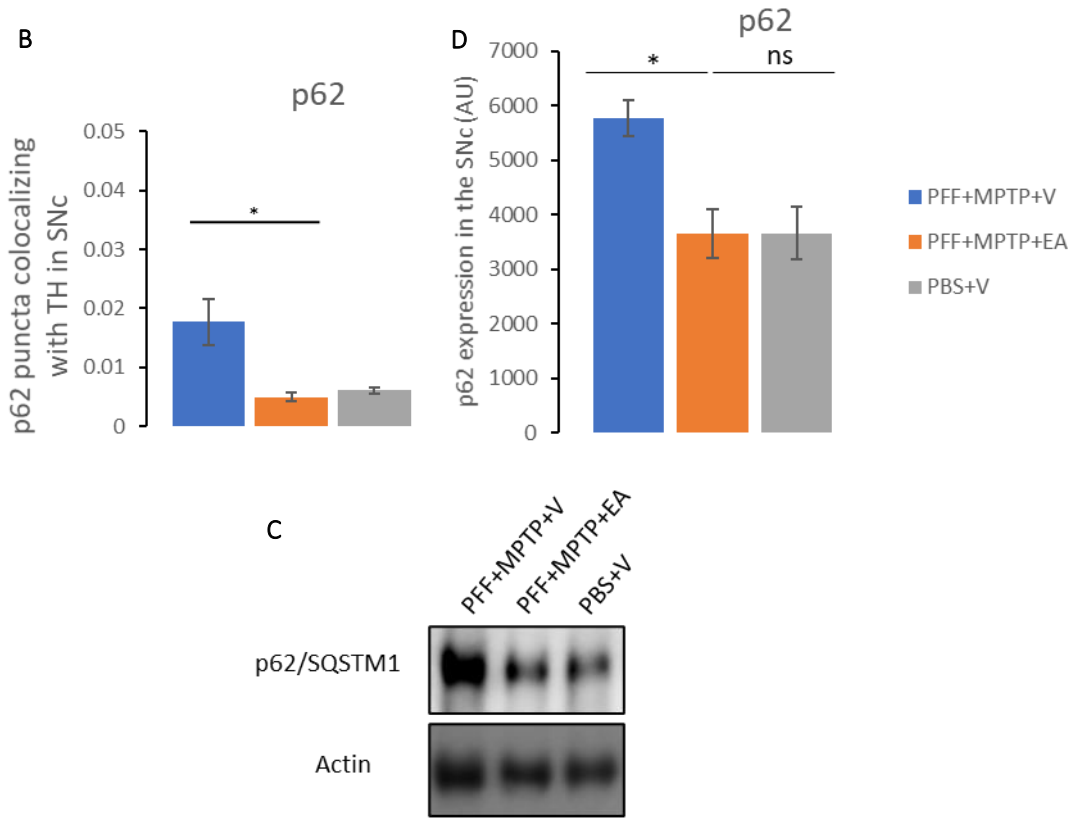

Figure 14: Assessment of Autophagy in TH Neurons in the SNc (II). (A) Representative results showing the p62 in the SNc in both PFF+MPTP treated groups in comparison with a control group, EA significantly decreases p62 puncta colocalized in the TH neurons of the SNc as compared with vehicle only treated group (+PFF+MPTP) (B). (C) Detection of lower levels of p62 in the EA treated group is indicative of a functional autophagy as opposed to control group as represented in (D). Data represented as p-value ± standard error of the mean (n = 3-4). Scale bar 25µm (A). (*p < 0.05, one-way ANOVA and Bonferroni post hoc test). p62/SQSTM1: Sequestosome 1; TH: Tyrosine Hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle (continued)

3.1.5 Oral Administration of EA: Testing the Translational Potential

The efficacy of the oral administration of EA was also tested under the same conditions as IP treatment. This investigation aims to explore the practical implications of EA as a potential therapeutic agent and assess its feasibility for oral delivery in translational medicine. The literature indicates a high degradation pattern associated with the oral administration of the EA molecule. In response to this, we doubled the oral dose to 20 mg/kg b.wt. and administered it using oral gavage, maintaining the similar conditions of

the IP dosing scheme. This adjustment aimed to enhance EA bioavailability. However, our observations revealed that mice exhibited significant resistance to the EA treatment compared to the non-treated groups (without EA). This resistance could potentially be attributed to the pungency of EA.

