

## Introduction

Neurodegenerative diseases such as Alzheimer's and Parkinson's are on the rise due to people living longer as a result better quality healthcare and advancement of science and technology. The number of individuals with age-related neurodegenerative diseases is expected to double, if not triple, by 2050 [2-4]. To date, no therapies exist to halt or slow the progression of neurodegenerative disease, and many pharmacotherapies only have a temporally limited efficacy.

While neurodegenerative diseases differ in cell-type and brain region affected, many share common occurrence of aberrant processes both inside degenerating neurons (cell autonomous) and outside degenerating neurons (non-cell autonomous) in other neuronal and non-neuronal cell types. Because many of these diseases result from a culmination of age-related intracellular and extracellular changes exacerbated by environmental stimuli and genetics, developing a multipronged therapeutic approach is of the utmost need to improve upon current therapeutic strategies. In section 1, I will provide an overview of aberrant cell-autonomous and non-cell autonomous mechanisms common to neurodegenerative diseases. In section 2, I will discuss how these mechanisms contribute specifically to degeneration of the nigrostriatal system in Parkinson's disease. Finally, in section 3 I will briefly overview existing therapies for Parkinson's disease conclude with suggestions for a switch to a more multi-pronged approach.

## Part 1: Cell Autonomous and Non-Cell Autonomous Mechanisms of Neurodegenerative Diseases

Neurodegenerative diseases such as ALS, Alzheimer's, Huntington's, and Parkinson's disease share many similar mechanisms contributing to disease progression, although they differ in associated genetic causes, aggregated protein species, and cell type/brain region affected (Table 1; adapted from [5]). What determines selective vulnerability of distinct cell populations in these diseases still remains unclear, but aberrant mechanisms within degenerating neurons and in the vicinity of degenerating neurons are being elucidated.

Disease	Aggregated Protein	Primary Cell Type Affected	Associated Genetic Mutations
Alzheimer's	Amyloid-beta, Tau	Hippocampal and cortical neurons	APOE4, PSEN1, PSEN2, APP
ALS	FUS, DP-43, DPTN, UBQLN2	Motor Neurons	SOD1
Huntington's	Huntingtin	Striatal Medium Spiny Neurons	PolyQ (CAG) repeat
Parkinson's	Alpha-synuclein	Nigrostriatal Dopamine Neurons	SNCA, LRRK2, DJ-1, PINK-1

Table 1: Adapted from [5]

### Cell Autonomous Mechanisms:

#### Protein Aggregation and Mitochondrial Dysfunction/Oxidative Stress

A common feature of many neurodegenerative disorders is accumulation of aggregated proteins. Within cells, the ubiquitin-proteasome system (UPS) is responsible for removing excess or damaged proteins. Ubiquitin is activated by a ubiquitin activating enzyme (E1) which then bonds to a ubiquitin conjugating enzyme (E2). Following bondage to the ubiquitin ligase enzyme (E3), the protein substrate undergoes addition of a polyubiquitin chain, which targets the protein for degradation by the proteasome. As cells age, the UPS becomes less efficient in degrading unwanted or toxic protein species, which can result in the accumulation of oxidized or nitrated proteins that are characteristic pathological hallmarks of many neurodegenerative

disorders [7]. An imbalance in the amount of misfolded protein and the ability of the UPS to degrade it can result in accumulation of toxic protein species, such as tau and amyloid beta in Alzheimer's, huntingtin in Huntington's disease, SOD1 in ALS, and alpha-synuclein in Parkinson's disease and other synucleinopathies [5].

It is widely recognized that oxidative stress plays a role in many neurodegenerative diseases and increases as a byproduct of normal aging. Although the brain only accounts for 2% of body weight, it utilizes 20% of the body's oxygen intake, making neurons susceptible targets of oxidative damage [8]. Oxidative stress occurs as mitochondria become damaged or less efficient and produce more reactive oxygen species which can damage DNA, alter proteins and membranes [9]. Mitochondrial dysfunction and subsequent oxidative stress are highly involved in Parkinson's disease pathogenesis and can be exacerbated by intracellular inclusions of alpha-synuclein, as will be discussed in Part 2.

