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Corresponding Author: YOUNGSEOK LEE

Authors: Binod Aryal[1], YOUNGSEOK LEE[1,*]

Institution: [1]Bio and Fermentation Convergence Technology, Kookmin University,

Disease model organism for Parkinson Disease: *Drosophila melanogaster*

(Running title: Parkinson Disease: *Drosophila melanogaster*)

Binod Aryal[1] and Youngseok Lee[1]*

[1]Department of Bio and Fermentation Convergence Technology, Kookmin University, BK21 PLUS Project, Seoul 02707, Republic of Korea

*Correspondence should be addressed to:

Y.L. (Tel. no: +82.2.910.5734; email: ylee@koomin.ac.kr)

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Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by selective and progressive loss of dopaminergic neurons. Genetic and environmental risk factors are associated with this disease. The genetic factors are composed of approximately 20 genes, such as *SNCA, parkin,* PTEN-induced kinase1 (*pink1),* leucine-rich repeat kinase 2 (*LRRK2), ATP13A2, MAPT, VPS35,* and *DJ-1*, whereas the environmental factors consist of oxidative stress-induced toxins such as 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), rotenone, and paraquat. The analyses of their functions and mechanisms have provided important insights into the disease process, which has demonstrated that these factors cause oxidative damage and mitochondrial dysfunction. The most invaluable studies have been performed using disease model organisms, such as mice, fruit flies, and worms. Among them, *Drosophila melanogaster* has emerged as an excellent model organism to study both environmental and genetic factors and provide insights to the pathways relevant for PD pathogenesis, facilitating development of therapeutic strategies. In this review, we have focused on the fly model organism to summarize recent progress, including pathogenesis, neuroprotective compounds, and newer approaches.

Key words: Parkinson's disease, mitochondrial dysfunction, oxidative stress, environmental toxins, genetic factors

Introduction

Parkinson disease (PD) is the second-most common human neurodegenerative (ND) disorder after Alzheimer's disease. The pathological features involve slow degeneration of the dopaminergic neurons in the substantia nigra (SN) and formation of intracytoplasmic Lewy body (LB) inclusion structures. Moreover, PD is characterized by neuronal inclusions composed of abnormal α-synuclein, which is generally referred to as the Lewy-related pathology (1). It leads to cellular toxicity and, eventually, PD pathogenesis. Most PD cases are idiopathic, which appears to be involved in multiple processes such as neuroinflammation, excitotoxicity, oxidative stress, environmental toxins, and accumulation of misfolded proteins from proteasome impairment (2).

Over the past 15 years, several gene mutations have been definitively shown to mediate familial PD. For instance, *SNCA* mutations (encoding α-synuclein to PARK1 (3) and PARK4 (4), *LRRK2* (PARK8) (5), *VPS35* (PARK17) (6), *HtrA2* (PARK13) (7), and *EIG4G1* (PARK18) (8) cause autosomal dominant forms of PD. Moreover, mutations in *parkin* (PARK2) (9), *DJ-1* (PARK7) (10), *pink1* (PARK6) (11), *DNAJC6* (PARK19) (12), *SYANJ1* (PARK20) (13), and *ATP13A2* (PARK9) (14) are associated with autosomal-recessive forms of PD.

Mitochondrial dysfunction and oxidative stress are the symptoms of PD pathogenesis (15). Recent demonstrations that *pink1*, *parkin,* and *DJ-1* play crucial

roles in mitochondrial function and resistance to oxidative stress, reinforcing the central importance of these themes in PD pathogenesis. Moreover, it allows us to understand PD processes at the molecular and cellular levels.

Drosophila melanogaster, commonly known as the fruit fly, is a powerful organism for modeling human ND diseases. Nearly 75% of all human disease genes have *Drosophila* homologues (16). *Drosophila* models have successfully provided valuable insights into the elucidation of pathomechanisms and development of therapies for neurodegenerative diseases. The causal relationship among PD abnormalities, such as dopaminergic cell degeneration, inclusion body formation, and locomotion dysfunction, have been elucidated with the expression of α-synuclein in *Drosophila* models (17). Most recently, *SPG7* mutants showed a short life span, progressive locomotion defects, and sensitivity to chemical and environmental stressors (18). Here, we reviewed in detail how these genetic and environmental factors are involved in PD with model organisms, especially *D. melanogaster*.

