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Outlook of PINK1/Parkin signaling in molecular etiology of Parkinson's disease, with insights into *Pink1* knockout models

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ABSTRACT

Parkinson's disease (PD) relates to defective mitochondrial quality control in the dopaminergic motor network. Genetic studies have revealed that *PINK1* and *Parkin* mutations are indicative of a heightened propensity to PD onset, pinpointing mitophagy and inflammation as the culprit pathways involved in neuronal loss in the substantia nigra (SNpc). In a reciprocal manner, *LRRK2* functions in the regulation of basal flux and inflammatory responses responsible for PINK1/Parkin-dependent mitophagy activation. Pharmacological intervention in these disease-modifying pathways may facilitate the development of novel PD therapeutics, despite the current lack of an established drug evaluation model. As such, we reviewed the feasibility of employing the versatile global *Pink1* knockout (KO) rat model as a self-sufficient, spontaneous PD model for investigating both disease etiology and drug pharmacology. These rats retain clinical features encompassing basal mitophagic flux changes with PD progression. We demonstrate the versatility of this PD rat model based on the incorporation of additional experimental insults to recapitulate the proinflammatory responses observed in PD patients.

Keywords: Parkinson's disease; Mitophagy; Inflammatory response; Genetic model; *Pink1* KO rats

INTRODUCTION

Parkinson's disease (PD) is a progressive, age-related neurodegenerative disease and is pathologically characterized by the loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc). Overwhelming evidence indicates that mitophagic defects are a central factor contributing to PD pathophysiology (Moon & Paek, 2015; Wright, 2022; Winklhofer & Haass, 2010). PD is estimated to affect approximately ten million people worldwide. Most cases are considered sporadic in nature, with only a small fraction attributed to genetic factors, raising questions about the existence of a unified genetic cause of this disease (Liu et al., 2019). Nonetheless, given its significant global prevalence in the aging population, clinical genome-wide association studies (GWAS) have identified a collection of genetic loci that implicate many genes in the modulation of mitochondrial functions and are known to result in clinical manifestations of the disease (Billingsley et al., 2019). Without an etiological consensus, the disease is considered multifactorial in nature with idiosyncratic presentations. Hence, current genetic approaches have shown little success in predicting disease onset and severity.

Moreover, recent genome-wide studies have identified additional risk loci in non-coding regions (Yao et al., 2021), suggesting limited use of traditional exome sequencing-based GWAS datasets. As a good starting point to study the genetic components of PD, it is widely believed that the same unified class of mitochondrial genes and their proximal cis-regulatory regions are implicated in PD progression, exerting key survival signals in neurons within the human SNpc. Although the mechanism underlying PD pathogenesis remains unclear, mitochondrial dysfunction is considered a vital contributor

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(Moon & Paek, 2015). Natural turnover of damaged mitochondria through mitophagy involves the selective priming of these organelles by ubiquitin labeling (Lavie et al., 2018). A range of genes and loci associated with PD phenotypes in a Mendelian fashion have been named as "*PARK*" genes. Among these genes, *PARK2*/Parkin (Okarmus et al., 2020; Song et al., 2016) and *PARK6*/PINK1 (Buneeva & Medvedev, 2022) are deemed major players in lysosomal degradation of ubiquitin-labeled organelles, suggesting a tight relationship between organelle turnover and disease occurrence.

PTEN-induced kinase 1 (PINK1), a serine/threonine ubiquitin kinase, acts in coordination with Parkin, an E3 ubiquitin ligase, to label damaged mitochondria for degradation via fusion with lysosomes, forming autophagosomes. (Quinn et al., 2020). This specific form of lysosomal autophagy involves the ubiquitination of mitochondrial proteins as the signaling cue for organelle degradation, thus named "mitophagy". Importantly, PINK1 involvement was initially established in the first linkage analysis of pedigrees with early-onset autosomal recessive PD, with *PINK1* mutations found to account for 4%–9% of cases with early onset PD in the Asian population, much higher than that in the Caucasian population (Bonifati et al., 2005).

Despite the identification of many susceptibility loci, a unified molecular mechanism leading to PD has not yet been proposed. Almost all subtypes of PD patients fall victim to the classical motor symptoms (rigidity, bradykinesia, postural instability, and tremor) (Magrinelli et al., 2016). Three long-recognized neuropathological signs of PD include the presence of α-synuclein-positive neuronal inclusions (also known as Lewy bodies), gradual but significant loss of SNpc DAergic neurons that project to the striatum, and subsequent striatal dopamine (DA) depletion, which manifests in motor defect phenotypes (Dauer & Przedborski, 2003). The direct relationship between these pathological features and motor phenotypes has been confirmed by postmortem examination of PD patients, demonstrating the degeneration of up to 60% of their SNpc DAergic neurons, which govern the mesostriatal neural network for motor control (Fearnley & Lees, 1991).

To study the molecular mechanism for specific neuronal loss, *in vitro* studies suggest that activation of mitophagy is essential for cell homeostasis (Liu et al., 2019), with PINK1 acting as a sensor for damaged mitochondria with lowered membrane potential and activating Parkin for ubiquitin labeling to target organelle degradation (Matsuda et al., 2010; Narendra et al., 2010). As clinical genetic studies have already correlated the genetic status of *PINK1* and *Parkin* genes to the manifestation of all three neuropathological signs, *in situ* mitochondrial defects in the DAergic neurons are believed to be an important trigger of PD. Notably, a series of defective mechanisms, including disruption of mitochondrial electron transport chain (ETC) (particularly complex I), decreased mitochondrial membrane potential (depolarization), and impaired organelle turnover, can lead to the accumulation of faulty mitochondria, which triggers neuronal cytotoxicity (Ge et al., 2020). The close association of PD etiology with mitochondrial dysfunction has led to its classification as a "mitochondrial disease".

In this review, we discuss current progress in both the general and moonlighting functions of PINK1 and Parkin for protecting various types of cells from mitochondrial stress and inflammation. In addition to the emphasis on mitophagy, we explore the use of the global *Pink1* knockout (KO) rat as a self-sufficient model to study the implications of mitochondrial-mediated inflammation in neurodegenerative diseases, particularly addressing the proinflammatory aspects of PD. This spontaneous PD rat model can also be applied to pharmacological studies on a spectrum of novel PD therapeutics, particularly the class of inhibitors for overactive leucine-rich repeat kinase 2 (*LRRK2*), given the overt linkage of *PINK1* in reciprocal regulation over other genes.

Of course, we do not advocate for the complete replacement of conventional mouse transgenic models for PD, given the strong merits of coupling double KO transgenesis with DAergic neuron-selective chemicals. We argue that many current experimental approaches involve the layering of multiple triggers, either multiple cross-bred genetic defects or chemical inductions, to supplement the single mitophagic gene KO model. These models often involve the layering of functionally deficient complex I machinery of the ETC, resulting in acute deterioration without inflammation, which may undermine the multi-faceted mitochondrial sensing functions for cell fate determination by PINK1.

REVISITING THE ELUSIVE MOLECULAR ETIOLOGY OF PD

A unique functional feature of DAergic neurons is the maintenance of central-acting DA release, which is heavily dependent on their characteristic dual firing pattern, consisting of intertwined single spikes and bursts (Zhai et al., 2018). These mixed clusters of multiple action potentials, alternated by single action potentials, are essential for maintaining the appropriate DA tone directed toward the dorsal striatum for motor control. All cytotoxic insults that may lead to a rise in intracellular calcium levels block the sustenance in this tone, resulting in reduced DA release, which gradually prevents effective motor control (Zhai et al., 2018).

Small-conductance Ca^{2+}-activated K^{+} (SK) channels are widely expressed throughout the central nervous system (CNS) (Adelman et al., 2012). In general, action potentials trigger calcium influx by activating voltage-gated calcium channels, leading to the opening of SK channels. In many neurons, the firing frequency of these action potentials can be reduced by the resultant hyperpolarization (Faber & Sah, 2007). In addition, SK channels are located in mitochondrial-enriched fractions, thus preventing mitochondrial dysfunction and neuronal cell death (Dolga et al., 2013). Recent data have indicated that SK channels modulate electrophysiological properties of dopaminergic cells in the SNpc by regulating the frequency and precision of pacemaker spiking, while electrical imbalance can ultimately lead to alterations in the cell survival signaling pathways. A recent brain slice study reported DAergic neuronal loss *in vitro* based purely on local firing regularity (Farassat et al., 2019; Lee et al., 2020), whilst advances in the field are moving toward recapitulating altered single-cell action potentials (Kaku et al., 2020).

These studies all pinpoint the importance of membrane-based SK channels as *de facto* regulators of firing pattern homeostasis. The opening of these channels somehow becomes spontaneously random as the subject ages, wherein associated bursts lead to an accelerated DA tone. Eventually, these irregular tonic responses warrant a persistent surge in intracellular calcium (Zaichick et al., 2017). The increase in calcium may affect cell survival through mitochondrial-mediated programmed cell death (Gandhi et al., 2009) and/or

generating a cytosolic environment in favor of synuclein aggregation (Rcom-H'cheo-Gauthier et al., 2014). Because α-synuclein in the misfolded confirmation is highly insoluble, it blocks synaptic vesicle trafficking near presynaptic sites, thus disrupting DA release (Burré, 2015). These deposited α-synuclein aggregates contribute to the formation of Lewy bodies, a well-known hallmark of PD.

