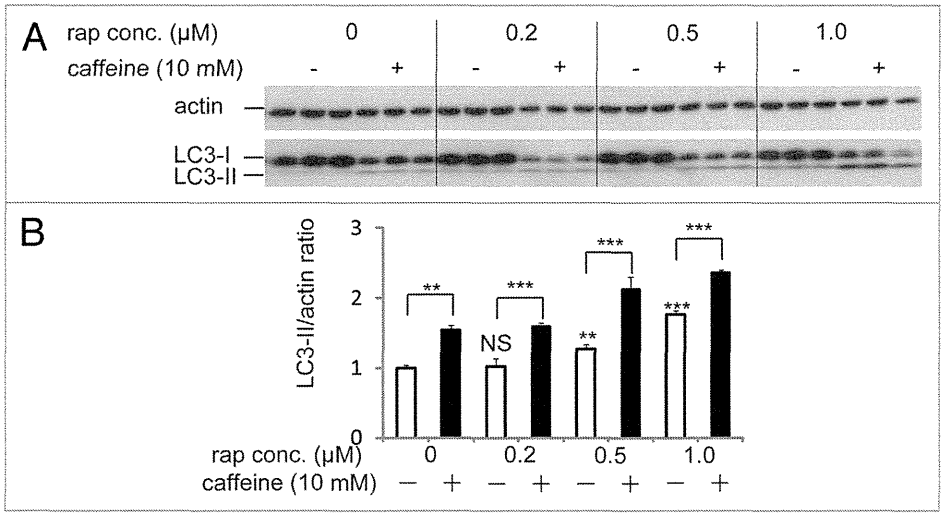


Figure 5. Rapamycin treatment with caffeine has an additive effect on enhancement of autophagy. (A) SH-SY5Y cells treated with various concentrations of rapamycin with or without 10 mM caffeine for 48 hours were analyzed by immunoblotting. (B) Densitometry analysis was performed using three independent experiments. Error bars, SD; *p < 0.05; **p < 0.01; N.S., not significant.

Materials and Methods

Cell line. HeLa cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 100 U/ml penicillin/streptomycin (Sigma) at 37°C and 5% CO_2. PC12D and SH-SY5Y cells were maintained in DMEM (Sigma) supplemented with 10% FBS (Sigma), 5% horse serum and 100 U/ml penicillin/streptomycin at 37°C and 5% CO_2. All experiments with PC12D were performed after differentiation with NGF treatment for 48 hours. *Atg7*$^{+/+}$ and $^{-/-}$ MEFs were maintained in DMEM (Sigma) supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 1% sodium pyruvate (Gibco, 11360), 1% non-essential amino acid (NEAA) and 4.2 μl 2% beta-mercaptoethanol at 37°C.

