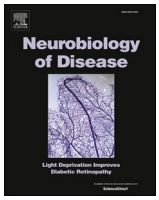
Neurobiology of Disease 175 (2022) 105920

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi



Interactions of dopamine, iron, and alpha-synuclein linked to dopaminergic neuron vulnerability in Parkinson's disease and Neurodegeneration with Brain Iron Accumulation disorders

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ARTICLE INFO

Keywords:
Parkinson's disease
Neurodegeneration with Brain Iron Accumulation
Dopamine
Alpha-synuclein
Iron
Neurodegeneration

ABSTRACT

Dopamine metabolism, alpha-synuclein pathology, and iron homeostasis have all been implicated as potential contributors to the unique vulnerability of substantia nigra dopaminergic neurons which preferentially decline in Parkinson's disease and some rare neurodegenerative disorders with shared pathological features. However, the mechanisms contributing to disease progression and resulting in dopaminergic neuron loss in the substantia nigra are still not completely understood. Increasing evidence demonstrates that disrupted dopamine, alpha-synuclein, and/or iron pathways, when combined with the unique morphological, physiological, and metabolic features of this neuron population, may culminate in weakened resilience to multiple stressors. This review analyzes the involvement of each of these pathways in dopamine neuron physiology and function, and discusses how disrupted interplay of dopamine, alpha-synuclein, and iron pathways may synergize to promote pathology and drive the unique vulnerability to disease states. We suggest that elucidating the interactions of dopamine with iron and alpha-synuclein, and the role of dopamine metabolism in driving pathogenic phenotypes will be critical for developing therapeutics to prevent progression in diseases that show degeneration of nigral dopamine neurons such as Parkinson's disease and the rare family of disorders known as Neurodegeneration with Brain Iron Accumulation.

1. Unique physiology of substantia nigra dopaminergic neurons

The substantia nigra (SN) is part of the midbrain cluster of subcortical nuclei known as the basal ganglia that regulate movement and reward networks. It is divided into two primary regions based on morphology and function: the more dorsal substantia nigra pars compacta (SNpc) which contains the pigmented, neuromelanin (NM)-dense dopaminergic (DAergic) neurons from which the nucleus gets its name, and the more ventral substantia nigra pars reticulata (SNpr), with fewer NM-containing neurons and largely comprised of inhibitory gamma-aminobutyric acid-containing (GABAergic) neurons (Lee and Tepper, 2007). The SNpc is further subdivided into dorsal and ventral tiers, with the latter showing greater neuronal loss in Parkinson's disease (PD) (Damier et al., 1999; Fearnley and Lees, 1991; Gibb and Lees, 1991). The SNpc provides a steady supply of dopamine (DA) neurotransmitter to the GABAergic spiny projection neurons of the striatum (Beckstead et al.,

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https://doi.org/10.1016/j.nbd.2022.105920
Received 29 April 2022; Received in revised form 21 October 2022; Accepted 4 November 2022
Available online 6 November 2022

1979), and these nigrostriatal projections regulate the direct and indirect pathways of motor control. This activity subsequently regulates the basal ganglia control of thalamocortical projections to the motor cortex, generating a net effect of activating the direct pathway to initiate voluntary movement while inhibiting the indirect pathway to suppress involuntary movement.

In addition to the SNpc, the mammalian midbrain houses two other major DAergic nuclei: the ventral tegmental area (VTA) and the retrorubral field (Björklund and Dunnett, 2007). However, the SNpc DAergic neurons display several unique morphological and physiological attributes that make them distinct from these other nuclei which may explain why this DAergic neuron population shows particular vulnerability in pathological conditions including aging, environmental toxins, PD, and subtypes of the rare Neurodegeneration with Brain Iron Accumulation (NBIA) family of disorders. The most prominent of these traits is their long, mostly unmyelinated axons with highly-branched and complex axonal arbors (Gauthier et al., 1999; Matsuda et al., 2009) that are 2- to 3-fold larger and more complex than DAergic neurons of the VTA (Giguère et al., 2019). This axonal complexity may also result in increased expression of the presynaptic protein alpha-synuclein (aSyn), a highly enriched constituent of Lewy bodies (LB). Interestingly, the arborization of rodent SNpc neurons is an estimated 10-fold less than that of humans and may explain, at least in part, the difficulty in recapitulating pathological phenotypes in PD animal models. Indeed, when rodent SNpc axonal arbors are expanded using either pharmacological (Parish et al., 2002; Tripanichkul et al., 2003), or genetic (Giguère et al., 2019; Tinsley et al., 2009) approaches, these normally resilient neurons exhibit increased intrinsic susceptibility. Moreover, the high energetic demands required to maintain this extensive arborization and its vesicle turnover create substantial proteostatic and oxidative burden on DAergic axons. Compared to the VTA, DAergic neurons of the SNpc exhibit increased numbers of axonal mitochondria, a higher rate of mitochondrial oxidative phosphorylation (OXPHOS), higher basal levels of superoxide production, and elevated levels of oxidative stress markers (Pacelli et al., 2015).

Another potential factor contributing to vulnerability may be the autonomous pacemaking activity of the SN. DAergic neurons of this brain nucleus exhibit a slow, rhythmic spiking at 2-10 Hz, which is accompanied by oscillations in intracellular calcium (Ca^{2+}) that is primarily driven by voltage-dependent L-type Ca^{2+} channels (LTCC) (Guzman et al., 2009). Relative to VTA neurons, those in the SNpc contain a higher density of LTCC, leading to a larger influx of Ca^{2+} and higher Ca^{2+} load (Philippart et al., 2016), as well as a relatively low expression of Ca^{2+} buffering proteins including calbindin (Foehring et al., 2009; German et al., 1992; Hurley et al., 2013; Mosharov et al., 2009). This, in turn, drives OXPHOS and ATP production necessary to maintain regulation of the indirect pathway. However, this physiological combination also leads to the production of reactive oxygen species (ROS) capable of damaging proteins, lipids, and DNA. Indeed, Ca^{2+}-dependent mitochondrial oxidant stress has been found to be elevated in SNpc DAergic neurons (Dryanovski et al., 2013; Guzman et al., 2010).

While none of the morphological and physiological characteristics of DAergic neurons in the SNpc alone appear to account for their selective vulnerability, their unique combination of traits may contribute to heightened susceptibility to genetic, epigenetic, and/or environmental aberrations seen in common neurodegenerative disease states like PD and in rare conditions like NBIA disorders.

2. Selective vulnerability in Parkinson's disease

2.1. Pathological hallmarks of Parkinson's disease dopaminergic neurons

Over 200 years ago, James Parkinson published his pioneering report on the "shaking palsy", the first description of the disease now known as PD. Today, PD is the fastest growing neurodegenerative disease, affecting 1% of those over 60 years old (Collaborators, 2018), but effective preventive therapies remain elusive. Most cases of PD are sporadic, with only 5–10% attributed to various autosomal dominant (e.g. *LRRK2, GBA1, SNCA*) or autosomal recessive (e.g. *PINK1, Parkin, DJ-1*) mutations (Bandres-Ciga et al., 2020). While neuronal loss is also seen in the locus coeruleus (LC), raphe nuclei (RN), pedunculopontine nuclei (PPN), and dorsal motor nucleus of the vagus (DMV) (Del Tredici et al., 2002; Gai et al., 1991; Halliday et al., 1990), the characteristic motor symptoms including slowed movement (bradykinesia), rigidity, and resting tremor arise primarily from the progressive degeneration of DAergic neurons in the SNpc, of which 50–70% are already lost in PD patients at symptom onset (Damier et al., 1999).

A prominent diagnostic hallmark of PD is the accumulation of the intracellular inclusions known as LBs. Post-mortem analyses of PD patient brains revealed that much of Lewy pathology (LP) occurs in the brain stem, as well as other brain regions and peripheral neurons (Sulzer and Surmeier, 2013). Following the groundbreaking report by Polymeropoulos and colleagues linking mutations in the *SNCA* gene, which encodes aSyn, to familial PD cases (Polymeropoulos et al., 1997), Spillantini et al. identified aSyn as the main protein component of LBs in sporadic PD patient postmortem brain tissue, thus confirming its importance in PD research (Spillantini et al., 1997). aSyn is abundantly expressed in the nervous system, and its close proximity to the presynaptic membrane indicates a role in neurotransmission that has been confirmed for SN axons (Somayaji et al., 2020). This protein also has the potential to undergo a conformational change into insoluble fibrils through a variety of oligomeric intermediates (Chen et al., 2015; Iljina et al., 2016; Lashuel et al., 2013). Studies suggest that these soluble oligomeric and prefibrillar species are the most cytotoxic, rather than the fibrils themselves, and that the process of LB inclusion formation may drive neuronal cell death via pathogenic interaction with lipids, organelles and membrane structures (Mahul-Mellier et al., 2020; Winner et al., 2011). Braak and colleagues first proposed the idea that LP progresses through a series of defined stages (Braak et al., 2004). In their seminal study of postmortem tissue, authors developed a 6-stage model of the spread of LP in which the pathology first appears in the olfactory bulb, gradually spreading to the cortex at later stages of the disease. However, this characterization may be too simplistic as some patients do not show aSyn aggregates, while others manifest LP in the absence of clinical PD (Surmeier et al., 2017a). Thus, while progressive LP may be a significant component of neuronal demise in some cases, this alone does not explain the full complexity of PD pathology.

