		White series		Asian series		Arab-Berber series	
	Aminoacid	Patients (n=6995)	Controls (n=5595)	Patients (n=1376)	Controls (n=962)	Patients (n=240)	Controls (n=372)
(Continued from previous page)							
rs79546190	V2390M	1 (0·01%)	1 (0·02%)	..	..	..	..
rs78964014	E2395K	1 (0·01%)	0	..	..	..	..
rs60545352	M2408I	1 (0·01%)	0	..	..	0	2 (0·54%)

Data are number (%). PD=Parkinson's disease. +=a variant that was noted with a minor allele frequency of at least 0·5% and as such was analysed as a common variant. ..=a variant that was not noted in the series. NA=a variant that was out of the Hardy-Weinberg equilibrium in the specific series. *Pathogenic variants for which the number (%) of carriers is summarised for the entire sample; any carriers of these pathogenic variants were removed from the summaries provided for each of the remaining non-pathogenic variants.

Table 4: LRRK2 rare variants

activity for the R1398H variant, suggesting this Roc domain substitution might be the most likely functional allele on the haplotype.

Although the results of our study have identified an association of PD only with common variants, they also draw attention to the many rare variants in *LRRK2* that could contribute to disease risk. Genetic loci that contribute to disease risk might do so through variants that span the whole range of minor allele frequencies, from rare mutations to frequent single nucleotide polymorphisms.[28] Despite the very large sample size, we noted only three of seven previously described pathogenic *LRRK2* mutations. Hence, the search for mutations contributing to familial PD should include an analysis of single pedigrees, with further assessment in very large population studies. Single pedigrees might result in some false-positive results, which can be filtered out with large population samples. For example, two variants (I1371V and T2356I) have been proposed as pathogenic and to account for the clinical and functional features of *LRRK2*-associated parkinsonism.[29,30] However, in our study, both variants were noted in patients and controls at the same frequency (table 4). Conversely, we noted other possible rare risk (E334K, R1325Q, and T1410M) and protective (A211V and A1151T) variants; however, because of their low frequency, large meta-analytical approaches are necessary to define their roles fully.

In this study, we focused on exonic variants because all pathogenic variants identified in *LRRK2* so far have been single nucleotide missense changes. However, silent, synonymous variants were also included because they can result in alternative splicing and, since protein translation is a function of codon use and transfer RNA abundance, could affect the rate of protein domain folding and secondary modifications.[31] Neither copy number variants nor other risk factors in non-coding regions that regulate *LRRK2* expression or alter splicing were assessed in our study.

As new loci for susceptibility to diverse diseases are continuously being discovered in genome-wide association and whole-genome sequencing studies, the results of our study show the importance of revisiting loci at which rare or common variants have been identified, since they could harbour many more independent signals of genetic risk in different populations.[25,32,33] Furthermore, *LRRK2* sequencing studies in under-represented populations (eg, from South America, sub-Saharan Africa, Middle East, and western Asia) will undoubtedly show novel ethnic-group-specific risk variants and could clarify the role of variants that were rare or absent in our study. *LRRK2* variants, including novel exonic variants, were reported as part of the 1000 Genome Project, lending support to this hypothesis.[34]

Large-scale parallel resequencing (targeted genomic capture of the specific regions—eg, gene-specific, exome, transcriptome, and whole-genome sequencing) is likely

Panel: Research in context

Systematic review

We searched PubMed with the terms "*LRRK2*" and "Genetics Parkinson's disease" and identified all *LRRK2* coding variations reported up until April 1, 2010. We also contacted our global network of collaborators and the members of the Genetic Epidemiology Of Parkinson's Disease (PD) Consortium for unreported variants.

Interpretation

By focusing on the role of *LRRK2* variation in PD, we have identified a common risk factor in the white population (M1646T), the third common risk factor in Asian populations (A419V), and a common global protective haplotype (N551K-R1398H-K1423K). This work complements the meta-analysis of PD genome-wide association,[25] which suggests a possible association at the *LRRK2* locus. We define some of the genetic variation that is likely to be contributing to the association noted in recent genome-wide association efforts and nominate potential functionally and clinically relevant variants. We show modulation of the underlying toxic effect is possible because of the protective nature of the N551K-R1398H-K1423K haplotype. The identification of common variants that affect risk clearly shows a greater role for *LRRK2* in idiopathic disease than previously thought.

to identify many more variants in candidate genes that might predispose to PD. Characterisation of each variant will require this type of collaborative international effort to define their pathogenicity, frequency in different populations, and contribution to disease pathogenesis through genotype–phenotype assessment.

Contributors

OAR and MJF were the principal investigators and were responsible for the concept and design of the study. AIS-O, JAB, OAR, and CVG were responsible for the technical aspects of the study. MGH and NND were responsible for all the analyses; OAR and MJF were responsible for drafting the report. All authors participated in study design and approach, sample collection, data acquisition, and critical revision and final approval of the report.

Conflicts of interest

JOA, MJF, and ZKW report holding a patent on *LRRK2* genetic variability and MJF has received royalties for licensing of genetically modified *LRRK2* mouse models. DMM declares a patent pending entitled *Methods to treat PD*. CK and RK declare receiving payment in their role as consultants for Centogene and Takeda Pharmaceutical, respectively. All other authors declare that they have no conflicts of interest.