Brain samples were collected and subjected to the same evaluations that proved the efficacy of the IP administration of EA. Assessment of the spreading of the α-syn was performed in this study. While 20 mg/kg b.wt. oral administration of EA significantly prevented the pS129 α-syn species spreading as shown in Figures 15-A and 15-B, filamentous α-syn species was reduced in a statistically insignificant manner (Figures 16-A and 16-B). The neuroprotective effect of EA observed in the IP route of administration (10 mg/kg b.wt.) was not translated in the oral route at a dose of 20 mg/kg b.wt., neither the DAT in the striatum (Figures 17-C and 17-D) nor the TH neuronal (Figures 17-A and 17-B) soma in the SNc were preserved from α-syn-induced toxicity.

Collectively, these data suggest that the lack of neuroprotective effects observed with oral administration of EA may be attributed to factors such as the high first-pass effect, intestinal degradation, and the low hydrophilic nature of EA. Future research endeavors should take these factors into consideration for more robust and insightful outcomes.

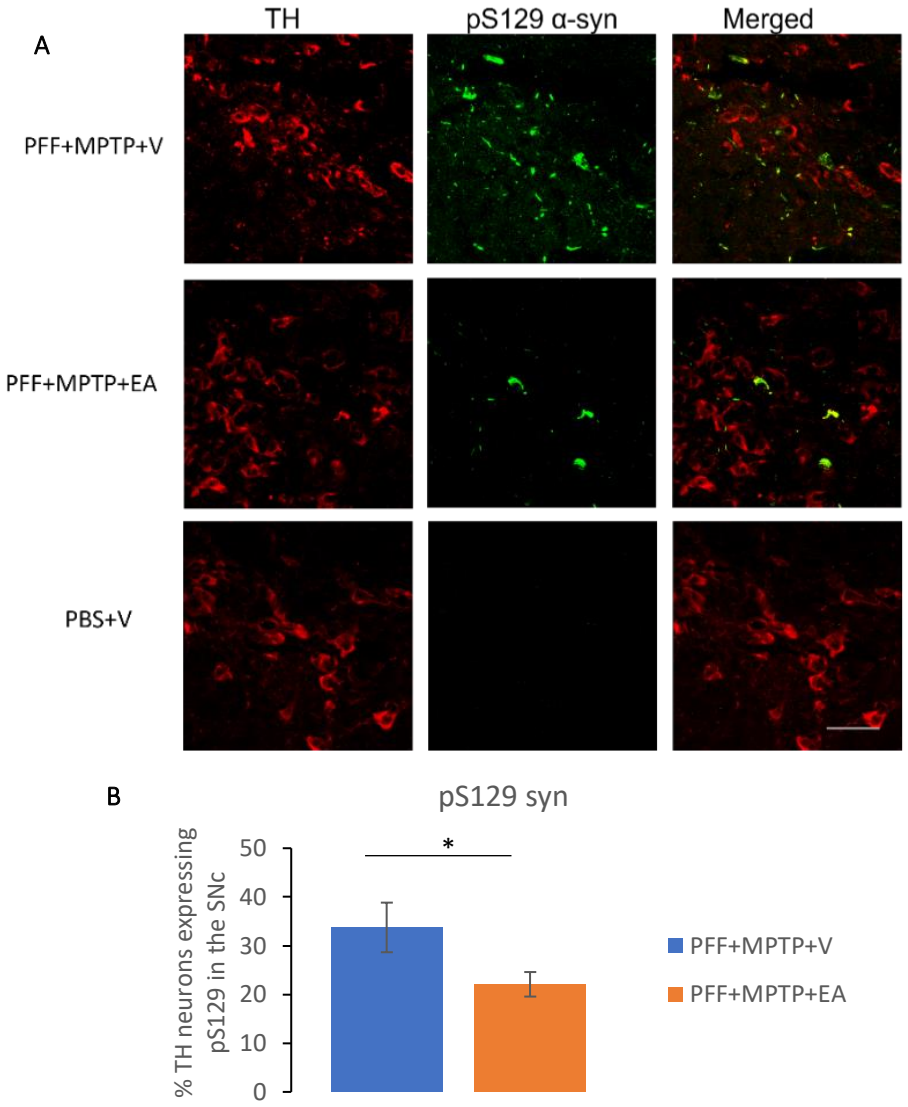

Figure 15: Assessment of α-Syn Spreading from Site of Injection (striatum) to TH Neurons in the SNc Upon Oral Administration of EA (20 mg/kg b.wt.). (A) Representative results showing the pS129 mouse α-syn spreading in the SNc in both PFF+MPTP treated groups in comparison with a control group, oral dose of EA20 mg/kg confers significant protection against synuclein spreading (B) detected in this experiment as the phosphorylated form of α-syn a primary component found in Lewy bodies. Data represented as percentage ± standard error of the mean (n = 3-4). Scale bar 50 µm (A). *p < 0.05 (B) compared with the control group (one-way ANOVA and Bonferroni post hoc test). pS129 α-syn: Phosphorylated α-synuclein; TH: Tyrosine Hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle

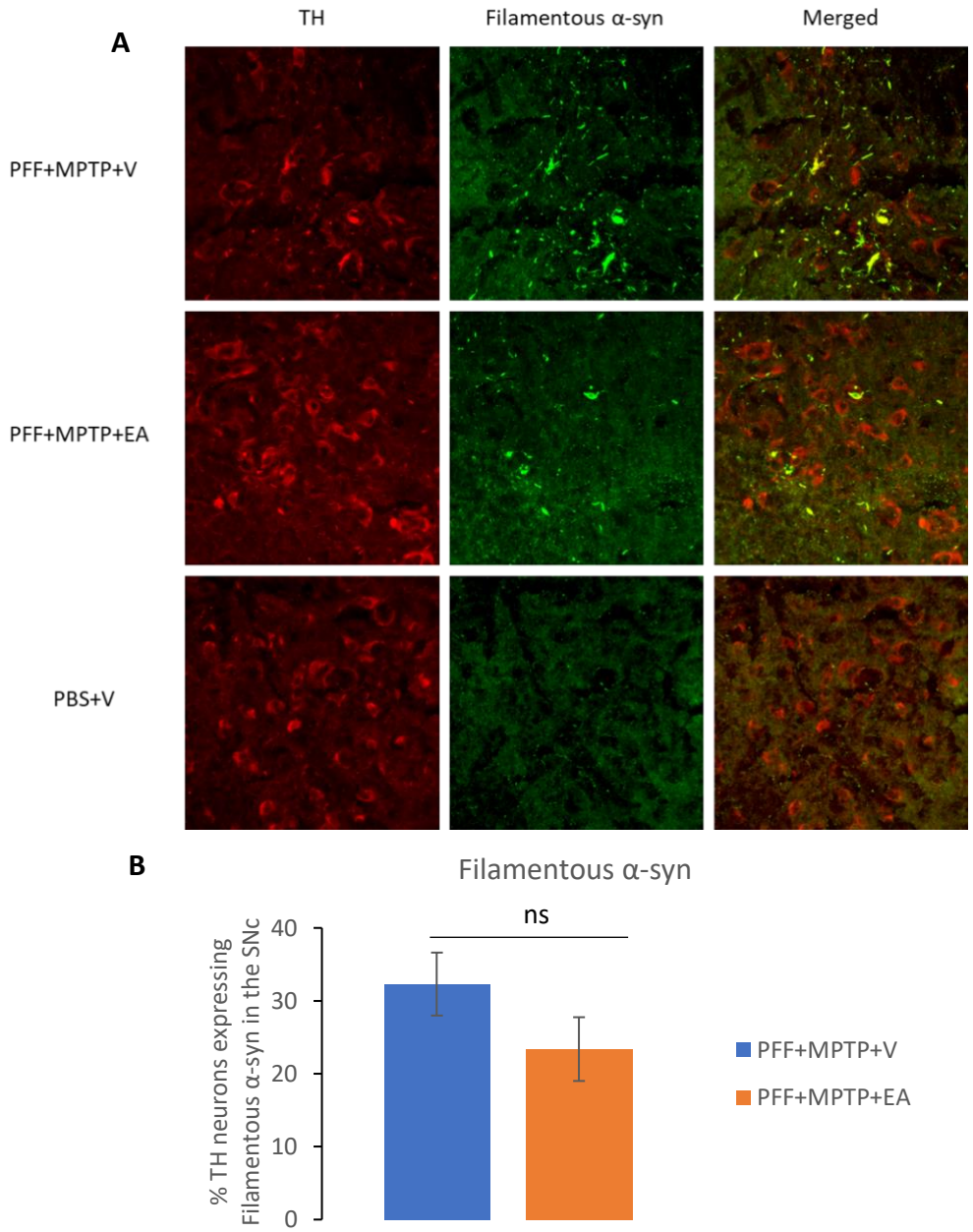

Figure 16: Assessment of α-Syn Spreading from Site of Injection (Striatum) to TH Neurons in the SNc. (A) Representative results showing the spreading via detection of Filamentous α-syn in the SNc in both PFF+MPTP treated groups in comparison with a control group, oral dose of 20 mg/kg of EA does not confer significant protection against synuclein spreading (B) detected in this experiment as the PK digestion resistant form of α-syn. Data represented as percentage ± standard error of the mean (n = 3-4). Scale bar 50 µm (A). ns denotes not significant (B) compared with the control group (one-way ANOVA and Bonferroni post hoc test). PK: Protease K; TH: Tyrosine Hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle

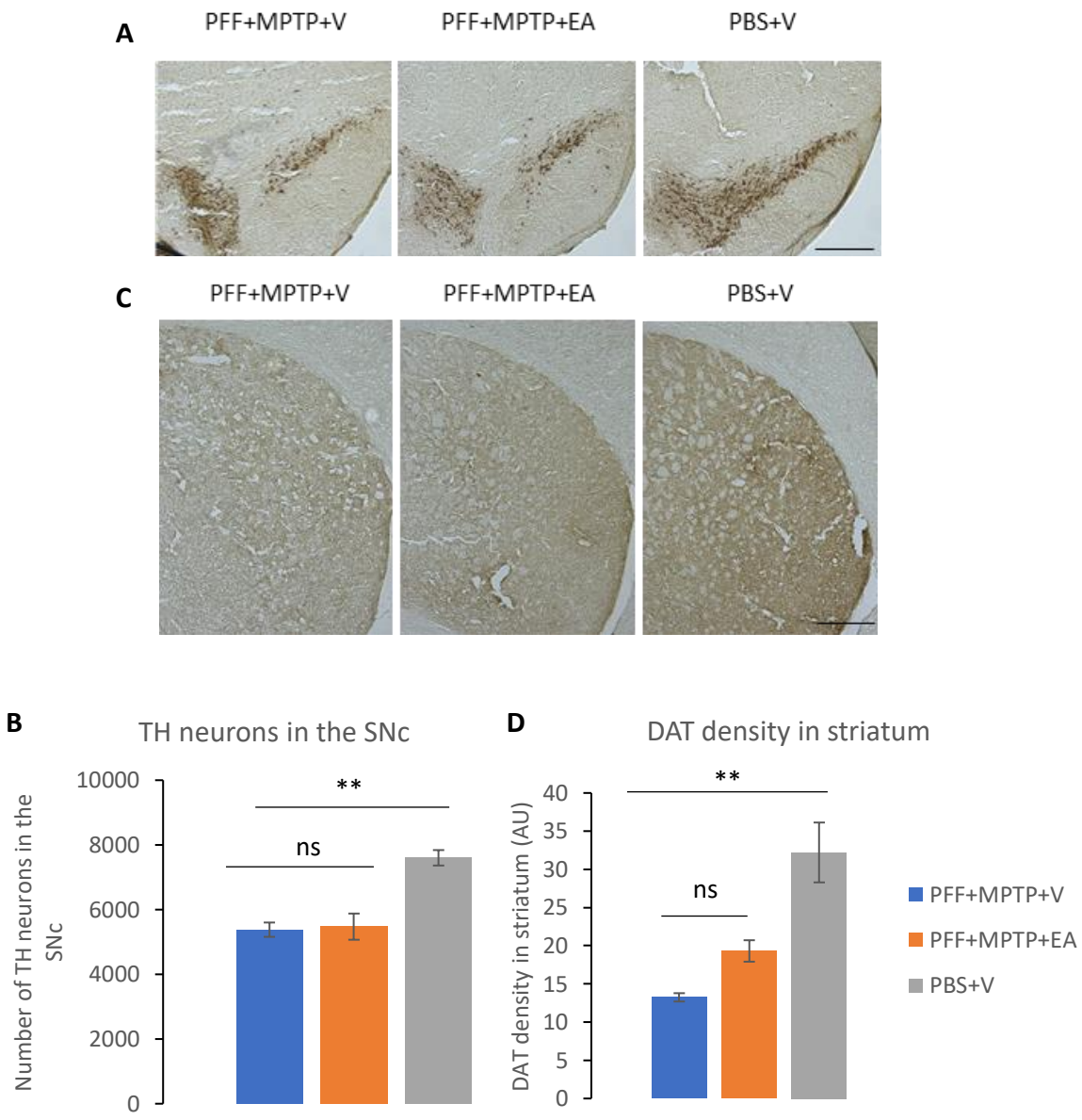

Figure 17: Assessment of Dopaminergic Neuronal Loss in PD Model Upon Oral Administration of EA. Evaluation of neuronal cell body in both the SNc (A) as well as the striatum (C) to evaluate the neuronal cell loss. Protection against dopaminergic cell loss was not significant by EA oral 20 mg/kg treatment (+PFF+MPTP) as compared to the counterpart vehicle treated group (+PFF+MPTP). Quantification dopaminergic neurons as the total number of TH neurons in the SNc using unbiased stereoinvestigator software represented in (B), in addition to measurement of DAT density in the STM (D). Data represented as averages ± standard error of the mean (n = 3-4). Scale bar 500 µm (A & C). **p < 0.001, *p < 0.05, ns not significant (B & D) compared with the control group (one-way ANOVA and Bonferroni post hoc test). STM: Striatum; SNc: Substantia Nigra compacta; DAT: Dopamine Transporter; TH: Tyrosine Hydroxylase; PFF: α-synuclein Preformed Fibrils; MPTP: 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine; EA: Ellagic Acid; V: Vehicle

3.2 Discussion

PD is the second most common neurodegenerative disease. The increment in the global prevalence of PD population had directed efforts to seek therapeutic targets beyond replacement therapies (concerned mainly with the external supplementation of dopamine precursors) and symptomatic treatments.

PD is pathologically characterized by the loss of dopaminergic neurons in the SNc area. This loss results in the depletion of the dopamine neurotransmitter production and manifests as the cardinal PD symptoms: tremors, rigidity, postural instability and muscle stiffness (Sveinbjornsdottir, 2016). Accumulation of α-syn, a presynaptic protein has been hypothesized to play a role in neurotransmitter release, in a form of insoluble moieties known as LBs is one of the hallmarks of PD (Spillantini et al., 1997). The aggregation of α-syn has been significantly associated with the development of PD pathology. The notion of limiting α-syn aggregation had been of great interest in PD research (Brás & Outeiro, 2021).