Another striking feature of the vulnerable SN neurons is their high iron content and metabolism. Iron is essential for many processes in the brain, including oxygen transport, ATP generation, neurotransmitter synthesis and axon myelination (Rouault, 2013). During healthy aging, iron levels increase in various brain areas, including the SN (Zecca et al., 2001; Zecca et al., 2004). In the SN of PD patients, iron accumulation is unusually enhanced and appears to correlate with disease severity (Dexter et al., 1987; Genoud et al., 2017; Ghassaban et al., 2019; Riederer et al., 1989). Excess iron can induce oxidative stress and cell death due to its catalytic function in the production of hydroxyl radicals and nonenzymatic oxidation of cytosolic DA to form DA-o-quinones (DAQs) and other toxic DA derivates, highly reactive species which react with proteins, leading to insoluble complexes that become sequestered in the NM pigment (Ferrari et al., 2017; Segura-Aguilar et al., 2014). NM appears in SNpc neurons within 3 years of birth (Cowen, 1986; Fenichel and Bazelon, 1968), increasing linearly with age (Zecca et al., 2002; Zecca et al., 2004), and has been recapitulated in human induced pluripotent stem cell (iPSC)-derived neuronal model systems (Burbulla et al., 2017; Chumarina et al., 2019; Jo et al., 2016; Miller et al., 2013). Notably, rodent SN neurons usually lack or have very low content of NM pigment (DeMattei et al., 1986; Marsden, 1961), though this can be induced in murine DAergic neurons *in vitro* following treatment with the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) (Sulzer et al., 2000). A recent model overexpressing human tyrosinase, the rate-limiting enzyme in peripheral melanin synthesis, in rat SN induced the

accumulation of melanin pigment which coincided with loss of TH-positive neurons (Carballo-Carbajal et al., 2019). However, there is no evidence supporting brain expression of tyrosinase, and its involvement in NM synthesis is still under debate (Ikemoto et al., 1998; Plum et al., 2016; Tribl et al., 2007; Zucca et al., 2018) and extensive characterization of the pigment generated in this model is needed.

In certain conditions, extracellularly released NM and aSyn can activate microglia which subsequently stimulate the expression of major histocompatibility complex class I (MHC-I) in catecholaminergic neurons, targeting these neurons for T-lymphocyte mediated degeneration. This autoimmune process contributes to the neuroinflammation commonly observed in both human PD brains and animal models (Cebrián et al., 2014). Thus, iron, DA, NM, and aSyn seem to interact in multiple pathways in aging SN DAergic neurons, but how exactly they contribute to neuronal vulnerability and ultimately drive neuronal death in PD is not yet fully defined. The following review will discuss in detail the individual and interactive roles that DA metabolism, aSyn accumulation, and iron homeostasis may play in the heightened vulnerability and selective atrophy of SN DAergic neurons seen in both common (PD) and other neurodegenerative disease states (NBIA).

2.2. Disrupted dopamine metabolism as a cause of neuron vulnerability

DA is a catecholamine neurotransmitter first identified in 1957 by a team of researchers led by Arvid Carlsson (Hornykiewicz, 2006), which earned them the Nobel Prize for Physiology or Medicine in 2000. Employed by several major central nervous system (CNS) signaling networks, it plays a well-recognized role in a variety of physiologic functions such as movement, cognition, mood, and reward. The dynamic regulation of DA signaling, distribution, and degradation is crucial, as dysregulation of DAergic neuronal function can contribute to several disorders including PD, attention deficit hyperactivity disorder, and substance abuse. DA production, vesicular sequestration, release, and extracellular concentration are largely regulated by the coordinated activity of tyrosine hydroxylase (TH), vesicular monoamine transporter 2 (VMAT2), and DA transporter (DAT) (Fig. 1). Monoamine oxidase (MAO)-A, MAO-B, and aldehyde dehydrogenase 1A1 (ALDH1A1) are well known for their roles in DA catabolism (Meiser et al., 2013), although other enzymes are involved in DA degradation pathways, as outlined below. TH is the rate-limiting enzyme in DA production (Nagatsu et al., 1964) that converts dietary L-tyrosine to L-DOPA, which,

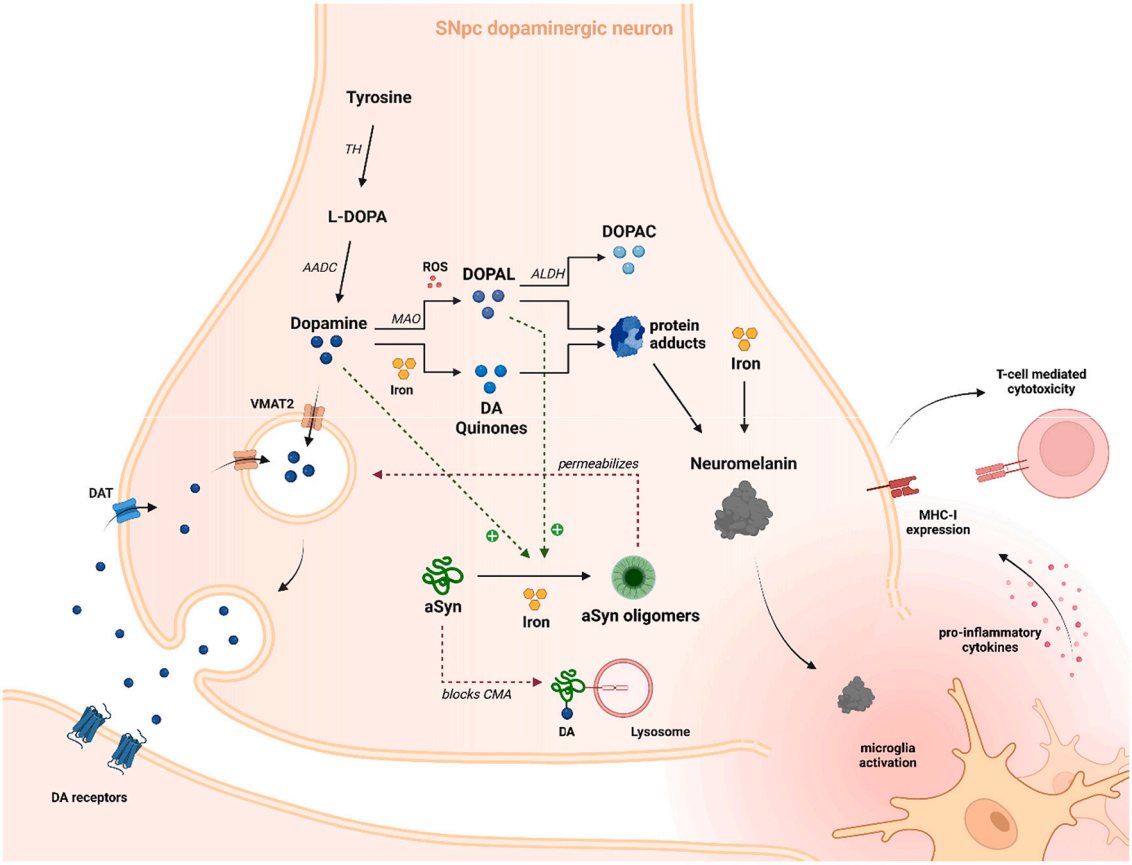

Fig. 1. Interplay between iron, alpha-synuclein, and dopamine **in substantia nigra neurons.** DA is synthesized from tyrosine through the sequential enzymatic activity of TH and AADC. Due to its highly reactive nature, the synthesis, metabolic breakdown, vesicular packaging, release, and reuptake of DA is tightly regulated through 1) loading into synaptic vesicles by VMAT2, 2) metabolism by MAO into the highly reactive DOPAL, a process which generates ROS, and further by ALDH into the much less reactive DOPAC, and 3) reuptake through DAT. Excess cytosolic DA, or disequilibrium of DA intermediates, pose significant problems for the neuron. Accumulated DOPAL causes protein modifications, including the promotion of aSyn oligomerization. These aSyn oligomers subsequently compromise synaptic vesicle membranes, resulting in DA leakage. Moreover, altered aSyn has been shown to display altered ferrireductase activity resulting in disrupted iron homeostasis. Finally, aSyn can form adducts with DA and oxidized derivates of DA, and these adducts have demonstrated impaired lysosomal translocation during chaperone-mediated autophagy (CMA), leading to buildup of CMA substrates. If not sequestered in synaptic vesicles or further metabolized, cytosolic DA is oxidized by iron to form DA quinones. Oxidized derivates of DA (e.g. DOPAL and DA quinones) can form adducts with proteins, a process ultimately leading to formation of neuromelanin (NM), so that these compounds can be removed from the cytosol. Moreover, NM can also bind large amounts of reactive/toxic iron (and other metals), and is therefore proposed to be neuroprotective. However, high NM content is correlated with high MHC-I expression in DAergic neurons, which may target them for destruction by cytotoxic T-cells. Furthermore, degenerating neurons release NM, activating microglia which then release inflammatory mediators to potentiate neuroinflammation and enhance neuronal MHC-I expression. Excess of reactive iron promotes DA oxidation, but can also directly bind the C-terminus of aSyn – a process that is exacerbated when aSyn is phosphorylated at serine 129 – making it more prone to oligomerization.

in turn, is converted by aromatic amino acid decarboxylase (AADC) to DA. After synthesis, VMAT2 transports DA (and other monoamine neurotransmitters, i.e., norepinephrine, serotonin, and histamine) from the cytoplasmic space into synaptic vesicles. Thereby, VMAT2 activity largely dictates the scale of neurotransmitter release (Omiatek et al., 2013; Pothos et al., 2000). Comprehensive understanding of the functional roles these proteins play in DA regulation, and more importantly dysregulation, will provide insight into the molecular mechanisms driving pathology in DAergic neurons, and may reveal novel avenues for therapeutic intervention and neuroprotection.

