

Table 1 Genetic and clinical characteristics of hereditary Parkinson's disease

Locus	Inheritance	Gene	Type of mutation	Clinical features
PARK1/PARK4	AD	SNCA	Missense, duplication, triplication	A30P: late onset, L-dopa responded parkinsonism; A53T: typical parkinsonism with rapid progression; E64K: DLB-like symptoms; duplication: typical parkinsonism; triplication: early onset parkinsonism with rapid progression
PARK2	AR	PRKN	Nonsense, frameshift, missense	Early onset, symmetric, slowly progressed parkinsonism with spasticity and sleep benefits
PARK3	AD	Unknown	—	—
PARK5	AD	UCH-L1	Missense	Similar to sporadic PD
PARK6	AR	PINK1	Nonsense, frameshift, missense	Early onset typical parkinsonism with psychiatric symptoms and L-dopa associated dyskinesia
PARK7	AR	DJ-1	Missense	Early onset parkinsonism with psychiatric symptoms, occasionally with scoliosis and blepharospasm
PARK8	AD	LRRK2	Missense	Middle to late onset typical parkinsonism with response to L-dopa
PARK9	AR	ATP13A2	Missense, deletion, insertion, duplication	Rapidly progressed parkinsonism with dementia and pyramidal features
PARK10	Sporadic	Unknown	—	—
PARK11	AD	Unknown	—	—
PARK12	Sporadic	Unknown	—	—
PARK13	AD	Omi/HtrA2	Missense	Typical parkinsonism
PARK14	AR	PLA2G6	Missense	Early onset parkinsonism with rapid progression, cognitive decline and brain atrophy (cerebellum and cerebrum)
PARK15	AR	FBX07	Missense, frameshift	Early onset parkinsonism with spasticity and response to L-dopa
PARK16	Sporadic	Unknown	—	—

AD, autosomal dominant; AR, autosomal recessive; DLB, dementia with Lewy bodies; PD, Parkinson's disease.

dependent manner by restricting conformational fluctuations of α -synuclein.¹⁶ Recent advances in research on the protein degradation system associated with PD revealed the importance of ubiquitin proteasome and the autophagy-lysosome pathway in disease pathogenesis.¹⁷ Wild-type α -synuclein is degraded by both chaperone mediated autophagy and macroautophagy, while A30P and A53T are degraded mainly by the latter.^{17–19} Furthermore, macroautophagy itself is blocked by α -synuclein via Rap1a dysregulation.²⁰

Several lines of evidence have shown that permeabilised α -synuclein from a neuron may be toxic to neurons and/or glias they are next to. Actually, grafted healthy neurons can gradually develop the same pathology as host neurons in PD brains.²¹ These findings have suggested that non-cell autonomous cell death as well as cell autonomous cell death may have an important role in disease pathogenesis.

Parkin (PARK2)

Clinicogenetics

The first genetic locus for autosomal recessive juvenile parkinsonism was mapped to chromosome 6, and the disease gene named parkin (*PRKN*) was identified in consanguineous families.^{22–24} Mutations in the *PRKN* gene are most common in autosomal recessive juvenile parkinsonism and many mutations have been reported.³ The clinical picture is similar to that of sPD except for earlier onset, dystonic features, brisk reflexes and sleep benefit. Pathologically, no Lewy bodies were seen in most cases.^{25–27} Whether or not heterozygous *PRKN* mutations may cause or increase the susceptibility to late onset typical PD remains controversial. [18F]Fluorodopa uptake by positron emission tomography was reduced in heterozygous carriers without symptoms.^{28 29} In addition, heterozygous carriers of *PRKN* mutations have been reported to have either minor motor signs or present with late onset parkinsonism, suggesting a link between heterozygous mutations and disease pathogenesis.^{27 30 31} On the other hand, screening for *PRKN* mutations in late onset PD and healthy controls revealed similar frequencies of genetic variants.^{32 33}

Molecular biology

Parkin is associated with the ubiquitin proteasome system as an E3 ubiquitin ligase.³⁴ The C terminal binds with ubiquitin E2 enzymes and recognises a substrate whereas the N terminal interacts with the 19S subunit of proteasome. A nonsense mutation lacking the rear RING finger motif had no E3 activity and sole IBER-RING2 retained E3 activity, and thus most parkin mutations do not lead to loss of kinase activity.³⁵ α -Synuclein and synphilin-1 were identified as parkin substrates and consist of Lewy bodies.^{36 37} Parkin mainly localises in the cytoplasm as well as in plasma membranes and partly in mitochondria. Under physiological or pathological conditions, parkin is involved in mitochondrial maintenance and recent evidence revealed that parkin with PINK1 physically associate and functionally cooperate to identify and label damaged mitochondria for selective degradation via autophagy (mitophagy).^{38–42} Protein-protein interactions between parkin and other PD related genes are detailed in each gene section.

PINK1 (PARK6)

Clinicogenetics

PARK6 was first identified on chromosome 1p36.⁴³ The disease gene was identified as *PINK1* (PTEN induced kinase 1) containing eight exons.⁴⁴ The clinical characteristics are autosomal recessive, early onset, slow disease progression and L-dopa responsive parkinsonism. Most mutations were missense mutations, but whole gene deletions were also reported.^{45 46} Many putative pathogenic mutations were also observed in a heterozygous state in familial and sPD patients as well as in healthy controls. However, most of the studies have not checked the copy number variants, causing the mutation pathogenicity to remain controversial.² Lewy bodies, neuronal loss and astrocytic gliosis in the substantia nigra were detected in a patient with *PINK1* compound heterozygous mutations.⁴⁷

Molecular biology

PINK1 has eight exons encoding 581 amino acids, including a mitochondrial targeting sequence, transmembrane domain and

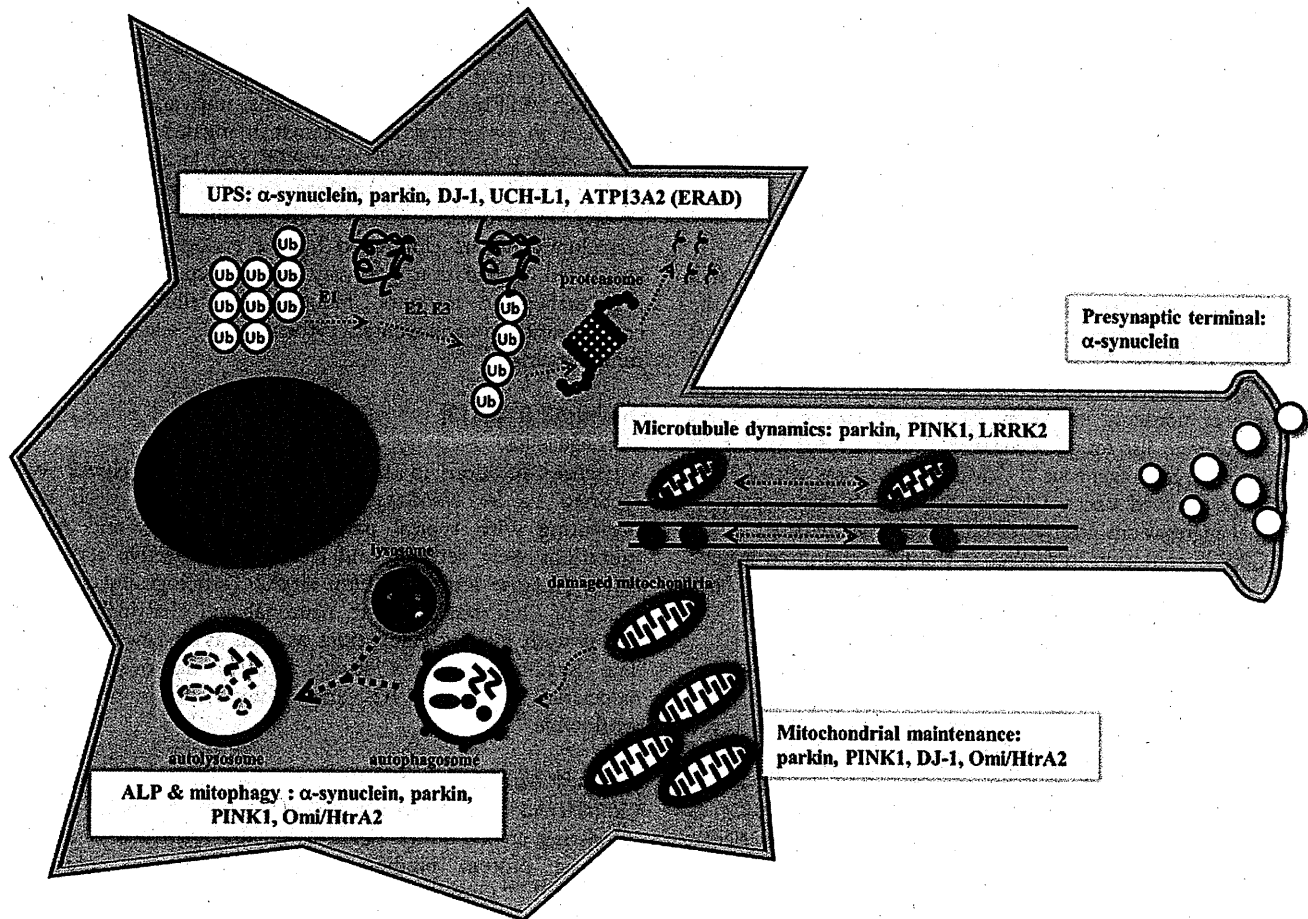


Figure 1 Schematic representation of the possible pathogenesis in hereditary Parkinson's disease. ALP, autophagy–lysosome pathway; ERAD, endoplasmic reticulum associated degradation; Ub, ubiquitin; UPS, ubiquitin proteasome system.