## **Non-Cell Autonomous Mechanisms**

### Microglia-Mediated Neuroinflammation and Protein Clearance

It is widely recognized that glia play a key role in non-cell autonomous toxicity in neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's [10]. Microglia comprise the brain's innate immune macrophages, responsible for migrating to the site of injury, phagocytizing cellular debris, providing trophic support, and interacting with astrocytes. After repeated insult or chronic accumulation of toxic protein species, microglia can become chronically activated and release proinflammatory cytokines, recruit cells from the periphery, and trigger release of reactive oxygen

species from astrocytes [11]. In some cases such as Parkinson’s, microglial activation and neuroinflammation occur prior to overt degeneration; conversely in Alzheimer’s, it is proposed that microglial dysfunction in protein clearance rather than general activation contributes to disease progression [12].

**Part 2: Cell Autonomous and Non-Cell Autonomous Toxicity of Alpha-Synuclein in Parkinson’s disease**

Parkinson’s disease (PD) is the second most-common neurodegenerative disorder, affecting approximately 1% of the population over the age of 65. PD presents clinically with motor symptoms such as

Cell Autonomous Mechanisms	Non Cell Autonomous Mechanisms
Mitochondrial Dysfunction	Inflammation
Oxidative Stress	Protein Propagation
Alpha-Synuclein Aggregation	
Altered Calcium Buffering	
Apoptosis signalling	

**Table 2. Cell autonomous and non-cell autonomous mechanisms in the pathogenesis of Parkinson’s disease [1, 2]**

resting tremor, bradykinesia, and rigidity although a host of non-motor symptoms may also be present. Clinical diagnosis of PD is made through a battery of motor tests and neuroimaging of the midbrain; however, confirmed diagnosis of PD cannot be made until post-mortem examination of the brain is performed. Diagnosis of PD is established by 1) Observed degeneration of dopaminergic (DA) neurons in the nigrostriatal system and 2) presence of proteinaceous cellular inclusions of alpha-synuclein ( $\alpha$ -syn) called Lewy Bodies. While genes implicated in PD are widely expressed in cells across brain regions and  $\alpha$ -syn pathology eventually becomes widespread throughout the brain, the substantia nigra (SN) is particularly vulnerable to degeneration, but the “why” is still unclear. In this section, I will discuss 1) intrinsic properties of SN DA neurons that make them especially susceptible to degeneration (mitochondrial dysfunction and oxidative stress) and how alpha-synuclein exacerbates the dysfunction in these mechanisms; 2)

non-cell autonomous mechanisms (alpha-synuclein propagation, neuroinflammation) that contribute to a feed-forward cycle of degeneration.

### Alpha-Synuclein Aggregation, Dopamine, and Oxidative Stress: A Symphony of Destruction

Dopaminergic neurons in the substantia nigra pars compacta (SNpc) are particularly vulnerable to degenerate in Parkinson's disease, despite widespread alpha-synuclein pathology. Recent studies have begun to highlight intrinsic properties of SNpc DA neurons that may render them predisposed to degeneration when genetic or environmental factors come into play [13]. While cell autonomous mechanisms of neurodegenerative disease were discussed as separate entities in Part 1, it is important to keep in mind that these aberrations in cell physiology do not act in isolation or independently of one another. While many of these cellular processes become less efficient or are altered during normal aging, aggregation of alpha-synuclein is proposed not only to exacerbate these factors, but also to initiate a feed forward cycle that further potentiates protein aggregation and cell death. In this section I will introduce the role of alpha-synuclein in PD, and discuss how expression of this protein in dopamine neurons renders them particularly vulnerable to degeneration.