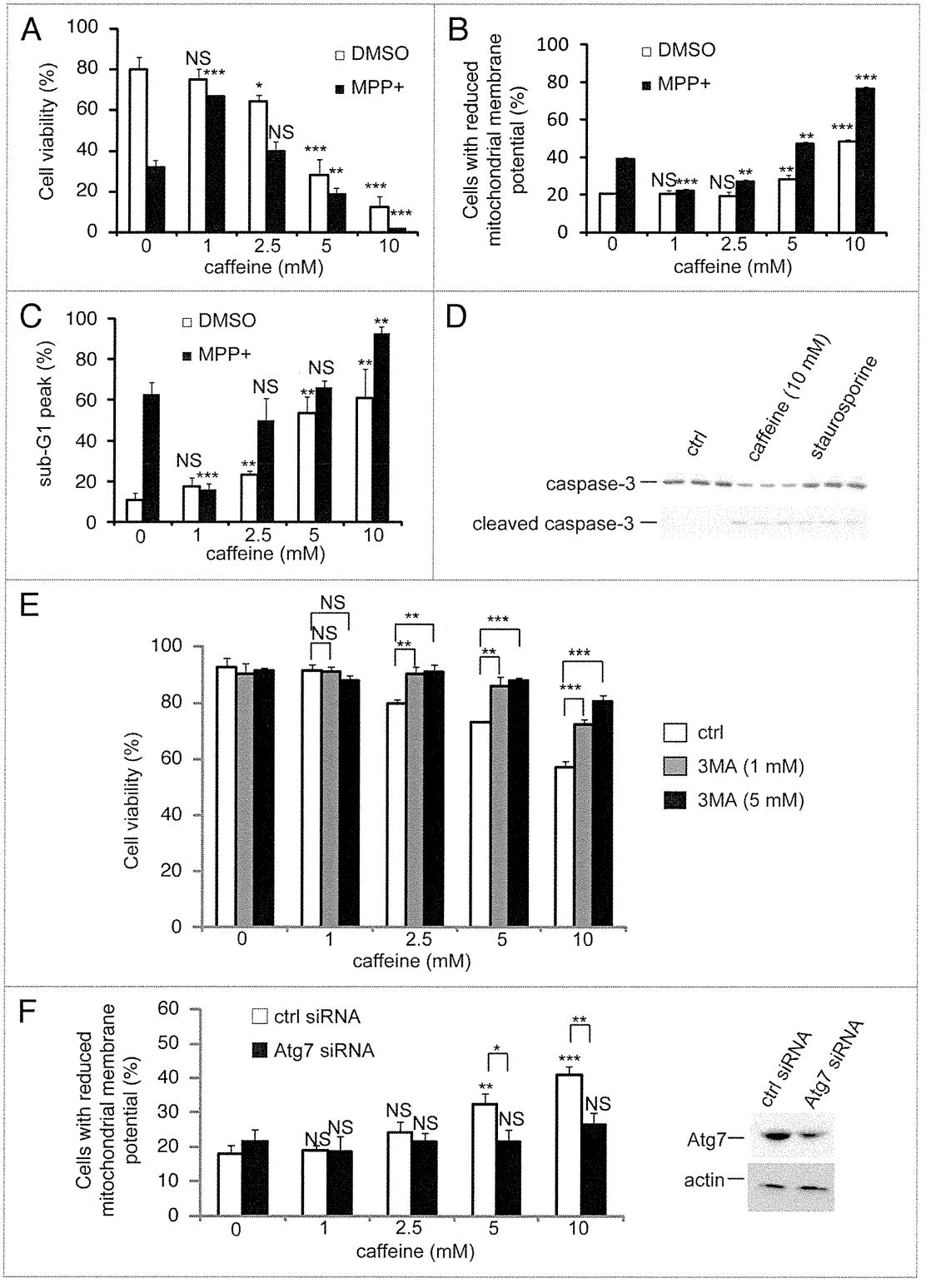
To establish a HeLa GFP-LC3 stable cell line, proliferating HeLa cells were transfected with a GFP-LC3 plasmid.[14] Forty-eight hours post-transfection with Lipofectamine 2000 (Invitrogen), positive stable clones were selected by growing cells with G418 (400 μg/ml) for 2 weeks and maintained in DMEM (Sigma) supplemented with 10% FBS (Sigma), 100 U/ml penicillin/streptomycin and 200 μg/ml G418 at 37°C and 5% CO_2. All cellular experiments were performed with cells cultured in complete medium with FBS as explained above.

Cell viability assays. A trypan blue dye (Invitrogen, 15250-061) exclusion assay was used to examine cell viability and performed according to previously reported protocols.[40,41] Changes of mitochondrial membrane potentials were assessed also with the lipophilic cationic membrane potential-sensitive dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraehylbenzimidazolylcarbocyanineiodide) (Wako, 106-00131) according to the manufacturer's protocol. Detection of early apoptotic cells was determined using an annexin V/propidium iodide (PI) detection kit (Invitrogen), according to the manufacturer's protocol. Briefly, 0.5 x 10^6 *Atg7*$^{+/+}$ or $^{-/-}$ MEFs were exposed to caffeine (0–25 mM) for 24 hours and washed twice. Then, they were incubated at room temperature with annexin V/Alexa488 and PI for 15 minutes. Annexin V$^+$PI$^-$ cells, considered as early apoptotic cells, were enumerated using FACScan (BD Biosciences). Data were analyzed with CellQuest (BD Biosciences) and FlowJo softwares (Tree Star Inc.). Cells positive or negative for annexin V were regarded as apoptotic or non-apoptotic cells, respectively.