kinase domain.⁴⁸ The gene product is ubiquitously expressed in the brain and systemic organs. The protein mainly localises in mitochondria, especially in the outer membrane. PINK1 is a serine–threonine kinase and several pathological mutations in PINK1 have been reported to change their kinase activities.^{49–52} In addition, Rictor (a component of mTORC2),⁵³ tumour necrosis factor receptor associated protein 1 (TRAP1; a mitochondrial chaperone),⁵⁰ Omi (PARK13 gene product) and parkin (PARK2 gene product) were identified as substrates for PINK1.^{54–55}

PINK1 regulates mitochondrial dynamics and respiratory functions.^{38–53–56–58} Mitochondrial fission is accelerated by PINK1 overexpression accompanied by parkin.^{59–60} PINK1 ablation with siRNA in neurons reduces resistance against oxidative stress while its overexpression provides resistance.⁶¹ Using genetically modified *Drosophila* models, we see that PINK1 deficiency causes the same phenotype as parkin deficiency and the PINK1 deficiency phenotype is rescued by parkin complementation, suggesting that parkin is downstream of PINK1.^{62–64} Several lines of evidence have provided new aspects of the PINK1/parkin pathway associated with mitochondrial elimination via macroautophagy (mitophagy). When mitochondrial membrane potentials are lost, endogenous PINK1 is accumulated followed by parkin recruitment, and subsequently the depolarised mitochondria were eliminated by mitophagy.^{40–41–65–66} Mitochondrial targeting sequence, kinase activity of PINK1 and the linker domain of parkin are indispensable for the PINK1/parkin mediated mitophagy.

DJ-1 (PARK7)

Clinicogenetics

Clinical features of *PARK7* are characterised by early onset parkinsonism with scoliosis, blepharospasm and psychiatric symptoms, similar to those of *PARK2* and *PARK6*. The disease gene was identified as *DJ-1*, which has eight exons encoding 189 amino acids. Three missense mutations (L166P, M26I, E64D) in exons 1–5 of the gene have been identified in Italian, Dutch and Uruguayan families. *DJ-1* protein was detected around Lewy bodies, suggesting *DJ-1* is not in the main structure of Lewy bodies. However, the protein was detected in astrocytes and in a part of the cytoplasmic inclusions positive to tau in brains with corticobasal degeneration, progressive supranuclear palsy and multiple system atrophy.^{67–69}

Molecular biology

DJ-1 is almost ubiquitously expressed in organs, including the brain. Endogenous *DJ-1* is present in synaptic terminals, mitochondria and membranous organelles.^{70–71} *DJ-1* with the L166P mutation lost more stability compared with the wild-type and mutant *DJ-1* (M26I, E64D).⁷² In *DJ-1* knockout mice, no significant loss of dopaminergic neurons and decreased susceptibility to oxidative stress were noted.⁷³ *DJ-1* is a multifunctional redox sensitive protein regulating mitochondrial oxidative stress and increases expression levels of SOD1 in an Erk1/2-Elk1 pathway dependent manner,⁷⁴ and facilitates prosurvival factor Akt, leading to suppression of apoptosis.⁷⁵ Also, the protein

inhibits TRAIL induced apoptosis by blocking Fas associated protein death domain mediated pro-caspase-8 activation.⁷⁶ Along with parkin and PINK1, DJ-1 has various cellular functions such as regulation of mitochondrial morphology as well as misfolded protein degradation by forming an E3 ligase complex with those proteins.⁷⁷

LRRK2 (PARK8)

Clinicogenetics

Clinical features of PARK8 are essentially similar to those of sPD except for earlier onset age. The disease gene was identified as the leucine rich repeat kinase 2 gene (*LRRK2*) linked to autosomal dominant inherited PD encoding 2517 amino acids.^{78–80} PARK8 is the most common form of hPD in the world. Until now, 20 missense or nonsense mutations have been reported.⁸¹ *LRRK2* mutations were also found in some sPD cases; neuropathological findings were heterogeneous.^{82–83} Most of the cases with *LRRK2* mutations showed various degrees of Lewy bodies but intraneuronal aggregations positive to tau were rarely detected.^{79–84–85} The G2019S mutation in *LRRK2* is the most common genetic cause of PD, accounting for a significant proportion of both autosomal dominant and sPD cases.

Molecular biology

LRRK2 protein, containing a GTPase domain, a Ras of complex domain, a C terminal of Ras complex domain and a mitogen activated kinase domain, is highly expressed in the brain, and mRNA levels are rich in the striatum and hippocampus compared with other regions.⁸⁶ Intracellular *LRRK2* is mainly distributed in the plasma membrane and vesicular structures.^{87–88} Immunoprecipitation techniques have revealed that *LRRK2* interacts with parkin.⁸⁹ In transgenic flies, neurodegeneration by *LRRK2* with or without a mutation is modified by overexpression or siRNA knockdown of parkin, PINK1 or DJ-1, suggesting genetic interaction between them.^{90–91} Activity changes of *LRRK2* kinase and GTPase have been suspected as a key factor in *LRRK2* pathogenesis. Changes in *LRRK2* activity cause alterations in mitogen activated protein kinase, translational control, tumour necrosis factor α /Fas ligand and Wnt signalling pathways with the cell biological functions of *LRRK2* such as vesicle trafficking.⁸⁰ The most common pathological mutation in *LRRK2*, G2019S *LRRK2*, causes neurite retraction by activation of Rac1 small GTPase.⁹² *LRRK2* mutations inhibit an endogenous peroxidase by phosphorylation promoting dysregulation of mitochondrial function and oxidative damage.⁹³ G2019S human *LRRK2* transgenic rat models specifically expressed in the nigrostriatal system have shown progressive degeneration of nigral dopaminergic neurons.⁹⁴ In terms of *LRRK2* control, PKA has been identified as a potential upstream kinase of *LRRK2* at S935, on which binding of 14-3-3 with *LRRK2* depends.⁹⁵ However, the exact biological function of *LRRK2* remains largely unclear because no physiological substrates have been identified to date.

ATP13A2 (PARK9)

Clinicogenetics

PARK9, also known as Kufor–Rakeb syndrome, is an autosomal recessive parkinsonian disorder characterised by early onset (14–16 years old), good response to L-dopa treatment, pyramidal feature, supranuclear gaze palsy and dementia.⁹⁶ The gene locus was mapped to 1p36 and the disease gene was identified as *ATP13A2*, which localises in lysosomal membranes.⁹⁷ Various types of mutations in the *ATP13A2* have been reported.

Molecular biology

ATP13A2 is predicted to be a lysosomal P5-type ATPase that plays important roles in regulating cation homeostasis. Although *ATP13A2* function remains unclear, it might be involved in protecting cells against manganese and mutant α -synuclein toxicity.⁹⁸ Wild-type *ATP13A2* localises mainly in lysosomes whereas three separate mutants with a mutation involved in PD cause retention of the protein in the endoplasmic reticulum, and are eliminated by the endoplasmic reticulum associated degradation pathway.⁹⁹ Wild-type *ATP13A2*, but not pathogenic mutants, reduced intracellular manganese concentration and prevented cytochrome C release from the mitochondria.¹⁰⁰

Omi/HtrA2 (PARK13)

Clinicogenetics

Missense mutations in the gene coding for Omi/HtrA2 were reported to be associated with four patients with sPD, presenting with typical parkinsonism.⁵⁵ G399S and A141S mutations were detected and resulted in defective activation of the protease activity of Omi/HtrA2. Pathologically, accumulation of Omi was found in neuronal and glial inclusions in brains with α -synucleinopathies as well as in Lewy bodies.¹⁰¹ The largest association study revealed no overall strong association of Omi/HtrA2 variants with sPD in populations worldwide.¹⁰²

Molecular biology

Omi/HtrA2 is a nuclearly encoded mitochondrial protein consisting of 458 amino acids, originally identified as a proapoptotic protein binding with an apoptosis inhibiting protein.^{103–104} Omi knockout mice presented with neuronal loss in the striatum and died within 30 days of birth.¹⁰⁵ Cells overexpressing Omi mutant with G399S have shown mitochondrial morphological changes followed by dysfunction and increased susceptibility against oxidative stress.⁵⁵ Interestingly, wild-type Omi/HtrA2, not protease defective mutant, activates autophagy through digestion of Hax-1, a Bcl-2 family related protein that represses autophagy via Beclin-1 inhibition, suggesting an insufficient protein degradation system may play a key role.¹⁰⁶

PLA2G6 (PARK14)

Clinicogenetics

PARK14 is an autosomal recessive parkinsonian syndrome characterised by early onset rapidly progressive parkinsonism, dystonia, cognitive decline, and cerebral and cerebellar atrophy. Through homozygosity mapping and direct sequencing, two different homozygous mutations in *PLA2G6*, which also causes infantile neuroaxonal dystrophy and neurodegeneration with brain iron accumulation, were identified.^{107–108} Cranial MRI did not detect iron accumulation in the basal ganglia in most cases with this disorder.^{108–109}

Molecular biology

The *PLA2G6* gene encodes a group VIA calcium independent phospholipase A2, also known as calcium independent phospholipase A2 β , which hydrolyses the sn-2 acyl chain of phospholipids, generating free fatty acids and lysophospholipids. In an in vitro assay, wild-type *PLA2G6* associated with infantile neuroaxonal dystrophy/neurodegeneration with brain iron accumulation failed to catalyse fatty acid release from phospholipids, while PARK14 associated mutations ((R741Q, R747W and R632W) did not, implying that other functions of *PLA2G6*

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include interactions with calmodulin and that PLA2G6 might also be associated with calcium/calmodulin dependent protein kinase II- β .^{110 111}

FBX07 (PARK15)

Clinicogenetics

Only three families with mutations in *FBX07* have been reported.^{112 113} Affected individuals had juvenile onset (10–19 years old) of progressive parkinsonism associated with spasticity, and variable response to L-dopa. No pathological studies have been reported.