Acknowledgments

This report is dedicated to the memory of J Mark Gibson (1953–2010). The work in this study was supported by a grant from the Michael J Fox Foundation for Parkinson's Research (OAR and MJF). Original funding for GEO-PD was supported by a grant from the Michael J Fox Foundation for Parkinson's Research Edmond J Safra Global Genetics Consortia programme. The Mayo Clinic is a Morris K Udall Center of Excellence in Parkinson's Disease Research (P50 NS072187) and was supported by a gift from the family of Carl Edward Bolch Jr and Susan Bass Bolch (DWD, RJU, ZKW, and OAR). This research was undertaken, in part, thanks to funding from the Canada Excellence Research Chairs programme (MJF and CV-G). Leading Edge Endowment Funds, provided by the Province of British Columbia, LifeLabs, and Genome BC, support the Dr Donald Rix BC Leadership Chair (MJF). Studies at individual sites were supported by different funding agencies worldwide—the Italian Ministry of Health (Ricerca Corrente 2010, Ricerca Finalizzata 2006); Fondazione Livio Patrizi; Swedish Parkinson Academy; the Swedish Parkinson Foundation; Lund University Research Fund, American Fidelity Assurance Insurance and the Royal Physiographic Society, Lund (AP and CN); Federal Ministry for Education and Research (BMBF, NGFNplus; 01GS08134; RK); NGFNplus (Neuron-Parkinson-subproject 7; SG); South African Medical Research Council and the University of Stellenbosch (SB, JC); Centre Hospitalier Régional Universitaire (CHRU) de Lille, University Lille 2 INSERM; French Ministry Programme Hospitalier de Recherche Clinique (1994/2002/1918, 2005/1914); Association France Parkinson (2005); Fondation de France 2004-013306; Fondation de la Recherche Médicale (2006); Le Programme Pluri-Formations (synucléothèque 2005–2009); Centres de Ressources Biologiques (L'Institut Pasteur de Lille, CHRU-Lille) and their scientific committee; the Agence Nationale de la Recherche (ANR-05-NEUR-019 and ANR-08-MNP-012; AB, SL); grant ES10758 from the National Institutes of Health; Swedish Research Council; Swedish Society for Medical Research; Swedish Society of Medicine; funds from the Karolinska Institutet and the Parkinson Foundation in Sweden (KW); Special Research Fund of the University of Antwerp; Research Foundation Flanders (Fonds Wetenschappelijk Onderzoek–Vlaanderen [FWO]); the Agency for Innovation by Science and Technology in Flanders (IWT); Interuniversity Attraction Poles Program P6/43 of the Belgian Federal Science Policy Office; Methusalem Excellence Grant of the Flanders Government and the Medical Research Foundation Antwerp and Neurosearch, Belgium; National Institutes of Health and National Institute of Neurological Disorders and Stroke 1RC2NS070276, NS057567, P50NS072187; Mayo Clinic Research Committee Clinical Research programmes (MCF and ZKW); Geriatric Medical Foundation of Queensland (GDM); a career development award from the Volkswagen Foundation and from the Hermann and Lilly Schilling Foundation (CK); Research Committee of University of Thessaly (code 2845); and Institute of Biomedical Research and Technology, CERETETH (code 01-04-207; GH and ED); and GlaxoSmithKline for past sponsorship of research into familial parkinsonism in Tunisia (RG and FH). DC is a holder of an FWO PhD fellowship and JT receives an FWO postdoctoral fellowship. For their contributions to make this work possible, we acknowledge Ferdinanda Annesi, Patrizia Tarantino (Institute of Neurological Sciences, National Research Council, Piano Lago di Mangone, Cosenza, Italy); Chiara Riva (Department of Neuroscience and Biomedical Technologies, University of Milano-Bicocca, Monza, Italy); Roberto Piolti (Department of Neurology, Ospedale San Gerardo, Monza, Italy); Magdalena Boczarska-Jedynak (Department of Neurology, Medical University of Silesia, Katowice, Poland); Aurélie Duflot, (UMR837 INSERM-University Lille 2, CHRU de Lille); Jean-Philippe Legendre, Nawal Waucquier (Neurologie et Pathologie du Mouvement, Clinique de Neurologie du CHU de Lille); Anna Rita Bentivoglio, Tamara Ialongo, Arianna Guidubaldi, Carla Piano (Institute of Neurology, Catholic University, Rome, Italy); Karen Nuytemans (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; Laboratory of Neurogenetics, Institute Born-Bunge and University of Antwerp, Belgium); Sebastiaan Engelborghs; Peter De Deyn (Department of Neurology, ZiekenhuisNetwerk Antwerpen Middelheim and Laboratory of Neurochemistry and Behaviour, Institute Born-Bunge and University of Antwerp); David Crosiers, Patrick Cras (Department of Neurology, University Hospital Antwerp and Laboratory of Neurobiology, Institute Born-Bunge and University of Antwerp, Belgium); Phil Hyu Lee (Department of Neurology, Yonsei University College of Medicine, Seoul, South Korea); Susanne Lindskov (Department of Geriatrics and Neurology, Central Hospital Kristianstad, Northeast Skåne Health Care District, Kristianstad, Sweden); Karin Nilsson (Department of Clinical Science, Section of Geriatric Psychiatry, Lund University, Sweden); Jan Reimer (Department of Neurology, Skåne University Hospital, Sweden); Manabu Funayama, Yuanzhe Li, Hiroyo Yoshino (Juntendo University School of Medicine, Tokyo, Japan); and we acknowledge all the patients and controls who kindly donated DNA to make collaborative studies like these possible. A full list of GEO-PD consortia is provided in webappendix pp 27–30.

References

1 Dachsel JC, Farrer MJ. *LRRK2* and Parkinson disease. *Arch Neurol* 2010; 67: 542–47.

2 Nuytemans K, Meeus B, Crosiers D, et al. Relative contribution of simple mutations vs. copy number variations in five Parkinson disease genes in the Belgian population. *Hum Mutat* 2009; 30: 1054–61.

3 Lesage S, Condroyer C, Lannuzel A, et al. Molecular analyses of the *LRRK2* gene in European and North African autosomal dominant Parkinson's disease. *J Med Genet* 2009; 46: 458–64.

4 Mata IF, Kachergus JM, Taylor JP, et al. Lrrk2 pathogenic substitutions in Parkinson's disease. *Neurogenetics* 2005; 6: 171–77.

5 Di Fonzo A, Tassorelli C, De Mari M, et al. Comprehensive analysis of the *LRRK2* gene in sixty families with Parkinson's disease. *Eur J Hum Genet* 2006; 14: 322–31.

6 Paisan-Ruiz C, Nath P, Washecka N, Gibbs JR, Singleton AB. Comprehensive analysis of *LRRK2* in publicly available Parkinson's disease cases and neurologically normal controls. *Hum Mutat* 2008; 29: 485–90.