Alpha-synuclein is a 140 amino-acid protein that exists in a naturally unfolded confirmation and is expressed throughout the central nervous system in the cytoplasm and axonal terminals [14]. Although its exact function is unknown, it is proposed to play roles in vesicular transport and lipid oxidation [14]. Furthermore, post-translational modifications can cause alpha-synuclein to undergo conformational changes into

dimers, higher order toxic oligomers [15] and fibrils, and insoluble aggregates [14]. Genetic alterations including point mutations (A53T, A30P, and E46K) and duplication/triplication of the SNCA gene are associated with early-onset or autosomal dominant familial forms of PD and increased levels of aggregation [16]. While genes associated with increased protein or mutant protein expression are found across brain regions, the substantia nigra is particularly vulnerable to degeneration, begging the question “What is it about accumulation of aggregated  $\alpha$ -syn in SN DA neurons that makes them more likely to die?”

In 2002, Xu et al. proposed a mechanism for selective degeneration of the SN by examining the relationship between  $\alpha$ -syn toxicity and dopamine. The authors found that alpha-synuclein transfection of human dopamine neurons, but not non-dopaminergic hippocampal neurons, resulted in 1) toxicity as measured by TUNEL labeling and 2) the production of reactive oxygen species. Furthermore, they found that when tyrosine hydroxylase was inhibited thus reducing dopamine production,  $\alpha$ -syn-mediated apoptosis was prevented [17]. These results suggest that alpha-synuclein expression specifically in dopamine neurons can render dopamine toxic, resulting in apoptosis and production of reactive oxygen species.

Mitochondrial damage and oxidative stress have long been implicated in the PD pathogenesis, due to high iron and neuromelanin content as well as the high-energy demand of dopamine metabolism in the substantia nigra [18]. Cell culture and animal models utilizing chronic, low-dose exposure to rotenone, a mitochondrial complex 1 inhibitor, result not only in oxidative damage and SN DA degeneration, but also an increase in  $\alpha$ -syn cytoplasmic inclusions [19, 20]. Interestingly, alpha-synuclein can

also exacerbate mitochondrial dysfunction and oxidative stress. Parihar and colleagues showed in both SHSY cells and isolated rat mitochondria that alpha-synuclein can interact with the mitochondrial membrane and exacerbate the signaling cascade associated with oxidative stress, including increased release of cytochrome C and alteration of mitochondrial proteins [21]. More recent evidence has suggested that oxidative stress in turn imposes specific post-translational modifications to alpha-synuclein in DA neurons, catalyzing their conversion to toxic conformations [22]. Taken together, evidence suggests that alpha-synuclein can exacerbate mitochondrial damage and the production of reactive oxygen species. In turn, oxidative stress can impose nitration, oxidation, and phosphorylation of alpha-synuclein causing it to misfold and oligomerize, rendering the protein toxic to neurons and initiating cell death.

## **Non-Cell Autonomous Effects of Alpha-Synuclein: Protein Propagation and Neuroinflammation**

### Alpha-Synuclein Propagation

In addition to degeneration of the nigrostriatal system, the second major pathological hallmark of PD is widespread deposition of proteinacious inclusions composed of alpha-synuclein ( $\alpha$ -syn), termed Lewy bodies and Lewy neurites (LBs, LNs). Early post-mortem examination of PD brains indicate a common pattern of spread of  $\alpha$ -syn inclusions, beginning in the vagus nerve and olfactory nucleus, progressing to the brainstem, up through the midbrain and subcortical structures, and finally throughout the cortex, although some degree variability between patients has been noted [23].

Two separate studies that spawned the idea of a prion-like spread of alpha-synuclein observed that after over a decade post-implantation, grafted fetal DA neurons in the striatum of PD patients developed  $\alpha$ -syn Lewy Body-like aggregates, suggesting that transfer of  $\alpha$ -syn can develop even in young, healthy cells in a similar manner to those observed in host tissue. However, the specific mechanisms by which this transfer occurs remain to be elucidated [24, 25]. These findings were further replicated in rats to determine whether observed alpha-synuclein aggregates in the grafted tissue were the result of host-to-graft transfer or the result of inflammation in the host. Fetal mesencephalic grafts were placed into the striatum of rats who had undergone striatal dopamine depletion by injection of the neurotoxin 6-hydroxydopamine. Following the graft, animals were injected in a distal site with a viral vector overexpressing human alpha-synuclein or GFP. Five months post-injection, they found that a subset of grafted cells were immunoreactive for alpha-synuclein but not GFP, further corroborating evidence in human PD patients that alpha-synuclein can propagate from diseased to healthy tissue [26]