PINK1 is a mitochondrial serine/threonine-protein kinase that protects cells from stress-induced mitochondrial dysfunction. Studies regarding electrophysiological and animal behavioral tests have indicated that elimination of PINK1 enhances neural transmission from the presynaptic part of DAergic neurons (Creed et al., 2021). Specifically, PINK1 is necessary for altering the input and output of protocerebral posterior medial region 3 (PPM3) neurons in fruit flies, an analogous type of neuron to human SNpc DAergic neurons (Qiao & Mao, 2020). Firing properties of action potentials are altered in *Pink1* KO genotypes, evoking mild abnormal motor ability (Qiao & Mao, 2020). Functional DAergic neurons are believed to constantly fine-tune their regulation of neuronal firing, coined as "pacemaker activity", but such activity is disrupted by the loss of *Pink1* (Iyer et al., 2017), probably due to disrupted ionic mechanisms with reduced after-hyperpolarization evoked by the abrupt opening of SK channels.

Moreover, PINK1 exerts another layer of control over intracellular calcium levels by controlling ion efflux to the cytosol via the mitochondrial Na($^+$)/Ca($^{2+}$) exchanger, although the relevant molecular mechanism is unclear (Jung et al., 2020). It has been suggested that PINK1 may modulate mitochondrial Ca^{2+} homeostasis through the mitochondrial Na^+/Ca^{2+} exchanger (Gandhi et al., 2009). PINK1 deficiency results in mitochondrial Ca^{2+} overload, which stimulates reactive oxygen species (ROS) production and inhibits glucose transport to impair mitochondrial respiratory action (Gandhi et al., 2009). Recent research also identified the direct phosphorylation of the leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) by PINK1, leading to defects in mitochondrial bioenergetics, metabolic signaling, and sensitizations to neuronal cell death ((Huang et al., 2017). Interestingly, Akundi et al. (2011) suggested that loss of PINK1 enhances sensitivity to Ca^{2+}-induced mitochondrial permeability transition, which is responsible for increased cell death susceptibility and consequently to DAergic neuronal loss.

Other important channels that may affect Ca^{2+} levels, and therefore assist in defining DA tone, are adenosine triphosphate (ATP)-sensitive K^+ channels (KATP), which are activated by energy status (Choudhury et al., 2022). In addition to regulating calcium flux, these channels also modulate the synuclein-induced reduction of excitability in DAergic neurons (Choudhury et al., 2022). Although there is no direct connection between PINK1 and KATP channels, DAergic neurons, which have a highly branched dendritic arbor and require high energy demand, are often under heightened metabolic stress due to active mitochondrial phosphorylation machinery coupled to ROS generation. Intensive mitochondrial activity leads to organelle damage and turnover, matching the observation of relatively higher PINK1 activity in these DAergic neurons (Zorov et al., 2014). These relentless physiological oxidative insults can eventually accelerate tissue degeneration.

In addition to age-related changes in firing patterns, experimental studies modeling motor disease frequently involve the acute administration of toxins to the same mesostriatal DA neuronal network (González-Hernández et al., 2010). Damage is often created via one of several mechanisms, including abolishing spontaneous firing of neurons, decreasing regularity of action potential firing, and potentiating *N*-methyl-D-aspartate-type glutamate receptor (NMDAR)-dependent currents (Hisahara & Shimohama, 2011). These electrophysiological changes are possible when animals are subjected to artificially stimulated gamma-aminobutyric acid (GABA) release (Błaszczyk, 2016) or pharmacological gating of K_{ATP} channels (Herrik et al., 2010; Schiemann et al., 2012). The high tendency of insults to hyperpolarize the membrane potential eventual leads to a persistent increase in intracellular calcium levels, which promotes the slow-onset of PD phenotypes.

MITOPHAGY AND NATURAL PROTEIN FUNCTION OF PINK1 AND PARKIN

Mitophagy can be described as a general cellular function for "the elimination of dysfunctional or superfluous mitochondria via cellular autophagy" (Wang & Klionsky, 2011). This process is compromised in PD patients and models and is associated with accelerated neurodegeneration (Clark et al., 2021; Malpartida et al., 2021; Fivenson et al., 2017). PINK1/Parkin coordination is a major step in the regulation of this dynamic mitochondrial quality control mechanism (Ge et al., 2020). Along this organelle-specific lysosomal process, ubiquitin-labeled mitochondrial fragments are further subjected to proteasomal degradation for material recycling (Jin & Youle, 2012; Vincow et al., 2013). This specific organelle-directed feature distinguishes mitophagy from other forms of autophagy, whereby the whole damaged organelle is loaded for elimination.

In humans, inherited or *de novo* mutations in both *Parkin* and *PINK1* that predispose an individual to early-onset PD are sufficient to express the full spectrum of micro pathologies (Brooks et al., 2009; Zhang et al., 2010). *PINK1* and *Parkin* mutations are responsible for more than 50% of autosomal recessive juvenile parkinsonism cases (Bonifati et al., 2002). Interesting, due to their physiologically compelled actions, mutations in *PINK1* and *Parkin* pairs may either complement each other or interact with other somatic mutations in the genome to develop more severe PD phenotypes with varying disease outcome (Creed & Goldberg, 2020; Kitada et al., 2009; Moisoi et al., 2014). Impaired mitochondrial respiration is observed in the striatum but not in the cerebral cortex, suggesting specificity of this defect for dopaminergic circuitry (Yang & Lu, 2009). *Parkin* KO mice exhibit an increase in extracellular DA concentration in the striatum, with a reduction in synaptic excitability in spiny neurons (Goldberg et al., 2003). Microscopically, abnormally damaged mitochondria show no accumulation when investigating cell physiology in these models. For example, *Parkin* and *Pacrg* double KO mice show no gross abnormalities of the dopaminergic system in the SNpc and no loss of neurons (Stephenson et al., 2018).

As a mitochondrial-targeted kinase, PINK1 primes itself in the proximity of the outer mitochondrial membrane (OMM), which is reinforced by a plethora of other kinase substrates in the cytosol to participate in the organelle degradation cascade (Narendra et al., 2010). Under healthy circumstances, PINK1 is subjected to proteasomal degradation following continuous translocation across the OMM via translocase of the outer

membrane (Deas et al., 2011; Greene et al., 2012). When the maintenance of mitochondrial membrane potential is disrupted, import of PINK1 into the mitochondrial matrix is prevented. Consequently, the protein kinase accumulates on the OMM, where it facilitates the phosphorylation of nearby proteins and the polymerization of ubiquitin (Ub) chains, which, in turn, activates E3 ubiquitin ligase Parkin at serine 65. The phosphorylated ubiquitin signal further triggers the autophagy receptor to initiate autophagosome formation (Kazlauskaite et al., 2014; Koyano et al., 2014) (Figure 1).

Parkin is a cytosolic Ring-between-Ring E3 ligase that uses a hybrid mechanism of ubiquitin transfer. PINK1 acts upstream of Parkin and is required for Parkin activation and recruitment to depolarize mitochondria. PINK1 also directly phosphorylates the key substrate known as GTPase mitofusin (Mfn), which serves as a direct linkage between PINK1 and Parkin (Gegg & Schapira, 2011). A related phosphorylation event also occurs on another mitofusin, Mfn2, which induces itself to act as a receptor for docking and recruiting Parkin in a feedforward manner. Cytosolic Parkin is then tethered to mitochondria and coats the organelle for autophagy receptor attachment (Chen & Dorn II, 2013). As the same feedforward cycle also accelerates degradation of ubiquitin-labeled Mfn1/2, these mitochondrial fusion proteins no longer become active and the mitochondrial network shifts toward fission instead, which is necessary to separate damaged components for degradation (Chen & Dorn II, 2013).

The shuttling transport of mitochondria is mediated by cargo-transport proteins belonging to the cytoskeleton machinery (Kruppa & Buss, 2021; Sugiura et al., 2014). Notably, the OMM protein Miro safeguards organelle trafficking by recruiting the adapter protein Milton, which loads mitochondria in the form of cargo onto the kinesin-1 heavy chain (Glater et al., 2006). After Miro is labeled for degradation by PINK1/Parkin coordination, mitochondrial motility is arrested. The Mfn family of proteins may physically interact with Miro, indicating coordinated control of all different mitochondrial dynamics by activation of mitophagy (Fatiga et al., 2021). Furthermore, through continuous fission and fusion, concatenated mitochondria establish a network that enables organelle self-renewal to maintain energy status in almost every nucleated cell (Youle & Van Der Bliek, 2012). Of note, the PINK1/Parkin pathway also promotes mitochondrial fusion in mammalian cells, where knockdown of PINK1 leads to elongated, interconnected mitochondria, while overexpression of PINK1 yields fragmented mitochondria (Yu et al., 2011), indicating that PINK1 plays a key role in regulating mitochondrial dynamics, including controlling constant size variation and interacting with the ubiquitin-directed lysosomal degradation pathway.