Several studies have shown the accumulation of cytosolic DA to be neurotoxic through the generation of ROS and DAQs (i.e. DA-o-quinone, aminochrome, indole-quinone) (Rabinovic et al., 2000; Segura-Aguilar et al., 2014; Sulzer and Zecca, 2000). DAQs are not stable in the cytosol at physiological pH and therefore undergo modifications into more stable forms (i.e., 5,6-dihydroxyindole, 5,6-indolequinone) and finally polymerize forming adducts with proteins and leading to the synthesis of the dark NM pigment (Ferrari et al., 2017; Munoz et al., 2012). Indeed, DAQs can form toxic adducts with amino acid residues (mainly cysteine residues) of different proteins (Belluzzi et al., 2012; Bisaglia et al., 2010; Burbulla et al., 2017; Kuhn et al., 1999; LaVoie et al., 2005; Zahid et al., 2011). Hence, iron dyshomeostasis may be playing a key role in neurodegeneration, in part by catalyzing the conversion of DA to toxic and highly reactive oxidized derivates, and direct promotion of ROS production. Further, this pathological process may be self-perpetuating, as it has been shown that specific o-quinones (i.e. aminochrome) are able to modify expression of proteins involved in iron homeostasis, namely, increasing the iron transporter divalent metal transporter 1 (DMT1 or SLC11A2) and decreasing the iron exporter ferroportin (FPN) (Aguirre et al., 2012), thereby enhancing iron accumulation and possibly subsequent DA oxidation.

Sequestration of cytosolic DA by VMAT2 is also of critical importance for survival of DAergic neurons, preventing cytosolic DA accumulation and its subsequent conversion to neurotoxic species (Goldstein et al., 2013; Mosharov et al., 2009; Sulzer and Zecca, 2000). As a 12-transmembrane domain antiporter, VMAT2 is predominantly localized to both dense core and small synaptic vesicles. Using the vesicular electrochemical gradient to exchange two H^+ ions for one monoamine molecule (Knoth et al., 1981), VMAT2 drives the packaging of cytosolic neurotransmitter into vesicles (Erickson and Eiden, 1993; Erickson et al., 1992; Liu et al., 1992). H^+-ATPase activity establishes the high intravesicular H^+ concentration upon which VMAT2 transport depends, and reducing H^+-ATPase function or neutralizing vesicular pH impairs VMAT2 transport activity (Yaffe et al., 2016). Thus, VMAT2 serves two primary functions: to avert intracellular toxicity and to mediate monoamine neurotransmission. Increasing the vesicular packaging of DA in VMAT2-overexpressing mice proved neuroprotective against the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), leading to reduced SNpc cell loss (Lohr et al., 2014) and is protective against methamphetamine neurotoxicity in dopaminergic neurons (Larsen et al., 2002). Enhanced VMAT2 also blocked the formation of NM in primary DAergic neurons (Sulzer et al., 2000), consistent with its increase of DA packaging inside synaptic vesicles (Pothos et al., 2000). In line with these reports, a study on a gain-of-function haplotype within the VMAT2 promoter region was shown to increase VMAT2 expression and was associated with a protective role in sporadic PD (Glatt et al., 2006). Consistently, loss of VMAT2 function in various VMAT2-deficient mouse models resulted in reduced DA packaging and nigrostriatal degeneration often coupled with aSyn accumulation (Caudle et al., 2007; Lohr et al., 2016; Taylor et al., 2014; Taylor et al., 2011; Taylor et al., 2009). Interestingly, isolated DA storage vesicles from the striatum of PD patient brains show diminished vesicular DA uptake compared to controls, suggesting VMAT2 protein itself as the cause of DA storage impairment (Pifl et al., 2014). This theory is supported by evidence of a VMAT2 mutation in humans being linked to an infantile parkinsonism-like condition (Rilstone et al., 2013). Furthermore, several environmental toxins are known to interfere with the proper function of VMAT2 suggesting that perturbation of VMAT2 may lead to DAergic neuron damage (Ascherio et al., 2006; Bemis and Seegal, 2004; Hatcher-Martin et al., 2012; Mariussen and Fonnum, 2001; Richardson and Miller, 2004), and this may represent a target for potential neuroprotective therapies to address both DA deficit neurotransmission and DA-derived neurotoxicity in PD (Segura-Aguilar et al., 2019).

If not sequestered into synaptic vesicles, enzymatic degradation of cytosolic DA also protects DAergic neurons from neurotoxicity by preventing accumulation of DA in the cytosol and subsequent generation of toxic DAQs. Accumulation of cytosolic DA induces neurotoxicity *in vitro* (Fuentes et al., 2007; Lieberman et al., 2017; Mosharov et al., 2009; Mytilineou et al., 1993; Pardo et al., 1995; Sulzer et al., 2000) and in rodents *in vivo* (Bucher et al., 2020; Burbulla et al., 2017; Caudle et al., 2007; Chen et al., 2008), although clinical studies indicate that L-DOPA toxicity in human subjects seems to be absent (Chan et al., 2007; Fahn et al., 2004; Holford et al., 2006). Additionally, toxin-based PD models demonstrate an involvement of DA in mediating neurodegeneration (Choi et al., 2015b; Izumi et al., 2014; Lieberman et al., 2017; Watabe and Nakaki, 2008). The main intra-neuronal DA catabolic pathway is initiated by oxidative deamination via MAOs. However, such metabolism can be problematic as MAO activity also generates oxidative stress via the production of toxic and reactive products, including hydrogen peroxide (H_2O_2) and 3,4-dihydroxyphenylacetaldehyde (DOPAL) (Eisenhofer et al., 2004; Meiser et al., 2013). The MAO enzymes, located in the outer mitochondrial membrane, consist of two separate isoforms encoded by two separate genes, MAO-A and MAO-B, which are differentially expressed in neurons and glia cells, respectively. DOPAL undergoes carbonyl metabolism primarily via aldehyde dehydrogenase (ALDH) enzymes, yielding the less toxic 3, 4-dihydroxyphenylacetic acid (DOPAC). Previous studies have shown that cellular ALDH enzymes are sensitive to oxidative stress and lipid peroxidation, which are thought to be elevated during PD pathogenesis (Florang et al., 2007; Jinsmaa et al., 2009). Inhibition of ALDH and the resulting accumulation of DOPAL is concerning as DOPAL is toxic to DAergic cells, readily modifying proteins and triggering their aggregation, damaging synaptic vesicles, and increasing DAergic cell loss *in vitro* and *in vivo* (Burke et al., 2008; Burke et al., 2003; Mattammal et al., 1995; Panneton et al., 2010; Plotegher et al., 2017). DOPAL may exert toxicity by protein cross-linking (Rees et al., 2009), oxidation to quinones (Anderson et al., 2011) and production of hydroxyl radicals (Li et al., 2001). Specifically, DOPAL potently oligomerizes and precipitates aSyn (Burke et al., 2008) leading to insoluble oligomeric and fibrillar aSyn aggregates in SN neurons. These aSyn-DOPAL oligomers have been shown to permeabilize synaptic vesicles *in vitro*, causing DA leak and potentiating the cycle of DOPAL formation (Plotegher et al., 2017). According to the 'catecholaldehyde hypothesis' (Goldstein et al., 2013), DOPAL causes or contributes to the neurotoxicity in PD. Recent reports of reduced ALDH expression levels and enzymatic activity in SN of PD patient brains suggest its involvement in defective DA metabolism (Galter et al., 2003; Meyer et al., 2004; Molochnikov et al., 2012). ALDH genes are comprised of 19 members in the human genome, but only ALDH1A1 is predominantly expressed by DA neurons of the more vulnerable, ventral tier of the SNpc (Liu et al., 2014; McCaffery and Dräger, 1994; Wu et al., 2019). Consistent with this, certain single nucleotide polymorphisms (SNPs) in *ALDH1A1* were associated with altered susceptibility to PD (Fan et al., 2021), whereas post-mortem analysis of PD brains revealed diminished ALDH1A1 expression in the ventral DA subpopulations (Liu et al., 2014; Pereira Luppi et al., 2021). Therefore, ALDH inhibition may be a mechanistic link between altered DA metabolism and pathologic events underlying neurodegeneration.

2.3. Intersection of iron and dopamine: the neuroprotective and neurodegenerative role of neuromelanin

The synthesis, metabolism, and synaptic dynamics of the

neurotransmitter DA are tightly regulated to both facilitate the unique electrochemical activity of SN DA neurons and prevent its cytosolic accumulation, which can lead to the production of toxic derivatives and amplify the vulnerability of these neurons to both external and internal stressors. The aforementioned enzymes and regulatory processes work together to maintain steady DA levels, but when one or more of these regulatory mechanisms falter, SN DA neurons employ a unique downstream process to prevent the cytotoxic consequences of excess cytosolic DA.

NMs are a family of compounds that accumulate during aging in neurons of several brain regions of humans and some animals (Marsden, 1961; Zecca et al., 2008a). NMs are insoluble dark brown pigments comprising melanic, peptide, lipid and metal components (Engelen et al., 2012). The most abundant metal in NM is iron, followed by zinc, copper and others in lower amounts (Biesemeier et al., 2016; Zecca et al., 2008a). NM pigment accumulates in autolysosomal organelles with proteins and lipid bodies mainly containing dolichols (Ward et al., 2007; Zucca et al., 2018). In aging there is a continuous accumulation of NM in catecholaminergic neurons, and this is particularly high in DAergic neurons of the SNpc. In PD, the severe degeneration of the SNpc occurs preferentially in the highly pigmented DAergic neurons which have amassed the highest concentrations of NM, substantially higher than DAergic neurons of the SNpr and VTA (Fearnley and Lees, 1991; González-Hernández and Rodríguez, 2000; Liang et al., 2004; Nair-Roberts et al., 2008; Pakkenberg et al., 1991).

The synthesis of NM begins with iron-mediated oxidation of DA to DAQs that react with proteins (with native or fibril structure) to give iron-DA-protein adducts which are further oxidized and polymerize to form iron-melanin-protein complexes in which reactive/toxic iron is entrapped. This complex is further processed into autophagosomes that fuse with lysosomes to form the NM-containing autolysosomal organelle where undegraded NM, protein, lipids, and metals accumulate over the lifetime of neurons (Zucca et al., 2018). This complex, however, is not saturated with iron and can further bind reactive/toxic iron (Shima et al., 1997). This NM-iron complex was demonstrated to induce the main effect in the contrast mechanisms of magnetic resonance imaging (MRI) of NM in human SN, enabling the imaging of loss of DAergic neurons containing NM and diagnosis of PD (Cassidy et al., 2019; Trujillo et al., 2017). Furthermore, NM can bind other potentially toxic metals arising from environmental exposure (Zecca et al., 2008a; Zucca et al., 2022), and a number of xenobiotics that can invade neurons, including paraquat, MPTP, β-carbolines, quinolines and neuropharmaceuticals (Capucciati et al., 2021), making NM synthesis a neuroprotective process.