Phytochemicals have always set the foundation in the development of active therapeutic agents. One of the prominent phytochemicals in PD research, belonging to the polyphenol group, is EA. The capacity of EA to elicit an antioxidative action in many diseases had highlighted the potential of this compound in therapeutic context (Amor et al., 2020; Mansouri et al., 2020; Rosillo et al., 2012). EA has a molecular structure that permits a buffering mechanism in many redox reactions, thus potentially decreasing the ROS which plays a great role in the propagation of the neuroinflammatory component in PD. In addition to its scavenging property, the polyphenolic compound was reported to induce the major antioxidative pathways such as Nuclear factor erythroid 2–related factor 2 (Nrf2) (Xiao et al., 2022) and decrease inflammatory markers such as inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase-2 (COX-2) (Ardah et al., 2020).

Autolysosomal machinery was reported to be induced by EA administration in cell culture model of PD (Ardah et al., 2021), which is substantially responsible for the degradation of toxic α-syn protein aggregates. Collectively, literature promotes the potential of EA as a therapeutic compound in the intervention of PD. However, the role of EA in preventing

alpha-synuclein spreading and aggregation has not been investigated in in vivo model of PD.

The recently developed mouse model of PD (Merghani et al., 2021) has two essential features such as 1) enhancement of synuclein spreading upon low dose of MPTP treatment and 2) significant loss of DA neurons in the SNc. Thus, in the current study, we utilized this model to evaluate the neuroprotective action of EA, firstly by investigating the status of α-syn propagation from the site of induction (striatum) to the dopaminergic neurons in the SNc area. The presence of two different forms of α-syn were examined in this study; namely the pathological pS129 α-syn (Figures 9-A and 9-E) as well as the filamentous form of α-syn (Figure 10-A). Interestingly, it has been found that EA significantly reduced the spreading of both these pathological forms of α-syn. Additionally the levels of endogenous α-syn (Figure 9-C) was significantly reduced in the EA-treated group (PFF+MPTP+EA) in comparison to the counterpart control group (PFF+MPTP+V).

Secondly, the evaluation of the dopaminergic neuronal soma in the SNc, and status of their nerve terminals in the striatum was performed. It has been reported that the loss of the neuronal synaptic plasticity preceding the α-syn pathological accumulation (Villar-Piqué et al., 2016), this results in the earlier disintegration of the dopaminergic nerve terminals which finally leads to the retrograde damage of the neurons as associated with the α-syn spreading. This point was accounted in our mouse model of PD pathology where the inoculation of human α-syn fiber seeds induced aggregation, propagation as well as the induced neurotoxicity of the indigenous α-syn. The relatively short half-life of the inoculated α-syn PFF seed in the striatum allowed endogenous triggering of the α-syn aggregation while clearing over the study period facilitating estimation of the endogenous α-syn spreading and aggregation. Additionally, the induction of MPTP neurotoxicity allowed mimicry of the ROS induced neuronal damage on an accelerated scale. EA IP administration at a dose of 10 mg/kg b.wt. succeeded in conferring significant neuroprotection of the SNc dopaminergic neuronal soma (Figure 11-A) as well as the nerve terminals in the striatum (Figure 11-C). These results were further substantiated by the detection of apoptotic neurons in the SNc area (Figure 12-A) which revealed

significantly lower rates of apoptosis upon EA treatment as opposed to control group (PFF+MPTP+V).

Finally, investigation of the autophagic flux was performed in this study via the detection of LC3 and p62. LC3II/I ratio was significantly increased upon EA treatment as detected by the western blotting analysis (Figure 13-C). Furthermore, the colocalization of LC3 with TH expressing dopaminergic neurons in the SNc was significantly higher in these samples than in control group (PFF+MPTP+V) (Figure 13-A). Additionally, the levels of p62, a chaperone in the mediation of the autophagic flux rendering it a substrate of autophagy, was compared between both the EA treated and control groups (PFF+MPTP). The accumulation observed in the p62 inclusions in control group (PFF+MPTP+V) was indicative of dysfunctional autophagy. EA treatment significantly reduced the p62 inclusions accumulation in the dopaminergic neurons in the SNc, the assessment of this data was confirmed by both the colocalization immunofluorescence via TH and p62 co-staining (Figure 14-A) and the western blotting assays (Figure 14-C).