Dopaminergic (DA) Neurons in Parkinson Disease

PD is characterized by the death of DA neurons in the substantia nigra (SN) region of the brain. Oxidative stress plays a key role in the DA neurons' degeneration. The susceptibility of the brain, especially the SN to oxidative stress, is augmented by various factors such as high oxygen demands, higher rates of oxidative metabolism,

lower levels of protective antioxidant system, and an abundant neuronal network (19). These pathways produce abundant quantities of ROS species. Moreover, mitochondrial dysfunction and the impaired protein degradation pathway align to the degeneration of dopaminergic neurons which further influence PD-related protein expressions, such as LRRK2, α-Syn, PINK1, UCH-L1, and DJ-1 (20-22). The misexpression or overexpression of the above parameters in *D. melanogaster* was examined to unscramble the root cause and mechanisms of DA neuronal loss. Therefore, studies of molecular and cellular mechanisms between mitochondrial dysfunction and different genes are essential for establishing therapeutic treatment for PD.

Mitochondrial dysfunction in PD

Most mitochondrial dysfunction results from damage to complex I or nicotinamide adenine dinucleotide phosphate (NADH): ubiquinone oxidoreductase—which forms a part of the oxidative phosphorylation system (23). PD pathogenesis results from impairment to complex I and complex I-mediated dopaminergic cell death resulting from Bax transcription activation (24). Furthermore, a clear correlation exists between ND diseases and impaired electron transport chain function. Iron containing cytochromes-associated movement plays a particularly prominent role in the mitochondrial membrane (25). As a result of this dysfunction, increased free radicals have been recorded, which is harmful to the proper functioning of cells. Oxidants,

including hydrogen peroxide and superoxide radicals, are produced as byproducts of oxidative phosphorylation, making the mitochondria the main site of ROS generation within a cell. However, in pathological situations where mitochondrial respiratory defects occur, the amount of ROS produced by the electron transport chain increases dramatically, swamping the antioxidant protection mechanisms. PD has been shown to produce these conditions (Figure 1). Evidence that oxidative stressors, such as ROS, are the culprits in these mitochondrial dysfunctions has recently emerged. The generation of oxidizing agents, such as hydrogen peroxide or superoxide, recapitulates the mitochondrial dysfunction (26).

Excess free radicals are scavenged by enzymes such as glutathione peroxidase, catalase, and superoxide dismutase in normal mitochondria. However, when ROS build up, they interact with the membrane lipids and proteins, altering their conformations and, ultimately, disrupting their functioning. Furthermore, complex I inhibitors, like MPTP or rotenone, demonstrate preferential cytotoxicity to the DA neurons (27). The MPP^+ (oxidized form of MPTP that is toxic) accumulates in the mitochondria, where it inhibits complex I in the mitochondrial electron transport chain complex (METC), thereby disrupting the flow of electrons along the METC (Figure 1). This event results in decreased ATP production and increased ROS generation (28). Similar to MPTP, rotenone is another mitochondrial complex I inhibitor. Interestingly, rotenone toxicity is involved in oxidative damage to proteins and Lewy body-like inclusions (29). Other evidence for mitochondrial dysfunction related to oxidative

stress and DA cell damage comes from findings that mutations in protein genes like *α-synuclein*, *parkin, DJ-1*, or *pink1* are linked to the familial forms of PD (Figure 1). Indeed, the latest study provides evidence that elevated mitochondrial Ca^{2+} is responsible for mitochondrial damage and neuronal death, which is controlled by a mitochondrial trafficking protein, Miro (30). The intercorrelated role of these proteins on mitochondrial dynamics reveals a common function in the mitochondrial stress response, which may provide a significant physiological basis for PD pathology (31).