Indeed, it has long been debated whether NM is protective or toxic for neurons in aging and PD; however, 20 years of research has revealed that specific cellular conditions may determine which role NM plays in cell fate (for a recent review, see (Zucca et al., 2022)). Interestingly, NM shares common features with β-amyloid protein, a major component of the hallmark plaques found in the Alzheimer's disease brain: both are insoluble complexes that builds up with age, they are capable of binding metals, and their synthesis sequesters neurotoxic precursors (Rao et al., 2006). Furthermore, once present in the extracellular milieu due to release from dying neurons, NM is widely thought to induce neurodegeneration by activating microglia (Beach et al., 2007; Langston et al., 1999; Viceconte et al., 2015; Wilms et al., 2003; Zecca et al., 2008b; Zhang et al., 2011).

The synthesis of NM is neuroprotective. In DAergic neurons of rat SN treated with L-DOPA, which is rapidly converted to DA, it was demonstrated that NM synthesis is driven by excess cytosolic DA not loaded into synaptic vesicles (Sulzer et al., 2000). High cytosolic DA autooxidizes to neurotoxic quinones, extremely reactive species able to modify proteins affecting their structure and function (Monzani et al., 2019). Primary rat nigral neurons overexpressing VMAT2 have low level of cytosolic DA and accumulate very low NM (Sulzer et al., 2000). Examination of human midbrain regions found that NM concentration and neuronal vulnerability are inversely related to VMAT2 expression (Liang et al., 2004). Compared to the fairly resilient DAergic neurons of the VTA, those in the SNpc have lower levels of VMAT2, higher accumulation of NM, and increased susceptibility in neurodegenerative disease states. This inverse correlation clearly shows a protective role of VMAT2 against PD neurodegeneration (Liang et al., 2004; Sulzer et al., 2000). In PD and parkinsonian syndromes, the pigmented DAergic neurons of the SN degenerate and thereby release large amounts of accumulated NM into the extracellular space. The NM-containing organelles left by dying neurons then release the reactive/toxic metals and organic toxins previously accumulated into NM. Such release of toxic compounds is also induced by microglial phagocytosis and degradation of NM. The activated microglia contribute with production and release of reactive species like H_2O_2 and peroxynitrite (Zhang et al., 2011), as well as cytokines that exacerbate microglia-mediated toxicity. Around these NM deposits there is also evidence of reactive astrocytosis (Imamura et al., 2003; McGeer et al., 1988). Both reactive astrocytes and microglia indicate a robust inflammatory state of SN in PD brain (Doorn et al., 2014; Iannaccone et al., 2013; Imamura et al., 2003; Kouli et al., 2020). Activated microglia have been observed in close proximity to extracellular deposits of NM in the SN of patients with PD and parkinsonism with dementia (Imamura et al., 2003; McGeer et al., 1988) or toxin-induced parkinsonism (Langston et al., 1999). This situation has been reproduced in microglia-neurons co-cultures treated with human NM, where activated microglia release reactive molecules and proinflammatory cytokines causing neuronal death while rapidly phagocytosing and degrading NM (Zhang et al., 2011). In addition, human NM injected into rat SNpc induces reactive microgliosis, astrocytosis, and selective death of DAergic neurons (Zecca et al., 2008b; Zhang et al., 2011).

There is also an autoimmune mechanism of neuronal death in PD explaining the selective vulnerability of NM-containing catecholaminergic neurons. Mouse and human DAergic neurons upregulate MHC-I expression in response to high cytosolic DA and/or oxidative stress, providing a possible link between disrupted DA homeostasis and neuroinflammation observed in PD (Cebrián et al., 2014). DAergic neurons containing NM have high expression of the MHC-I, likely induced by interferon-gamma from T cells or other cell types, which can bind foreign peptides and present them to T lymphocytes. These pigmented neurons presenting MHC-I-peptides complexes are then available for targeting by antigen-specific $CD8^+$ cytotoxic T-cells for destruction (Cebrián et al., 2014). As has been described with microglia (Zhang et al., 2011), dendritic cells can also phagocytose NM *in vitro*, subsequently becoming activated and releasing proinflammatory cytokines which induce T-cell proliferation (Oberländer et al., 2011). This likely impairs the integrity of the blood-brain barrier, promoting the entry of additional peripheral immune cells into the brain parenchyma. However, whether dendritic cells *in vivo* can interact with NM remains to be seen, since they occur mainly in tissue near cerebrospinal fluid and their number in the SNpc is much lower than resident microglia.

In conclusion, NM synthesis can be protective under physiological conditions as this compound can sequester reactive/toxic compounds, metals, and various types of xenobiotics from the cytosol, immobilizing them as stable adducts. Conversely, NM can bind and accumulate foreign peptides inside autolysosomes where they can bind to MHC-I, followed by presentation of these complexes on neuronal membrane of catecholaminergic neurons and induction of an autoimmune response. Another neurotoxic mechanism induced by NM, when released by dying neurons, is the activation of microglia which can kill neurons by releasing reactive/toxic species and proinflammatory cytokines. The continuous release of NM by degenerating neurons in PD fuels a continuous mechanism of neuroinflammation and neurodegeneration that contributes to disease progression. Accumulation of NM during the lifetime of neurons and the role of NM in neurodegeneration show an important link between PD and aging - the largest risk factor of PD.

2.4. Synergistic toxicity of alpha-synuclein and dopamine

Because of the selective vulnerability of SNpc DAergic and LC norepinephrine-containing neurons, both containing the highest concentrations of NM in the aged brain (Zecca et al., 2004), dysregulation of catecholamine homeostasis has long been proposed to play a role in PD neurodegeneration (Edwards, 1993; Gainetdinov et al., 1998; Lotharius and Brundin, 2002; Sulzer, 2001; Uhl, 1998). Catecholamines are synthesized from the non-essential amino acid tyrosine by the enzymes TH and AADC and, interestingly, aSyn has been shown to co-immunoprecipitate with both TH (Lou et al., 2010; Perez et al., 2002) and AADC (Tehranian et al., 2006). This interaction leads to decreased enzymatic activity, suggesting that a loss of soluble aSyn due to reduced expression or aggregation may increase catecholamine synthesis.

Ca^{2+} levels positively regulate the activity of both TH and AADC, providing a direct connection between synaptic activity and DA synthesis. However, as a result of Ca^{2+}-driven pacemaking in SNpc and LC neurons (Surmeier et al., 2017b), elevated levels of Ca^{2+} also lead to chronically elevated levels of cytosolic catecholamines. Consistent with this, L-DOPA treatment produces higher concentration of cytosolic catecholamines in cultured SNpc (Mosharov et al., 2009) and LC (Mosharov et al., unpublished data) compared to VTA neurons, which translates into higher susceptibility of these neurons to L-DOPA-induced degeneration. Similarly, SNpc neurons are significantly more sensitive to the parkinsonian neurotoxin MPP^+ than VTA neurons, which is also associated with cell-type specific increase in cytosolic Ca^{2+} that requires the activity of LTCC (Choi et al., 2015a; Lieberman et al., 2017). The difference in susceptibility of SNpc and VTA neurons to both stressors was minimized by pharmacological or genetic blockade of the $Ca_v1.3$ channel, confirming its role in selective PD-like degeneration. Importantly, SNpc neuronal death could also be rescued by aSyn deletion, which provided a similar, but not additive, degree of protection as the blockade of LTCC, suggesting that aSyn and LTCC may act in concert (Lieberman et al., 2017).

A hypothesis that increased cytosolic DA may lead to PD-like nigrostriatal neurodegeneration was examined in mice that displayed a 95% reduction of VMAT2 expression due to a hypomorphic allele (Caudle et al., 2007). Surprisingly, the first generation of these mice (VMAT2-deficient KA1 line (Mooslehner et al., 2001)) did not show any PD phenotype despite an ~85% reduction in brain levels of DA, norepinephrine, and serotonin. It was subsequently discovered, however, that this mouse line had a spontaneous deletion of the *SNCA* gene leading to loss of aSyn expression (Colebrooke et al., 2006; Specht and Schoepfer, 2001). After further breeding to reintroduce the wild-type (WT) aSyn gene, the resulting mice had diminished VMAT2 (VMAT2-LO) and showed signs of PD-like progressive neurodegeneration, including L-DOPA-responsive motor deficits, oxidative stress and protein damage, decreased DA, DAT and TH levels in the striatum, pathological accumulations of aSyn, and reduced number of DAergic neurons in the SNpc (Caudle et al., 2007; Taylor et al., 2011). Thus, the VMAT2-LO mouse model not only demonstrated that a reduced capacity of cells to sequester cytosolic DA is sufficient to cause PD-like degeneration, but also that this effect requires the presence of aSyn.

The oxidation products of DA and other catecholamines are able to interact with aSyn, producing DA metabolite-modified aSyn, which is less likely to fibrilize and instead forms soluble oligomers (Bisaglia et al., 2010; Burke et al., 2008; Conway et al., 2001; Pham et al., 2009). This interaction is non-covalent, reversible, and occurs at the $Y_{125}EMPS_{129}$ pentapeptide in the C-terminal region of aSyn with an additional long-range electrostatic interaction with E83 in the non-amyloid β-component (NAC) region (Herrera et al., 2008; Mazzulli et al., 2007). Using fluorescence-lifetime imaging microscopy to monitor the relative position of the N- and C- terminals of aSyn, it was shown that DA induces a conformation where the termini are closer together, which may inhibit fibril formation (Outeiro et al., 2009). Additionally, DOPAL may cross-link aSyn lysine residues, also facilitating its aggregation (Werner-Allen et al., 2016). Intracellular aSyn oligomeric species can be cytotoxic by a variety of mechanisms, including permeabilization of vesicular and plasma membranes by pore-forming fibrils (Ding et al., 2002; Gosavi et al., 2002; Lashuel et al., 2002; Mosharov et al., 2006; Pacheco et al., 2015; Parres-Gold et al., 2020), disruption of proteasomal protein clearance, chronic endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and inhibition of SNARE complex formation and neurotransmitter release (Choi et al., 2013; Ebrahimi-Fakhari et al., 2011; Zaltieri et al., 2015). The presence of aSyn was shown within the NM organelle (Zucca et al., 2018). This likely derives from reaction of fibril aSyn with DOPAL in early steps of NM synthesis to form a DOPAL-aSyn adduct which is then oxidized to non-toxic melanin-protein complex. The latter is taken into autophagic vacuoles that are further processed to generate the final NM organelle, as discussed above .