Recent corroborating evidence in vitro and in vivo has proposed that toxic, misfolded forms of  $\alpha$ -syn 1) are released from affected cells, 2) taken up by other neuronal or non-neuronal cells in the immediate vicinity 3) and subsequently recruit endogenous soluble  $\alpha$ -syn to form insoluble intracellular LB-like aggregates that are ubiquitin-positive and proteinase-k resistant [27-29]. Furthermore, pathological  $\alpha$ -syn has been shown to spread through connected networks [30]. In mice harboring the A53T mutation, intracerebral inoculation with fibrillar  $\alpha$ -syn resulted in robust and progressive spread of  $\alpha$ -syn pathology, whereas inoculation in  $\alpha$ -Syn<sup>-/-</sup> mice resulted in



relatively weak immunostaining of phosphorylated  $\alpha$ -syn [31]. Furthermore, intrastriatal injection of fibrillar  $\alpha$ -syn into non-transgenic mice resulted in widespread pathology, selective degeneration of SN DA neurons, and decreases in striatal dopamine .[31,32]. These findings suggest that pathological  $\alpha$ -syn can propagate through interconnected networks, requires endogenous  $\alpha$ -syn for seeding of aggregates, and contributes to selective degeneration of neurons in the nigrostriatal system.

Recent evidence has also suggested that  $\alpha$ -syn pathology may originate in the enteric nervous system and self-propagate through the vagus nerve to the brainstem. Routine gastrointestinal biopsies taken up to 8 years prior to onset of motor symptoms in patients who eventually developed PD vs. normal patients revealed intense staining of phosphorylated alpha-synuclein in mucosal and submucosal nerve fibers in the upper and lower gastrointestinal tract, and similar findings have been replicated elsewhere [33]. In addition, studies in mice have shown that exposure to rotenone results in release of alpha-synuclein from enteric neurons with subsequent retrograde transport and accumulation in the soma [34]. Given that the gut may be the largest site in terms of surface area for exposure to pesticides and other environmental toxins present in the food we eat, further investigation into environmental toxin exposure and its relationship to  $\alpha$ -syn release and propagation in this area is of need and interest. The presence of gastrointestinal  $\alpha$ -syn has been proposed as a biomarker for PD, however results have been conflicting and require further investigation.

In summary, it is proposed that alpha-synuclein spreads in a self-propagating, prion-like manner. Pathological conformations are released from cells, taken up by neighboring cells and seed aggregates through recruitment of endogenous alpha-

synuclein. This non-cell autonomous process contributes to the spread of pathology not only within the substantia nigra but also through interconnected networks.

### Alpha-Synuclein-Induced Neuroinflammation

Early examination of post-mortem SN and CSF from PD patients revealed high expression of microglial inflammatory markers and pro-inflammatory cytokines, respectively, when compared to healthy age-matched controls [35-37]. Gene expression analysis of isolated microglia revealed a region-specific increase in expression of TNF- $\alpha$  (a key mediator in the inflammatory response) in the substantia nigra, which may also contribute to selective vulnerability of SN DA neurons [38]. Furthermore, administration of lipopolysaccharide, a known inflammogen, resulted in selective degeneration of dopaminergic neurons in vitro and in vivo [39]. Given these early findings and that that the SN is home to one of the densest populations of microglia in the brain, neuroinflammation has recently garnered interest by PD researchers as a possible contributor to disease progression, specifically through the direct interaction of  $\alpha$ -syn with the brain's resident immune cells (microglia) [40] and the subsequent production of reactive oxygen species by astrocytes [41].