7 Farrer M, Ross OA. *LRRK2*-related Parkinson disease. GeneReviews. Seattle, WA: University of Washington, 2006. http://www.ncbi.nlm.nih.gov/books/NBK1208/ (accessed Aug 22, 2011).

8 Aasly JO, Vilarino-Guell C, Dachsel JC, et al. Novel pathogenic *LRRK2* p.Asn1437His substitution in familial Parkinson's disease. *Mov Disord* 2010; 25: 2156–63.

9 Gonzalez-Fernandez MC, Lezcano E, Ross OA, et al. Lrrk2-associated parkinsonism is a major cause of disease in Northern Spain. *Parkinsonism Relat Disord* 2007; 13: 509–15.

10 Hulihan MM, Ishihara-Paul L, Kachergus J, et al. *LRRK2* Gly2019Ser penetrance in Arab-Berber patients from Tunisia: a case-control genetic study. *Lancet Neurol* 2008; 7: 591–94.

11 Lesage S, Durr A, Tazir M, et al. *LRRK2* G2019S as a cause of Parkinson's disease in North African Arabs. *N Engl J Med* 2006; 354: 422–23.

12 Di Fonzo A, Wu-Chou YH, Lu CS, et al. A common missense variant in the LRRK2 gene, Gly2385Arg, associated with Parkinson's disease risk in Taiwan. *Neurogenetics* 2006; **7**: 133–38.

13 Farrer MJ, Stone JT, Lin CH, et al. Lrrk2 G2385R is an ancestral risk factor for Parkinson's disease in Asia. *Parkinsonism Relat Disord* 2007; **13**: 89–92.

14 Tan EK, Peng R, Teo YY, et al. Multiple LRRK2 variants modulate risk of Parkinson disease: a Chinese multicenter study. *Hum Mutat* 2010; **31**: 561–68.

15 Ross OA, Wu YR, Lee MC, et al. Analysis of Lrrk2 R1628P as a risk factor for Parkinson's disease. *Ann Neurol* 2008; **64**: 88–92.

16 Gelb, DJ, Oliver E, Gilman S. Diagnostic criteria for Parkinson disease. *Arch Neurol* 1999; **56**: 33–39.

17 Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease. A clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry* 1992; **55**: 181–84.

18 DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177–88.

19 Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med* 2002; **21**: 1539–58.

20 Li B, Leal SM. Methods for detecting associations with rare variants for common diseases: application to analysis of sequence data. *Am J Hum Genet* 2008; **83**: 311–21.

21 Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protocols* 2009; **4**: 1073–81.

22 Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet* 2002; **70**: 425–34.

23 Dudoit S, van der Laan MJ, Pollard KS. Multiple testing. Part I. Single-step procedures for control of general type I error rates. *Stat Appl Genet Mol Biol* 2004; **3**: article 13.

24 Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res* 2003; **31**: 3812–14.

25 Nalls MA, Plagnol V, Hernandez DG, et al. Imputation of sequence variants for identification of genetic risks for Parkinson's disease: a meta-analysis of genome-wide association studies. *Lancet* 2011; **377**: 641–49.

26 Ioannidis JP. Population-wide generalizability of genome-wide discovered associations. *J Natl Cancer Inst* 2009; **101**: 1297–99.

27 Lee BD, Shin JH, Vankampen J, et al. Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's disease. *Nat Med* 2010; **16**: 998–1000.

28 Panagiotou OA, Evangelou E, Ioannidis JP. Genome-wide significant associations for variants with minor allele frequency of 5% or less—an overview: a HuGE review. *Am J Epidemiol* 2010; **172**: 869–89.

29 Deng J, Lewis PA, Greggio E, Sluch E, Beilina A, Cookson MR. Structure of the ROC domain from the Parkinson's disease-associated leucine-rich repeat kinase 2 reveals a dimeric GTPase. *Proc Natl Acad Sci USA* 2008; **105**: 1499–504.

30 Goldstein DS, Imrich R, Peckham E, et al. Neurocirculatory and nigrostriatal abnormalities in Parkinson disease from LRRK2 mutation. *Neurology* 2007; **69**: 1580–84.

31 Kimchi-Sarfaty C, Oh JM, Kim IW, et al. A "silent" polymorphism in the *MDR1* gene changes substrate specificity. *Science* 2007; **315**: 525–28.

32 Ormond KE, Wheeler MT, Hudgins L, et al. Challenges in the clinical application of whole-genome sequencing. *Lancet* 2010; **375**: 1749–51.

33 McCarthy MI, Abecasis GR, Cardon LR, et al. Genome-wide association studies for complex traits: consensus, uncertainty and challenges. *Nat Rev Genet* 2008; **9**: 356–69.

34 Durbin RM, Abecasis GR, Altshuler DL, et al. A map of human genome variation from population-scale sequencing. *Nature* 2010; **467**: 1061–73.

Autophagy 7:2, 176-187; February 2011; © 2011 Landes Bioscience

Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition

Shinji Saiki,[1] Yukiko Sasazawa,[2] Yoko Imamichi,[1] Sumihiro Kawajiri,[1] Takahiro Fujimaki,[2] Isei Tanida,[3] Hiroki Kobayashi,[2] Fumiaki Sato,[4] Shigeto Sato,[1] Kei-Ichi Ishikawa,[1] Masaya Imoto[2] and Nobutaka Hattori[1,*]

[1]Department of Neurology; Juntendo University School of Medicine; Bunkyo, Tokyo; [2]Department of Biosciences and Informatics; Faculty of Science and Technology; Keio University; Kohoku, Yokohama; [3]Department of Biochemistry and Cell Biology; National Institute of Infectious Diseases; Shinjyuku, Tokyo; [4]Research Institute for Disease of Old Age; Juntendo University School of Medicine; Tokyo, Japan

Key words: apoptosis, autophagy, PI3K/Akt/mTOR/p70S6K, ERK1/2, caffeine

Abbreviations: PI3K, phosphoinositide-3 kinase; 4E-BP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; 3-MA, 3-methyladenine; MEFs, mouse embryonic fibroblasts; p70S6K, 70-kDa ribosomal protein S6 kinase; PI, propidium iodide; MPP^+, 1-methyl-4-phenylpyridinium