Monomeric DA-aSyn may also be toxic by interfering with protein degradation via a lysosomal pathway called chaperone-mediated autophagy (CMA) (Cuervo et al., 2004). CMA cytosolic substrates contain a KFERQ-like motif that can be recognized by the chaperone protein heat shock cognate 70 kDa protein (cyt-Hsc70) that delivers them to a lysosomal associated membrane protein (LAMP2A). LAMP2A forms a translocation complex once bound to a substrate and the unfolded protein crosses into the lysosomal lumen where it can be degraded. While aSyn, oxidized aSyn, and DA-aSyn complexes show similar LAMP2A binding levels, the latter binds to the lysosome without evidence of translocation. Furthermore, DA-aSyn blocks both the binding and uptake of a CMA substrate GAPDH, suggesting stronger binding to LAMP2A. A mutation in the DA-interacting region of aSyn ($Y_{125}EMPS_{129}$ to $F_{125}AAFA_{129}$) nullifies the effect, further demonstrating that the interaction of DA and oxidized forms of DA with aSyn leads to this change in CMA. In primary neuronal cultures, the same CMA blockade was demonstrated after exposure to a high dose of L-DOPA, but not in neurons derived from aSyn null animals (Martinez-Vicente et al., 2008). Importantly, six PD-associated proteins have been reported to cause CMA dysfunction, resulting in accumulation of CMA substrates including aSyn: leucine-rich repeat kinase 2 (LRRK2), ubiquitin C-terminal hydrolase 1 (UCHL1), vacuolar protein-sorting associated protein 35 (VPS35), DJ-1, β-glucocerebrosidase (GCase) and aSyn itself (Kabuta et al., 2008; Kuo et al., 2022; Orenstein et al., 2013; Tang et al., 2015; Wang et al., 2016), providing a possible common mechanism of disease initiation and progression.

The toxic interaction between aSyn and DA was studied *in vivo* by combining a common familial PD aSyn mutation with elevated cytosolic DA (Mor et al., 2017). Mice that overexpress the PD mutant A53T aSyn were injected with a lentivirus containing mutant TH which is unresponsive to feedback inhibition by DA, resulting in increased neurotransmitter production in the cytosol (Nakashima et al., 2002). In A53T aSyn-overexpressing, but not WT, mice this elevation of cellular DA levels induced progressive motor impairment accompanied by nigrostriatal degeneration and increased formation of aSyn oligomers. Furthermore, in *C. elegans* overexpressing A53T aSyn, DA toxicity was prevented if DA-interacting residues of aSyn were mutated (Mor et al., 2017). Another recent study of DA- and aSyn-mediated toxicity in human iPSC-derived DA neurons from both idiopathic and familial (mutant DJ-1) PD patients provided more evidence for the involvement of multiple factors in mediating PD-like neurotoxicity (Burbulla et al., 2017). The authors identified an oxidized DA- and Ca^{2+}-dependent toxic cascade that started with mitochondrial oxidative stress leading to lysosomal dysfunction, reduced GCase activity, and aSyn accumulation. This toxic pathway was not present in DJ-1 deficient mice or their iPSC-derived DAergic mouse neurons unless either DA production or aSyn expression was increased.

Overall, both *in vitro* and *in vivo* data suggest that DA and aSyn have a synergetic effect on DAergic neuron vulnerability, and that decreasing the levels of either compound is neuroprotective.

2.5. Interaction of iron and alpha-synuclein

Free unbound iron is highly reactive, necessitating precise homeostatic mechanisms to tightly control cytosolic iron levels in the cell to ensure proper function and survival (Rouault, 2013). Iron homeostasis plays a fundamental role in a variety of metabolic processes, including oxygen transport, deoxyribonucleic acid (DNA) synthesis, electron transport, and DA synthesis. However, abnormal metabolism and accumulation of iron can be observed during aging and both are characteristics shared by common (e.g. PD) and rare (e.g. NBIA disorders) neurodegenerative conditions exhibiting targeted decline of DAergic neurons. Iron accumulation in the SN of PD patients has been reported by several studies (Chen et al., 2019; Dexter et al., 1987; Graham et al., 2000; Griffiths et al., 1999; Li et al., 2022; Liu et al., 2017; Riederer et al., 1989), with nigral DAergic neurons showing increased levels of intracellular iron (Friedrich et al., 2021; Oakley et al., 2007) and increased redox-active iron in NM aggregates (Faucheux et al., 2003) being highest in those patients with the most severe neuronal losses. Importantly, both iron deposition and aSyn aggregation are neuropathological hallmarks of PD, and iron has been shown to facilitate protein aggregation. Indeed, while aSyn is the major component of LBs, redox-active iron is highly enriched in LB cores of the resilient DAergic neurons of the SNpc, suggesting this may be a protective mechanism (Castellani et al., 2000). Interestingly, iron-laden LBs were unique to the SNpc neurons in PD brains, while LBs in cortical neurons of Alzheimer's patient brains remained iron-negative (Castellani et al., 2000).

Intracellular iron and aSyn are closely related to each other and may act in a vicious feedforward cycle of toxicity that contributes to the vulnerability of DAergic neurons. aSyn undergoes a variety of post-translational modifications including oxidation and nitration that are induced by excess iron and iron-induced oxidative stress. Iron, both ferrous and ferric, directly binds to the C-terminus of aSyn (Binolfi et al., 2006; Davies et al., 2011) with increased binding affinity of ferrous, but not ferric, iron when the S129 residue is phosphorylated (Lu et al., 2011) and the N-terminus is acetylated (Abeyawardhane et al., 2018). This ability of aSyn to bind iron (Bharathi et al., 2007; Bharathi and Rao, 2007; Peng et al., 2010), in turn promotes its aggregation into fibrils by inducing conformational changes (Golts et al., 2002; Li et al., 2011; Ostrerova-Golts et al., 2000) facilitating aSyn oligomerization (Hashimoto et al., 1999; Jinsmaa et al., 2014; Uversky et al., 2001). In detail, for polyvalent cations such as iron, cross-linking or ligand bridging has been proposed to be an important factor in inducing conformational changes in aSyn (Uversky et al., 2001). One study reported that it is the effect of trivalent metal ion impurities, such as the ferric form of iron, which mediates oligomerization rather than oxidation of aSyn *per se* (Kostka et al., 2008). On the other hand, aSyn has been suggested to act as a ferrireductase regulating the balance of ferrous and ferric iron under basal conditions (Davies et al., 2011). However, disease-associated variants in aSyn disrupt its physiological ferrireductase activity and concomitantly lead to dysregulation of iron metabolism (Davies et al., 2011; McDowall et al., 2017).

In summary, striking evidence supports the direct structural links between iron and aSyn and their reciprocal interactions contributing to oxidative stress, dysfunction, and ultimately vulnerability of DAergic neurons. Further studies are needed to validate how iron specifically participates in aSyn pathology and whether targeting iron and aSyn interactions may provide a possible therapeutic target for DAergic neuron degeneration in the SNpc.

3. Selective vulnerability in neurodegeneration with brain iron accumulation disorders

While iron accumulation is a typical process that accompanies brain aging, as discussed above this accumulation is disproportionately high in the SNpc of PD patients and coincides with aSyn oligomerization and accumulation, dysregulated DA metabolism, and the preferential decline of DAergic neurons that leads to the progressive motor symptoms. Importantly, the distinctive susceptibility of this neuronal subpopulation is not unique to PD. NBIA is a group of genetically-defined rare disorders connected by the shared pathological features of iron accumulation in the basal ganglia and neuronal deterioration in defined brain nuclei (Spaull et al., 2021). These disorders are caused by an array of genetic mutations impacting diverse cellular pathways, including coenzyme A biosynthesis (*PANK2*, *COASY*), lipid metabolism (*PLA2G6, C19orf12, FA2H, SCP2, CRAT*), autophagy (*WDR45, ATP13A2, AP4M1, REPS1*), iron metabolism (*FTL, CP*), and some with presently unknown functions (*DCAF17, GTBP2*), detailed in two recent reviews (Iankova et al., 2021; Levi and Tiranti, 2019). Despite having varied genetic etiologies, modes of inheritance, and clinical manifestations, three subtypes of NBIA share important pathological features with PD: Beta propeller protein-associated neurodegeneration (BPAN), Mitochondrial membrane protein-associated neurodegeneration (MPAN), and PLA2G6-associated neurodegeneration (PLAN). Each subtype also displays prominent pathology in the SN, including iron accumulation, early and/or preferential degeneration of nigral DA neurons, the development of early-onset, progressive parkinsonism, and/or diffuse LP.

While the field of NBIA research is still in its infancy, information on pathological mechanisms shared by these three disorders which have diverse yet defined genetic etiologies may yield important insight into the molecular underpinnings of DAergic neuron vulnerability. The impact of this has far-reaching potential, as the question of mechanisms driving DAergic neuronal degeneration in distinct disease states—particularly PD—have puzzled clinicians and scientists.