Evidence from two-hit animal models suggests that  $\alpha$ -syn and microglia act synergistically to potentiate disease progression [42]. In response to an acute insult, microglia proliferate, secrete anti-inflammatory cytokines and trophic factors and phagocytize cellular debris in a regulated manner that promotes tissue repair. However, in response to chronic insult such as  $\alpha$ -syn aggregation, microglia become chronically active, secrete pro-inflammatory cytokines, and further potentiate nigrostriatal

degeneration, suggesting a secondary, but contributory role for microglial-mediated neuroinflammation in the selective vulnerability of SN DA neurons in PD [11, 43].

The innate immune response mediated by microglia occurs through activation of Toll-like receptors (TLRs). Recently, neuron-released oligomeric  $\alpha$ -syn has been shown to act as an endogenous agonist of TLR-2, which subsequently initiates a signaling cascade causing increased production of proinflammatory cytokines [44, 45]. Elevated expression of TLR-2 has also been observed in the brain and blood of PD patients and mice overexpressing  $\alpha$ -syn [46, 47]. Furthermore, blocking this signaling cascade reduced production of proinflammatory cytokines, which, in the case of mice overexpressing  $\alpha$ -syn, resulted in less robust degeneration and restoration of some motor deficits [44].

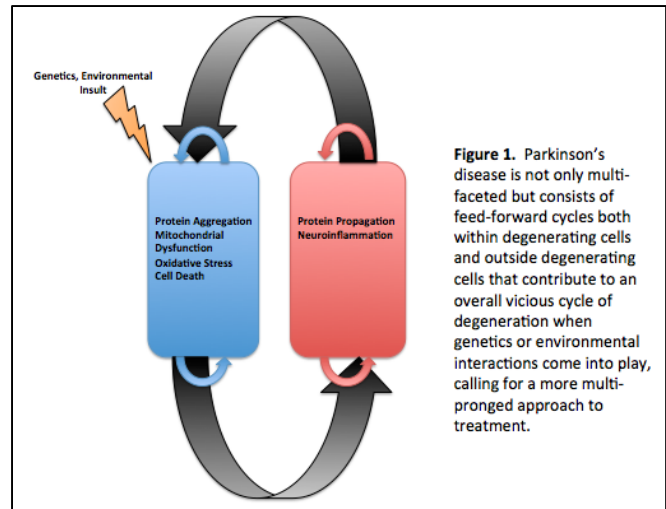
In summary, PD is multifaceted; a culmination of abnormal cell and non-cell autonomous mechanisms that not only contribute to disease progression in and of themselves, but affect each other to initiate a feed forward cycle that contributes to degeneration.

### **Part 3: Therapeutic Approaches to PD: The Need for Disease-Modifying Therapies**

#### Neurobiological Basis for PD Pathogenesis and Existing Treatments

The number of individuals living with Parkinson's disease is expected to double by 2050 [48]. The main pathological hallmarks of PD are 1) degeneration of dopamine neurons in the substantia nigra, resulting in denervation of the striatum and depletion of dopamine stores and 2) presence of intracellular inclusions composed of fibrillar alpha-synuclein. Given that motor symptoms of PD do not manifest until approximately 80% of

nigral DA neurons have degenerated, it is reasonable to suggest that the cycle of protein aggregation, cellular dysfunction (i.e., oxidative stress, mitochondrial turnover, calcium signaling), excitotoxicity, neuroinflammation, and protein propagation have been occurring for years, even decades prior to diagnosis. Collier et



Adapted from [6], [2]

**Figure 1.** Parkinson's disease is not only multifaceted but consists of feed-forward cycles both within degenerating cells and outside degenerating cells that contribute to an overall vicious cycle of degeneration when genetics or environmental interactions come into play, calling for a more multi-pronged approach to treatment.

al. (2011) suggest that as a byproduct of normal aging, processes within SN DA neurons including mitochondrial turnover, oxidative stress production, and efficacy of the ubiquitin-proteasome system are altered [2]. Senescence of these mechanisms renders this neuronal population more susceptible to degeneration when genetic or environmental factors further exacerbate these alterations, which also concurs with the multiple hit hypothesis of PD development [6]. As depicted in Figure 1, PD is a culmination of changes both within degenerating neurons and outside degenerating neurons that are exacerbated by genetic or environment interactions, contributing to multiple feed forward cycles to promote degeneration. The recognition of PD as a multifaceted disease is of utmost importance to revolutionizing current treatment strategies.