Caffeine is one of the most frequently ingested neuroactive compounds. All known mechanisms of apoptosis induced by caffeine act through cell cycle modulation or p53 induction. It is currently unknown whether caffeine-induced apoptosis is associated with other cell death mechanisms, such as autophagy. Herein we show that caffeine increases both the levels of microtubule-associated protein 1 light chain 3-II and the number of autophagosomes, through the use of western blotting, electron microscopy and immunocytochemistry techniques. Phosphorylated p70 ribosomal protein S6 kinase (Thr389), S6 ribosomal protein (Ser235/236), 4E-BP1 (Thr37/46) and Akt (Ser473) were significantly decreased by caffeine. In contrast, ERK1/2 (Thr202/204) was increased by caffeine, suggesting an inhibition of the Akt/mTOR/p70S6K pathway and activation of the ERK1/2 pathway. Although insulin treatment phosphorylated Akt (Ser473) and led to autophagy suppression, the effect of insulin treatment was completely abolished by caffeine addition. Caffeine-induced autophagy was not completely blocked by inhibition of ERK1/2 by U0126. Caffeine induced reduction of mitochondrial membrane potentials and apoptosis in a dose-dependent manner, which was further attenuated by the inhibition of autophagy with 3-methyladenine or *Atg7* siRNA knockdown. Furthermore, there was a reduced number of early apoptotic cells (annexin V positive, propidium iodide negative) among autophagy-deficient mouse embryonic fibroblasts treated with caffeine than in their wild-type counterparts. These results support previous studies on the use of caffeine in the treatment of human tumors and indicate a potential new target in the regulation of apoptosis.

Introduction

Caffeine has a diverse range of pharmacological effects.[1] In addition to its various effects on the cell cycle and growth arrest, higher (4–10 mM) concentrations of caffeine can induce apoptosis in several cell lines, such as 10 mM caffeine in human neuroblastoma cells,[2] 4 mM caffeine in human pancreatic adenocarcinoma cells[3] and 5 mM caffeine in human A549 lung adenocarcinoma cells.[4] Although caffeine has been reported to modulate cell cycle checkpoints and perturb molecular targets of the cell cycle, the exact mechanism of caffeine-induced apoptosis remains unclear.[1]

Autophagy is a key mechanism in various physiopathological processes, including tumorigenesis, development, cell death and survival.[5,6] It has also been shown to have a complex relationship with apoptosis, especially in tumor cell lines.[7] Several reports have shown that autophagy not only enhances caspase-dependent cell death, but is also required for it.[8] In contrast, it has also been shown that autophagy plays an important role in promoting cell survival against apoptosis.[7] Caffeine has been reported to inhibit some kinase activities, including various forms of phosphoinositol-3 kinase and mammalian target of rapamycin (mTOR).[9,10] Recently, in food spoilage studies involving yeast, caffeine has been shown to induce a starvation response,[11] which is a key regulator of autophagy causing its induction. However, the exact mechanism by which caffeine induces autophagy is still unknown.

Here we report that higher concentrations of caffeine enhance autophagic flux in a dose-dependent manner in various cell lines. Furthermore, we show that caffeine-induced autophagy is mainly dependent on PI3K/Akt/mTOR/p70S6 signaling and eventually results in apoptosis.

*Correspondence to: Nobutaka Hattori; Email: nhattori@juntendo.ac.jp
Submitted: 06/22/10; Revised: 10/27/10; Accepted: 11/02/10
Previously published online: www.landesbioscience.com/journals/autophagy/article/14074
DOI: 10.4161/auto.7.2.14074