Cell cycle analysis. To examine apoptosis, 1.0 x 10^4 cells/well PC12D cells were seeded onto 96-well culture plate and incubated for 48 h in DMEM with NGF and treated with caffeine for 72 h. The cells were harvested and washed with PBS and fixed with ice-cold 70% ethanol at 4°C for 2 h. The cells were then stained with PI solution according to previously reported protocol.[41] DNA content was analyzed by flow cytometry using FACScan and CellQuest software (BD Biosciences).

Compounds. Compounds used included caffeine (Wako, 031-06792), E64d (Sigma, E8640), pepstatin A (Sigma, P5318), rapamycin (LC Laboratories, R5000), CCI-779 (Selleck Chemicals, S1044), MPP$^+$ (Sigma, M0896), bafilomycin A1 (Sigma, B1793), 3-methyladenine (Sigma, M9281), insulin (Sigma, I0516), U0126 (Sigma, U120), Akt1/2 inhibitors (Sigma, A6730), staurosporine (Cell Signaling Technology, 9953) and DMSO (Sigma, D2650).

Figure 6 (See next page). Caffeine induces apoptosis by enhancement of autophagy. (A) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP$^+$ for 72 hours, cell viability was measured using trypan blue dye exclusion assay. Data are the means of triplicate experiments. (B) After cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP$^+$ for 48 hours, mitochondrial membrane potential was analyzed by JC-1 using a flow cytometry. Data are the means of triplicate experiments. (C) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP$^+$ for 72 hours, caffeine-induced sub G_1 area was analyzed by propidium iodide staining assay using a flow cytometry. Data are the means of triplicate experiments. (D) PC12D cells were treated with H_2O or caffeine for 24 hours or staurosporine (positive control) for 3 hours and analyzed with immunoblotting for levels of caspase-3 and cleaved caspase-3. (E) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with or without 1, 3 or 5 mM 3MA for 24 hours, cell viability was measured by trypan blue dye exclusion assay. (F) PC12D cells were transfected with control siRNA or siRNAs targeting Atg7. Forty eight hours later, they were treated with 0, 1, 2.5 or 10 mM caffeine for 24 hours and mitochondrial membrane potential was analyzed using JC-1. The knockdown effects on *Atg7* were confirmed by immunoblotting using antibodies against *Atg7* and actin. Data are the means of triplicate experiments. Error bars, S.D. NS, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.



Plasmid DNAs. Myrystoylated Akt (21–151), a constitutively active form of Akt, was purchased from Millipore.

siRNA knockdown experiments. PC12D cells were transfected with rat Atg7 siRNAs (Invitrogen, 10620318-9) using Lipofectamine RNAiMAX (Invitrogen, 13778-075) according to the manufacturer's protocol.

Western blotting. Cell pellets were lysed on ice in RIPA buffer for 20 minutes in the presence of protease inhibitor (Roche). Western blotting was performed according to a previously published report.[42] The antibodies used were as follows: anti-p70 ribosomal protein (Cell Signaling Technology, 2708), anti-ribosomal protein (Cell Signaling Technology, 2217), anti-4E-BP1