Molecular models for Parkinson disease (Table 1)

SNCA (*α-synuclein*: *αS*)

SNCA encodes a small protein called α-synuclein. α-Synuclein is abundant in the brain; small amounts are detected in the heart, muscles, and other tissues. PD correlates with the formation of insoluble fibrillar aggregates in the central nervous system that contain α-synuclein (3) and misfolding of α-synuclein resulting from point mutations in *SNCA* (Figure 2A). Aggregated monomeric α-synuclein generates β sheet-rich oligomers, inducing selective oxidation of the ATP synthase β subunit and mitochondrial lipid peroxidation. These oxidation events increase the probability of permeability transition pore opening, triggering mitochondrial swelling and, ultimately, cell death (32). A30P, A53T, and E46K (33) are three PD-related *αS* mutations. Among them, A30P and A53T are the most well-studied mutations. A53T transgenic mice displayed abnormally large accumulations of α-synuclein, causing rapid

neurodegeneration and leading to cell death. A30P α-synuclein transgenic animals exhibit similar physiological and phenotypic characteristics to those found in humans, including the slow degeneration of DA neurons, formation of LB-like inclusions, and loss of locomotor functions (17). Similarly, a *Drosophila*-expressing A30P mutant causes a more rapid loss of climbing ability (34). Cathespin D, glucocerobrosidase, and proteinase K actively participate in accumulation of α-synuclein in the brain, resulting in DA neuronal loss along with decreased locomotor activity (35-38). N-terminal 32 amino acids of human α-synuclein contains mitochondrial targeting signal that plays a role in the association of these proteins with the inner mitochondrial membrane. Aggregated α-synuclein in the mitochondrial membrane of DA neurons results in complex I impairment, increased ROS production, and decreased mitochondrial transmembrane potential (39).

parkin

parkin mutation leads to an early onset form of PD, and its product encodes an E3 ligase, including functional domains such as the ubiquitin-like domain and RING finger domains. The first *in vivo* indication that *parkin* regulates mitochondrial integrity arose from studies on *Drosophila parkin* mutants. *parkin* fly mutants exhibit locomotor defects, reduced longevity, male sterility, DA neurodegeneration, and mitochondrial defects in several energy-intensive tissues such as muscles and brain (40, 41). *D.*

parkin null mutants display degeneration of DA neurons in the PPL1 cluster and reduced TH- staining in the PPM1/2 cluster (Figure 2B), resulting in reduced DA content in the brain. *D. parkin* loss-of-function mutants exhibit shrinkage of DA neurons with a decrease of tyrosine hydroxylase (TH) level and locomotor defects (42).

pink1

This gene encodes a putative serine/threonine kinase with a mitochondrial targeting sequence (11). *pink1* mutants possess fragmentation in mitochondrial cristae and are very susceptible to oxidative stress. *pink1* mutants are characterized by reduced lifespan, locomotor defects, degenerated flight muscle, and loss of DA neurons (43). *D. pink1* mutants also have a defective thorax phenotype in three-day-old flies as well as age-dependent loss of DA neurons in the PPL1 cluster at the age of 30 days (44). Furthermore, *pink1* loss of function in mice models resulted in locomotor defects and degenerated DA neurons (45). These studies provide cellular and behavioral phenotypes of *pink1* mutant reproducing PD phenotypes.

The *pink1* mutant flies share marked phenotypic similarities with *parkin* mutants. A *pink1* mutant phenotype was rescued by *parkin* overexpression, whereas *pink1* overexpression had no effect on *parkin* mutant phenotypes (46, 47). These observations suggest that Parkin acts downstream of Pink1 in the same pathway, which is conserved between flies and mammals. Genetic epistasis analyses revealed that proteins function in the same pathway to maintain mitochondrial fidelity, although

they are localized differently; *pink1* localizes to the mitochondria, and *parkin* resides in the cytosol (40, 47, 48). Cell studies have revealed *parkin* is recruited from the cytosol to depolarized mitochondria to mediate selective autophagic removal of the damaged organelle (mitophagy) (49). Furthermore, in *Drosophila*, *pink1* directly phosphorylates *parkin* to control its translocation to the mitochondria (50). The above finding suggests that *pink1* and *parkin* act in a common pathway.

DJ-1

DJ-1 binds to the subunits of mitochondrial complex I and regulates its activity (51). It is present in the mitochondrial matrix and intermembranal space (52). Its translocation into the mitochondria is enhanced by oxidative stress. *DJ-1* KO mice elicit nigrostriatal DA neuron loss and accumulate defective mitochondria, which can be reversed by adenovirus-mediated *DJ-1* overexpression; this phenomenon demonstrates *DJ-1*'s specific role in mitochondrial function (53).