PINK1 is also instrumental in ETC functionality since fibroblasts from patients harboring *PINK1* mutations reveal diminished complex I activity and oxidative stress levels (Hoepken et al., 2007; Piccoli et al., 2008). The linkage to complex I dysfunction may be associated with the loss of phosphorylation at serine residue-250 within the subunit NdufA10 (Morais et al., 2014). Loss of Parkin also leads to deliberate activation of mitochondrial dysfunction (Pickrell & Youle, 2015). Interestingly, specific *Parkin* KO in mice SNpc alone is sufficient to induce progressive degradation of SNpc DAergic neurons, resulting in reciprocal up-regulation of the downstream proteins aminoacyl tRNA synthase complex-interacting multifunctional protein 2 (AIMP2) and parkin interacting substrate (PARIS) (Dawson & Dawson, 2014). Importantly, PARIS modulates nuclear transcription of mitochondrial proteins along the ETC, again reinstating defective mitochondrial energy flux as a prelude to DAergic neuronal loss (Shin et al., 2011). Furthermore, voltage-dependent anion channels (VDAC1) are a target for Parkin-mediated Lys 27 polyubiquitylation during mitophagy in neuroblastoma SH-SY5Y cells as a direct linkage for the gene to govern action potential changes (Geisler et al., 2010).

Other research has highlighted an indirect association between mitochondrial biogenesis and PARIS degradation, as

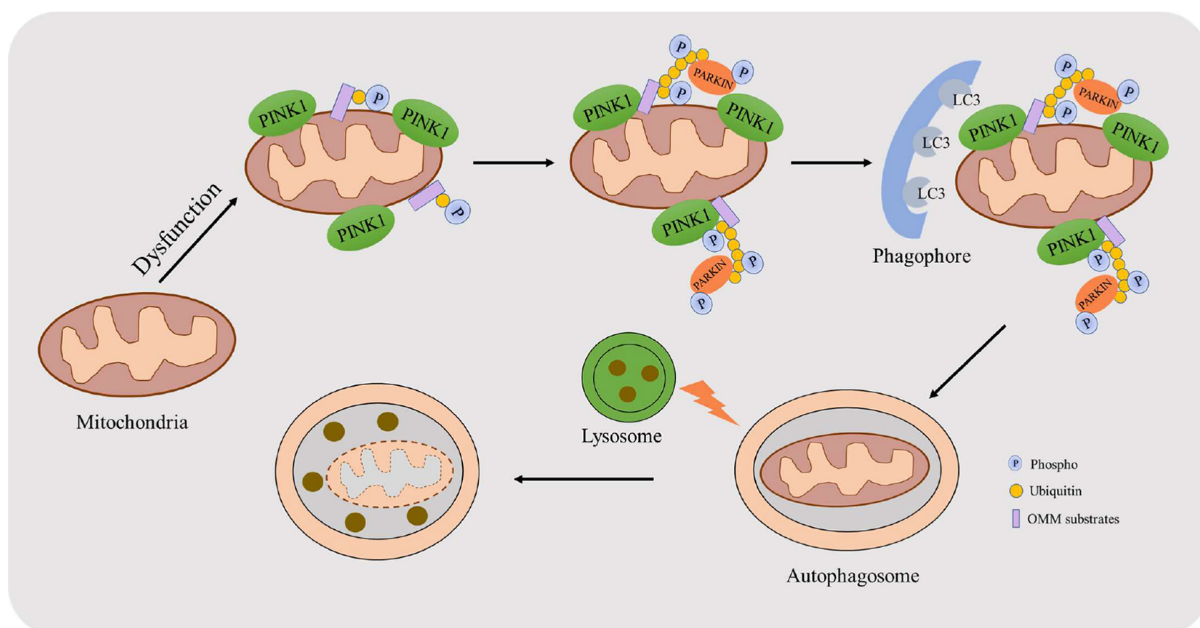

Figure 1 Overview of PINK1/Parkin-mediated mitochondrial quality control

When mitochondria undergo oxidative stress, genetic mutation, or functional dysfunction, PINK1 accumulates on the OMM. Once activated, PINK1 phosphorylates ubiquitinated substrates on the OMM and then recruits Parkin to active its E3 ubiquitin ligase. At the same time, the phosphor-ubiquitin recruits autophagy receptors to initiate autophagosome formation, leading to degradation of damaged mitochondria.

the latter process is influenced by mitochondrial activity and impacts the levels of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which serves as the primary regulator of biogenesis (Castillo-Quan, 2011). PGC-1α interacts with mitochondrial transcription factor A (TFAM) to prepare mitochondrial DNA (mtDNA) for redox insults, with the *Parkin* gene providing an additional layer of protection for organelle integrity (Nanjaiah & Vallikannan, 2019). Both Parkin and PINK1 respond to mild oxidative stress by signaling for mitochondrial-derived vesicle (MDV) formation destined for degradation, thus favoring repair of mitochondria over replacement (McLelland et al., 2014). Thus, the loss in any member within this gene pair can promote cellular aging.

PINK1 is considered the dominant regulator of mitophagy, with Parkin sometimes described as "dispensable". For instance, in mitoPARK mice with conditional TFAM deletion in DAergic neurons, Parkin is not recruited even upon overexpression (Ekstrand et al., 2007). When Parkin is silenced, *Drp1*-KO neurons *in vitro* undergo mitophagy with significant mitochondrial ubiquitination (Kageyama et al., 2014). Thus, Parkin exhibits a more subtle and specific function than PINK1 in promoting neuronal health by enhancing ubiquitination of toxic substrates for indirect regulation of mitochondrial quality by triggering mitophagy and vesicular transport (Borsche et al., 2021). While PINK1 exhibits other ubiquitous functions, it primarily serves as a sensor for mitochondrial damage under multiple stress insults that affect mitochondrial dynamics and turnover. However, in addition to its role as a sensor gene, PINK1 demonstrates novel moonlighting functions that control mtDNA integrity, as supported in several studies discussed below.

MOONLIGHTING FUNCTION OF PINK1 AND PARKIN IN INFLAMMATION

As well as its indispensable role in mitochondrial turnover, mitophagy is also involved in inter-organelle communication based on, at present, poorly understood machinery. In collaboration with the endoplasmic reticulum, mitochondria may also serve as a platform for RNA and DNA sensing. This "mitosensor" property plays a pivotal role in the general antiviral innate immune signaling strategy, which is highly evolutionarily conserved to enable adaptation to changes in cellular inflammation (West & Shadel, 2017).

Inflammation associated with mitochondrial damage and the subsequent mtDNA release may also serve as biomarkers of PINK1/Parkin-linked PD state and progression (Borsche et al., 2020). As discussed, *PINK1* and *Parkin* mutations can cause early onset of PD (Brooks et al., 2009; Kitada et al., 1998). Individuals harboring biallelic *PINK1*/*Parkin* mutations show increased levels of interleukin 6 (IL-6) and circulating cell-free mtDNA compared to idiopathic PD (Borsche et al., 2020). Primary human blood-derived macrophages obtained from PD patients with *Parkin* mutations exhibit a high level of NLR family pyrin domain containing monoallelic and biallelic 3 (NLRP3) and IL-1β when stimulated with lipopolysaccharide (LPS) or LPS-ATP (Mouton-Liger et al., 2018). In addition, elevated serum levels of proinflammatory cytokines, such as IL-6 and IL-1β, are observed in humans with monoallelic or biallelic *Parkin* mutations, but not in control serum (Sliter et al., 2018).

Sporadic PD is usually characterized by DAergic neurons with accumulated mtDNA deletions (Bender et al., 2006; Kraytsberg et al., 2006). Animal models harboring the mtDNA polymerase (*Polg*) mutation show increased nigrostriatal degeneration (Reeve et al., 2013). *Polg* mutant mice bearing the D257A mutation for exonuclease activity exhibit defects in the correction of mtDNA replication. These mtDNA mutations can give rise to multiple characteristics of premature aging, such as hair loss, weight loss, kyphosis, and organ damage (Moore et al., 2020). Compromised *Polg* function is implicated in certain mitochondrial-associated disorders, including PD (Hsieh et al., 2019; Orsucci et al., 2011). Another link between mtDNA and age-related diseases is the close proximity to sites of active ETC, a major source of cellular pro-senescent ROS (Nissanka & Moraes, 2018). When subjected to oxidative stress, mtDNA can serve as a potential danger signal, leaking out of the organelle due to the dissipation of mitochondrial membrane potential. In addition, mtDNA can also accumulate passive mutations under such insults. Given the essential role of mtDNA-encoded components in ETC, these mutations can lead to critical mitochondrial dysfunction. Furthermore, they may exacerbate the development of age-related diseases, such as cancer, diabetes, and aging (De Gaetano et al., 2021; Riley & Tait, 2020).