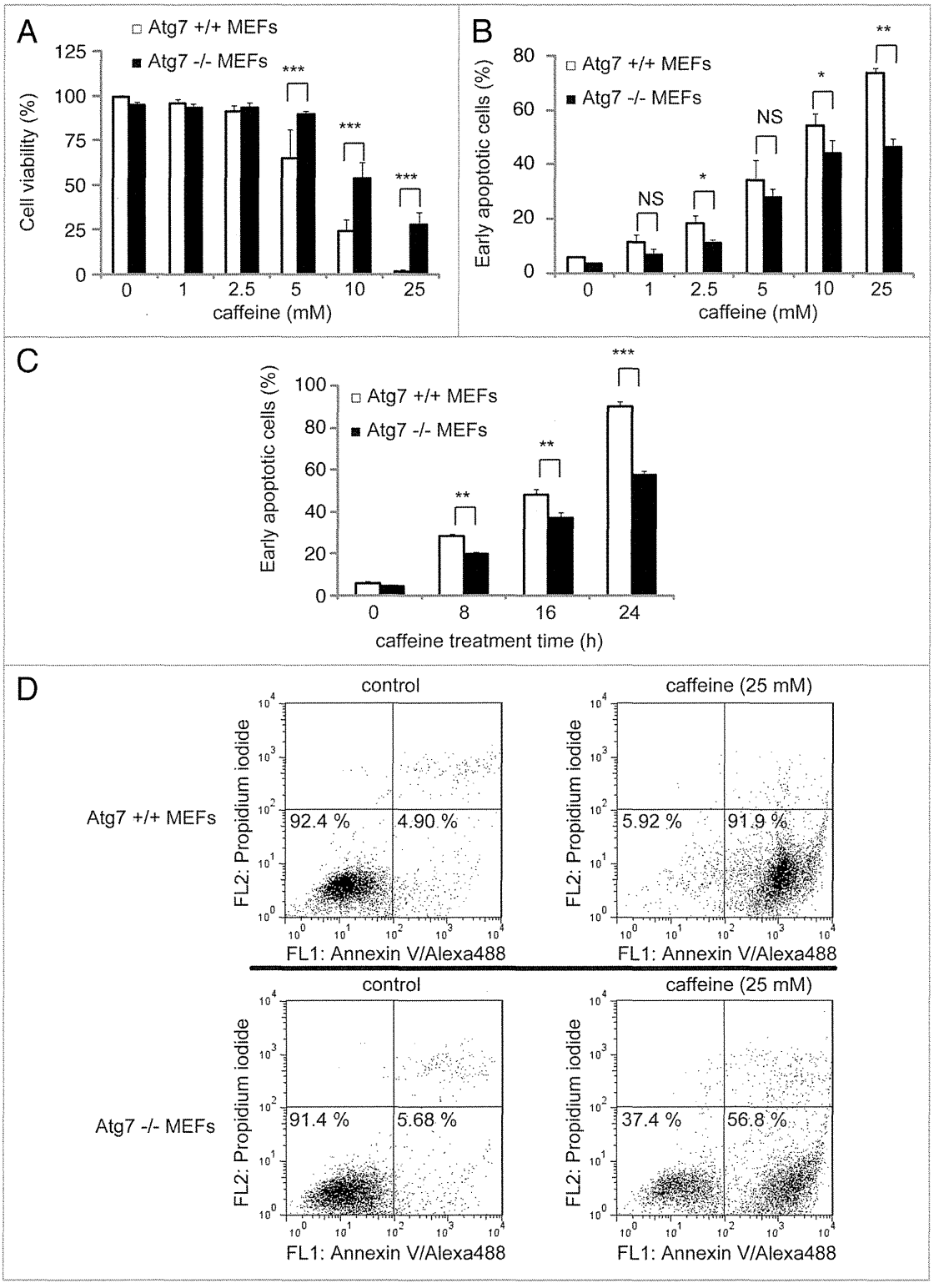


Figure 7. Cells without *Atg7* expression are more resistant to caffeine-induced apoptosis. (A) After *Atg7*$^{+/+}$ or $^{-/-}$ mouse embryonic fibroblasts (MEFs) were treated with 0, 1, 2.5, 5, 10, 25 mM caffeine for 24 hours, the cell viability was measured by trypan blue dye exclusion assay. Data are the means of triplicate experiments. (B–D) Fluorescence-activated cell-sorting analysis for annexin V/propidium iodide (PI). *Atg7*$^{+/+}$ or $^{-/-}$ MEFs were cultured with various concentrations of caffeine for 24 hours (B) or with 25 mM caffeine for various times (0, 8, 16 or 24 hours) (C and D). Annexin V/PI staining was subsequently performed to assess early or late apoptosis and necrosis. 5 x 10^3 cells were analyzed by flow cytometry and the percentage of early apoptotic cells (annexin V-positive and PI-negative cells, the lower right region in (D) was determined). Data are the means of triplicate experiments. Error bars, SD. NS, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