3.1. Beta propeller protein-associated neurodegeneration (BPAN)

Since the 2012 discovery of the mutation in the *WDR45* (WD Repeat Domain 45) gene (Haack et al., 2012), BPAN has become one of the most commonly encountered subtypes of NBIA (Gregory and Hayflick, 1993). WDR45, also known as WIPI4, has been shown to play an important role in the autophagy pathway (Chowdhury et al., 2018; Maeda et al., 2019; Zheng et al., 2017), though its full function remains undefined. While most NBIA disorders are inherited in an autosomal recessive pattern, BPAN is the only subtype with X-linked dominant inheritance, with predominantly simplex cases of *de novo* origin (Gregory and Hayflick, 1993). BPAN follows a biphasic clinical course beginning in early childhood with global developmental delay, intellectual disability, epilepsy, fine and gross motor impairment, and abnormal behavior reminiscent of autism spectrum disorder. The second phase begins in adolescence or early adulthood (average of 25 years (Gregory and Hayflick, 1993)) and is characterized by progressive cognitive decline, dementia, and movement disorders including parkinsonism and dystonia. The earliest and most prominent neuropathology is observed in the SNpc, and to a lesser extent in the globus pallidus, which also sets BPAN apart from many of the other NBIA disorders (Hayflick et al., 2013; Kim et al., 2017; Paudel et al., 2015; Saitsu et al., 2013). Brain MRI commonly reveals T2-weighted hypointensity in the SNpc due to accumulated iron coincident with a T1-weighted hyperintense "halo" signal, possibly from iron-NM complexes released from dead and dying pigmented neurons (Ginat and Meyers, 2012; Hayflick et al., 2013; Paudel et al., 2015; Saitsu et al., 2013). The loss of dopaminergic neurons has also been confirmed with DAT positron emission tomography (PET) technology (Kim et al., 2017). Additionally, diffuse tau-positive neurofibrillary tangles have been confirmed in at least two female patients (Hayflick et al., 2013; Paudel et al., 2015). Interestingly, while LB pathology is pervasive in PD, there are no reports to-date of synucleinopathy in BPAN (Paudel et al., 2015; Hayflick et al., 2013).

Animal models of BPAN have provided valuable insight into the roles of WDR45 in neurodevelopment and neurodegeneration (Biagosch et al., 2021; Noda et al., 2021; Wan et al., 2020; Zhao et al., 2015). WDR45-deficient mouse models have reliably demonstrated learning and memory defects, however, the recapitulation of other important disease

phenotypes including brain iron accumulation, degeneration of SNpc neurons, and development of motor impairment has been inconsistent. This may be due to different genetic modification strategies or to the important species-specific differences between mouse and human DA neurons (Burbulla et al., 2017). With that in mind, investigations using human cellular models of BPAN are critical for understanding the molecular drivers of pathogenesis. In contrast to animal models, iron accumulation is reliably seen across many human *WDR45* mutant cell types (Aring et al., 2022; Ingrassia et al., 2017; Lee et al., 2021; Saitsu et al., 2013; Seibler et al., 2018; Xiong et al., 2021), clearly indicating disturbed iron homeostasis. While the precise iron homeostatic mechanisms involved in pathology remain debated, the elevated iron observed across human BPAN cellular systems appears to correlate with elevated ROS, mitochondrial dysfunction, and suppressed ATP production (Aring et al., 2022; Lee et al., 2021; Seibler et al., 2018). Importantly, in the only investigation to-date using BPAN patient iPSC-derived DA neurons, Seibler and colleagues confirmed iron accumulation, mitochondrial dysfunction, and signs of oxidative stress in the cell type most impacted by WDR45 mutation (Seibler et al., 2018). While the apparent lack of accumulated aSyn in the BPAN brain contrasts starkly with PD, the elevation of iron and parallel degeneration of SN DAergic neurons, which have been confirmed in an animal model (Wan et al., 2020) and is a consistent feature of human *in vitro* models, clearly demonstrates that disruption to iron and DA metabolism has severe consequences for this uniquely vulnerable subpopulation of neurons. However, further investigation into DA pathways and NM accumulation, and how excess iron intersects with both, are warranted to comprehensively define the neuronal pathology in disease-relevant cell types.

3.2. Mitochondrial membrane protein-associated neurodegeneration (MPAN)

MPAN is another relatively common subtype of NBIA (Gregory and Hayflick, 1993; Kolarova et al., 2022), resulting from the autosomal recessive inheritance of mutant *C19orf12* (Chromosome 19 Open Reading Frame 12), a small transmembrane protein with unknown function. In addition to autosomal recessive transmission, several cases with seemingly autosomal dominant inheritance have been reported (Fraser et al., 2021; Gregory et al., 2019; Rickman et al., 2021). Disease onset typically ranges from childhood to early adulthood, and pathology progresses slowly in most cases. Clinical manifestations of MPAN include gait abnormalities, signs of upper and lower motor neuron dysfunction, neuropsychiatric changes, and progressive dystonia, parkinsonism, and cognitive decline. Brain MRI of these patients frequently reveals large iron deposits in the basal ganglia, particularly in the globus pallidus and SN (Hartig et al., 2011; Hogarth et al., 2013). Similar to BPAN, brain tissue from one MPAN patient revealed extensive tau pathology (Hartig et al., 2011). However, in contrast to BPAN, MPAN patients demonstrate diffuse aSyn-positive LP which is also prevalent in the SN, even showing higher aSyn-containing LBs and Lewy neurites (LN) than PD patient brains (Hartig et al., 2011; Olgiati et al., 2017). This correlates with massive degeneration of nigral DAergic neurons (Hogarth et al., 2013).

To date, MPAN pathology has been investigated in drosophila (Iuso et al., 2014), zebrafish (Mignani et al., 2020), and human cellular models (Shao et al., 2022; Venco et al., 2015) which have provided critical insight into protein subcellular localization (Venco et al., 2015), neurodevelopment (Iuso et al., 2014), and pathogenesis. While the function of *C19orf12* remains elusive, Venco and colleagues showed that the protein localizes to mitochondrial and ER membranes and responds to oxidative stress by translocating to the cytosol (Venco et al., 2015). However, when pathogenic mutations are transduced into HeLa and HEK-293 cells, it becomes insensitive to oxidative stress signals and does not relocate to the cytosol, suggesting that this function may play a major role in MPAN pathophysiology. Most recently, Shao and colleagues used both *C19orf12* knockout (KO) M17 neuroblastoma cells and MPAN patient fibroblasts to demonstrate that the combination of elevated redox active iron and mitochondrial abnormalities culminated in heightened oxidative stress and susceptibility to erastin-induced ferroptosis, proposing this as a potential mechanism driving loss of DAergic neurons in MPAN patients (Shao et al., 2022). Authors further demonstrated that some pathological phenotypes could be prevented by pretreatment with the iron chelator deferoxamine, highlighting the importance of iron in MPAN pathology. Interestingly, knockdown of *C19orf12* orthologs in drosophila led to impaired locomotor activity and shortened lifespan, but did not manifest brain iron accumulation (Iuso et al., 2014). However, much more work is needed to fully define the roles of C19orf12 in the CNS, particularly in the most vulnerable midbrain DAergic neurons, and to elucidate pathogenic mechanisms leading to their specific demise.

3.3. PLA2G6-associated neurodegeneration (PLAN)

PLAN defines a group of three neurodegenerative disorders resulting from mutation of the Ca^{2+}-independent phospholipase PLA2G6 (Phospholipase A2 Group VI) which functions in a number of cellular processes, primarily phospholipid metabolism (Burke and Dennis, 2009; Ramanadham et al., 2015). These three disorders are defined by their differing clinical phenotypes, and are classified as INAD (infantile neuroaxonal dystrophy), ANAD (atypical neuroaxonal dystrophy), and PLA2G6-related dystonia-parkinsonism. INAD typically begins between six months and three years of age and displays the most severe clinical course, resulting in rapid decline in motor function, spasticity, and cognitive impairment, and death usually within the first decade (Gregory et al., 2008). The age of onset for ANAD is later than INAD and presents with a slower disease progression until a rapid neurological decline between seven and 12 years of age (Gregory et al., 2008). Both INAD and ANAD show iron accumulation in the globus pallidus and cerebellar atrophy, but do not always exhibit iron accumulation in the SN or degenerative pathology in the SN (Kurian et al., 2008); thus, this section will focus on the third form of PLAN: the adult-onset PLA2G6-related dystonia-parkinsonism.

PLA2G6-related dystonia-parkinsonism exists on a continuum with PD (PARK14), with some patients exhibiting abnormal iron accumulation in the basal ganglia, namely the globus pallidus, SN, and/or striatum; however this finding is not consistent and is therefore not used as a diagnostic criterion (Magrinelli et al., 2022; Paisan-Ruiz et al., 2008; Sina et al., 2009). While the precise delineation between PLA2G6-depenedent PD and NBIA remains unclear, many preclinical investigations have revealed that loss or reduction of PLA2G6 function leads to mitochondrial abnormalities and elevated ROS production (Chiu et al., 2017; Chiu et al., 2019; Ke et al., 2020; Kinghorn et al., 2015), DAergic neuron atrophy (Chiu et al., 2017; Chiu et al., 2019; Iliadi et al., 2018; Ke et al., 2020; Mori et al., 2019; Yeh et al., 2021; Zhou et al., 2016), aSyn-positive LP in the SN (Chiu et al., 2019; Mori et al., 2019), and progressive motor dysfunction (Chiu et al., 2019; Iliadi et al., 2018; Kinghorn et al., 2015; Mori et al., 2019; Yeh et al., 2021; Zhou et al., 2016). Taken together, this suggests that while PLA2G6 function is necessary for survival of SN DAergic neurons, the role of iron and aSyn in this NBIA is undefined and may not be crucial for the manifestation of pathological phenotypes. Thus, further investigation in both patient-derived neuronal models and *in vivo* systems will be crucial in determining which PLA2G6 cellular functions correlate with iron homeostasis, aSyn regulation, and DA neuron degeneration.