Previous studies have linked dysregulated complex I/ETC to oxidative DNA damage and programmed cell death (Liguori et al., 2018). However, recent findings suggest that PINK1 also regulates the proinflammatory mitosensor pathway via its interactions with cyclic guanosine monophosphate-adenosine monophosphate (GMP-AMP) synthase (cGAS)-stimulator of interferon genes (STING) (Chen et al., 2016). The activation of STING occurs when cGAS binds to cytosolic double-stranded DNA (dsDNA), including mtDNA, and result in STING-mediated inflammation due to an accumulation of mtDNA mutations in mutated mice. These results indicate that mtDNA is a crucial inflammatory signal in the absence of Parkin (Chen et al., 2016; Sliter et al., 2018). While previous research has suggested that this proinflammatory mitosensor pathway signals a non-canonical form of type I interferon (IFN) activity, a recent report identified its involvement in mitophagy crosstalk via PINK1 (Zhong et al., 2022). Under inflammation without autophagy, accumulated mtDNA in the cytosol may trigger increasing production of multiple inflammatory cytokines, including tumor necrosis factor (TNF/TNF-α) and IL-6, through activation of the nuclear factor kappa B (NF-κB) pathway via Toll-like receptor 9 (TLR9) (De Gaetano et al., 2021).

Similar to dsDNA fragments in the cytosol, leaked mtDNA binds to the cytosolic DNA sensor cGAS, which, in turn, promotes TMEM173/STING-dependent type I IFN induction by synthesizing cyclic GMP-AMP (Riley & Tait, 2020). PINK1/Parkin-dependent mitophagy also limits IFN production via the same STING-mediated DNA-sensing pathway following activation of mitochondrial stress. Sliter et al. (2018) demonstrated a critical connection between mitochondrial stress and inflammation in the context of PD via activation of cGAS-STING and other immune receptors, such as TLR9 or Nlrp3 inflammasomes. This proinflammatory pathway also signals DAergic neurons to undergo programmed cell death in PD, notably through sensing mtDNA changes. Compromised *Pink1*$^{-/-}$ and *Parkin*$^{-/-}$ mice show higher levels of type I IFN in their serum after performing exhaustive exercise, which also induces acute stress in mitochondria and serum cytokine concentrations in *Pink1* KO mice, but not wild-type mice (Sliter et al., 2018). Loss of Parkin in human subjects can also evoke an increase in circulating inflammatory cytokines as a prelude

to symptomatic neurodegeneration (Magnusen et al., 2021). As a component of a reciprocal feedback relationship, cellular stress can induce mtDNA release, which, in turn, triggers TMEM173-dependent proinflammatory IL-6 production, especially when mitophagy triggered by PINK1/Parkin activation is abolished (Li et al., 2021a).

Nevertheless, one should not disregard the established link between mitochondrial dysfunction and chronic inflammation, where PINK1 deficiency disrupts the feedback loop necessary for homeostasis under inflammatory conditions. Loss of mitochondrial membrane potential indirectly triggers inflammation via the NLRP3 inflammasome, while PINK1 acts as the *de facto* sensor for such changes in membrane potential, as discussed above (Zhou et al., 2011). Moreover, overexpression of Parkin appears to enhance mitochondrial transcription and inhibits mitochondrial damage through interaction with TFAM, contributing to the integrity of the mitochondrial genome (Rothfuss et al., 2009). Thus, activation of mitophagy in response to mtDNA damage is another mechanism by which PINK1/Parkin prevent overload and eliminate inflammatory stress. This crosstalk is important in preventing DAergic neuronal loss and associated motor defects in aged mice with increased mtDNA damage.

In conclusion, *PINK1* and *PARKIN* not only exhibit interconnected activity within the unified cascade of mitophagy, but also demonstrate individual connections with proinflammatory signals, expanding our knowledge of their function in organelle renewal. They are also involved in crosstalk with cGAS and NLRP3 signaling for stress-induced mitophagy, making them a crucial sensor hub for mitochondrial stress and inflammation (Figure 2). Proper functioning of these genes can protect highly energetic cells, such as DAergic neurons, from inflammation-induced cytotoxicity. To date, however, research on similar moonlighting functions of PINK1 in the context of DAergic neurons is lacking. We suggest that *Pink1/Parkin* KO serve as good models for uncovering the uncharted facets of PD etiology and revealing the pathophysiological actions of age-related mtDNA damage.

INTERACTION BETWEEN LRRK2 AND PINK1 IN PD PATHOGENESIS

PD is primarily regarded as a sporadic disease; however, approximately 10% of cases have a familial origin (Liu et al., 2019; Sellbach et al., 2006). Mutations in *PARK8/LRRK2* are an important autosomal dominant trait, particularly the G2019S substitution variant common in Caucasians (Squillaro et al., 2007). LRRK2, which encodes a large multidomain protein of the ROCO family, combining both kinase and GTPase activities, is considered a genetic contributor to PD (Berwick et al., 2019; Klein et al., 2009). LRRK2 has also been identified as an upstream kinase that phosphorylates a subset of Rab GTPases for membrane trafficking to prevent lysosomal overload (Steger et al., 2017).

The LRRK2 protein participates in cellular vesicle trafficking, controlling microtubule dynamics and autophagy (Godena et al., 2014; Orenstein et al., 2013; Shin et al., 2008), as well as in inflammation signaling (Barrett et al., 2008; Gardet et al., 2010; Kim et al., 2012). In particular, LRRK2 is involved in the regulation of immune cell responses in the brain and is implicated in microglial involvement in late-onset PD. Transgenic mice with the G2019S mutation in *LRRK2* show reduced neuronal excitability as early as eight months of age with reduced firing (Chou et al., 2014). The *LRRK2* R1441C

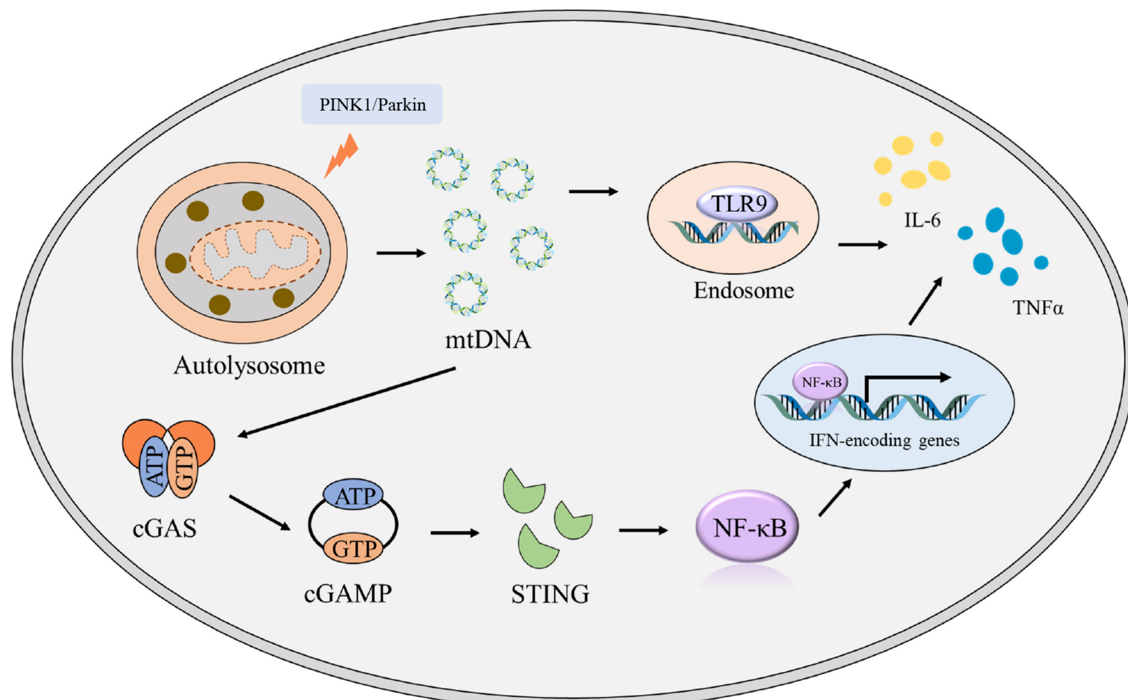

Figure 2 PINK1/Parkin-mediated mitophagy generates mtDNA to activate different proinflammatory pathways, including cGAS-STING and TLRs

Accumulated mtDNA mutations or mitochondrial stress result in mtDNA release into the cytosol. Undegraded mtDNA interacts with cytosolic DNA sensor cGAS to promote a conformational change from cGAS to cGAMP, which activates STING signaling. Activation of STING triggers the production of type I IFN and NF-κB signals, leading to activation of proinflammatory genes. Conversely, mtDNA associates with TLR9 to promote endosomal processing and signal transduction to induce cell TNFα genes and release of proinflammatory cytokines (IL-6, TNFα).

mutation causes reduced neuronal firing and bursting in SNpc DAergic neurons (Tong et al., 2009). Correspondingly, G2019S substitution in cell models disturbs mitochondrial function by affecting membrane potential (Kim et al., 2012), ATP generation (Kim et al., 2012), and modulation of oxidative stress (Grünewald et al., 2019), key molecular features that largely overlap with the routine sensor functions of PINK1. Genetic screening of a cynomolgus monkey with spontaneous PD identified missense mutations in *LRRK2* and *ATP13A2* (Li et al., 2021b). This PD monkey also exhibited a sensitive response to classic PD treatment drugs, suggesting that genetically modified monkeys with *LRRK2* mutations may be a feasible model for novel drug discovery in PD preclinical studies (Li et al., 2021d).