(Cell Signaling Technology, 9452), anti-Akt (Cell Signaling Technology, 9272), anti-p44/42 MAP kinase (Cell Signaling Technology, 9102), anti-phospho-p70 ribosomal protein (Thr389) (Cell Signaling Technology, 9205), anti-phospho-S6 ribosomal protein (Ser235/236) (Cell Signaling Technology, 2211), anti-phospho-4E-BP1 (Thr37/46) (Cell Signaling Technology, 9459), anti-phospho-p44/p42 MAPK (Thy202/Tyr204) (Cell Signaling Technology, 9101), anti-Atg7 (Cell Signaling Technology, 2631), anti-phospho-Akt (Cell Signaling Technology, 4060), anti-actin (Millipore, clone C4), anti-LC3 (MBL, clone 4E12), anti-p62 (Progen Biotechnik, GP62-C) antibodies. Antibody signals were enhanced with chemifluorescent methods from GE HealthCare.

Immunofluorescent microscopy. Cells were embedded with 4% paraformaldehyde for 20 minutes. Following this, they were permeabilized with 0.1% Triton-X in 1x PBS. After incubation with 10% FBS and 1% bovine serum albumin in 1x PBS for 30 minutes, cells were immunostained with anti-LC3B (x500) (Sigma, L7543), anti-LAMP2 (x50) (Development Studies Hybridoma Bank, clone H4B4) overnight and incubated with anti-rabbit IgG tagged with AlexaFluor 488 or anti-mouse IgG tagged with AlexaFluor 546 for 1 hour. The cover slips were embedded with VectaShield, stained with DAPI and images were acquired on a Zeiss LSM510 META confocal microscope (63 x 1.4 NA) or a Leica TCS SP5 confocal microscope at room temperature using Zeiss LSM510 v.3.2 software or Leica LAS AF software. Adobe Photoshop 7.0 (Adobe Systems Inc.) was used for subsequent image processing. For colocalization assay in HeLa cells, an appropriate confocal image was taken with Leica LAS AF software. Then, these images were analyzed automatically with the ImageJ "Colocalization" Plugin (Settings: Each threshold: 25, Ratio: 75%) followed by "Analyze particles" (Settings: threshold 25; Pixel: 1) between endogenous LC3 positive and LAMP2 vesicles. Experiments were done in triplicate at least twice.

Quantification of cells with GFP-LC3 vesicles. HeLa cells stable expressing GFP-LC3 were treated with various concentrations of caffeine for 24 or 48 hours and then fixed as described above. Analyses in triplicate were done for counting the proportion of GFP-positive cells with GFP-LC3 vesicles as previously described in reference 43.

Electron microscopy. SH-SY5Y cells treated with various concentrations of caffeine were prefixed in 2% glutaraldehyde in PBS at 4°C, treated with 1% OsO_4 for 3 hours at 4°C, dehydrated in a graded series of ethanol and flat embedded in epon. Ultra-thin sections were doubly stained with uranyl acetate and observed using a JEOL JEM-2000EX electron microscopy at 80 kV.

Statistical analysis. Densitometry analysis was performed using ImageJ 1.43 on immunoblots from three independent experiments. A t-test was performed with SYSTAT software (Hulinks).