DJ-1 encodes a highly conserved protein belonging to the ThiJ/PfPI superfamily of molecular chaperones (54). Two *DJ-1* orthologs exist in *Drosophila*: *DJ-1α* and *DJ-1β*. *DJ-1α* is predominantly expressed in the male testis and, at a lower level, in the brain than *DJ-1β. DJ-1α* exhibits a role in oxidative stress, generating DA neurodegeneration, although the *DJ-1β* mutant contributes more to DA neuronal degeneration (55). *DJ-1β* decreases climbing ability (41) and increases sensitivity to environmental toxins such as H_2O_2, paraquat, and rotenone (56). *DJ-*

1β loss of function results in accumulated ROS in adult brains, elevated levels of lipid peroxidation, and an increased catalase enzymatic activity (57). In *Drosophila,* both the aging process and oxidation challenge promote *DJ-1β* overoxidation at cysteine 104 (which is analogous to cysteine 106 in human DJ-1) which, in turn, irreversibly inactivates the protein *DJ-1* (58). Aged flies demonstrate further vulnerability to oxidative stress, which suggests that *DJ-1*'s protective function against oxidative stress could be progressively lost through aging, increasing the risk of DA neuron loss. Recently, our group reported that the *DJ-1β* mutant has low sugar sensitivity and reduced taste-associative memory (59), which are relevant phenotypes because >30% of PD patients have dementia. Our group also showed recovery from reduced memory defect by feeding health supplements such as omija. The fly model organism can be used for drug discovery in behavioral as well as cellular studies.

LRRK2

The most common form of sporadic PD occurs due to mutations in the gene encoding *LRRK2*, which comprises a large domain GTPase and kinase activity. *LRRK2* has been associated with a diverse set of cellular functions and signaling pathways, including mitochondrial function, vesicle trafficking, together with endocytosis, retromer complex modulation, and autophagy (60). The study in mice showed that the degeneration of dopamine neurons is enhanced due to combined effects of

LRRK2G2019S mutation with environmental toxins such as MPTP (61). The overexpression of *LRRK2* or *LRRK2-G2019S* lead to retinal degeneration, selective loss of DA neurons, decreased climbing activity, and early mortality in flies (62). *LRRK2*-induced neuronal degeneration is mediated by *hemipterous* (*hep* or JNKK). The expression of RNA interference of JNKK or dominant-negative form of JNK, a downstream kinase of JNKK, increases fly survival, locomotor activity, and decreases DA neuronal degeneration in *LRRK2-G2019S* mutant (63).

Environmental risk factors for PD

MPTP

MPTP is the most commonly used toxin to generate a PD model. It is one of the first models to link the inhibition of mitochondria complex I to PD (64). Several animal species, such as sheep, cats, mice, rats, and monkeys have been treated with MPTP to recapitulate the phenotype of a PD model. Both monkeys and mice treated with MPTP have shown selectively progressive loss of DA neurons, but no LBs (65). Loss of DA neurons leads to reduced motor abilities, although there are no LBs. MPTP induces a high level of NO in flies. Resveratrol decreases MPTP-mediated oxidative stress in flies and increases their life span. Therefore, resveratrol can be used as a therapeutic agent against PD (66), which indicates that a MPTP toxin-induced model in *D. melanogaster* is a useful tool for PD pathophysiology.

Rotenone and Paraquat

Several studies have looked at rotenone and paraquat (PQ) (a proposed mitochondrial complex I inhibitor) in *Drosophila* to investigate the susceptibility of PD genetic models and their role in neuronal cell death. Not only do these models induce DA neuron loss, but also show evidence of behavioral and histological changes, completing the pathological picture of PD (67). Paraquat causes oxidative stress in cells through the ROS generation. Rotenone blocks the mitochondrial electron transport chain through inhibition of complex I, as seen in MPTP. Rotenone also blocks mitosis and inhibits cell proliferation, which is caused by the perturbation of microtubule assembly and decreases the GTP hydrolysis rate (68). Chronic systemic exposure to rotenone in rats led to the development of several features of PD, including nigrostriatal DA degeneration. This model has been shown to reproduce almost all PD features, including the formation of intracellular inclusions that resemble LB (69).