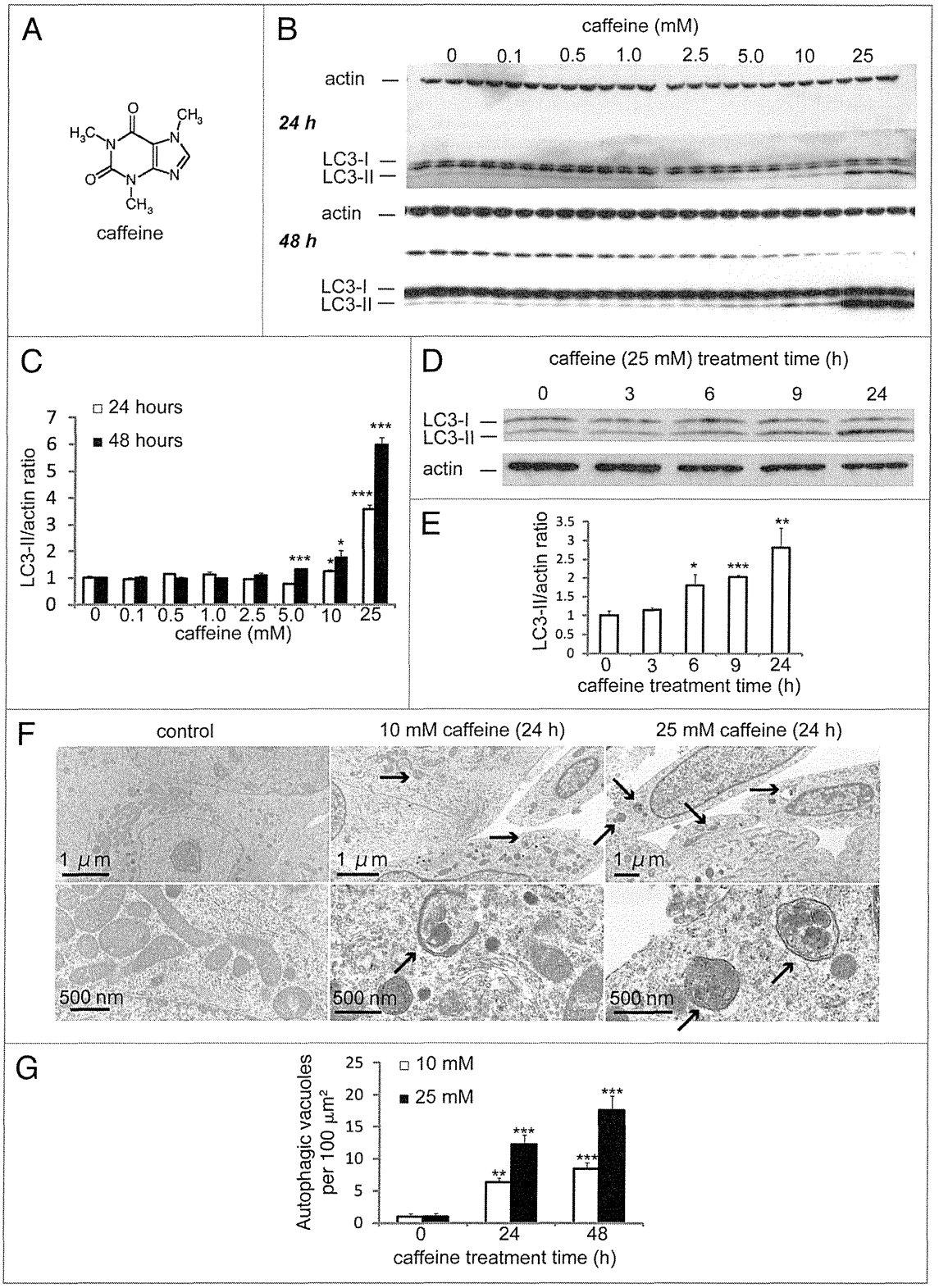
Results and Discussion

Caffeine (**Fig. 1A**) is a widely used psychoactive drug that has been used for centuries to increase alertness and energy. It has been reported that caffeine induces autophagy in *Zygosaccharomyces bailii* in association with a starvation response, caused by a unknown mechanism.[11] However, it remains unknown whether caffeine affects autophagy in mammalian cells. To determine if caffeine regulates autophagy at a steady state, we first examined levels of the microtubule-associated protein 1 light chain 3 (LC3)-II, which is an LC3-phosphatidyl-ethanolamine conjugate and a promising autophagosomal marker.[12] LC3-II levels (compared to actin loading controls) increased with 5–25 mM caffeine treatment over 48 hours in SH-SY5Y (**Fig. 1B and C**), PC12D and HeLa cells (**Suppl. Fig. S1A and B**). The LC3-II/actin ratio also increased in a time-dependent manner in SH-SY5Y (**Fig. 1D and E**) and HeLa cells (data not shown). Using an electron microscopy technique, the numbers of autophagic vacuoles (AVs) were markedly increased in SH-SY5Y cells treated with 10 or 25 mM caffeine, but not in the control (**Fig. 1F and G**). Morphometric analysis revealed that the number of AVs per 100 μm^2 of SH-SY5Y cytoplasm in control (Mean ± standard deviation: 1.3 ± 0.50), whereas that in caffeine-treated cells (10 mM: 8.0 ± 0.82; 25 mM: 15 ± 1.9) for 24 hours. Expression levels of p62, a well-known autophagic substrate, were also decreased by caffeine treatment in SH-SY5Y (**Fig. 1H and I**) and HeLa cells (**Suppl. Fig. S1C and D**). Furthermore, 10 mM caffeine treatment markedly increased the number of EGFP-LC3-positive vesicles in SH-SY5Y cells transiently transfected with EGFP-LC3 (data not shown) and HeLa cells stably expressing EGFP-LC3 (**Figs. 1J and K**).[12,13] This effect was confirmed by the observation that caffeine administration also increased the number of vesicles positive to endogenous LC3 (**Suppl. Fig. S1E**).

Endogenous LC3 is post-transcriptionally processed into LC3-I, which is found in the cytosol. LC3-I is in turn lipidated to LC3-II, which then associates with autophagosome membranes.[14] LC3-II can accumulate due to increased upstream autophagosome formation or impaired downstream autophagosome-lysosome fusion. To distinguish between these two possibilities, we assayed LC3-II in the presence of E64D plus pepstatin A or bafilomycin A1, which inhibits lysosomal proteases or blocks downstream autophagosome-lysosome fusion and lysosomal proteases, respectively.[15,16] Caffeine significantly increased LC3-II levels in the presence of E64d plus pepstatin A or bafilomycin compared to E64d plus pepstatin A or bafilomycin alone in (**Fig. 2A and B; Suppl. Fig. S1F and G**) and HeLa cells (**Fig. 2C and D; Suppl. Fig. S1H and I**). A saturating dosage of bafilomycin A1 was used in this assay and no further increases in LC3-II levels were observed when cells were treated with higher concentrations. Similar results were observed in PC12D cell lines (data not shown). To confirm the caffeine effect on autophagic flux, we assessed the numbers of autolysosomes and autophagosomes in HeLa cells. The ratio of the numbers of autolysosomes (positive to both LC3 and LAMP2) to autophagosomes (positive to LC3) was increased by 10 mM caffeine treatment for 48 hours (**Fig. 2E**). Quantification data using ImageJ also showed significant increase of the ratio (**Fig. 2F**). These results strongly indicate that high concentration of caffeine treatment enhances autophagic flux.

The class I phosphatidylinositol 3-phosphate kinase (PI3K)/Akt/mTOR/p70ribosomal protein S6 kinase (p70S6K) signaling pathway and the Ras/Raf-1/mitogen-activated protein kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway are two well-known pathways involved in the regulation of autophagy. Both are associated with tumorigenesis and often activated in numerous types of tumors.[17] Therefore, we examined the effect of caffeine on both of these pathways, using western blotting, according to the protocol by Inoki and colleagues.[18] After a 24 hour treatment with caffeine, there was a significant decrease in the levels of phosphorylated p70 S6 kinase, S6 ribosomal protein and 4E-BP1, compared with total normal levels in SH-SY5Y (**Fig. 3A**), HeLa and PC12D cells (data not shown). Consistent with these results, nonphosphorylated 4E-BP1 proteins were increased by caffeine treatment (**Fig. 3A**). To further investigate the upstream inhibition of mTOR by caffeine, we examined Ser473 phosphorylation of Akt, which measures both Akt/mTOR and mTORC2 activity. As shown in **Figure 3B**, treatment with caffeine also decreased the level of phosphorylated Akt in SH-SY5Y cells, which was consistent with a previous report.[19] Similar findings were obtained in HeLa (**Suppl. Fig. S2A**) and PC12D cells (data not shown). Subsequently, we examined whether caffeine increases the phosphorylation of ERK1/2, a key regulator of autophagy downstream of Akt. As shown in **Figure 3C**, treatment with caffeine increased phosphorylated ERK1/2. The effects of caffeine on mTOR inhibition were initially detected 3 hours after the addition of caffeine and reached a maximal level after 6 hours in SH-SY5Y (**Fig. 3D**) and 9 hours in HeLa cells (**Suppl. Fig. S2B and C**).