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Note

Supplementary materials can be found at:
www.landesbioscience.com/journals/autophagy/article/14074

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REVIEW

Molecular pathogenesis of Parkinson's disease: update

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ABSTRACT

Parkinson disease (PD) is a neurodegenerative disease characterised by progressive disturbances in motor, autonomic and psychiatric functions. Much has been learnt since the disease entity was established in 1817. Although there are well established treatments that can alleviate the symptoms of PD, a pressing need exists to improve our understanding of the pathogenesis to enable development of disease modifying treatments. Ten responsible genes for PD have been identified and recent progress in molecular research on the protein functions of the genes provides new insights into the pathogenesis of hereditary as well as sporadic PD. Also, genome wide association studies, a powerful approach to identify weak effects of common genetic variants in common diseases, have identified a number of new possible PD associated genes, including PD genes previously detected. However, there is still much to learn about the interactions of the gene products, and important insights may come from chemical and genetic screens. In this review, an overview is provided of the molecular pathogenesis and genetics of PD, focusing particularly on the functions of the PD related gene products with marked research progress.

INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease, named after James Parkinson's who provided a classic account of the condition in 1817. Affecting 1–2% of the population over the age of 65 years, the prevalence of PD increases by approximately 4% in those older than 85 years. Ten genes that contribute to the genetic aetiology of hereditary PD (hPD) were identified, mainly through positional cloning strategies in inherited PD patients and families (table 1).[1 2] Several responsible genes for hPD have been identified, and based on functional studies in vitro and in vivo of gene products, some have been found to interact with each other in various cellular systems for homeostasis, such as synaptic homeostasis (α-synuclein), mitochondrial maintenance (PINK1, parkin, DJ-1, Omi/HtrA2), autophagy–lysosome pathway (α-synuclein, parkin, PINK1, Omi/HtrA2), axonal transport (LRRK2) and ubiquitin proteasome systems (α-synuclein, parkin, DJ-1, UCH-L1). Impairments in a number of cellular systems have been suggested to underlie hPD (figure 1). Also, more recent studies revealed that mutations in the same genes can be involved in familial PD and be risk factors for sporadic PD (sPD), suggesting that inherited and sPD could have common pathological mechanisms.[3] Therefore, understanding the function of the proteins encoded by hPD genes will hopefully further our understanding of the mechanisms leading to inherited and sPD.

In this review, we will summarise the latest research progress in the molecular mechanisms of hPD and genetic association studies of sPD.

HEREDITARY PD

α-Synuclein (PARK1 and PARK4)

Clinicogenetics

SNCA was the first causal PD gene identified in a large Italian family.[4] Mutations (A30P, E46K and A53T), duplications and triplications of the *SNCA* gene have been reported.[2] Clinical features of patients with the E46K mutation are similar to those of dementia with Lewy bodies, while A30P is not associated with severe dementia. Individuals with *SNCA* triplication developed an early onset form of PD with rapid progression and more extended neurodegeneration.[5]

Recent genome wide association studies (GWAS) have demonstrated a strong association between common single nucleotide polymorphism within the *SNCA* locus and PD in European and Japanese population, consistent with the finding that variation at the *SNCA* locus increases PD susceptibility.[6–9] Although the *SNCA* single nucleotide polymorphism associated with sPD show a low OR (1.2–1.4), these findings are consistent with α-synuclein aggregation pathology.

Molecular biology

α-Synuclein is mainly expressed in the presynaptic terminal of the CNS. The protein binds with lipids and unfolds in the steady state. Although the exact function remains unclear, it regulates dopamine homeostasis in presynaptic vesicle cycling.[5] The phenotype of α-synuclein knockout mice is unremarkable and only shows a mild decrease in dopamine levels in the striatum and a mild decrease in synaptic vesicles in the hippocampus. Compared with the wild-type α-synuclein, mutant forms easily aggregate in neuronal cells in vitro and in vivo.[10 11] Transgenic mice with wild or mutant α-synuclein under various promoters have shown neuronal inclusions, mitochondrial abnormalities and neurodegeneration.[12–14] Which type of α-synuclein species is the most toxic to cells remains unclear but some studies assert that mature aggregates are not themselves the toxic moiety but rather an attempt by the cell to clear small toxic oligomers.[15] Hsp90 modulates the assembly of α-synuclein in an ATP