Therapeutics Approach in Parkinson Disease

Vitamin K_2 acts as an electron carrier and enhances ATP production in the mitochondria. Defective mitochondria are also found in Parkinson's patients with a *pink1* or *parkin* mutation. Vitamin K_2 may offer hope for a new PD treatment (70). Vitamin K_2 is essential to electron transfer in *Drosophila* mitochondria. *Heix* mutants show severe mitochondrial defects that are rescued by vitamin K_2, which serves as a

mitochondrial electron carrier, helping to maintain normal ATP production. A major breakthrough in PD drug development was L-dopa, which protects the brain from oxidative stress and free radicals (71). Most pharmacological approaches to PD treatment are symptomatic and target the nigrostriatal dopaminergic pathway. The gold-standard drug is L-dopa—a precursor of dopamine—which crosses the blood–brain barrier and is converted to dopamine. Other drugs are used as monotherapy or combined with L-dopa to enhance its efficacy, including dopamine receptor agonists, catechol-O-methytransferase (COMT) inhibitors, and monoamine oxidase (MAO) inhibitors (72). Zinc is an essential trace metal and a component of serval enzymes and transcriptional regulators. Unlike copper and iron, zinc is not redox-active and, under most conditions, it serves as an antioxidant. The condition of *parkin* mutants raised on zinc-supplemented food is greatly improved. *Parkin* mutants perform best at high zinc concentrations, where controls begin to show adverse effects as a result of the metal supplement. This is manifested in a higher frequency of reaching adulthood, extended lifespan, and improved motor abilities (73).

Conclusion and Future Perspective

Drosophila mutants and transgenic models have been used to study the genetics and environmental factors responsible for PD. More than 20 genes are associated with PD, which shows interaction between genetics and environmental factors. The common endpoint of gene and toxins are believed to initiate mitochondrial dysfunction, which

results in lower ATP and oxidative stress. Various antioxidants, such as zinc and vitamin K_2, have shown good medicinal value in PD. Similarly, omija feeding has also helped resolve taste memory problems and learning defects. Until now, most studies have focused on mitochondrial dysfunction and correlated genes. In addition to mitochondrial dysfunction and oxidative stress, endoplasmic reticulum (ER) stress is another demanding model to study PD pathogenesis in *D. melanogaster*. ER stress can be reduced with piperine, which increases mesencephalic astrocyte-derived neurotrophic factor expression that ameliorates spinocerebrellar ataxia 17 (SCA17)-associated neuropathology in the TBP-105Q knock-in mouse model (74). The study of piperine's involvement in controlling neurodegeneration would be a fascinating approach for effective prophylaxis. More powerful clinical treatments than L-dopa (a precursor of dopamine) are needed for PD patients, especially in an aging society.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Toxins and genetic factors responsible for PD.

Schematic illustrations for related genes of PD and toxins in the mitochondria.

Figure 2. Clinical presentation of pathogenesis in PD and fly dopaminergic neuronal clusters.

(A) Dopaminergic neurons in the substantia nigra and PD pathology related with Lewy body. (B) Dopaminergic neuronal clusters in a fly brain.

Table 1. Parkinson diseases and their phenotypic expressions in animal models
PD genes and their phenotypic expressions in animal models, especially *Drosophila melanogaster*.

PD gene/ locus	Mammalian/mouse	*Drosophila melanogaster*
SNCA/ PARK1	Expression of Human α-Syn (A53T): ↑Accumulation of α-synuclein, ND and leading to cell death (75). Expression of Human α-Syn (A30P): Progressive motor disorder accompanied by accumulation of α–synuclein in the soma and neurite (76).	Expression of Human α-Syn (A30P and A53T) in pan-neuron: Dopaminergic cell degeneration, LB inclusion formation and locomotor dysfunction (17).
parkin/ PARK2	Expression of C-terminally truncated *parkin* in DA neuron: Motor deficit, nigrostriatal degeneration, α-synuclein accumulation (77).	KO mutants: ↓Lifespan and locomotion, and male sterility (40). Loss of proper morphology of DA neurons and deficit in motor function (42).
PARK3	ND in SN of brain and LB formation, presence of neurofibrillary tangles and Alzheimer plaques (78).	-
SNCA/ PARK4	Nigral degeneration with LB, vacuoles in neurons of the hippocampus and other brain parts (78).	-
UCH-L1/ PARK5	Rotenone induced mouse models: S-Nitrosylation of UCH-L1, ↑α-synuclein aggregation(79).	KD mutants: ↓ Dopamine in the brain results in locomotor dysfunction (80).
pink1/ PARK6	KO mouse: Impairment in hindlimb and forelimb steps(81).	KO mutants: Mitophagy of flight muscle cells and dopaminergic neuron with aging (82).
DJ-1/ PARK7	KO mouse: Loss of DA neurons in SN of brain (83).	*DJ-1β* mutant; ↓Climbing activity (41). Exhibit taste impairment and memory defect (59).