Caffeine has been shown to inhibit PI3K and components of the PI3K/Akt pathway.[9,20] Next, we performed experiments to confirm whether caffeine-induced autophagy is activated through the PI3K/Akt pathway. Insulin or insulin-like growth factor upregulates PI3K and its downstream targets including Akt and mTOR, resulting in the inactivation of autophagy.[21-23] As shown in **Figure 4A and B**, insulin treatment for 30 minutes significantly phosphorylated Akt at Ser473, whereas the phosphorylation was completely abolished by additional treatment with caffeine. No significant differences of the LC3-II/actin ratio between caffeine treatment and caffeine treatment with insulin were observed. Also, caffeine and Akt1/2 inhibitors did not have additive effects on the levels of LC3-II/actin ratio compared to the single treatment of caffeine or Akt inhibitors (**Fig. 4C and D**). To further confirm the caffeine effects on this pathway, cells were transiently transfected with myristoylated Akt (myr-Akt), a constitutively active form of Akt.[24] Caffeine treatment of both cells transfected with control vector and myr-Akt markedly decreased the levels of the phosphorylated Akt (**Fig. 3E**), indicating that caffeine directly inhibits the Akt phosphorylation. If caffeine facilitates autophagy through PI3K/Akt and ERK1/2 signalings, the autophagy should be partially blocked by ERK1/2 inhibition using the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor, U0126. U0126 significantly but mildly



reversed the levels of LC3-II/actin ratio (**Fig. 4F and G**). The failure of U1026 to reverse completely the caffeine effect can be explained by the autophagy induction through Akt/mTOR signaling. In addition, only Akt knockdown with inducible short hairpin RNAs (shRNAs) to specifically and stably knock down all three Akt isoforms sufficiently increases autophagic flux.[25] Therefore, we concluded that the caffeine-induced autophagy is mainly dependent on the PI3K/Akt/mTOR pathway.

Figure 1A–G (See opposite page). Caffeine increases autophagic flux in various cell lines. (A) Structural formula of caffeine. (B and C) SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours were analyzed by immunoblotting (B) with antibodies against LC3 and actin. Densitometry analysis of LC3-II levels relative to actin (C) was performed using three independent experiments. (D and E) SH-SY5Y cells treated with 25 mM caffeine for 3–24 hours were analyzed by immunoblotting (D) with antibodies against LC3 and actin. Densitometry analysis of LC3-II levels relative to actin (E) was performed using three independent experiments. (F) Electron microscopic examination of SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours. Autophagic vacuoles accumulating in the cytoplasm are shown by arrows. (G) Morphometric analysis of autophagic vacuoles was performed with 30 different areas of the cytoplasm of control and caffeine-treated cells.

Because caffeine induces autophagy dependently of mTOR inhibition, we hypothesized that combination treatment of caffeine with rapamycin would not have additive effects on autophagy. However, caffeine and rapamycin showed an additive effect on the enhancement of LC3-II/actin ratio compared to the single treatment of caffeine or rapamycin (**Fig. 5A and B**). Several lines of evidences support the hypothesis that resistance to rapamycin results from a positive feedback loop from mTOR/S6K1 to Akt, resulting in enhancement of Akt phosphorylation at Ser 473.[26-28] Recently, mutual suppression of the PI3K/Akt/mTOR pathway by combination of rapamycin with perifosine, an Akt inhibitor, induces synergistic effects on autophagy-induced apoptosis as well as enhancement of autophagy, suggesting that dual inhibition of the PI3K/Akt/mTOR by rapamycin with caffeine would be also a rational treatment for cancer.[29]

Several anti-cancer agents are known to inhibit the PI3K/Akt/mTOR/p70S6K pathway and simultaneously activate ERK1/2, resulting in induction of autophagy in tumor cell lines.[30,31] The upregulation of this process has beneficial effects in neurodegenerative diseases, such as Parkinson and Huntington diseases, whereas an excess of autophagy can lead to cell death.[32,33] Therefore, we decided to investigate whether caffeine-induced autophagy rescues or induces cell death. Using PC12D cells treated with 1-methyl-4-phenylpyridinium (MPP^+), a well-established Parkinson disease model,[34] we determined that 1 mM caffeine treatment was not sufficient for the induction of autophagy (**Suppl. Fig. S4 and B**) and promoted increased cell viability, whereas >2.5 mM caffeine decreased cell viability (**Fig. 6A**). In addition, a significant decrease in cell viability was noted in cells treated with >2.5 mM caffeine without MPP^+. Also, mitochondrial membrane potentials assessed by JC-1 were significantly preserved by 1 mM caffeine treatment compared to the control with MPP^+, while those were lost by >5 mM caffeine treatment (**Fig. 6B and Suppl. Fig. S5A**). These data suggest that caffeine-induced autophagy is not protective in these cell lines and leads to cell death.