In addition to PINK1 and Parkin, LRRK2 also acts as an important player in mitophagy. Hyperactivity of the *LRRK2* genetic variant dictates cellular mitophagy (Bonello et al., 2019; Wauters et al., 2020; Yakhine-Diop et al., 2022). Fibroblasts from PD patients bearing the *LRRK2* G2019S mutation show obvious loss of mitochondrial membrane potential, with increased autophagic flux and markers (Korecka et al., 2019). The gene function of *LRRK2* includes modulation of mitochondrial dynamics through its kinase activity, which promotes the tethering of Drp1 to mitochondria and affects fission through phosphorylation cascades (Ho et al., 2018). As Drp1-dependent mitochondrial fission is crucial for segregating damaged parts from healthy network clusters in mitochondria, LRRK2 activity is considered essential for classical mitophagy (Ho et al., 2018; Wang et al., 2012). Furthermore, LRRK2 and its kinase activity attenuates common PINK1/Parkin-dependent mitophagy by disrupting interactions between Parkin and Drp1 in the early stage of mitophagy (Ho et al., 2018; Buhlman et al., 2014).

Spontaneous LRRK2 activation is also found in both rotenone-induced and α-synuclein-overexpressed rat models of PD (Di Maio et al., 2018). The genetic status of *LRRK2* affects PINK1-dependent stress-induced mitophagy and mitochondrial network aggregation and clearance through a collection of protein-protein interactions and kinase substrate activity (Singh & Ganley, 2021). Interestingly, *in vitro* study further suggests an antagonistic effect of PINK1 and LRRK2 through independent LRRK2 phosphorylation of Rab8a at serine-111 by PINK1 and threonine-72 (Singh et al., 2021). Related LRRK2 kinase substrate RAB10 also naturally binds to optineurin to promote its accumulation in depolarized mitochondria in a PINK1- and Parkin-dependent manner (Vieweg et al., 2020).

LRRK2 is gaining prominence for its regulation of mitochondrial homeostasis and participation in basal mitophagy (Singh & Ganley, 2021), which is reported to be independent of the genetic status of PINK1 (McWilliams et al., 2018). Using mitophagy (mito-QC) reporter mice, transgenic mice with a pH-sensitive fluorescent mitochondrial signal, McWilliams et al. (2016) found that pathogenic G2019S *LRRK2* mutation results in increased kinase activity, with reduced basal mitophagy in cells and tissues of clinical relevance, including midbrain DA neurons and microglia cells. With further breeding of these mito-QC mice with *Pink1* KO mice, loss of *Pink1* did not influence basal mitophagy, especially in tissues of high metabolic demand, indicating that basal mitophagy occurs independently of *Pink1* (McWilliams et al., 2018). Other research in *Drosophila* has also demonstrated that loss of *Pink1* and *Parkin* does not affect basal mitophagy (Lee et al., 2018). Furthermore, studies on human and monkey brains have revealed that PINK1 causes neurodegeneration without affecting mitochondrial protein expression and morphology, indicating that PINK1 kinase activity rather than mitochondrial function is essential for neuronal survival, with kinase dysfunction potentially contributing to the pathogenesis of PD (Yang et al., 2022).

In terms of neuroinflammation, LRRK2 may also interact with PINK1 in the moonlighting aspect. Immune cells, such as monocytes, neutrophils, and dendritic cells, exhibit relatively higher expression of LRRK2 (Cook et al., 2017; Russo et al., 2022; Wallings & Tansey, 2019). As previously mentioned, basal mitophagy is relatively active in microglial cells, which act as the first barrier of innate immune system in the CNS. Inhibiting LRRK2 activity in microglia reduces the level of proinflammatory cytokines, such as IL-6, IL-1β, and TNFα (Moehle et al., 2012). In addition, stimulation of TLRs results in phosphorylation, dimerization, and membrane translocation of LRRK2, reinforcing its function in immune regulation (Moehle et al., 2012). In parallel, elevated mtDNA deletions have been reported in *LRRK2* mutation carriers (Delcambre et al., 2020), suggesting a missing link between LRRK2 and "mitosensor" activity. *LRRK2* G2019S mutation causes an increase in microgliosis and astrogliosis in synuclein-overexpressed transgenic mice (Lin et al., 2009). Reciprocally, overexpression of α-synuclein in *LRRK2* G2019S transgenic rats results in neuroinflammation and neurodegeneration, as evidenced by microglial activation in the SNpc (Daher et al., 2015). Genetic ablation of *LRRK2* is also reported to protect against dopaminergic neurodegeneration induced by LPS, as well as against α-synuclein-induced neuroinflammation (Daher et al., 2014). Certain animal models may be useful for assessing the pharmacokinetics and structure-activity relationships (SARs) of this novel class of potent drugs, particularly in terms of kinase selectivity, blood-brain barrier penetration, and body distribution.

As mitophagic defects in patient cells with *LRRK2* mutations can be rescued by specific knockdown or direct kinase inhibition (Wauters et al., 2020), LRRK2 kinase inhibition may be a potential therapeutic target for correcting mitophagic defects, even in idiopathic PD (Bonello et al., 2019). In a recent study, Li et al. (2022b) showed that application of the LRRK2 inhibitor PF-06447475 significantly reduces neuronal damage in rat models with or without G2019S mutation. Another pharmacological effect of the LRRK2 inhibitor is its role in modulating the protein kinase A (PKA) signaling pathway. Activation of PKA can prevent neurite shortening caused by mutated LRRK2, as evidenced by the suppression of autophagy through phosphorylating microtubule-associated protein light chain 3 (Dagda & Das Banerjee, 2015). However, on-target adverse effects in other organs have hindered overall progress in studies on LRRK2 inhibitors. For example, preclinical models have revealed a disappointing toxic profile for LRRK2 kinase inhibition due to undesirable on-target side-effects in peripheral tissues, such as the lung and kidney (Taymans & Greggio, 2016). Non-ATP-competitive LRRK2 kinase inhibitors may provide a different safety profile for the development of novel drugs (Azeggagh & Berwick, 2022). As more LRRK2 inhibitors are designed, more well-tolerated drug candidates may be developed for the treatment of PD (Estrada et al., 2012; Henderson et al., 2015).

CHEMICAL TREATMENT RECAPITULATES FACETS OF PD SYMPTOMS

In addition to electrophysiological modulations, catecholaminergic neurons are vulnerable to oxidative stress and mitochondrial damage due to the necessary monoamine oxidase (MAO) action during neurotransmitter synthesis, which generates ROS (Sawada et al., 2013). This enhanced oxidative environment also reduces the activity of complex I of the ETC in PD patients (Keeney et al., 2006). The highly oxidative environment within the organelle matrix can also affect the mitochondrial genome, as a prelude to subsequent mutations (Nakabeppu et al., 2007).

Participating in a similar metabolic pathway as catecholamine DA, 6-hydroxydopamine (6-OHDA) is commonly adopted to prompt acute SNpc DAergic neuronal loss by producing toxic by-products when directly injected to various central sites (Simola et al., 2007; Tanguay et al., 2021). Nonetheless, aside from high specificity toward DAergic neurons, the toxicological effects of 6-OHDA are largely idiosyncratic. Indeed, the extent of damage is highly dependent on the dosage and site of toxin application across various animal models. Electrophysiological recordings show a general permanent effect for a toxin dosage bolus (Qu et al., 2014), as further characterized by Ca^{2+} imaging (Trevathan et al., 2021). The altered electrophysiology phenocopies PD pathologies, with a marked reduction in spontaneous firing activity and regularity shifts from steady pacemaking activity to irregular bursting events after damage-enhanced Ca^{2+} influx (Qu et al., 2014).

Another chemical-induced PD model is based on the discovery of toxin-induced motor defects in heroin drug addicts dosed with synthetic side-product 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) (Langston et al., 1983). MPTP is selective to DAergic neurons and is commonly used as an experimental tool due to its high lipophilicity, which enables it to cross the blood-brain barrier, where it is metabolized to MPP+ by monoamide oxidase B (Wiemerslage et al., 2013). While it is not entirely clear how these compounds spare the mesolimbic system while selectively impairing mesostriatal neurons involved in motor functions, their effects may be related to redox cycling activity exerted by repeated ETC decoupling that renders the mitochondrial complex I activity vulnerable (Meredith & Rademacher, 2011). Similarly, another noxious complex I toxin, named rotenone, can also elicit PD-like pathology in fruit flies, mice, and even higher mammals (Betarbet et al., 2000). As a lipophilic isoflavonoid, rotenone can efficiently inhibit complex I, leading to the general degeneration of multiple types of neurons. Administration of rotenone can also prompt the formation of synuclein-containing intracellular inclusions in nigral DAergic cells, reinforcing the importance of ETC integrity in PD progression (Rocha et al., 2022; Sherer et al., 2003).