Molecular biology

Fbx07 is a member of the F box containing protein (FBP) family with an F box domain. F box containing proteins are expected to function as molecular scaffolds in the formation of the protein complex; however, the exact function of *FBX07* remains unclear.

OTHER GENES ASSOCIATED WITH PARKINSON'S DISEASE

GWAS have uncovered a number of candidate genes involved in PD in European and Japanese populations, indicating a substantial contribution of genetics underlying susceptibility to both early onset and late onset PD.^{6 7 114–119} These studies have shown repeatedly a common variation in *SNCA* and an inversion of the region containing the *MAPT*. Recent genetic studies revealed mutations in the *GBA* gene, the most widespread genetic risk factor for parkinsonism identified to date.^{120–124} In this section, we summarise the molecular mechanisms of the two genes, *MAPT* and *GBA*.

MAPT

Mutations in *MAPT*, encoding microtubule associated tau, result in tauopathies, including progressive supranuclear palsy; corticobasal degeneration and frontotemporal lobar degeneration.¹²⁵ Tau is a soluble protein, but insoluble aggregates are produced during the formation of neurofibrillary tangles which disrupts microtubule associated dynamics and neuronal functions. Considering the interplay between α -synuclein and tau reported previously,¹²⁶ it is interesting that there would be a common pathogenesis associated with aggregation formations.

GBA

Early observed patients with Gaucher disease and their heterozygous relatives present with parkinsonism.¹²⁷ In addition, autopsy studies have shown the presence of mutant glucocerebrosidase (GCase) in α -synuclein positive Lewy bodies in Gaucher disease patients and carriers with α -synucleinopathies.¹²⁸ GCase is a lysosomal hydrolase with 497 amino acids that catalyses the metabolism of the glycolipid glucosylceramide to ceramide and glucose. Cells overexpressing mutant GCase promoted α -synuclein accumulation in a dose and time dependent manner.¹²⁹ α -Synuclein GCase interacts selectively under lysosomal solution conditions (pH 5.5) and the interaction site was mapped to the α -synuclein C terminal residues 118–137.¹³⁰ Insufficient functions of the lysosomes may have an effect on chaperone mediated autophagy or macroautophagy.

CONCLUDING REMARKS

In the 14 years since the first causative gene (α -synuclein) in PD was discovered, great advances have been made in understanding the biology of the disease. Recent evidence shows that the environment plays no role in the aetiology of PD.¹³¹ In addition, GWAS suggest that a number of genes influence susceptibility.³

The PD associated genes provide valuable clues regarding the molecular pathogenesis of PD because the pathomechanism for sPD would have certain pathways in common with those of hPD. Importantly, basic biological studies in PD have led to numerous potential therapeutic strategies. For example, a specific inhibitor for LRRK2 phosphorylations at Ser910 and Ser935 was recently developed.¹³² In the future, it becomes more important to translate laboratory data, including molecular pathogenesis as well as genetic associations, into clinical treatments, leading to disease modifying therapies to conquer the disease onset and/or progression.

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Review Article

Genetic Mutations and Mitochondrial Toxins Shed New Light on the Pathogenesis of Parkinson's Disease

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The cellular abnormalities in Parkinson's disease (PD) include mitochondrial dysfunction and oxidative damage, which are probably induced by both genetic predisposition and environmental factors. Mitochondrial dysfunction has long been implicated in the pathogenesis of PD. The recent discovery of genes associated with the etiology of familial PD has emphasized the role of mitochondrial dysfunction in PD. The discovery and increasing knowledge of the function of PINK1 and parkin, which are associated with the mitochondria, have also enhanced the understanding of cellular functions. The PINK1-parkin pathway is associated with quality control of the mitochondria, as determined in cultured cells treated with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which causes mitochondrial depolarization. To date, the use of mitochondrial toxins, for example, 1-methyl-4-phenyl-tetrahydropyridine (MPTP) and CCCP, has contributed to our understanding of PD. We review how these toxins and familial PD gene products are associated with and have enhanced our understanding of the role of mitochondrial dysfunction in PD.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, affecting 1% of the population above the age of 60. The classical form of the disease is characterized clinically by rigidity, resting tremor, bradykinesia, and postural instability. In addition to these four cardinal symptoms, many nonmotor symptoms frequently appear in PD, such as cognitive impairment, hallucinations, delusion, behavioral abnormalities, depression, disturbances of sleep and wakefulness, loss of smell, pain, and autonomic dysfunctions such as constipation, hypotension, urinary frequency, impotence, and sweating. The pathological hallmarks of PD are the preferential loss of dopaminergic neurons of the substantia nigra (SN) pars compacta and formation of Lewy bodies. Exposure to environmental factors inducing mitochondrial toxin like 1-methyl-4-phenyl-tetrahydropyridine (MPTP) produces selective degeneration of dopaminergic neurons in SN and results in an irreversible Parkinsonism [1–3]. The active metabolite of MPTP, 1-methyl-4-phenylpyridinium ion (MPP⁺), is an inhibitor of complex I, and

it accumulates in dopaminergic neurons because it is actively transported via dopamine transporter (DAT) [4–6]. The inhibition of the electron transport induces oxidative damage by increasing the formation of reactive oxygen species (ROS) and leads to further mitochondrial dysfunction [7]. These findings were supported by evidence of oxidative damage including an increase in lipid peroxide [8], decrease in glutathione [9], increase in hydroxynonenal-modified proteins [10], and increase in 8-hydroxy-deoxy guanine [11] in SN. ROS impair mitochondrial proteins, further aggravating mitochondrial function. Ultimate outcomes are dissipation of mitochondrial membrane potential and the release of cytochrome *c* into the cytoplasm and activation of the apoptotic cascade. A biochemical link between MPTP toxicity and Parkinsonism was confirmed with the finding of low levels of complex I in the SN, skeletal muscle, and platelets in patients with PD [12, 13]. In contrast, it remains unknown whether this systemic deficiency of complex I is crucially related to dopaminergic cell loss in PD. Rats administered rotenone (an inhibitor of complex I) developed neuronal degeneration and formation of synuclein-positive

inclusions; however, the degree of complex I inhibition was not severe enough to induce brain mitochondrial dysfunction [14]. Although inhibition of complex I and production of free radical result in increased oxidative stress, it remains unclear whether such dysfunction is a primary or a secondary process in the pathogenesis of the disease.

2. Involvement of Two Mitochondrial Toxic Pathways in Synuclein, DJ-1, and Parkin Mice Model

Several mutations of the synuclein gene (*SNCA*) at the *PARK1* locus induce autosomal dominant Parkinsonism. Three missense mutations: A53T [15], A30P [16], and E46K [17], duplications [18–21], and triplications [22, 23] of *SNCA* have so far been described. Triplications are associated with Parkinsonism and dementia, and the age of onset is younger than the other mutations, and the neuropathological changes are those of diffuse Lewy body disease. Regarding the pathogenesis of *PARK1*-linked PD, accumulation of normal synuclein is likely to predispose nigral neurons for protofibril formation. Toxicity associated with increased synuclein expression is an important cellular event that enhances the genetic predisposition to sporadic PD. At present, indirect evidence suggests a relationship between synuclein and oxidative stress, including protein carbonylation and lipid peroxidation. Furthermore, synuclein-deficient mice were found to have striking resistance to MPTP-induced degeneration of dopaminergic neurons, and this resistance appeared to be related to failure of the toxin itself. Interestingly, there was dissociation in the resistance between MPTP- and rotenone-induced cell vulnerability of synuclein-null dopaminergic neurons [24]. This result suggests that MPTP associates with synuclein through another pathway independent of complex I inhibition (mitochondrial dysfunction), to finally induce dopaminergic cell death. Several mutations of the DJ-1 gene at the *PARK7* locus induce autosomal recessive Parkinsonism [25]. Clinical phenotype is characterized by an onset in the midthirties, good levodopa response, and slow disease progression. Several lines of evidence suggest that it plays a role in the oxidative stress response [26, 27]. Subcellular localization studies have shown DJ-1 to be present in the cytosol, mitochondria, and nucleus [26, 28, 29]. Junn et al. [30] showed that in response to oxidative stress, some of the DJ-1 protein is translocated from its major cytosolic pool to mitochondria and nucleus. DJ-1 null mice are vulnerable to MPTP [31]. On the other hand, Thomas et al. [32] reported that the susceptibility of SN to MPTP in mice is independent of parkin activity. In short, the absence of parkin does not seem to increase the vulnerability of dopaminergic neurons to MPTP intoxication. Another study also found that oxidative stress, including MPTP, altered parkin solubility, causing parkin aggregation, thereby suggesting parkin dysfunction as a pathogenic mechanism of sporadic PD [33].