Autophagy and apoptosis may act independently in parallel pathways or may influence one another.[7] To confirm the relationship between these pathways in cells treated with caffeine, we examined caffeine effects on the cell cycle with a propidium iodide (PI) staining assay. Treatment with 2.5–10 mM caffeine increased the percentage of cells in the sub-G_1 peak, which is indicative of

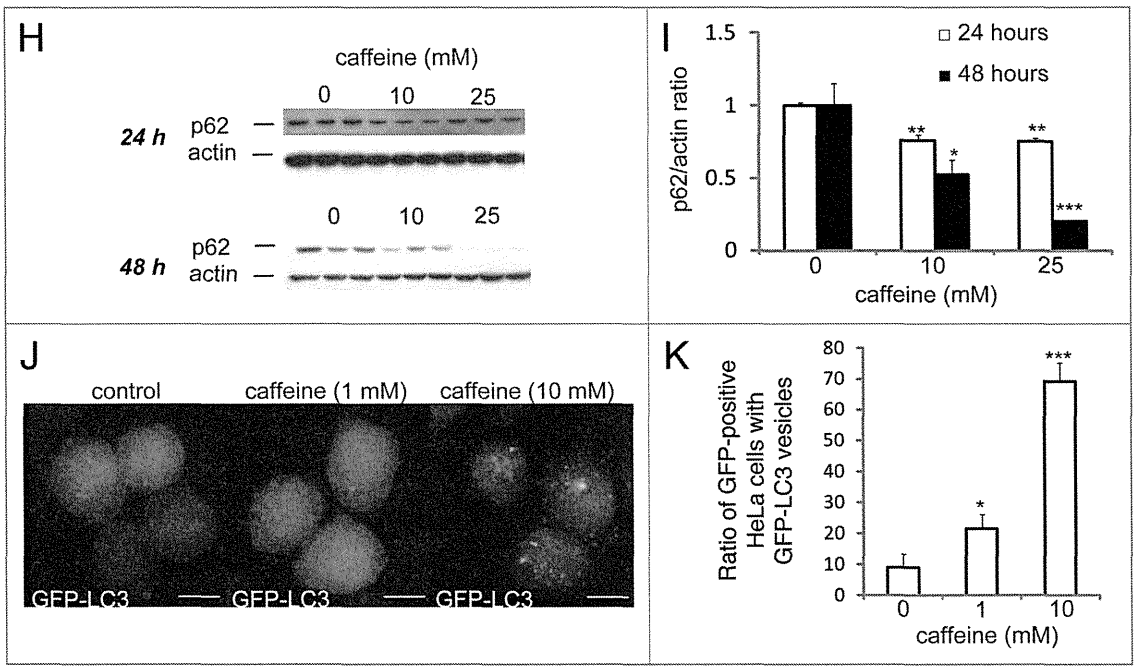


Figure 1H–K. Caffeine increases autophagic flux in various cell lines. (H and I) SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours were analyzed by immunoblotting with antibodies against p62 and actin. Densitometry analysis of p62 levels relative to actin (I) was performed using three independent experiments. (J and K) HeLa cells stably expressing EGFP-LC3 were treated with various concentrations of caffeine for 24 hours and analyzed using confocal microscopy. The percentage of EGFP-positive HeLa cells with >5 EGFP-LC3 vesicles was assessed (K) described previously in reference 43. Error bars, S.D.; *p < 0.05; **p < 0.01.