3.4. Summary

Some of the most significant challenges facing PD researchers are the lack of early biomarkers preceding neuronal decline, the incomplete or suboptimal disease recapitulation in preclinical models, the genomic variability of idiopathic cases, and the limited understanding of the pathological cascade which culminates in diagnosable PD. Patients with

BPAN, MPAN, and PLAN display some of the most well-defined pathological features observed in PD, most notably: iron accumulation and neurodegeneration in the SN, progressive movement disorders, and/or robust accumulation of aSyn. Therefore, these NBIA disorders represent promising genetically-defined models of DAergic vulnerability and degeneration, and may generate critical knowledge about disease mechanisms in NBIA and PD alike.

4. Current therapeutic landscape for Parkinson's disease and neurodegeneration with brain iron accumulation disorders

4.1. Parkinson's disease

4.1.1. Dopamine replacement therapy

Sixty years after its introduction (Birkmayer and Hornykiewicz, 1962), the oral substitution therapy using the DA precursor L-DOPA still dominates the therapeutic landscape for people with PD. L-DOPA, unlike DA, is able to cross the blood-brain barrier (BBB) and is commonly co-administered with an AADC inhibitor like carbidopa to prevent its peripheral catabolism and amplify the amount delivered to the CNS. Once in the brain, L-DOPA is metabolized into DA and able to supplement the waning endogenous DA signaling, providing relief for some motor symptoms of PD. Interestingly, L-DOPA has demonstrated iron-chelating ability, and a recent report in a preclinical model suggests that this property may contribute to the compound's efficacy through the sequestration of iron and mitigation of DA oxidation (Billings et al., 2019). Conversely, L-DOPA may function as a siderophore by forming a complex with human siderocalin; this limits the amount of free L-DOPA available for DA synthesis thus diminishing its therapeutic benefits (Alhassen et al., 2022).

One of the seminal trials to understand its effects was the ELLDOPA (Earlier versus Later Levodopa Therapy in Parkinson's disease) study that investigated L-DOPA vs. placebo, demonstrating a clear dose-dependent efficacy with a favorable risk profile (Fahn et al., 2004). Despite this early success, L-DOPA therapy also revealed the eventual emergence of motor complications, especially L-DOPA induced dyskinesias (LID), sparking a long-standing discussion on the risk/reward tradeoffs with this therapeutic strategy (Fahn, and Parkinson Study, G, 2005). Later research showed that patients develop motor complications regardless of how early L-DOPA was initiated, arguing against the "L-DOPA sparing" approach of delaying L-DOPA treatment (Cilia et al., 2014; Katzenschlager et al., 2008). Moreover, despite the motor fluctuations associated with L-DOPA treatment, the motor symptoms and disease progression were still significantly less severe when compared with the natural clinical course of PD, pointing to a beneficial long-term effect (Cilia et al., 2020). The 'Levodopa in Early Parkinson's Disease' (LEAP) trial detected neither protective nor harmful effects of effective early L-DOPA treatment (Verschuur et al., 2019) on the severity of parkinsonian symptoms compared to the delayed treatment group, again supporting the early intervention strategy of L-DOPA for PD patients. A pragmatic perspective on the treatment with L-DOPA was provided by the 'Parkinson's Disease Medicines' (PDMED) trial project for 1620 people with PD. This seven-year longitudinal trial compared early L-DOPA intervention with early DA agonist intervention (L-DOPA sparing regimen) (Group et al., 2014), and revealed that 93% of patients randomized to early L-DOPA remained on this treatment, but only 50% and 28% remained on DA agonist or MAO-inhibitor therapy, respectively, indicating L-DOPA effectiveness and lack of serious side effects. This confirmed results from earlier long-term studies which demonstrated that L-DOPA resulted in less side effects, better patient-reported activities of daily living, and better motor outcomes in direct comparison studies versus DA agonist such as pramipexole (Holloway et al., 2004), or ropinirole (Rascol et al., 2000).

Unlike L-DOPA, which can induce motor complications like LID, D2/3/4-family DA agonists frequently cause significant non-motor side effects including sleepiness, nausea, hallucinations, and impulse-control disorders, limiting their clinical use. Clinical trials are ongoing with D1/5-family agonists which might avoid such side effects. While phase I has demonstrated good safety and tolerability profiles (Gurrell et al., 2018; Sohur et al., 2018), achievement of significant pharmacodynamic and clinical endpoints has been variable (Gurrell et al., 2018; Huang et al., 2020; Riesenberg et al., 2020), and two phase II trials were discontinued (NCT02687542, NCT02847650). Thus, L-DOPA remains the clinical gold standard for symptomatic treatment of PD.

4.1.2. Inhibition of dopamine degradation

A clinically validated approach for maintaining DAergic signaling in PD patients is the inhibition of key metabolic enzymes that are involved in DA degradation: MAO and catechol-O-methyl transferase (COMT). These enzymes are differentially expressed in neurons and glial cells; however, both are responsible for the breakdown of DA into DOPAL and homovanillic acid (HVA), thus, their inhibition results in increased DA available for neurotransmission.

MAO inhibitors are a class of drugs that inhibit the activity of one or both MAO enzymes (MAO-A and MAO-B). Currently, the only FDA-approved MAO-B-selective inhibitors, selegiline and rasagiline, are irreversible, forming covalent adducts to the flavin adenine dinucleotide (FAD) cofactor within the MAO-B active site, and are mainly used in early PD phases (Rascol et al., 2005). Because of numerous neuroprotective outcomes with these compounds, a disease modifying mechanism was initially assumed from a large randomized delayed onset trial, but subsequently disproven in follow-up studies (Rascol et al., 2011; Rascol et al., 2016).

COMT is mainly expressed in postsynaptic neurons and glial cells, and thus plays a limited role in the clearance of DA within catecholaminergic neurons (Meiser et al., 2013; Monzani et al., 2019) (Fig. 1). Nevertheless, COMT inhibitors have long provided a clinically useful pharmacological tool in the treatment of PD as they increase the availability of extracellular DA, thus achieving therapeutic effects (Brooks, 2004). In the reaction catalyzed by COMT a methyl residue is attached to one of the hydroxyl groups of the catechol ring preventing its possible auto-oxidation to quinone. The latest member of COMT inhibitors, opicapone, demonstrated significant alleviation of motor symptoms in large multi-center trials (Ferreira et al., 2015). Tolcapone, with the highest efficacy of the three marketed COMT inhibitors for its ability to penetrate the blood-brain barrier, has shown remarkable success in ameliorating tremor severity in a number of cases with otherwise therapy-resistant tremor (Napolitano et al., 1999).

4.1.3. Prevention of alpha-synucleinopathy

Recent preclinical research and early-phase clinical trials have suggested several approaches that may help to overcome the limitations of current therapeutics for PD, which are primarily restricted to symptomatic relief. Such developments include targeting the misfolded or aggregated aSyn through reducing its expression (antisense therapy (Alarcón-Arís et al., 2020)), preventing the formation of toxic aSyn aggregates (antiaggregatives (Levin et al., 2014; Levin et al., 2022), chelators (Devos et al., 2014)), dissolving or elimination of intra- or extracellular toxic aSyn aggregates (active immunotherapy (Volc et al., 2020), passive immunotherapy (Pagano et al., 2021), and anti-aggregatives), enhancement of cellular clearance mechanisms to eliminate toxic forms of aSyn (autophagy (Pagan et al., 2020; Pagan et al., 2021), lysosomal function (Mullin et al., 2020)), and modulation of neuroinflammatory processes (Athauda et al., 2019; Athauda et al., 2017). Regardless of the approach, the ultimate goal of targeting aSyn is to reduce the intracellular aSyn burden and the toxicity associated with synuclein-laden LBs and LNs to reduce the propagation of the disease. While many of these strategies have demonstrated promise in preclinical and/or early-phase clinical trials, each comes with its own set of concerns and limitations which impede their large-scale implementation. Prominent among these is the incomplete understanding of aSyn function, making manipulation of its expression, structure, and dynamics

potentially very challenging and unpredictable. These therapeutic strategies are also limited by poor BBB penetrance, the potential for off-target effects on physiological forms of aSyn, and the need to balance aSyn homeostasis rather than eliminate it altogether.

4.1.4. Iron chelation therapy

Substantial evidence supports the role of iron accumulation in midbrain pathology in PD; thus, iron chelators have been explored extensively in preclinical models. Iron chelating agents bind to oxidized iron ions, forming a stable complex which can then be eliminated from the body. The use of the brain-penetrant iron chelator deferiprone (DFP) has been explored in two clinical trials of PD to-date and both trials demonstrated specific reduction of iron load in the SN with treatment (Devos et al., 2014; Martin-Bastida et al., 2017). These results showed significant promise to warrant further investigation in the form of a large multi-center phase II clinical trial (FAIR-PARK-II) with over 350 European early-stage PD patients. This trial investigates the efficacy of moderate-dose DFP at mitigating motor and non-motor symptoms, however conclusions about the effectiveness of this intervention cannot be made until trial results are published (NCT02655315).

Of note, iron chelation has not been adopted clinically for a number of reasons, including adverse events in trial subjects, poor response of patients with neuroinflammatory markers, and the concern that long-term chelation therapy may disrupt iron homeostasis in the brain, particularly in non-neuronal cells (i.e. myelin-producing oligodendrocytes) which require large amounts of iron to function. These translational limitations have spurred development of next-generation iron chelation strategies, and these include the bis-hydroxyphenyltriazoles, hydroxypyridinones, the Metal-Protein Attenuating Compounds (MPACs), and multi-target drugs (for an in-depth review, please see (Ward et al., 2021).

4.2. Neurodegeneration with brain iron accumulation disorders

4.2.1. Beta propeller protein-associated neurodegeneration

In the early childhood phase of the disease, individually tailored epilepsy management is of the utmost importance, including antiepileptic drugs and facultatively ketogenic diet or vagus nerve stimulation. Developmental delay and intellectual disability may be alleviated by early intervention and education programs. Behavioral difficulties may require psychotherapeutic, sometimes also psychopharmacologic approaches.