3. Functional Interplay between PINK1 and Parkin to Maintain Mitochondrial Integrity

Many mutations of the parkin gene at the *PARK2* locus induce autosomal recessive Parkinsonism [34–38]. The usual age of onset is between 20 and 40 years. Clinical features consist of dystonia and sleep benefit, which are also characteristic symptoms. Despite affected patients responding well to levodopa, they soon develop motor fluctuations. Conversely, mutations of the PINK1 (PTEN-induced kinase 1) gene at the *PARK6* locus induce autosomal recessive Parkinsonism. The age of onset is slightly delayed relative to *PARK2*, that is, from 32 to 48 years [39]. The affected patients show levodopa-responsive Parkinsonism. PINK1 contains an N-terminal mitochondrial targeting signal and a highly conserved serine/threonine kinase domain, and many missense and nonsense mutations have been reported at the kinase domain [40–44]. In particular, the identification of PINK1 mutations has strongly implicated mitochondrial dysfunction in the pathogenesis of PD [40]. The activity of PINK1 kinase is crucial for mitochondrial maintenance via TRAP phosphorylation [45]. The loss of PINK1 function results in increased vulnerability to various stresses [46–48]. *Drosophila* models have demonstrated that PINK1 and parkin ensure stable mitochondrial function. Parkin null mutants show severe mitochondrial pathology associated with reduced lifespan, apoptosis, and muscle degeneration [49]. While the PINK1 mutant phenotype can be rescued by parkin gene overexpression [50, 51], the converse does not occur, suggesting that parkin acts downstream of PINK1 in a common pathway to maintain mitochondrial integrity. PINK1 loss-of-function results in reduced mitochondrial membrane potential [52], and the PINK1-parkin pathway is associated with mitochondrial elimination in cultured cells treated with the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which causes mitochondrial depolarization [53–58]. The exact mechanism underlying CCCP-induced mitochondrial depolarization, leading to mitochondrial autophagy, has been examined in detail. At steady state, parkin is localized throughout the cytosol but not in the mitochondria. However, parkin was rapidly recruited into the mitochondria when HeLa cells were treated with CCCP [55]. Furthermore, PINK1 recruits parkin from the cytoplasm to the low-membrane potential mitochondria, resulting in the mitochondrial degradation. Interestingly, the ubiquitin-ligase activity of parkin is repressed in the cytoplasm at steady state; however, PINK1-dependent mitochondrial localization triggered by mitochondrial depolarization liberates the potential enzymatic activity of parkin. While CCCP is well described, its mitochondrial toxic effects provide new insights on the functional interplay between PINK1 and parkin.

4. Accumulation of PINK1 in Damaged Mitochondria

PINK1 is localized in both the mitochondria [40, 59] and the cytoplasm [55, 60]. Treatment with CCCP results in gradual accumulation of PINK1 and translocation of the cytoplasmic

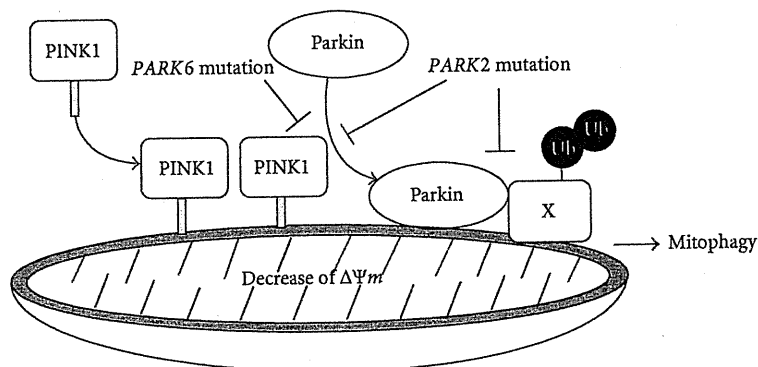


FIGURE 1: Schematic representation of PINK1-parkin-mediated mitophagy. In damaged mitochondria, PINK1 and parkin regulate mitochondrial elimination by inducing mitophagy. Under steady state, PINK1 is cleaved and degraded rapidly in the mitochondria. This process may be inhibited by the mitochondrial depolarization, resulting in PINK1 accumulation in the mitochondria. This accumulation is a crucial signal for parkin recruitment to the mitochondria. Parkin is presumed to ubiquitinate substrate (X), resulting in the induction of mitophagy.

PINK1 to the mitochondria. The subcellular localization of PINK1 is regulated by the mitochondrial membrane potential. Such accumulation may be the first trigger of PINK1-related parkin recruitment. Co-overexpression of PINK1 and parkin results in their colocalization in the mitochondria [61]. Even when these cells were not treated with CCCP, overexpression of PINK1 was associated with translocation of parkin to the cells, together with their mitochondrial aggregation.

Moreover, overexpression of both PINK1 and parkin in the cells resulted in the complete disappearance of the mitochondria. These results suggest that both PINK1 and parkin are indispensable for mitochondrial elimination and that accumulation of PINK1 in the mitochondria results in recruitment of parkin to the mitochondria even in the absence of CCCP [54].

5. PINK1 Kinase Activity Is Essential for Translocation of Parkin

PINK1 is composed of an atypical N-terminal mitochondrial targeting signal and transmembrane domain, kinase domain in the middle, and a conserved C-terminal domain, and deletion of the N-terminal amino acids abolished the mitochondrial localization of PINK1 [62]. Among other mutations, G309D, L347P, and G409V are associated with reduction in PINK1-kinase activity, and a C-terminal domain deletion mutant is associated with PINK1 dysfunction [63, 64]. The G309D/L347P/G409V mutants preserved mitochondrial localization, though their mitochondrial elimination was less compared to cells expressing both the wild-type PINK1 and parkin. When introduced into PINK1-deficient cells, the mutants were unable to complement the localization of parkin [55]. These results indicate that targeting the kinase activity and mitochondrial distribution of PINK1 is important for the mitochondrial recruitment of parkin (Figure 1).

6. PINK1 Deficiency Itself Causes Respiratory Chain Defects

Impaired mitochondrial respiration was observed in the brain of PINK1 null mice [65] although the mechanism linking PINK1 to mitochondrial membrane potential remains to be determined. Amo et al. [66] reported depletion of the mitochondrial membrane potential and cellular ATP levels (~80%) in PINK1-deficient mouse embryonic fibroblasts (MEFs) compared with those in littermate wild-type MEFs. However, loss of PINK1 did not alter mitochondrial proton leak, which reduces the membrane potential in the absence of ATP synthesis. Instead, the authors reported reduced activity of the respiratory chain, which produces the membrane potential by oxidizing substrates using oxygen. The H_2O_2 production rate by PINK1 null mitochondria was lower due to low oxygen consumption rate, while the proportion (H_2O_2 production rate per oxygen consumption rate) was higher. These results suggest that mitochondrial dysfunction in PD is not caused by proton leak, but by a defective respiratory chain. Furthermore, rate of free radical leak was significantly higher in PINK1-deficient MEFs than in wild-type MEFs. Because the differences disappeared with the addition of rotenone (inhibitor of complex I, which inhibits reverse electron flow from coenzyme Q to complex I), conceivably ROS generation enhanced by loss of PINK1 was mostly from complex I. With regard to PINK1-related PD, ROS may be an important factor. The above may also explain why cytoplasmic PINK1 protects neurons against MPTP [47]. Inhibition of complex I itself is associated with increased ROS production [67]. These results are at least in part consistent with those of previous studies, suggesting that MPTP and rotenone induce neuronal cell death by inhibiting complex I activity, leading to a PD-like phenotype [68–70] (Figure 2).