The above neurotoxins are commonly used to generate PD animal models, which cause acute neurodegeneration and cell death in SNpc. However, these studies have shown little success in mice, with abrupt disruption in homeostatic mitochondrial turnover. Successful animal models for studying PD pharmacology, such as via L-DOPA-induced dyskinesia, have been made possible with the recruitment of 6-OHDA-lesioned rats (Cenci & Lundblad, 2007). The main drawback of all these neurotoxin models is that they only evoke acute phenotypes, rather than the gradual progressive motor defects observed in clinical PD.

Recently, successful PD models have been generated through genetic alterations that selectively target the mitochondrial complex I genes *ND4* and *ND6* via base-editing. These models induce modest but progressive changes based on heteroplasmic mt-specific gene modifications that accelerate mitochondrial turnover, resulting in better modeling of PD phenotypic changes (Bender et al., 2006; Buneeva et al., 2020). However, despite the remarkable specificity of these artificial models, most have failed to recapitulate the distinct features in PD, particularly the chronic inflammatory phenotypes. Although inflammatory changes have long been observed in the CNS of PD patients, the implications of inflammation as a root cause of progressive disease outcome are becoming clearer, particularly new theories regarding the involvement of microglial activation.

It is reasonable to say that none of the aforementioned models is suitable for studying PD etiology, with the vast majority failing to even recapitulate Lewy bodies. Interestingly, postmortem examinations of PD patients bearing *PINK*1 mutations exhibit Lewy body pathology (Samaranch et al., 2010), although this hallmark may not be an obvious feature in most familial forms of PD (Kumazawa et al., 2008). Therefore, *PINK1* KO genetic models may be more suitable for studying the progressive deterioration of SNpc neurons.

GENE KO MOUSE MODELS DO NOT MANIFEST OVERT PD PHENOTYPES

Several animal models in which one of the mitophagy genes has been knocked out exhibit early onset of PD symptoms. Due to the loss-of-function mechanism associated with *PINK1* mutations in PD, various research groups have attempted to generate *PINK1* KO models in different animals. Taking the simplest genetic model with a catecholaminergic network, fruit flies (*Drosophila melanogaster*) demonstrate defects in mitochondrial homeostasis and strong oxidative stress when the orthologs of human *PINK1* and *Parkin* are knocked out (Lee et al., 2018; Zhou et al., 2011). CRISPR/Cas9 has been employed to target Parkin/DJ-1/PINK1 and generate a model of PD in Bama miniature pigs, which remained healthy and displayed normal behavior at the age of 10 months, with PD-related symptoms and pathological changes in the SNpc remaining unknown (Li et al., 2021c; Wang et al., 2016). In another study, PINK1 was found to promote mitochondrial fission in porcine preimplantation embryos, with knockdown of PINK1 resulting in mitochondrial dysfunction, oxidative stress, ATP deficiency, and increased autophagy and apoptosis (Niu et al., 2019).

As aging processes differ significantly among different animal genera, a shorter lifespan, as found in rodents, may not be synonymous with the gradual progression of neurodegeneration found in humans, which usually spans decades. Therefore, given their extended aging periods, PINK1 mutant monkeys may be a better surrogate model for investigating the unique functions of PINK1 in neuronal survival in primate brains. Several groups have attempted to create PD monkey models using CRISPR-mediated *PINK1* KO in fertilized monkey embryos (Li et al., 2021c; Sun et al., 2022; Yang et al., 2019a). For example, CRISPR-based deletion of *PINK1* in the prefrontal cortex and SNpc of rhesus macaques revealed its importance in physiology, resulting in concurrent deterioration of the cortex, striatum, and SNpc,

together with reported deaths in neonates (Yang et al., 2019b). Although *PINK1* knockdown monkeys exhibit increased lysosomal and phagocytic vacuoles, no mitochondrial abnormalities are observed (Sun et al., 2022). Compared to mid-aged monkeys, co-editing the *PINK1* and *DJ-1* genes in the SNpc of old-aged monkey results in all classic PD symptoms, accompanied by the pathological hallmarks of PD, such as nigral DAergic neuronal loss and α-synuclein accumulation (Li et al., 2021c). Chen et al. (2021) used single-strand nickases to target *PINK1* in monkeys, which did not produce PD symptoms. Moreover, most patients with *PINK1* mutations do not exhibit the severe phenotypes seen in *PINK1* KO transgenic monkeys, which cannot recapitulate synonymous *PINK1* mutation subtypes. Natural *PINK1* deletion in both human alleles has never been reported clinically. While these primate models demonstrate merits in their higher degree of similarity to humans over rodents, their application in large-scale field study is cost-preventive given the relatively low reproductive capability, lengthy period of sexual maturity, and husbandry and maintenance costs over a longer average lifespan (Li et al., 2022a).

Given their much shorter lifespans, mouse and rat *Pink1* KO models are still widely used for PD study. However, mouse models remain controversial as they cannot faithfully recapitulate the primary pathological characteristics of PD found in humans, such as DAergic neuronal loss or aggregation of α-synuclein (Kazlauskaite et al., 2015; Kumar et al., 2015). *Pink1* mice with targeted deletion of exons 4–7 show impaired corticostriatal long-term potentiation and depression, but no change in DA levels or degeneration of DAergic neurons (Kitada et al., 2007). Germline deletion of *Pink1* in mice significantly impairs mitochondrial functions, with decreased mitochondrial respiration activities but no gross changes in the ultrastructure or number of mitochondria (Gautier et al., 2008). *Pink1* KO mice with targeted deletion of exons 4–5 show no SNpc degeneration but display loss of DA in the striatum of aged mice, altered mitochondrial calcium storage, and aberrant innate immune response (Akundi et al., 2011). In other *Pink1* KO mice used to study PD, homozygous mutant descendants retain normal numbers of DAergic neurons and similar levels of striatal DA compared to wild type mice, indicating that even with null *Pink1*, there is no defect in the functionality of the motor network (Kitada et al., 2007). The characteristics of recently published *Pink1* KO mouse models are summarized in the context of neurodegeneration in Table 1.

Similar to *Parkin* KO mice, the absence of motor defects in *Pink1* KO mice does not necessarily indicate that the strain has no phenotype. In aged mice, loss of *Pink1* in the genome slowly manifests in enlarged and defective mitochondria in the striatum, coupled with loss of DA (Gautier et al., 2008). *Pink1* KO-induced mitochondrial defects are progressive, leading to a gradual decrease in cellular respiration in the cerebral cortex with age, and significant retracted dendrites in various neurons by 10 months old (Stauch et al., 2016).

The subtle mutation-led phenotypic changes observed in *Pink1* KO mice do not reflect the autosomal recessive changes seen in early onset human PD clinical studies. However, the dysregulation is marked by an age-dependent lower energy status due to reduced ATP generation, and aged mice with *Pink1* KO eventually exhibit a mildly reduced release of DA with subtle changes in striatal DA physiology (Kelm-Nelson et al., 2021). Studies have indicated that this reduction in energy state is due to dysregulated PKA (Das Banerjee et al., 2017). However, whether this global gene KO model provides a reference for the molecular etiology of PD remains uncertain due to contrasting reports on the roles of dysregulated PKA signaling in relation to PD (Dagda & Das Banerjee, 2015; Dagda et al., 2011; Wu et al., 2018).

As mentioned, *Parkin* KO mouse models do not develop significant motor phenotypes (Goldberg et al., 2003; Kitada et al., 2007; Perez & Palmiter, 2005), although several sporadic recessive mutations around the *Parkin* locus can disrupt mitophagy (Pickrell et al., 2015). *Parkin*-mutated neuronal cultures also do not manifest PD pathogenesis (Ashrafi et al., 2014; Bingol et al., 2014). The mismatch in genotype-phenotype concordance in these global KO transgenic mice makes it difficult to study gene function in PD, although there is a clear genetic linkage between both Parkin and PINK1 and early onset PD.

One way to fully utilize KO mouse models is by adding more mitochondrial stressors on top of the genetic perturbations to induce a neurodegenerative phenotype. Pickrell et al. (2015) recapitulated neurodegenerative phenotypes by crossing

Table 1 Pathological and behavioral characterization of *Pink1* KO mice

PINK1 KO	DAergic neurons	Mitochondria	Motor deficits	Other phenotypes	References
Deletion of exons 4–7	Unchanged DA numbers and levels of striatal DA/DA receptor	N/A	N/A	Striatal plasticity impairments; Increased excitatory transmission	Feligioni et al., 2016; Kitada et al., 2007
Germline deletion	N/A	Impaired mitochondrial respiration in striatum; Increased larger mitochondria	N/A	Reduced aconitase activity	Gautier et al., 2008
Deletion of exons 4–5	Loss of DA neurons in striatum; impaired DA release	Altered mitochondrial calcium storage	N/A	Aberrant innate immune response	Akundi et al., 2011
Deletion of exons 2–3	No DA neuronal neurodegeneration	Increased mitochondrial fragmentation	No motor deficits observed	Serotonergic neuronal loss	Glasl et al., 2012
G309D-PINK1 mutation	Deficiency of striatal DA, no neurodegeneration	Increased mitochondrial dysfunction with impaired mitochondrial fission	Reduction of locomotor activity	Absence of Lewy bodies in SNpc	Gispert et al., 2009
Deletion of exon 1	N/A	Fragmentation of mitochondria; Increased ROS level; Impaired mitochondrial ATP synthesis	N/A	Impaired calcium homeostasis	Oliveras-Salvá et al., 2014
Deletion of exons 2–3	Reduction of DA in SNpc with neurodegeneration	Increased mitochondrial stress	Impaired motor abilities	N/A	Moisoi et al., 2014

N/A: Not available. DAergic: Dopaminergic. DA: Dopamine. SNpc: Substantia nigra pars compacta.