For over five decades, the gold standard pharmacotherapy for PD has been administration of Levodopa (L-DOPA), which crosses the blood-brain-barrier and is converted to dopamine by the enzyme dopa decarboxylase. L-DOPA acts to replenish depleted dopamine stores and increase the efficacy of DA production by remaining dopamine neurons. When administered orally, L-DOPA can be converted to dopamine

prematurely before reaching the brain. Thus, L-DOPA is often administered in conjunction with carbidopa (combination L-DOPA/carbidopa: Sinemet), which acts to limit L-DOPA conversion to DA before reaching the BBB [49]. While L-DOPA is initially effective in alleviating motor symptoms of PD, it is not without adverse side effects. Many patients experience debilitating uncontrolled movements called dyskinesias after prolonged use, which poses a significant problem to early-onset PD patients who will typically require treatment for longer periods of time than those diagnosed with sporadic, late-onset PD [50]. In the case of early onset PD, dopamine agonists may be used in order to provide some symptomatic relief while delaying the need for L-DOPA.

Deep brain stimulation (DBS) is the most commonly used surgical treatment of Parkinson's disease. DBS acts as a pacemaker: an electrode is implanted into one of several regions in the brain (thalamus, internal globus pallidus, subthalamic nucleus), to modulate an imbalance in excitation and inhibition that results from nigrostriatal degeneration, although the exact mechanism by which patients receive symptomatic alleviation is currently under debate [51]. Given the risky nature of this procedure, especially in the elderly, this treatment is often used as a last resort when pharmacotherapies are no longer effective.

### Novel Therapeutic Approaches

A major roadblock preventing the implementation of disease-modifying therapies is that by the time PD patients present clinically with motor symptoms, a majority of SN DA neurons have already degenerated,  $\alpha$ -syn pathology is most-likely widespread, and neuroinflammation has been uncontrolled. This notion begs the questions "How

efficient are the remaining 20% of SN DA neurons and what are we really trying to protect?” Given the extent of nigrostriatal degeneration upon clinical diagnosis, it is unlikely that sparing the remaining 20% of DA neurons via neuroprotective strategies will have any noticeable symptomatic effect. Currently, there is a major drive to discover potential biomarkers for PD in at risk populations in an effort to allow for 1) earlier diagnosis 2) a more personalized approach to treatment regimen and 3) development of disease-modifying therapies [52]. While I firmly believe that earlier diagnosis of PD is key in the improvement, development, and implementation of disease modifying therapies, the field isn't quite there yet.

As discussed previously, the primary pharmacotherapy for PD is administration of dopamine agonists or dopamine precursors in an effort to replace depleted dopamine stores. While these drugs may offer some symptomatic benefit, they only target one piece of a feed-forward cycle in a multi-faceted disorder (See figure 1). I believe that a novel multi-pronged approach which targets both cell autonomous and non-cell autonomous mechanisms would have the best chance of modifying disease progression. Some of these approaches are currently being tested independently in experimental models. For instance, combining immunotherapy using antibodies against alpha-synuclein [53] with anti-inflammatory pharmacotherapy [54] in conjunction with lifestyle factors such as consuming foods high in antioxidants (berries), and avoiding those known to have inflammatory properties (i.e. gluten). Such a combination of therapies would target alpha-synuclein aggregation and propagation, neuroinflammation, and oxidative stress. Naturally, different therapeutic modalities will have to undergo extensive study in rodents and non-human primates before translation

to the clinic, however, I think moving PD treatment to a multi-targeted approach is crucial. While dopamine-replacement therapy may still be needed given the lack of available biomarkers for earlier diagnosis, it makes sense that a disease-modifying therapy should target an event upstream of DA neuron death, so as to slow or halt degeneration of remaining neurons.

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