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 blephalospasm
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	FBXO7	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 IBR-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

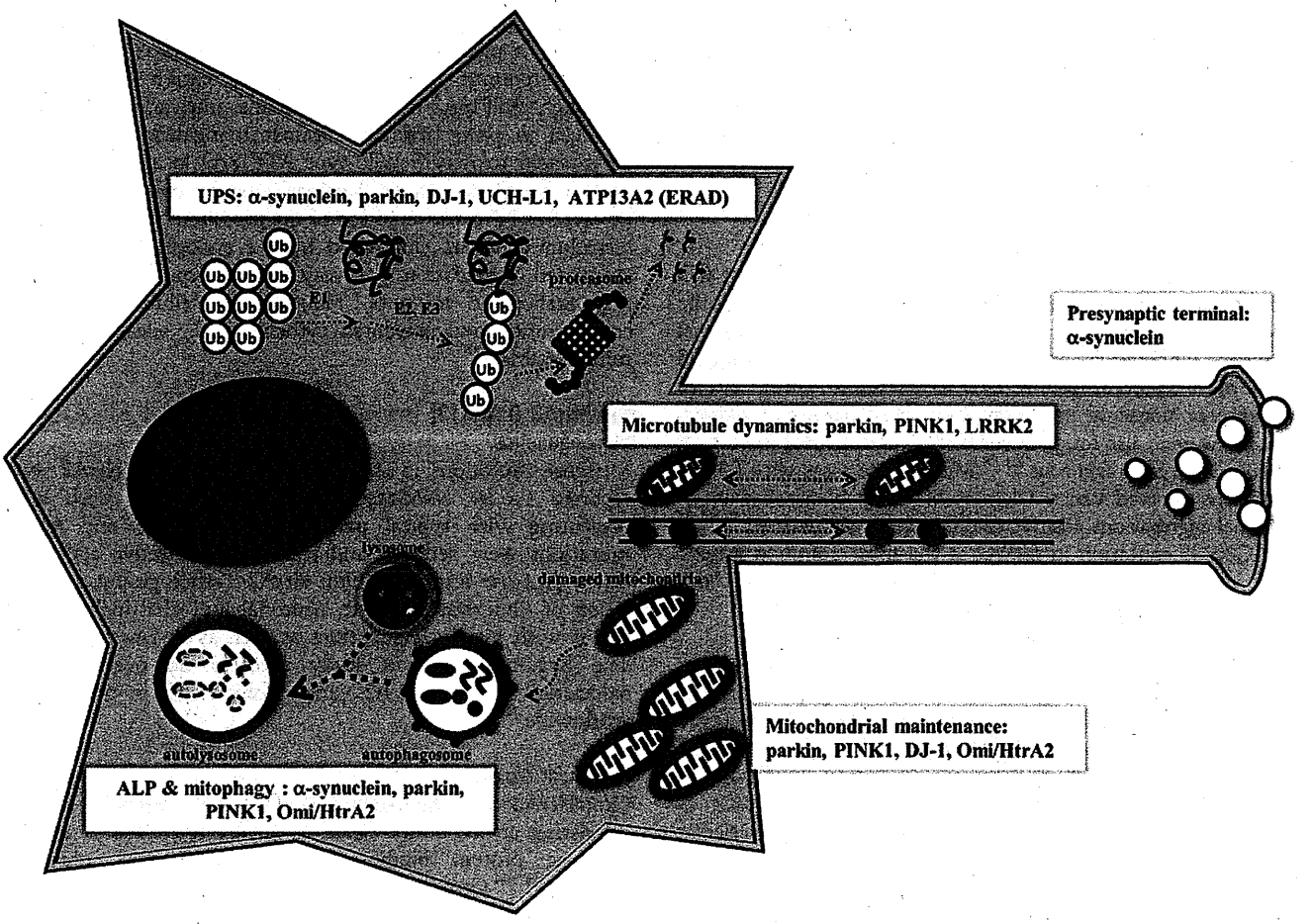

Figure 1 Schematic representation of the possible pathogenesis in hereditary Parkinson's disease. ALP, autophagy–lysosome pathway; ERAD, endoplasmic reticulum associated degradation; Ub, ubiquitin; UPS, ubiquitin proteasome system.