Parkin$^{-/-}$ mice with "Mutator" mice, which exhibit accumulated dysfunctional mitochondria due to accelerated generation of mtDNA mutations. These mice were born at the expected Mendelian ratios and appeared normal at birth, except for significant body weight loss in mice lacking Parkin (Pickrell et al. 2015). Moreover, Parkin expression can tolerate an increase in heteroplasmic point mutation levels in the brain, resulting in overt PD pathogenicity with these mtDNA mutations (Ahier et al., 2021). The loss of Parkin prevents stress-induced mitophagy, leading to PD-like pathologies with mtDNA mutations (Ahier et al., 2021).

Other researchers have crossed *Parkin* KO mice with α-synuclein overexpression models to simulate chronic PD. While the α-synuclein-based *Snca*$^{+/+}$ mouse model is capable of instigating nigral DAergic neurons to alter their spontaneous firing activity and promote neuronal loss through gene overexpression alone, it typically does not result in inflammation (Janezic et al., 2013; Möller et al., 2022). Previous studies have shown that mutant α-synuclein overexpression induces mitophagy, and this phenotype is exacerbated under loss of *Parkin* (Chen et al., 2015), suggesting that synuclein pathology may be the upstream activator of mitophagy itself. Locomotor deficits are exacerbated when *Pink1/Snca* double KO animal models are supplemented with a mutated *Snca* A53T (Valek et al., 2021). Furthermore, altered ubiquitination of α-synuclein under such mutation increases the formation of pSer129-alpha-synuclein aggregates (Gispert et al., 2015), while MAP1B/ANK2 phosphorylation events are implicated in Parkinsonian neurodegeneration in mitophagy-defective animals (Auburger et al., 2019). Similarly, *Pink1* KO mice with targeted deletion of exon 1 manifest mass DAergic neuronal loss when given ectopically overexpressed α-synuclein (Oliveras-Salvá et al., 2014). However, these approaches remain empirical and rely heavily on complex transgenic models.

TRANSGENIC *PINK1* KO RAT MODELS FOR STUDYING PD

Compared to the subtle mouse models, *Pink1* KO rat models show nigral neurodegeneration with 50% dopaminergic cell loss, striatal DA and serotonin content increase, and motor defects, including uncoordinated motor movement with loss of balance and gait (Dave et al., 2014). The first *Pink1* KO rat model was created at Sage Labs (established under a collaboration with the Michael J. Fox Foundation) by transgenic gene disruption using zinc-finger nuclease (ZFN) technology (Dave et al., 2014). Their discovery of homozygous *Pink1* KO rats sufficient to demonstrate PD phenotypes of motor impairment and dopaminergic cell loss formed the basis of studies on PD molecular etiology in rats. The applications of these *Pink1* KO rats from Sage Labs are summarized in Table 2.

Although phenotypically normal at birth, *Pink1* KO rats demonstrate gradual diminishment in motor ability in terms of gait, coordination, and strength as early as five weeks of age (Dave et al., 2014). The homozygous *Pink1*-null rats manifest increased foot slips on the tapered balance beam, in contrast to mice bearing the same mutation, which show reduced spontaneous locomotor activity at six months of age in the cylinder test and time taken for pole climbing (Kelm-Nelson et al., 2018). At that same age, rats already manifest abnormal paw positioning with a short stride and lengthened traverse time along the beam, with whole population developing hindlimb dragging due to decreased hindlimb muscle strength at eight months of age (Grant et al., 2015). In another study, *Pink1* KO rats exhibited significant gait deficits at the age of five months, although this effect disappeared at eight months due to some unknown compensation mechanism (DeAngelo et al., 2022). Consistent with the results reported by Sage Lab, these KO rats show apparent loss of tyrosine hydroxylase (TH)-positive neurons in the SNpc without alteration in

Table 2 Pathological and behavioral characterization of *Pink1* KO rat model from Sage Labs

PINK1 KO rat model	DA neurons	Mitochondria	Motor deficits	Other phenotypes	References
Sage Labs	N/A	N/A	N/A	Abnormal α-Syn localization; Protease resistance and aggregation	Creed & Goldberg, 2019
	Reduced TH-immunoreactivity in locus coeruleus	N/A	Progressive vocalization and oromotor deficits	α-Syn aggregation	Grant et al., 2015
	N/A	N/A	N/A	USV acoustic deficit impaired communication	Pultorak et al., 2016
	N/A	Reduced expression of mitochondrial genes	Impaired motor behavior	Reduced glutathione and ATP; Elevated oxidative stress	Ferris et al., 2018
	N/A	Impaired cognitive and motor deficits	N/A	Changes in putative gray matter microarchitecture	Cai et al., 2019
	No differences in DA, DOPAC, or HVA in dorsal striatum	N/A	Behavioral deficits with reduced rearing frequency and locomotor activity	N/A	De Haas et al., 2019
	Degeneration of DA neurons	Diminished mitochondrial respiration and glycolysis	Early motor deficits	Psychological distress induced anxiety and Parkinsonian symptoms	Grigoruţă et al., 2020
	N/A	N/A	Reduced intensity of vocalizations; No limb sensorimotor deficits	Anhedonia and anxiety	Marquis et al., 2020
	N/A	Decreased Atp13a2 expression in periaqueductal gray	N/A	α-Syn aggregation; Reduced glutamate decarboxylase 1 level; Lysosomal dysfunction	Kelm-Nelson et al., 2016b

N/A: Not available. DA: Dopaminergic. SNpc: Substantia nigra pars compacta. α-Syn: Alpha-synuclein. DOPAC: 3,4-dihydroxyphenylacetic acid. HVA: Homovanillic acid.

acetyltransferase-positive cells, indicating that dopaminergic rather than cholinergic cell loss contributes to gait deficits in the *Pink1* KO model (DeAngelo et al., 2022).

Another characteristic shared between the rat model and PD patients is the propensity to develop defects in oromotor activities. Rats recapitulate certain features, including defective vocalization, licking, and biting, as early as two months old (Grant et al., 2015), compared to only a modest defect in ultrasonic vocalizations in mice at four to six months of age (Kelm-Nelson et al., 2018). Furthermore, altered muscle tone with decreasing rearing behavior and open-field mobility also appear from eight months onwards (Dave et al., 2014; Glass et al., 2019). Compared to wild-type counterparts, *Pink1* null rats have a larger body size and two- to three-fold higher striatal DA and serotonin release in the early months (Dave et al., 2014), with degeneration of DA neurons reported as earlier as 2.5 months (Grigoruţă et al., 2020). A general loss in TH-positive neurons in the SNpc also occurs in KO rats (Gispert et al., 2009; Villeneuve et al., 2016), although other studies have reported no significant degeneration in the SNpc at eight months of age based on non-stereological quantification (De Haas et al., 2019; Grant et al., 2015). In addition to DAergic neuron-associated symptoms, *Pink1* KO rats also possess non-motor and non-dopaminergic symptoms of PD, with a loss of smell, olfactory circuitry defects, and anhedonia, as revealed by functional magnetic resonance imaging (fMRI) and altered diffusion tensor imaging (DTI) across the CNS (Kelm-Nelson et al., 2021). Therefore, *Pink1* KO rat models readily recapitulate the non-motor symptoms that precede actual DAergic neuronal loss.

The effects of *PINK1* KO on striatal DA still remains somewhat controversial (Ren & Butterfield, 2021). Although DAergic neuronal loss in the SNpc is associated with PD, Dave et al. (2014) found that the level of DA increased two- to three-fold at eight months of age in *Pink1* KO rats, although the number of TH-positive neurons decreased in the SNpc. In contrast, Kelm-Nelson et al. (2021) reported a slight decrease at the same age, correlating with slower traversal of the tapered balance beam. Quantitative autoradiography demonstrated subtle changes in the density of DAergic receptor subtypes in the striatum, with KO rats exhibiting a 26% and 19% increase in the D2 and D3 receptors, respectively (Sun et al., 2013). In addition, *Pink1* KO rats exhibit significant gait deficits, with apparent loss of TH-positive cells rather than choline acetyltransferase-positive cells (DeAngelo et al., 2022), highlighting the variability within the models.