In the second phase of BPAN, mostly beginning in adolescence or early adulthood, pharmacologic treatment of dystonia and spasticity may include oral baclofen, clonazepam and other benzodiazepines, sometimes intramuscular botulinum toxin injections to mitigate focal dystonia elements. Parkinsonism is responsive to DAergic medication, but treatment may be compromised by the development of motor fluctuations, dyskinesias, and dystonia (Wilson et al., 2021).

Iron accumulation is most likely a secondary phenomenon in BPAN. Only two patients were reported as treated with the iron chelator DFP, both without benefit (Lim et al., 2018; Fonderico et al., 2017). Accordingly, iron chelating treatment is not recommended outside of a clinical trial.

Potentially disease-modifying treatments under preclinical investigation comprise activation of autophagy and inhibition of ER stress. The BPAN-specific *WDR45* mutations lead to impaired autophagy. Rapamycin inhibits the protein kinase mTOR (mechanistic Target Of Rapamycin) and leads to disinhibition of autophagy (Bové et al., 2011). In a BPAN mouse model, rapamycin was able to reduce ER stress, partially restore autophagy and alleviate neuronal death (Wan et al., 2020). Clinical studies of mTOR inhibition are running in PD but not yet in BPAN (Iankova et al., 2021). The ER stress inhibitor tauroursodeoxycholic acid (TUDCA) which is under clinical investigation in amyotrophic lateral sclerosis can reduce cellular death in WDR45-deficient cells but has not been investigated clinically in BPAN so far.

4.2.2. Mitochondrial membrane protein-associated neurodegeneration

Symptom-oriented management includes baclofen, trihexyphenidyl, and intramuscular botulinum toxin for dystonia and spasticity, as well as DAergic agents in case of parkinsonism. Optic atrophy may be ameliorated by visual aids, dysphagia by gastric feeding tubes (Gregory and Hayflick, 1993).

Iron chelating treatment with DFP was reported only in two MPAN patients, one without clinical benefit, and one with discontinuation due to gastrointestinal side effects (Iankova et al., 2021).

The exact function of the *C19orf12* gene product is yet unknown but it has been hypothesized to play a major role in mitophagy (Venco et al., 2015). In MPAN patient fibroblasts, mislocalization of the C19orf12 protein leads to an increase of calcium in mitochondria with increased susceptibility to oxidative stress. A Drosophila model of MPAN partially recapitulates the human phenotype, the flies show climbing abnormalities, reduced survival and vacuolation in the brain (Iuso et al., 2014). Preclinical testing of potential therapies in flies and patient cell lines are underway.

4.2.3. PLA2G6-associated neurodegeneration

Symptom-oriented management in INAD and ANAD includes pharmacologic treatment of spasticity, visual aids, and psychiatric therapies for the common neuropsychiatric symptoms. In PLA2G6-related dystonia-parkinsonism, treatment with DAergic drugs is in most cases highly beneficial in the beginning, but response wears off over time often followed by prominent early dyskinesias.

Regarding disease-modifying therapies, deuterated polyunsaturated fatty acids (D-PUFAs) were found to reduce oxidative stress and restore mitochondrial membrane potentials in cultured fibroblasts and are currently being tested in patients with a variety of neurodegenerative disorders, including Friedreich's ataxia and progressive supranuclear palsy. A prospective open-label study is being conducted to test the efficacy and safety of RT001, a D-PUFA drug, in patients with INAD (Clinical Trials No: NCT03570931). Treatment with desipramine, an approved tricyclic antidepressant but with drug repurposing potential for other applications, in a PLAN fly model resulted in reduction of ceramides, lysosomal stress, and neurodegeneration, and has been tested in an open-label trial in four INAD patients (NCT03726996). Somatic gene replacement therapy has been tested in a knock-in mouse model of PLAN, and led to significant weight gain, prevention of motor decline, extension of lifespan, and reduced neurodegeneration. A study in patients is in preparation (Iankova et al., 2021).

5. Conclusion and perspectives

DAergic neurons of the SNpc form a critical component of the basal ganglia circuitry that regulates thalamocortical control of voluntary and involuntary movements. Their unique combination of structural and physiological properties generate high energetic demands which make these neurons more susceptible to the effects of aging, genetic variation, iron-mediated toxicity, and environmental toxins. Multiple lines of evidence suggest a complex interplay of cellular pathways whereby disrupted DA metabolism, iron homeostasis, and aSyn buildup converge to exacerbate pathological mechanisms, ultimately leading to death of this vulnerable neuron population in PD and NBIA (Fig. 2).

Although much progress has been made towards our understanding of pathogenesis, the development of disease-modifying therapies that counter progressive loss of nigral DAergic neurons is complicated by the limitations of available preclinical models, the species-specific differences between animal models and human neurophysiology, and the genetic heterogeneity of PD. Thus, investigations using disease models that more faithfully recapitulate human pathology will be of critical importance for advancing therapeutic discovery. The utilization of human patient iPSCs for *in vitro* disease modeling, combined with improved neuron-glia co-cultures and 3D brain organoids will provide novel insight into pathogenesis and pathological cascades at the

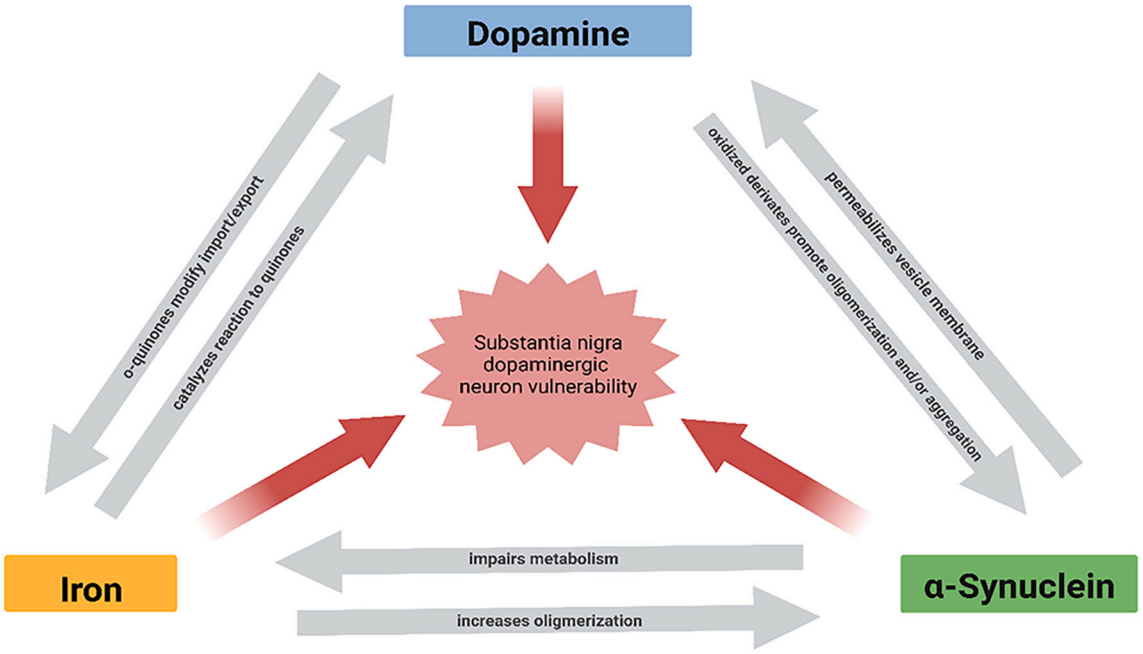

Fig. 2. The complex interactions of dopamine, iron, and alpha-synuclein which may contribute to the preferential vulnerability of substantia nigra dopaminergic neurons.

crossroads of DA, iron, and aSyn. Additionally, improving animal models to better represent pathological features seen in human brain, including LP and NM accumulation, will be crucial for our understanding of the cell autonomous and non-cell autonomous mechanisms driving SN vulnerability. Together, these efforts will provide important answers to untangle the complex interplay of cellular processes driving neurodegeneration and will serve as important resources to identify optimal translational strategies that could prevent, slow, or even stop the loss of SN DA neurons in both PD and NBIA.

Declaration of Competing Interest

T.K. served as coordinating investigator of the FORT trial; received research funding from Retrophin, Inc.; served as coordinating investigator of the deferiprone in PKAN randomized and extension trial; received research funding from ApoPharma Inc.; provided consulting services to CoA Therapeutics, Comet Therapeutics, and Retrophin, Inc.; received travel support from ApoPharma Inc.

Data availability

No data was used for the research described in the article.

Acknowledgements

L.F.B. is supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No. [948027]), by the DFG under the Heisenberg Programme and under Germany's Excellence Strategy within the framework of the Munich Cluster of Systems Neurology (EXC 2145 SyNergy—ID 390857198). **A.W.** is supported by a PhD stipend from the Hans and Ilse Breuer Foundation. **D.S** and **E.V.M** are supported by Aligning Science Across Parkinson's (ASAP) research grant, NIH R01NS095435 and the JPB Foundation. **F.A.Z** and **L.Z.** are supported by Grigioni Foundation for Parkinson's disease (Milano, Italy), and by the Italian Ministry of Education, University, and Research (MIUR) - Smart Cities and Communities and Social Innovation, SMART AGING Project - SCN_00442. **T.K.** acknowledges funding by the European Commission 7th Framework Programme (FP7/2007-2013, HEALTHF2-2011; Grant Agreement No. 277984) for the international collaborative project TIRCON (Treat Iron-Related Childhood-Onset Neurodegeneration). Subsequently to EU funding, TIRCON has been sustained through continuous donations from international patient advocacies (NBIA Alliance and its members, www.nbiaalliance.org) and through single donations from pharmaceutical companies including ApoPharma Inc. (Toronto, Canada), CoA Therapeutics (San Francisco, CA; USA) and Retrophin, Inc. (San Diego, CA; USA). **T.K.** is also a member of the European Reference Network for Rare Neurological Diseases (ERN-RND), co-funded by the European Commission (ERN-RND: 3HP 767231).

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