LRRK2/ PARK8	Overexpression of *LRRK2*^{R1441G}: Progressive motor deficit with immobility by 10-12 months (84).	Expression of RNA interference of JNKK or dominant-negative form of JNK increases fly survival time, locomotor activity, and decrease DA neuronal degeneration in *LRRK2*^{G2019S} overexpression in DA neurons (63).
ATP13A 2/PARK9	KD mouse: Impairment in lysosomal degradation, α-synuclein accumulation and neurotoxicity (85).	-
unknown /PARK10	-	-
GIGYF2/ PARK11	Heterozygous *Gigyf2*$^{+/-}$ mouse: Exhibits motor dysfunction by 12–15 months (86).	KO mutants: Locomotor defects and early mortality (87).
Unknown /PARK12	-	-
HtrA2/ PARK13	KO mouse: ↓Climbing ability, movement disorders, and tremor (88)	KO mutants: Mitochondrial defects, loss of flight and climbing ability, male infertility, and increase of sensitivity to oxidative stress (89).
PLA2G6/ PARK14	KO mouse: Loss of DA neurons in SN and rescue by feeding L-DOPA in motor dysfunction (90).	KO mutants: Mitochondrial dysfunction and oxidative stress (91).
FBOX7/ PARK15	KO mouse: ↓Proteasome activities and early-onset motor deficit (92).	Expression of FBXO7 rescues *parkin* mutant phenotypes, including locomotors dysfunction, DA neuron losses and muscle degeneration (93).
RAB7L1 (one of the candidat e gene)/ PARK16	KD rodent: DA neuron degeneration as *LRRK2* mutant phenotype. Overexpression of *RAB7L1* reduces *LRRK2*mutant induced DA neurodegeneration (94).	KD Mutants: DA neuron degeneration as *LRRK2* mutant phenotype. Overexpression of *RAB7L1*in DA neurons rescues DA neurodegeneration(94).
VPS35/ PARK17)	*VPS35*$^{+/-}$ mouse:	KD of *VPS35*: Locomotor impairments, mild compound eye disorganization,

	Mitochondrial fusion and cellular respiration function impairments and DA neuronal loss(95).	and interommatidial bristleloss (37).
EIG4G1/ PARK18	Mutation in *EIG4G1*(A502V, R1205H): Impairment in oxidative stress resistance (8)	-
DNAJC6/ PARK19	KO mouse: Early postnatal mortalities, and weight loss of surviving pups (96).	KD mutants: Loss of climbing abilities, decrease of lifespan, and DA neuron death (97).
SYNJ1/ PARK20	*SYNJ1*$^{+/-}$ mice: Progressive PD-like behavioral alterations and DA neurodegeneration (98). .	KD mutants: ↓Endogenous synaptic transmission at the neuromuscular junction, and 80% reduction of evoked transmission (99).

Notes: PD: Parkinson's disease; UCH-L1: ubiquitin carboxyl-terminal esterase L1; PINK1: PTEN-induced putative kinase 1; LRRK2: leucine-rich repeat kinase 2; HtrA2: High temperature requirement protein A2; FBOX7: F-box protein 7; LOF: Loss of function; KD: Knockdown; KO: Knockout; DA: dopamine; ↓: Decreased/Reduced; ↑: Increased/Enhanced; LB: Lewy body.