MOLECULAR FEATURES RECAPITULATING IN *PINK1* KO RAT BRAINS

Although the *PINK1* KO model spontaneously generates α-synuclein aggregates, the same model can be layered to higher synuclein deposition due to reduced mitophagic flux (Ren & Butterfield, 2021). Overexpression of α-synuclein can cause a reduction in spontaneous firing frequency in rats, altering the possibility of DA neuron to become excited (Dagra et al., 2021). Notably, α-synuclein containing aggregates in mutant rat brains appear as early as four months old (Creed & Goldberg, 2019). A steady decrease in the mRNA levels of *Atp13a2*, a lysosomal ATPase, can be found within the periaqueductal gray area containing densely aggregated synuclein complexes (Kelm-Nelson et al., 2016b). In parallel, a reduction in mitochondrial complex I expression can also be found in the striatum, suggesting the occurrence of mitochondrial dysfunction.

These *Pink1*-deficient rats exhibit a decrease in complex I-driven respiration, as well as an increase in complex II-mediated respiration, likely due to a compensation mechanism in the striatal nerve terminals (Stauch et al., 2016). These changes are followed by inadequate mitochondrial spare respiratory capacity and neuronal death (Yadava & Nicholls, 2007), allowing the study of molecular etiology and pharmacological testing, as well as the additional layering of genetic and chemical insults. In a *Pink1* KO rat model context, overexpression of the A53T mutant form of synuclein through adeno-associated virus (AAV) induction or intracranial injection of α-synuclein preformed fibrils (PFFs) can instigate higher endogenous α-synuclein aggregation (Gispert et al., 2015), with subsequent research demonstrating that accumulation of these PFFs in nigral DAergic neurons perturbs spontaneous firing activity in a bidirectional and time-dependent manner (Tozzi et al., 2021). Therefore, this animal model likely recapitulates PD features in a more comprehensive way compared to similar mouse models. The increase in α-synuclein aggregation in the *Pink1* KO context may be attributable to reduced mitophagic flux. As *Pink1* KO rats exhibit mitochondrial respiration followed by α-synuclein accumulation (although not in the form of Lewy bodies), the spontaneous nigral neuronal loss accompanied by α-synuclein aggregates make it an ideal model for restoring mitochondrial respiration to prevent other symptoms in PD (Creed & Goldberg, 2019).

Pharmacological treatments for PD typically involve drugs acting on DA pathways to compensate and restore neurotransmitter release levels or to sensitize the body response to reduced DA (Connolly & Lang, 2014; Smulders et al., 2016). In recent studies, levodopa has been administered to alleviate motor symptoms, resulting in a prominent restoration of gait movements but no restoration of lost hindlimb strength (Vazquez-Mayorga et al., 2022). Levodopa has also been administered in an attempt to restore oromotor activities and ameliorate vocalization in *Pink1*$^{-/-}$ rats by adjusting the dopaminergic network for behavioral change but showed no correction of vocal defects (Kelm-Nelson et al., 2016a), suggesting that vocalization deficits in PD may have an extra-DAergic origin. A recent study also demonstrated that *Pink1* KO rats do not exert DAergic neuronal loss in the SNpc unless co-injected with α-synuclein (Orr et al., 2017). Furthermore, the reintroduction of kinetin into KO rats with excessive α-synuclein does not significantly rescue the neurodegeneration deficits in the stratum, indicating different responses of PINK1 activators in rodents and clinical patients with *PINK1* mutation (Orr et al., 2017).

Other research groups have also studied PKA signaling in PD pathogenesis, with the application of forskolin as a potential drug candidate with anti-Parkinsonian effects (Vazquez-Mayorga et al., 2022). While the relationship between PKA signaling and PD pathogenesis remains somewhat obscure, results showed that forskolin treatment not only restores motor coordination but also improves hindlimb grip strength (Vazquez-Mayorga et al., 2022). Thus, the *Pink1* KO rat model is well-suited to study how specific PKA activation through forskolin administration in the absence of *Pink1* may contribute to its neuroprotective effects, with promising results.

CHALLENGE AND PERSPECTIVES OF *PINK1* KO MICE AND RATS

Apart from PD pathogenesis, *Pink1* KO mice have also been used as a model for investigating other gene functions relevant to innate immunity and cancer phenotypes, highlighting the versatility of the *Pink1* KO mouse model beyond the study of mitophagy alone (Akundi et al., 2011; Kang et al., 2019; Matheoud et al., 2016; Dai et al., 2021). Studies have shown that mice lacking either *Pink1* or *Parkin* do not exhibit substantial PD-relevant phenotypes in terms of inflammation (Goldberg et al., 2003; Kitada et al., 2007). However, acutely prepared brain slices from *Pink1* KO mice have demonstrated elevated levels of proinflammatory cytokines (Kim et al., 2013). Of note, while *Pink1* KO mice do not mimic the behavior and molecular changes observed in PD patients, they have received more attention in the field than *Pink1* KO rats, which share the same isogenetic defects.

As discussed, *Pink1* KO rat models demonstrate both motor and non-motor deficits, with slight loss of striatal DA levels at eight months of age, providing a link between the KO model and human PD (Dave et al., 2014). The age-related decrease in TH-positive neurons in the SNpc is consistent with early-stage Braak pathology (Dave et al., 2014). Most current PD studies have primarily focused on the signs/symptoms that match the clinical hallmarks of PD, often overlooking non-motor features such as vocalization, swallowing, and depression, which fall outside classical DAergic neuronal loss. A recent study found that *Pink1* KO rats demonstrate significant and progressive deficits in vocalization and swallowing (Grant et al., 2015), with abnormal nociceptive responses and ventilatory abnormalities (Glass et al., 2019, 2020). However, the *Pink1* KO rat model still has some limitations, such as controversial neurodegeneration, DA release, hindlimb issues, and less behavioral robustness than observed in the mouse model. Direct comparison of the rat versus mouse *Pink1* KO models is difficult, and perhaps unrealistic, as inherent species differences dictate behavioral deficits and model-specific tissue changes. Nevertheless, the rat model is noted for its ability to perform complex behaviors and learning and is well-established for presenting PD motor characteristics.

The experimental use of the rat and mouse models with the same genetic defect shows a clear dichotomy. While *Pink1* KO rats have larger brains, which facilitate easy collection of biopsy samples, other molecular tools, such as antibody-based assays and bulk sequencing techniques, are not as effective as those used in mice. It could be argued that the less comprehensive reference genomes available for rat species have hindered molecular research using the larger rodent. Indeed, the field of molecular studies has relied heavily on the smaller counterpart in various ways. For example, capture-based assays, such as exome-seq or methylation-seq, are currently unavailable for rats, despite their usefulness in studying epigenetic changes given the ease of collecting sufficient target cell DNA and RNA. Furthermore, while the mouse molecular mimicry model is commonly used, the rat PD-behavior mimicry model is not suitable for studying PD etiology. Thus, the *Pink1* KO rat model may be an ideal choice for studying basal mitophagy and related signaling. The rat model also has the potential to provide appropriate behavioral responses for pharmacological testing and pharmacokinetic evaluations of novel receptor target lead compounds, such as LRRK2 and GLP-1 antagonists, while maintaining safety and potency.

Another overlooked aspect regarding differences in the two models is their availability across labs. Currently, the *Pink1* KO rat is not widely bred worldwide, and reports on behavioral studies in this model are mostly produced by the Sage Lab. It is important to note that with the progression of CRISPR/Cas9 techniques, generating a new strain of *Pink1* KO rat is now easier compared to the previous ZFN/TALEN approach utilized over a decade ago. Moreover, there are no freedom-to-operate constraints in producing another *Pink1* KO rat strain. We hope to see increasing molecular and pharmacological studies once more rat strains are created. Indeed, regarding the development of AAV-directed neuronal labeling techniques, the rat is an ideal model animal for confirming optogenetic studies using a similar viral approach. As such, the skewed focus on behavioral (motor) activities in rat models is changing. With *Pink1* KO rats, it is possible to study brain-behavior relationships at any of these early stages.

CONCLUSIONS

We advocate for the use of the rat model as an effective tool in studying pharmacokinetics, drug effects, and possible side-effects of novel drug candidates for PD. By layering chemical or genetic insults, such as α-synuclein deposition or overexpression, the *Pink1* KO rat model can demonstrate well-rounded PD molecular pathophysiology. The *Pink1* KO rat also provides an excellent *in vivo* model for investigating the moonlighting functions of the innate immune response, exhibiting a robust feedback cycle that links immunological alterations, inflammation, and mitochondrial dysfunction, all of which contribute to the development of PD. Moreover, this autosomal recessively inherited Parkinsonism is a spontaneous disease that is embryonically viable

How can we expand the use of these rats in PD research? With recent studies revealing the crucial role of LRRK2 in basal mitophagic activity, the *PINK1* KO model can eliminate the idiosyncratic activation signals via stress-induced mitophagy and is thus an ideal *in vivo* model for studying background mitophagic flux. Therefore, we propose the adoption of this spontaneous PD model to study the pharmacological effects of non-toxic LRRK2 lead inhibitor candidates as a future therapeutic strategy for this motor disease.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.Z. conceptualized, wrote, and edited the manuscript. S.W.C. and K.K.M. assisted in the writing and critical review of the text. H.Z. provided financial support. W.Y.C. conceptualized, designed, edited, and provided financial support. All authors read and approved the final version of the manuscript.

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