Our results thus far show that the IP treatment of EA could elicit a neuroprotective effect in PD mouse model. The induction of the autophagic flux was observed in this study suggesting an active involvement of the catabolic process rather than the canonical antioxidant effect. In our study we wanted to test the translational capacity of EA administration using oral delivery. Unfortunately, the change of the route of administration from IP to the more therapeutically favored oral administration diminishes the neuroprotective effects of EA. The poor oral absorption of the EA molecule rendered the dose ineffective even in a dose two times higher than the IP dose (20 mg/kg b.wt.). Despite the lack of the significant translational effect of the EA in oral administration on dopaminergic neuronal protection (Figure 17-A and 17-C), it is important to note that there was a significant reduction in pS129 α-syn species (Figure 15-A) in addition to a non-significant trend in the reduction of filamentous α-syn species (Figure 16-A).

Chapter 4: Conclusion

4.1 Conclusion

This study investigated the effect of EA in a C57BL/6 male mice model of PD, focusing on the amelioration of PD pathology linked to the two major hallmarks of the disease. The first hallmark involves the retrospective spreading of α-syn from the site of injection to the SNc where dopaminergic neuronal soma resides. The second hallmark is the loss of dopaminergic neurons in the nigral region.

EA, administered via intraperitoneal route at the dose of 10 mg/kg b.wt., demonstrated efficacy in retarding the spreading of both forms of α-syn, including pS129 and pathological filamentous α-syn, from the striatum to the SNc. Additionally, EA administration prevented the loss of dopaminergic neurons in the SNc area. The study also reported the induction of autophagic flux by EA administration, suggesting a potential mechanistic link in the context of PD.

4.2 Study Limitations and Future Directions

The effect of EA administration on mitochondrial dysfunction and ROS production/clearance in our mouse model of PD was not assessed in this study and should be addressed in future studies. While this study highlights the enhanced autophagic flux in a mouse PD model stimulated by EA, the specific mechanism by which autophagy is activated was not assessed. However, this study is the first, to our knowledge, to demonstrate the involvement of enhanced autophagic flux in the context of EA treatment in vivo.

It is crucial to acknowledge factors contributing to the lack of significance in the oral administration of EA, including the limited oral bioavailability of the EA molecule and the challenges posed by the pungency of EA during oral dosing, leading to mice resistance compared to the groups that did not receive EA (PBS and PFF+MPTP). Future research should be designed to address these pitfalls encountered during the oral administration of EA.

Furthermore, to broaden the scope of understanding, it is recommended to replicate the efficacy of EA in a counterpart study focusing on a female mouse model of PD. These future directions aim to overcome current limitations and provide additional insights into the potential role EA could play in the future development of pharmacotherapeutics for PD.

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List of Publications

Radwan, N., Khan, E., Ardah, M. T., Kitada, T., & Haque, M. E. (2024). Ellagic Acid Prevents α-Synuclein Spread and Mitigates Toxicity by Enhancing Autophagic Flux in an Animal Model of Parkinson's Disease. *Nutrients*, 16(1), Article 1. https://doi.org/10.3390/nu16010085

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Parkinson's Disease (PD) is the second most common neurological disorder pathologically characterized by loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNc) as well as the formation of Lewy bodies composed mainly of α-synuclein (α-syn) aggregates. It has been documented that abnormal aggregation of α-syn is one of the major causes of developing PD. Ellagic Acid (EA) is a widely known dietary supplement found in pomegranates as well as a plethora of berries and nuts. EA has been shown to prevent α-syn aggregates' toxicity in cellular model of PD. In the current study we aim to evaluate whether EA can prevent α-syn spreading and associated toxicity in an animal model of PD.

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