It is not doubtful that ROS generation is harmful to the cells, but the process of cell death is supposed to be slow. The crucial point is how inhibition of complex I

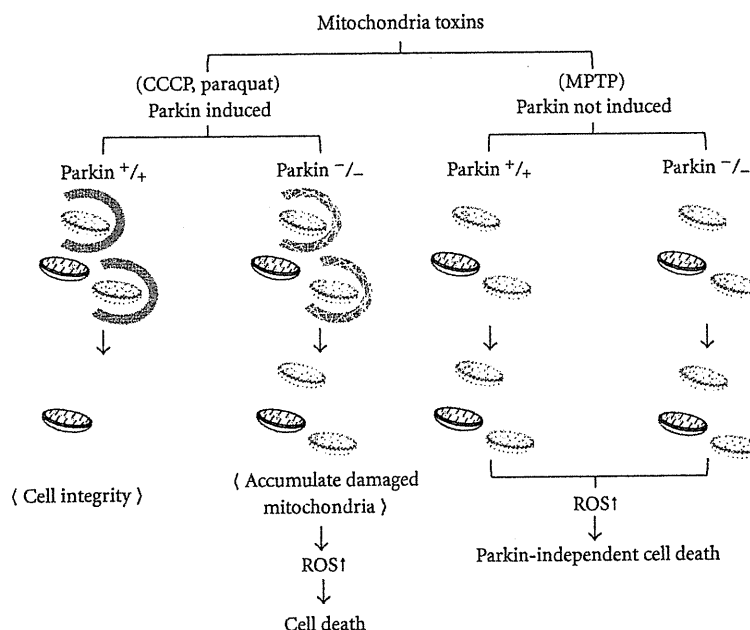


FIGURE 2: Two mechanisms of mitochondrial toxicity and parkin function. The effect of mitochondrial toxicity is different between CCCP and MPTP. Treatment with CCCP recruits parkin to the mitochondria resulting in mitophagy to keep mitochondrial integrity. Parkin deficiency is associated with accumulation of damaged mitochondria and accelerated cell death. Treatment with MPTP does not necessarily induce parkin. Parkin may be the sensor of damage-adaptive autophagy.

affects mitochondrial dysfunction including mitochondrial depolarization. Considering that the onset of *PARK6* (at 32–48 years) is slightly delayed relative to that of *PARK2* [39], some cases of *PINK1* mutation might not affect parkin recruitment and thus maintain at least part of mitochondrial integrity. This may explain the late onset of *PARK6*. On the other hand, parkin did not translocate into the mitochondria when cells were treated with MPTP (our unpublished data). This finding means that inhibition of complex I does not necessarily induce low membrane potential. Further research is needed to investigate two independent pathogenic mechanisms related to MPTP and CCCP (Figure 2).

7. Conclusion

Cell death of dopaminergic neurons is due to a combination of exogenous stress and genetic predisposition. The discovery of PD genes has provided important insight including an understanding of *PINK1*-parkin mediated mitophagy. Furthermore, mitochondrial toxins provided crucial clues: (1) CCCP directly affects mitochondrial dysfunction and induces mitophagy; (2) MPTP toxicity seems to alter ROS generation rather than mitochondrial depolarization. The effects of mitochondrial toxins do not seem to be a one-way manner. The information is available for understanding the pathogenesis in PD. Here, we touched on the fringes of molecular mechanisms of *PINK1*-parkin-mediated mitophagy. Further research will elucidate how this quality control system applies to neurons.

Abbreviations

CCCP:	Carbonyl cyanide m-chlorophenylhydrazone
DAT:	Dopamine transporter
MEFs:	Mouse embryonic fibroblasts
MPTP:	1-methyl-4-phenyl-tetrahydropyridine
PD:	Parkinson's disease
<i>PINK1</i> :	PTEN-induced putative kinase1
ROS:	Reactive oxygen species
SN:	Substantia nigra.

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Enhanced Hyperthermia Induced by MDMA in Parkin Knockout Mice

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Abstract: MDMA (3,4-methylenedioxyamphetamine) is reportedly severely toxic to both dopamine (DA) and serotonin neurons. MDMA significantly reduces the number of DA neurons in the substantia nigra, but not in the nucleus accumbens, indicating that MDMA causes selective destruction of DA neurons in the nigrostriatal pathway, sparing the mesolimbic pathway. Parkinson's disease (PD) is a neurodegenerative disorder of multifactorial origin. The pathological hallmark of PD is the degeneration of DA neurons in the nigrostriatal pathway. Mutations in the parkin gene are frequently observed in autosomal recessive parkinsonism in humans. Parkin is hypothesized to protect against neurotoxic insult, and we attempted to clarify the role of parkin in MDMA-induced hyperthermia, one of the causal factors of neuronal damage, using parkin knockout mice. Body temperature was measured rectally before and 15, 30, 45, and 60 min after intraperitoneal injection of MDMA (30 mg/kg) at an ambient temperature of $22 \pm 2^\circ\text{C}$. Significantly enhanced hyperthermia after MDMA injection was observed in heterozygous and homozygous parkin knockout mice compared with wildtype mice, suggesting that parkin plays a protective role in MDMA neurotoxicity.

Keywords: Hyperthermia, knockout, mice, MDMA, parkin.

INTRODUCTION

The amphetamine derivative 3,4-methylenedioxyamphetamine (MDMA) is abused by young adults despite its potentially neurotoxic effects and psychiatric complications. MDMA produces a rapid enhancement of serotonin and dopamine (DA) release in the brain [1, 2]. Administration of MDMA in mice is well known to produce acute hyperthermia and degeneration of striatal DA nerve terminals [3]. Recently, Granado and colleagues [4] reported that MDMA produces a significant decrease in the number of tyrosine hydroxylase (TH)-immunoreactive neurons in the substantia nigra. This decrease was accompanied by a dose-dependent decrease in TH- and DA transporter (DAT)-immunoreactivity in the striatum. MDMA significantly reduces TH- and DAT-immunoreactivity in the striatum, but not in the nucleus accumbens, indicating that MDMA causes selective destruction of DA neurons in the nigrostriatal pathway, sparing the mesolimbic pathway. The degree of long-term neurodegeneration produced by MDMA appears to be closely related to the magnitude of the hyperthermic response [5]. Attenuation of the hyperthermia alleviates MDMA-induced loss of striatal dopamine [3].

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. The major pathological hallmark of PD is the degeneration of DAergic neurons in the substantia nigra that innervate the striatum. The major symptoms of PD include tremor, bradykinesia, cogwheel rigidity, and postural instability, which arise from the degeneration of

DAergic neurons in the substantia nigra. PD is a neurodegenerative disorder of multifactorial origin, and mutations in the gene encoding parkin, an E3 ubiquitin-protein ligase [6], are frequently observed in autosomal recessive parkinsonism in humans. The loss of parkin function has been suggested to result in aberrant accumulation of parkin substrate proteins [6]. Accumulation of these proteins has been postulated to confer toxicity to DAergic neurons in the substantia nigra [7].

In the present study, we hypothesized that parkin protects against neurotoxic insult, and we attempted to clarify the role of parkin in MDMA-induced hyperthermia, one of the causal factors of neuronal damage, using parkin knockout mice.

MATERIALS AND METHODS

Mice

Wildtype, heterozygous, and homozygous parkin knockout mice were prepared from heterozygous/heterozygous parkin knockout mouse crosses (21-37 g, 12-29 weeks of age). Mice were housed in an animal facility maintained at $22 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ relative humidity under a 12/12 h light/dark cycle with lights on at 8:00 a.m. Food and water were available *ad libitum*. All behavioral testing was conducted during the light cycle. The experimental procedures and housing conditions were approved by the Institutional Animal Care and Use Committee of the Tokyo Institute of Psychiatry, and all animals were treated humanely in accordance with our institutional animal experimentation guidelines.

Body Temperature Measurement

Rectal temperature measurement was performed using a digital thermometer (BAT-12; Physitemp Instruments Inc.,

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Clifton, NJ, USA) with 0.1°C accuracy and a rectal probe for mice (RET-3, Physitemp Instrument Inc.). Each mouse was lightly restrained by hand for approximately 20 s while the probe was inserted approximately 2 cm into the rectum and a steady reading was obtained. Body temperature was measured rectally before and 15, 30, 45, and 60 min after intraperitoneal (i.p.) injection of MDMA (30 mg/kg) at an ambient temperature of $22 \pm 2^\circ\text{C}$.

Drugs

MDMA was synthesized at Matsuyama University College of Pharmaceutical Sciences and freshly dissolved in saline. MDMA and vehicle were administered in a volume of 0.1 ml/10 g body weight.

Statistical Analysis

Mean and standard error were calculated from the values of 12-17 subjects. Changes in body temperature were analyzed by repeated-measures analysis of variance (ANOVA) followed by Scheffe's *post hoc* test. Baseline temperature and changes in body temperature areas-under-the-curve (AUC) were analyzed by one-way ANOVA and Scheffe's *post hoc* test.

RESULTS

Baseline Body Temperature in Parkin Knockout Mice

Baseline body temperature was measured before MDMA injection at room temperature ($22 \pm 2^\circ\text{C}$). No significant difference in baseline body temperature was observed among wildtype, heterozygous, and homozygous parkin knockout mice (Fig. 1).

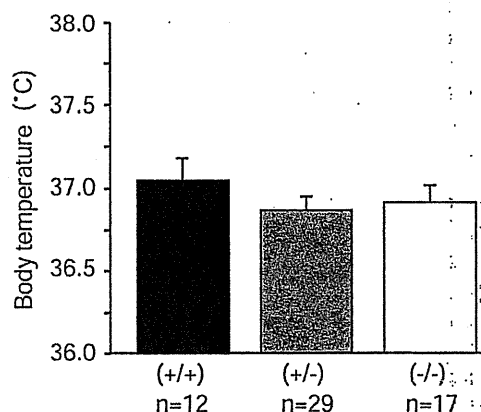


Fig. (1). No significant differences in baseline body temperature were observed among wildtype, heterozygous, and homozygous parkin knockout mice. Body temperature prior to MDMA injection did not significantly differ among genotypes. Baseline body temperature was analyzed by one-way ANOVA ($F_{2,55} = 0.629$, $p = 0.5369$) at an ambient temperature of $22 \pm 2^\circ\text{C}$.