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, blephalospasm 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 LRRK 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

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

FBXO7 (PARK15)

Clinicogenetics

Only three families with mutations in *FBXO7* 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

Fbox7 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 FBXO7 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 supuranuclear 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-phynyl-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 like1-methyl-4-phynyl-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 steadystate; 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

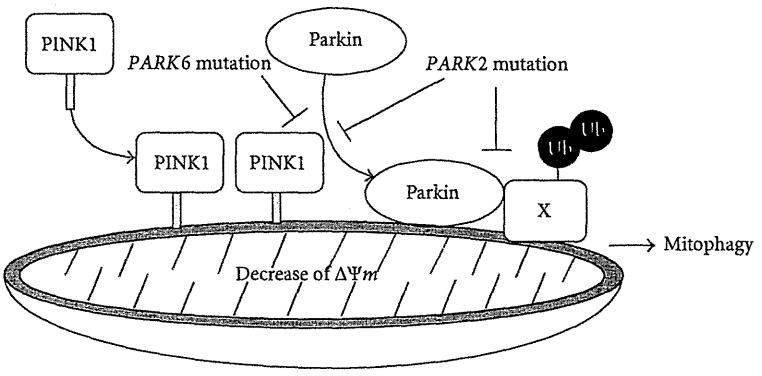

FIGURE 1: Schematic representation of PINK1-parkin-mediated mitophagy. In damaged mitochondria, PINK1 and parkin regulate mitochondrial elimination by inducing mitophagy. Under steady state, PINK1 is cleaved and degraded rapidly in the mitochondria. This process may be inhibited by the mitochondrial 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

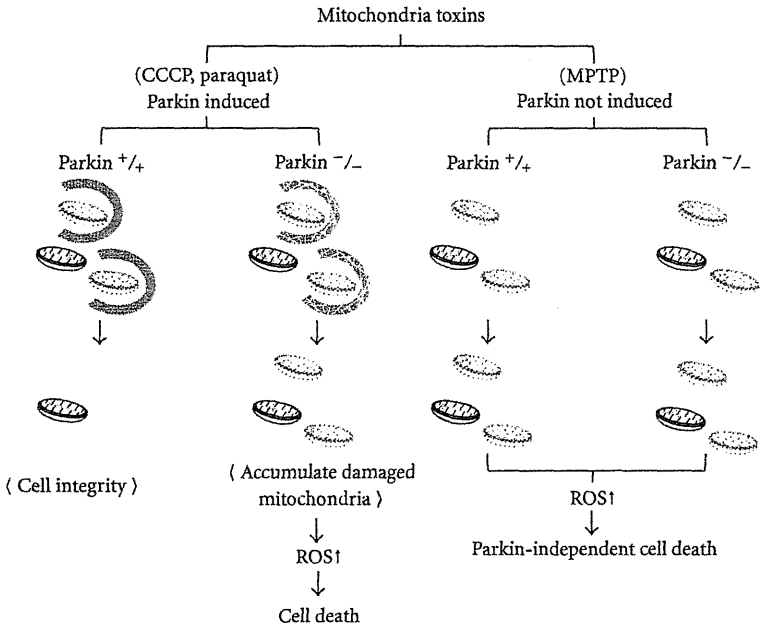

FIGURE 2: Two mechanisms of mitochondrial toxicity and parkin function. The effect of mitochondrial toxicity is different between CCCP and MPTP. Treatment with CCCP recruits parkin to the mitochondria resulting in mitophagy to keep mitochondrial integrity. Parkin deficiency is associated with accumulation 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-phynyl-tetrahydropyridine
PD: Parkinson's disease
PINK1: 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-methylenedioxymethamphetamine) 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 ± 2°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-methylenedioxymethamphetamine (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 ± 2°C and 55 ± 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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