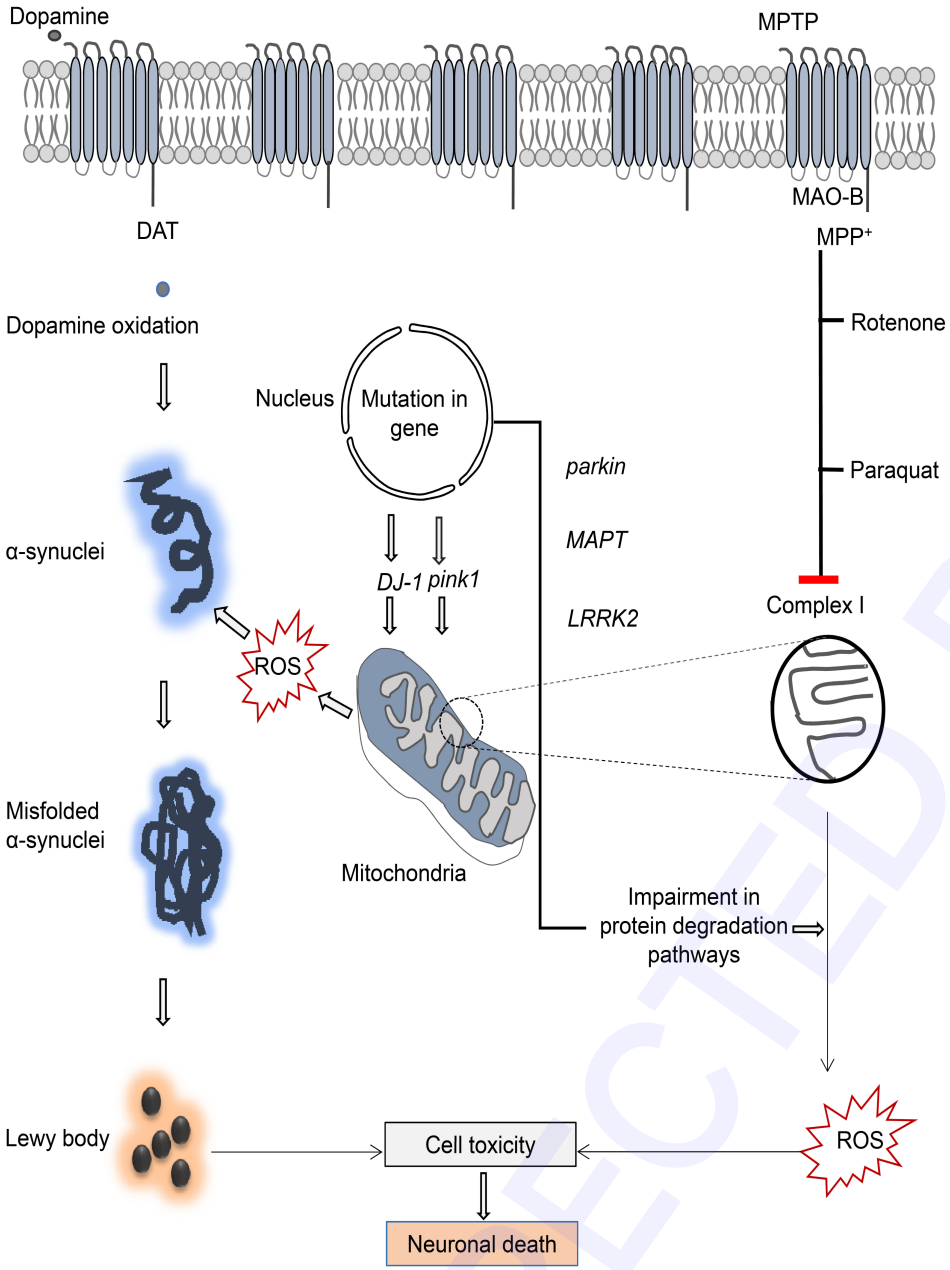
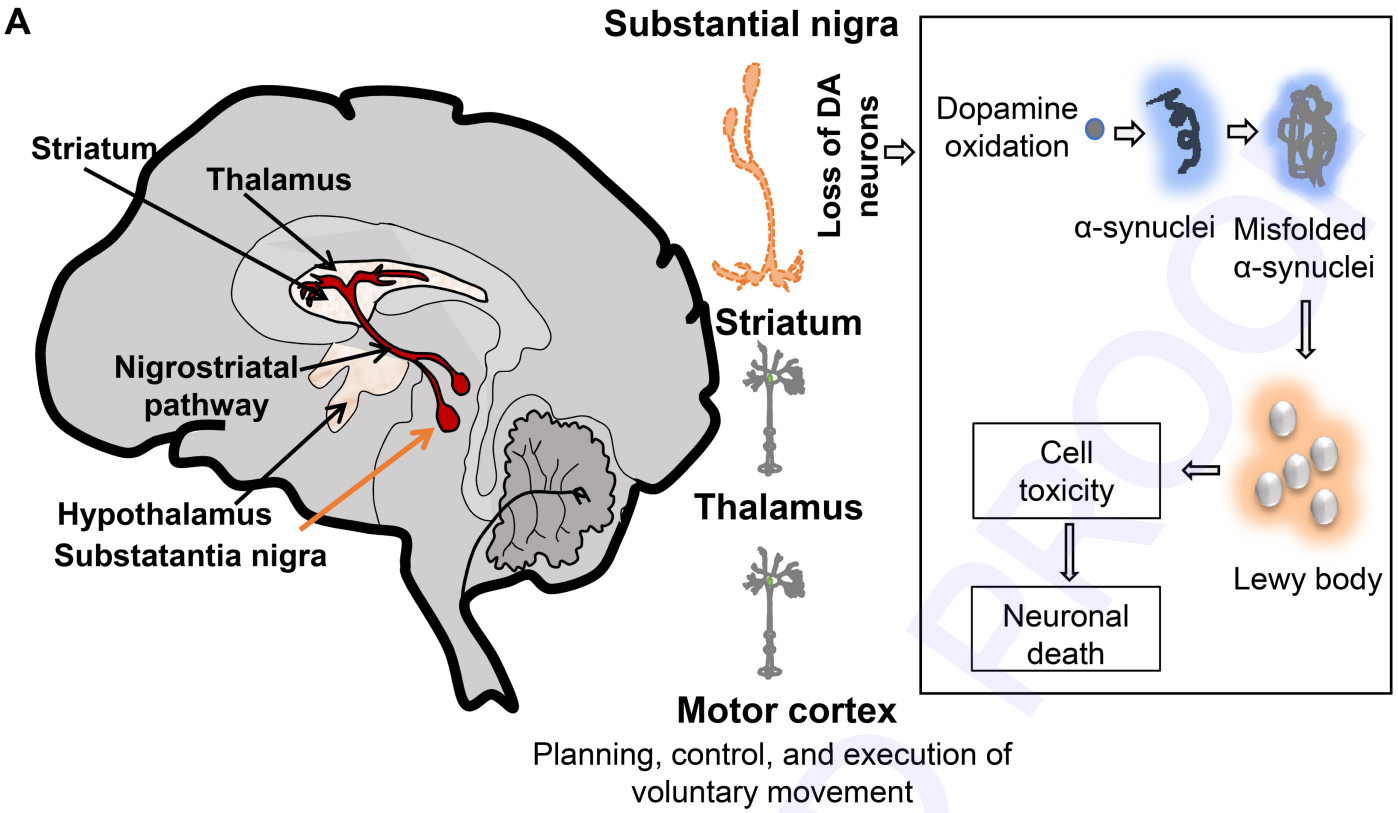