No Sex Differences in MDMA-Induced Hyperthermia

Body temperature was measured 15, 30, 45, and 60 min after i.p. injection of MDMA (30 mg/kg) at an ambient temperature of $22 \pm 2^\circ\text{C}$. No significant differences in MDMA-induced hyperthermia were observed between males and females within each genotype (Fig. 2).

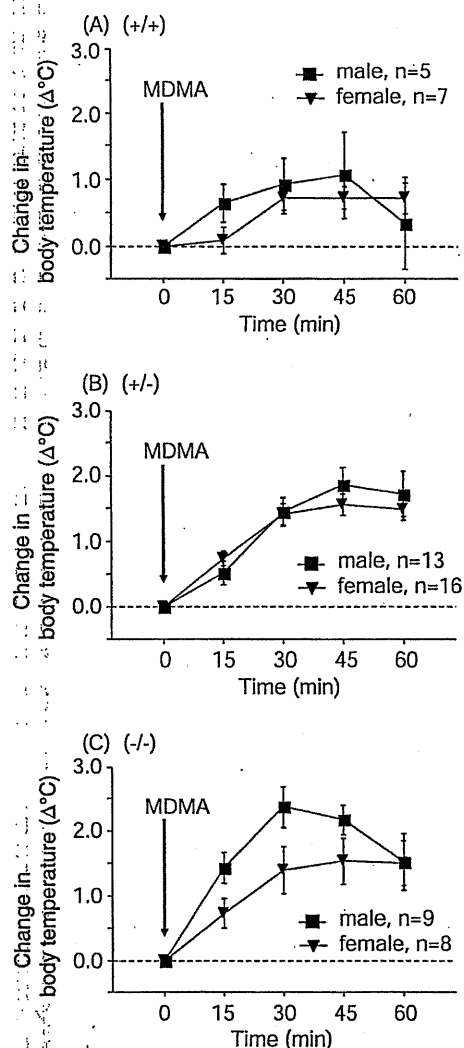


Fig. (2). Similar MDMA-induced (30 mg/kg, i.p.) hyperthermia was observed in male and female mice within each genotype. Body temperature areas-under-the-curve were analyzed by repeated-measures ANOVA. (A) Sex, $F_{1,10} = 0.181$, $p = 0.6796$; Time, $F_{4,40} = 4.741$, $p = 0.0032$; Sex \times Time interaction, $F_{4,40} = 1.124$, $p = 0.3587$. (B) Sex, $F_{1,27} = 0.134$, $p = 0.7170$; Time, $F_{4,108} = 62.705$, $p < 0.0001$; Sex \times Time interaction, $F_{4,108} = 1.231$, $p = 0.3021$. (C) Sex, $F_{1,15} = 2.350$, $p = 0.1461$; Time, $F_{4,60} = 26.22$, $p < 0.0001$; Sex \times Time interaction, $F_{4,60} = 2.059$, $p = 0.0974$.

Enhancement of MDMA-Induced Hyperthermia in Parkin Knockout and Heterozygous Mice

Body temperature gradually increased from baseline after MDMA injection in all genotype groups. MDMA significantly enhanced hyperthermia from 15 to 45 min after injection in parkin knockout mice and from 45 to 60 min after injection in heterozygous mice compared with wildtype mice (Fig. 3A). MDMA produced hyperthermia, with a maximum increase of 0.9°C (37.9°C) 45 min after injection in wildtype mice, 1.7°C (38.6°C) 45 min after injection in heterozygous mice, and 1.9°C (38.8°C) 30 min after injection in parkin knockout mice. Body temperature AUC values reflected significantly enhanced hyperthermia in parkin knockout and heterozygous mice compared with wildtype mice (Fig. 3B).

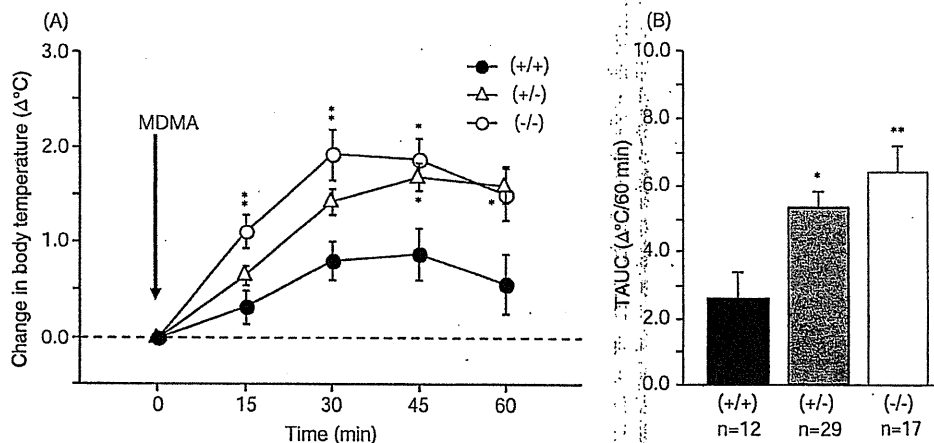


Fig. (3). Enhanced MDMA-induced hyperthermia in heterozygous and homozygous parkin knockout mice compared with wildtype mice. (A) Change in body temperature in mice injected with MDMA (30 mg/kg, i.p.). Body temperature areas-under-the-curve were analyzed by repeated-measures ANOVA (Genotype, $F_{2,55} = 6.746$, $p = 0.0024$; Time, $F_{4,220} = 61.267$, $p < 0.0001$; Genotype \times Time interaction, $F_{8,220} = 3.664$, $p = 0.0005$) followed by Scheffe's *post hoc* test ($*p < 0.05$, $**p < 0.01$). (B) Change in body temperature areas-under-the-curve (TAUC) shown as an integration of the temperature vs. time curve shown in panel A. TAUC values were analyzed by one-way ANOVA ($F_{2,55} = 6.746$, $p = 0.0024$) followed by Scheffe's *post hoc* test ($*p < 0.05$, $**p < 0.01$).

DISCUSSION

In the present study, significantly enhanced MDMA-induced hyperthermia was observed in parkin knockout and heterozygous mice compared with wildtype mice (Fig. 3B). The enhanced MDMA-induced hyperthermia in parkin knockout mice supports the hypothesis that parkin protects against MDMA-induced neurotoxic insult.

Hyperthermia is one of the major symptoms of acute MDMA-induced toxicity, which has been shown to be affected by body temperature [3]. MDMA produces a rapid enhancement of DA release in the striatum [1] and preoptic anterior hypothalamus [8]. MDMA-induced hyperthermia was blocked by a DA D_1 receptor antagonist [9]. Moreover, both the hyperthermia and augmented DA levels in the preoptic anterior hypothalamus after i.p. MDMA injection were significantly reduced by pretreatment with a D_1 antagonist [8]. Interestingly, Sato *et al.* (2006) [10] reported that D_1 receptor levels in the striatum in parkin knockout mice was higher than in wildtype mice, although no change in TH-positive substantia nigra neurons was found in parkin knockout mice, and no significant decrease in DAT levels was observed in the striatum. Therefore, the enhanced MDMA-induced hyperthermia observed in the present study may be attributable to increased levels of D_1 receptors in parkin knockout mice.

Sato *et al.* (2006) [10] also suggested that presynaptic neurons (i.e., DAergic neurons) are functionally impaired in parkin knockout mice. DA synthesis is significantly decreased and methamphetamine-induced DA release is reduced in parkin knockout mice. Considering that DAergic neurons in the substantia nigra are severely damaged in PD patients, the enhanced MDMA-induced hyperthermia in parkin knockout mice may be attributable to functional impairment of DAergic neurons, although the relationship between hyperthermia and DAergic neuron dysfunction remains to be elucidated.

Additionally, we found no significant difference in baseline body temperature among wildtype, heterozygous, and

homozygous parkin knockout mice (Fig. 1). These data suggest that parkin does not play a crucial role in the system that maintains basal body temperature.

In conclusion, MDMA-induced hyperthermia was enhanced in parkin knockout and heterozygous mice compared with wildtype mice. Parkin is hypothesized to be critical for protecting DAergic neurons from toxic insult, and the present results suggest that parkin plays a protective role against MDMA-induced DAergic neuron neurotoxicity.

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ABBREVIATIONS

ANOVA	=	Analysis of variance
DA	=	Dopamine
DAT	=	Dopamine transporter
MDMA	=	3,4-methylenedioxymethamphetamine
PD	=	Parkinson's disease
TH	=	Tyrosine hydroxylase

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DJ-1 associates with synaptic membranes

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder caused by loss of dopaminergic neurons. Although many reports have suggested that genetic factors are implicated in the pathogenesis of PD, molecular mechanisms underlying selective dopaminergic neuronal degeneration remain unknown. *DJ-1* is a causative gene for autosomal recessive form of *PARK7*-linked early-onset PD. A number of studies have demonstrated that exogenous DJ-1 localizes within mitochondria and the cytosol, and functions as a molecular chaperon, as a transcriptional regulator, and as a cell protective factor against oxidative stress. However, the precise subcellular localization and function of endogenous DJ-1 are not well known. The mechanisms by which mutations in DJ-1 contributes to neuronal degeneration also remain poorly understood. Here we show by immunocytochemistry that DJ-1 distributes to the cytosol and membranous structures in a punctate appearance in cultured cells and in primary neurons obtained from mouse brain. Interestingly, DJ-1 colocalizes with the Golgi apparatus proteins GM130 and the synaptic vesicle proteins such as synaptophysin and Rab3A. Förster resonance energy transfer analysis revealed that a small portion of DJ-1 interacts with synaptophysin in living cells. Although the wild-type DJ-1 protein directly associates with membranes without an intermediary protein, the pathogenic L166P mutation of DJ-1 exhibits less binding to synaptic vesicles. These results indicate that DJ-1 associates with membranous organelles including synaptic membranes to exhibit its normal function.

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder next to Alzheimer's disease and is characterized by motor symptoms as cardinal features such as resting tremor, rigidity, bradykinesia and postural instability. Pathological hallmarks of PD include marked cell loss of dopaminergic neurons in the substantia nigra pars compacta which causes dopamine depletion in the striatum and the presence of intracytoplasmic inclusions known

as Lewy bodies in the remaining neurons (Fearnley and Lees, 1991). Although most of the PD cases are sporadic, approximately 5% of PD patients have clear familial etiology. Thus, the presence of monogenic forms of familial PD tells us that genetic factors contribute to the pathogenesis of PD. Indeed, heterozygous and homozygous mutations in one of the responsible genes have been reported in sporadic cases, suggesting that genetic factors are implicated in the pathogenesis of PD. Until now, 9 genes for familial PD have been reported, and these include *α-synuclein*, *parkin*, *UCH-L*, *PINK-1*, *DJ-1*, *LRRK2*, *ATP13A2*, *PLA2G6*, and *FBXO7* (Hatano et al., 2009).

Previous reports have suggested that DJ-1 functions as a molecular chaperon (Lee et al., 2003), a transcriptional regulator (Kim et al., 2005; Niki et al., 2003; Shinbo et al., 2005; Takahashi et al., 2001), and as a cell protective factor against oxidative stress (Canet-Aviles et al., 2004; Taira et al., 2004b; Yokota et al., 2003). The localization of DJ-1 has been shown to be in mitochondria, cytosol, nucleus, and microsomes (endoplasmic reticulum (ER) and Golgi) (Bonifati et al., 2003; Canet-Aviles et al., 2004; Miller et al., 2003; Taira et al., 2004a). However, most studies have been performed by exogenous DJ-1 using overexpression systems. On the other hand, endogenous DJ-1 is present in synaptic terminals, in both axons and dendrites, as well as

Abbreviations: PD, Parkinson's disease; FRET, Förster resonance energy transfer; WT, wild type; ER, endoplasmic reticulum; KO, knockout; RT, room temperature; PBS, phosphate-buffer saline; FBS, fetal bovine serum; BSA, bovine serum albumin; Tfn-R, transferrin receptor; IR, immunoreactivity; HB, homogenizing buffer.

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in mitochondria (Olzmann et al., 2007; Zhang et al., 2005). However, the precise function and dynamics of DJ-1 related to vesicular trafficking remain unclear. In the present study, we demonstrate the association of endogenous DJ-1 with membranous organelles and the molecular interaction of recombinant DJ-1 protein with membranes in cultured cells. In addition, we examine whether pathogenic mutations found in *PARK7*-linked early onset PD patients may be affected by binding activities of DJ-1.

Materials and methods

Antibodies and recombinant proteins

Mouse monoclonal antibody (M043-3, Clone 3E8) and rabbit polyclonal antibody (NB300-270) for DJ-1 were obtained from Medical & Biological Laboratories Co. (MBL, Nagoya, Japan) and Novus Biologicals, Inc. (Littleton, CO), respectively. Rabbit polyclonal antibodies to Rab3A (sc-308), Rab4A (sc-312), Rab5B (sc-598), and Tom20 (sc-11415) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and Rab7B (R4779) was obtained from Sigma (St. Louis, MO). Mouse monoclonal antibodies to synaptophysin were purchased from Chemicon International, Inc. (MAB5258, Temecula, CA) (used for immunoblotting) and Progen Biotechnik (61012, Heidelberg, Germany) (used for immunocytochemistry). Synaptotagmin (610434) and NMDAR1 (556308) were obtained from BD Biosciences Pharmingen (San Diego, CA). Other primary antibodies were Rab3A (107111, Synaptic Systems, Gottingen, Germany), anti-human transferrin receptor (13-6800, Zymed Laboratories, South San Francisco, CA), Parkin (#4211) and Calnexin (#2679S) (Cell Signaling, Danvers, MA), VAMP2 (NB300-595, Novus Biologicals, Inc.), BIP2 (ab21685, Abcam, Cambridge, MA), Hsp70 (610608, BD Transduction Laboratories), Mito Tracker Red CMXRos (M-7512, Molecular Probes), and total OXPHOS rodent WB antibody cocktail (MS604; MitoSciences, Eugene, Oregon). Secondary antibodies conjugated to horseradish peroxidase were purchased from GE Healthcare Bio-Sciences (Piscataway, USA). From Invitrogen Molecular Probes, 488 and 546 conjugated secondary antibodies were purchased. The vectors encoding GST-tagged WT and mutants DJ-1 (M26I, A104T, D149A, and L166P) were kindly provided by Hiroyoshi Ariga (Laboratory of Pharmaceutical Science, Hokkaido University).

Experimental animals (DJ-1 KO mice)

The DJ-1 KO mice (F2) were a kind gift from The Laboratory of Pharmaceutical Science, Hokkaido University. The DJ-1 KO mice were generated at the Center for Neurologic Diseases, Brigham and Women's Hospital Program in Neuroscience, Harvard Medical School (Goldberg et al., 2005). F2 progeny were backcrossed for five generations to C57BL/6 mice, and heterozygotes were intercrossed to generate homozygous mice for the targeted *DJ-1* allele. For the experiments, C57BL/6j mice and DJ-1 KO mice were used at 7 to 9 weeks of age. All animal experiments were carried out in accordance with the Ethics Review Committee for Animal Experimentation of Juntendo University School of Medicine.

Cell culture and transfection

SH-SY5Y cells and HeLa cells were grown in Dulbecco's modified Eagle's medium (D-MEM, Sigma) with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin–streptomycin (PS; Invitrogen). SH-SY5Y cell culture medium was supplemented with 1% non-essential amino acid, 1% sodium pyruvate, and 1% L-glutamate (Invitrogen). The cells were cultured at 37 °C and 5% CO₂. PC12 cells were grown in D-MEM with 5% FBS and 10% horse serum. Primary cortical neurons containing glia cells were prepared from E15.5 C57BL/6j mice and cultured for growth on Fisher-brand cover glass (Fisher Scientific, Pittsburgh, USA) in starting

medium (F12 and Minimum Essential Medium with 10% FBS, 1% PS, and 0.001% insulin) for 3 days, and incubated sequentially for 5 days with 0.5 μM Ara-C (Sigma) in maintenance medium (F12 and Minimum Essential Medium with 5% calf serum, 5% horse serum, 1% PS, and 0.001% insulin). HeLa cells were transfected with expression vectors for FLAG-DJ-1 WT, M26I, A104T, D149A, or L166P by using FuGENE HD Transfection Reagent (Roche). After 24 h, immunocytochemistry was performed on the cells.

Immunocytochemistry

Cells were fixed for 10 min in 4% paraformaldehyde and 0.5% sucrose in phosphate-buffered saline (PBS). The cells were permeabilized with PBS containing 0.2% Triton X-100 (Sigma) for 5 min at RT. For blocking, 1× BlockAce (Yukijirushi Co., Osaka, Japan) was used for SH-SY5Y cells, and 10% FBS and 1% bovine serum albumin (BSA) in PBS (primary cortical neurons from mice) was used for primary cortical neurons for 30 min. Cells were incubated overnight with primary antibodies at 4 °C. The cells were washed 3 times with PBS and were incubated at RT for 1 h with secondary antibodies. After the cells were washed 3 times with PBS, the slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and analyzed using a Leica confocal microscopy.

Preparation of synaptosome fractions from mouse brain

Synaptic vesicles were prepared as described previously (Hatano et al., 2007; Hell, 1998), with some modification. Briefly, whole brains from 3 mice (C57BL/6j) at 7 to 9 weeks of age were placed into 8 ml ice-cold synaptosomal homogenizing buffer (HB) (0.32 M sucrose, 4 mM HEPES–NaOH, pH 7.4 with EDTA-free protease inhibitor cocktail Complete Mini, EDTA free). The tissues were homogenized using a glass-Teflon homogenizer (10 up and down strokes, 830 rpm). The homogenized brain sample was centrifuged at 1000g for 10 min at 4 °C. After the supernatant (S1-1) was removed, the pellet was resuspended in 5 ml HB and was homogenized and centrifuged at the same speed. The supernatant (S1-2) was removed, and the pellet was resuspended in 3 ml HB, and was homogenized and centrifuged in the same manner. The pellet was considered the P1 fraction, while the supernatant (mixed with S1-1, S1-2, and S1-3) was centrifuged at 12,000g for 15 min at 4 °C. The supernatant (S2) was removed and the pellet (P2) was resuspended with HB, and then centrifuged for 15 min at 13,000g at 4 °C. After removal of the supernatant (S2'), the pellet (P2') was collected as the crude synaptosome fraction. P2' was subsequently re-suspended with HB to a final volume of 1 ml. The P2' fraction was suspended with 4 ml of ice cold water in the EDTA-free protease inhibitor cocktail. The samples were homogenized by 6 up and down strokes of the glass-Teflon homogenizer at 830 rpm and mixed with 39 μl 1 M HEPES, pH 7.4, then centrifuged for 20 min at 33,000g at 4 °C. The lysate pellet was considered the LP1 fraction, and the supernatant (LS1) was centrifuged for 2 h at 260,000g at 4 °C. After the supernatant (LS2) was removed, the pellet (LP2) was resuspended with 300 μl of HB. To loosen the pellet, samples were extruded consecutively through a 23-gauge and a 26-gauge hypodermic needle attached to a 1 ml syringe. The concentration of protein in each of the fractions was calculated using the BCA protein assay kit (Pierce, Rockford, IL). Finally, the same amounts of proteins from each fraction were analyzed by SDS–PAGE followed by immunoblotting.