Fig. 1.



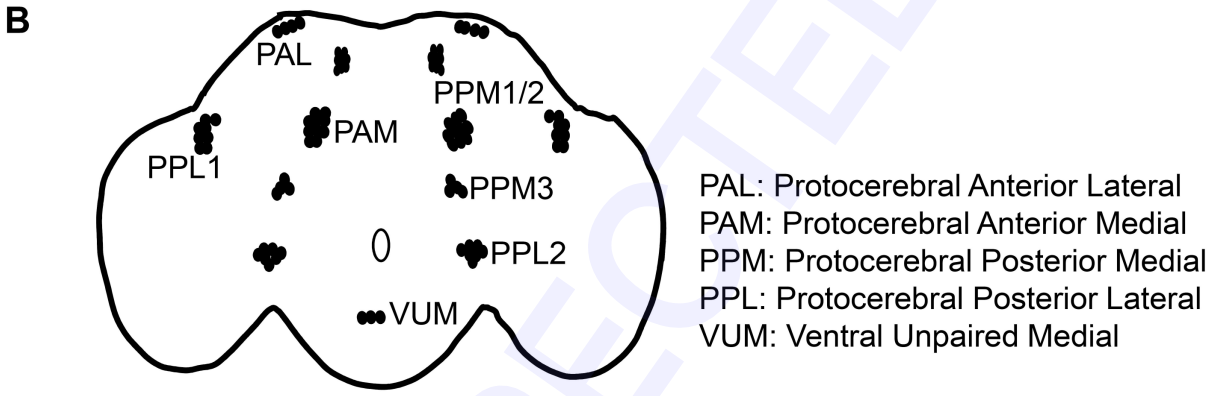


Fig. 2.