Sucrose gradients of LS1 fraction from mouse brain

The LS1 fraction was layered on top of a linear sucrose density gradient ranging from 0.2 to 2.0 M sucrose dissolved in HEPES buffer (pH 7.4), and ultra-centrifuged at 465,000g for 13 h at 4 °C. Each of the fractions (0.5 ml) was collected from the top of the gradient, and equal volumes of each fraction were subjected to SDS–PAGE followed by immunoblotting.

Preparation of magnetic beads cross-linked with antibodies

For the following experiments of immunoisolation and immunoprecipitation, the DJ-1 polyclonal antibody and the synaptophysin antibody, and the normal rabbit IgG and the normal mouse IgG as control, were cross-linked to protein G-coated magnetic beads (Dyna-beads Invitrogen). The beads were washed 3 times with citrate buffer, and then 50 μ l of magnetic bead slurry was combined with 50 μ g of each antibody by rotating for 1 h at RT. The antibody-bound beads were washed 3 times with 0.2 M sodium borate buffer (pH 9.0), and then resuspended in 0.2 M sodium borate buffer containing dimethyl pimelimidate (Pierce Biotechnology). After reacting by rotating the samples for 1 h at RT, the supernatants were removed and the Dyna-bead pellets were washed 3 times with 0.2 M triethanolamine buffer (pH 8.0). The washed beads were suspended with 0.2 M triethanolamine buffer containing 50 mM glycine, and were reacted for 2 h at RT. The supernatant was removed and the beads were washed 3 times with PBS, stored at 4 °C with PBS containing 0.05% Tween 20, and used within 1 week of the reactions.

Immunoisolation and immunoprecipitation of LS1 fraction containing synaptic vesicles from the mouse brain

Immunoisolation: beads cross-linked with DJ-1 antibody and synaptophysin antibody, or beads cross-linked with normal rabbit IgG and normal mouse IgG were washed 6 times with PBS and were blocked for 1 h at RT using PBS containing 10% BSA as nonspecific competitor, followed by washing in PBS 3 times. In addition, each of the 1 ml LS1 fraction samples were immunoisolated with 37.5 μ l of the beads cross-linked with antibody for a total of 12 h at 4 °C after blocking non-specific sites by rotating with the beads with the cross-linked normal rabbit IgG, or normal mouse IgG for 1 h at 4 °C. The pellets and supernatants were subjected to SDS-PAGE followed by immunoblotting using antibodies against the indicated proteins.

Immunoprecipitation: beads cross-linked with the antibodies, the same as in the immunoisolation protocol, were blocked using PBS containing 10% BSA for 1 h at RT. LS1 fractions (900 μ l) were dissolved in 100 μ l of 10 \times RIPA buffer (final concentration: 140 mM KCl, 20 mM HEPES-KOH (pH 7.3), 2 mM EDTA, protease inhibitors, and 1% Triton X-100), and then the samples were blocked by rotating with normal IgG for 1 h at 4 °C. The supernatants were immunoprecipitated with 12.5 μ l of each of the beads cross-linked with antibody overnight at 4 °C. The pellets and supernatants were subjected to SDS-PAGE followed by immunoblotting using antibodies against the indicated proteins.

Förster resonance energy transfer (FRET)

Synaptophysin-YFP and pCAGGS-CFP vector were a kind of gift from the Department of Cellular Neurobiology Graduate School of Medicine University of Tokyo. CFP-DJ-1 and CFP-VAMP2 were generated by fusing in frame to the DJ-1 N-terminal or VAMP2 N-terminal coding region in the pCAGGS-CFP vector. HeLa cells were transfected with expression vectors for CFP-DJ-1 or CFP-VAMP2, and synaptophysin-YFP using FuGene HD (Roche), according to the manufacturer's instruction. After 24 h, the cells were imaged with an IX70 inverted microscope (Olympus, Tokyo, Japan) equipped with BioPoint MAC5000 excitation and emission filter wheels (Ludl Electronic Products Ltd., Hawthorne, NY) and a Cool SNAP-HQ cooled CCD camera (Roper Scientific, Trenton, NJ). The filters used were purchased from Omega Optical Inc. (Brattleboro, VT): two excitation filters, XF1071 (440AF21) for CFP and Förster resonance energy transfer (FRET), and XF1068 (500AF25) for YFP; an XF2034 (455DRLP) dichroic mirror; two emission filters, XF3075 (480AF30) for CFP and XF3079 (535AF26) for FRET and YFP. Cells were illuminated with a 75 W xenon lamp through a 6% ND filter. Exposure times for 3 \times 3 binning were 100 ms to obtain fluorescence

images and 20 ms to obtain differential interference contrast image. MetaMorph software (Universal Imaging, West Chester, PA) was used to control the CCD camera and filter wheels, and also for the analysis of the cell image data.

Sensitized FRET measurement was performed using the method by Gordon et al. (1998). Briefly, fluorescence images for more than 20 cells were acquired sequentially through YFP, CFP, and FRET filter channels. The background was subtracted from raw images before FRET calculations. The fractions of the bleed-through of CFP and YFP fluorescence through the FRET channel were 0.502 and 0.385, respectively. Corrected FRET (FRET_C) was therefore calculated on a pixel-by-pixel basis for the entire image by using the equation: FRET_C = FRET - 0.502 \times CFP - 0.385 \times YFP, where FRET, YFP, and CFP correspond to background-subtracted images of cells co-expressing CFP and YFP. Calculated FRET_C values are expressed as box and whisker plots, where the highest and lowest boundaries of the box represent the 25th and 75th percentiles, respectively, and whiskers above and below the box designate the 10th and 90th percentiles, respectively; the line within the box indicates the median value. FRET_C images are also presented in the pseudocolor mode.

Alternatively, 293F cells (Invitrogen) were transfected with expression vectors for CFP-DJ-1 or CFP-VAMP2, and synaptophysin-YFP using 293fectin (Invitrogen) according to the manufacturer's recommendation. After 24 h, the cells were analyzed by a Flicyme-300 flow cytometer (Mitsui engineering and Shipment, Tokyo, Japan), which is equipped with a 445 nm semiconductor laser and is able to measure the fluorescence lifetime of CFP in the frequency domain at a single cell level. Data were acquired using the machine-bundled software, and exported to FlowJo flow cytometry analysis software (Tree Star, Inc., Ashland, OR). Using a gate tool, a population that expresses both CFP and YFP was selected, and FRET efficiency (*E*) of each cell was calculated by the following equation: $E = 1 - \tau_d' / \tau_d$, where τ_d' and τ_d are donor (CFP) lifetimes in the presence and absence of the acceptor chromophore, respectively. *E* values of all analyzed cells were plotted in box and whisker plots.

Confocal laser scanning microscopy

Confocal images were obtained using an FV-10i confocal microscope (Olympus, Tokyo, Japan). Image data were exported to MetaMorph software and fluorescence intensities on lines of interest were gauged by the "Line Scan" function and plotted.

Cell fractionation

For cell fractionation studies, cultured cells (PC12) were washed with PBS, scraped off the culture plate in PBS, and centrifuged at 600g for 5 min. Cell pellets were resuspended in homogenization buffer (20 mM HEPES pH 7.2 and 0.25 M sucrose) in the presence of a cocktail of protease inhibitor (Complete Mini, EDTA-free), and sonicated at 4 °C (10 s, 3 times). The nuclei and unbroken cells were then pelleted by centrifugation at 1000g for 10 min at 4 °C. The supernatant was centrifuged at 100,000g for 1 h at 4 °C to separate the cytosolic and membrane fractions. To study the effects of salts and non-ionic detergent on the solubilization of DJ-1, the membrane fractions were incubated on ice for 30 min with homogenization buffer with 50, 150, and 1000 mM sodium chloride or 1% Triton X-100. After separation of the soluble and insoluble materials by centrifugation (100,000g, for 1 h, at 4 °C), equal volumes of each fraction were subjected to immunoblot with DJ-1, Parkin, and Tfn-R antibodies.

Proteinase K (PK) digestion of PC12 cell membrane fractions

Membrane fractions were isolated from PC12 cells and incubated with 0, 20, 40, 60, 80, and 120 μ l of Proteinase K (PK)-agarose (Sigma) at 30 °C with rotation for 1 h. PK beads were removed from the