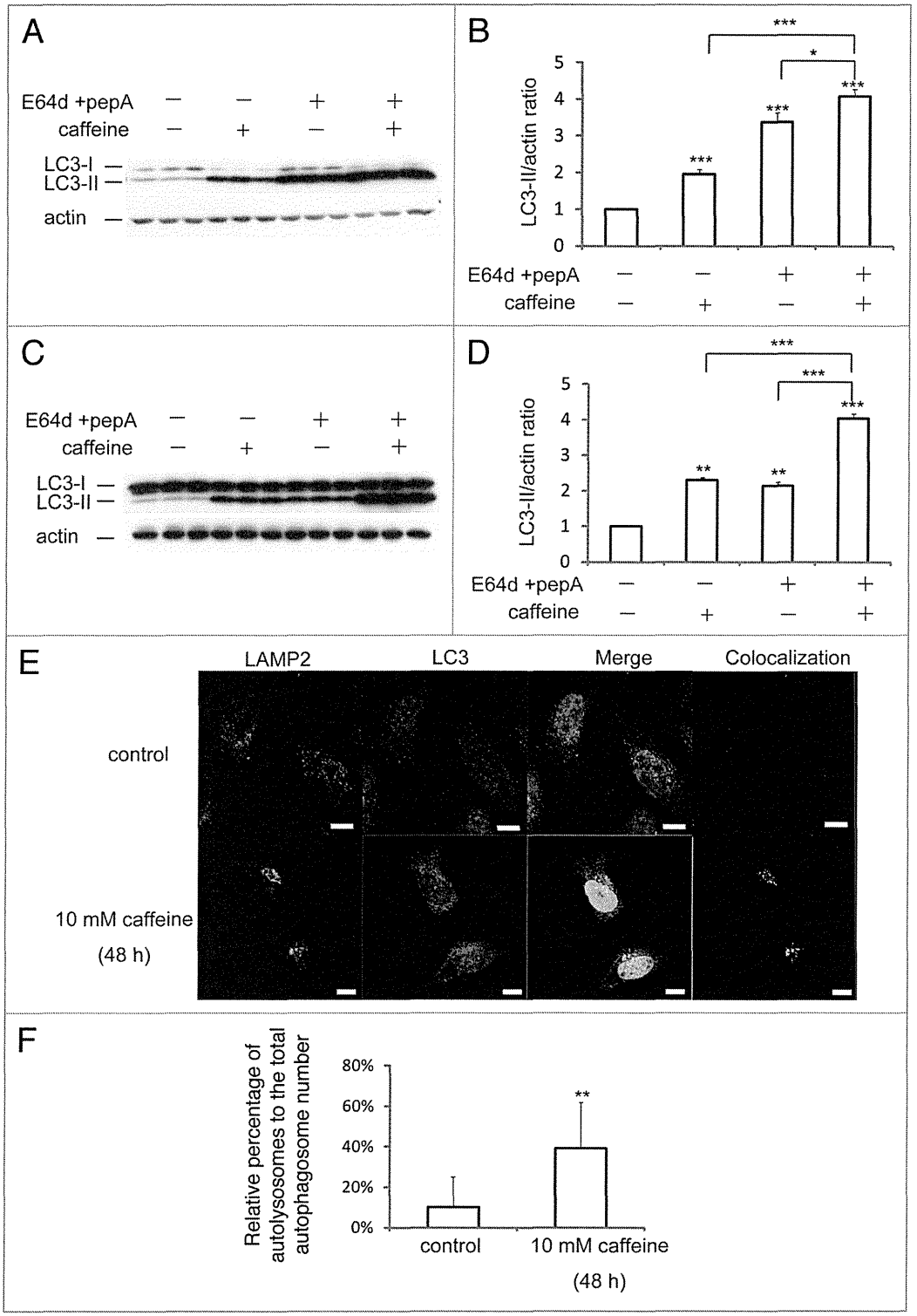


Figure 2. Caffeine does not block autophagosome-lysosome fusion. (A–D) SH-SY5Y (A) or HeLa (C) cells treated with 10 mM caffeine with or without E64d (10 μg/ml) and pepstatin A (10 μg/ml) were analyzed by immunoblotting with antibodies against LC3 and actin. Densitometry analysis of LC3 levels relative to actin in SH-SY5Y (B) and HeLa (D) cells was performed using three independent experiments. (E and F) HeLa cells treated with various concentrations of caffeine for 48 hours were analyzed using confocal microscopy (E). Number of the autolysosomes and autophagosomes were automatically counted using ImageJ "Colocalization" Plugin and the ratios were calculated (F).

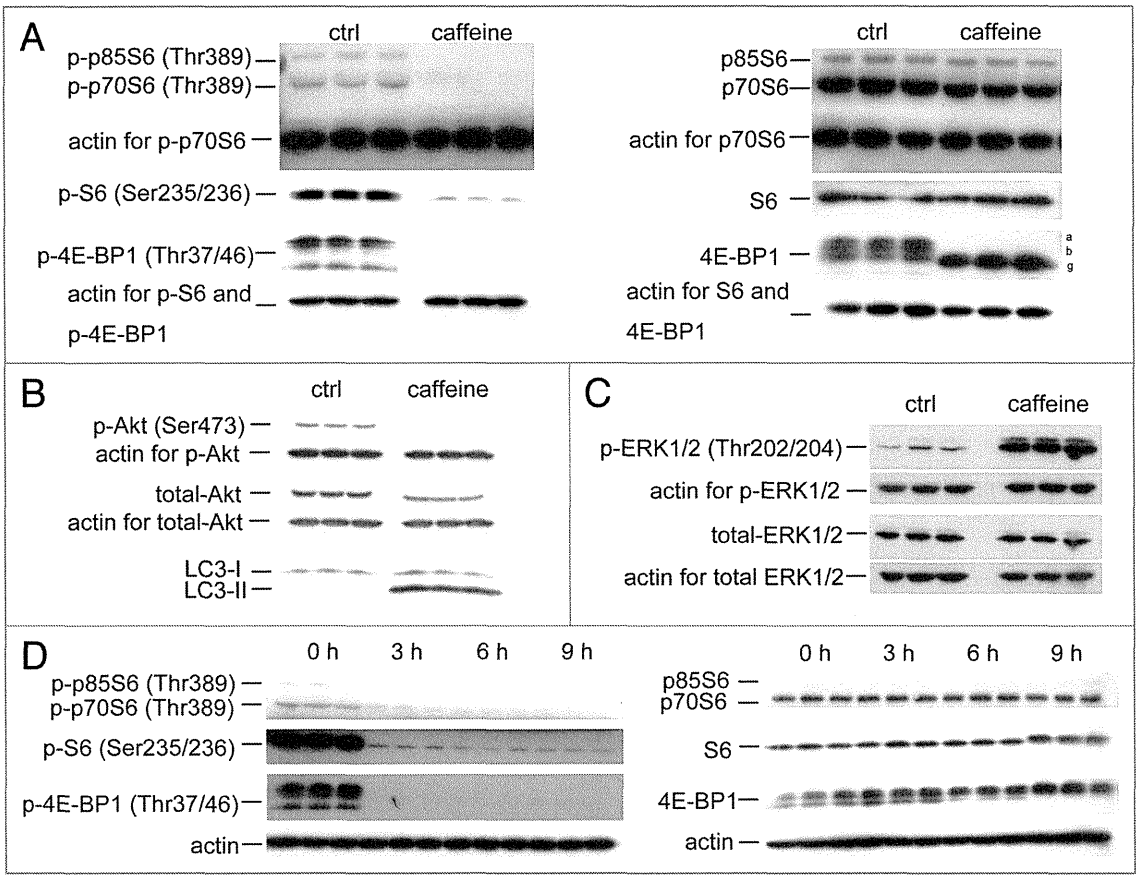


Figure 3. Caffeine inhibits the Akt/mTOR/p70S6 signaling pathway and activates ERK1/2 signaling. (A and B) SH-SY5Y cells treated with or without 10 mM caffeine for 24 hours were analyzed for mTOR activity by immunoblotting for levels of phosphor- and total p70 ribosomal S6 protein, S6, 4E-BP1 (A), Akt (B) and actin. (C) SH-SY5Y cells treated with or without 10 mM caffeine for 0, 3, 6 or 9 hours were analyzed by immunoblotting for levels of phosphor- and total ERK1/2 and actin. (D) SH-SY5Y cells treated with 10 mM caffeine for various time periods were analyzed by immunoblotting for levels of phosphor- and total p70 ribosomal S6 protein, S6, 4E-BP1 and actin.

apoptosis (**Fig. 6C**). To confirm whether caffeine-induced cell death is apoptotic, we examined the activity of caspase-3, a well-known inducer of apoptosis. Treatment with 10 mM caffeine markedly increased levels of cleaved caspase-3 and decreased full-length caspase-3 in PC12D cells (**Fig. 6D**), consistent with previous reports on the induction of apoptosis by caffeine.[35-37]

To test whether caffeine-induced apoptosis is dependent on autophagy, we determined whether the inhibition of autophagy by 3-methyladenine (3-MA) or Atg7 siRNA knockdown affects caffeine-induced cytotoxicity in PC12D cells. Treatment with 1 or 5 mM 3MA or Atg7 knockdown significantly decreased the percentage of cell death or cells with reduced mitochondrial membrane potentials caused by caffeine treatment (5 or 10 mM) (**Fig. 6E and F and Suppl. Fig. S6B**). As can be seen from the increased caffeine-induced apoptosis shown in **Figure 6A and C**, our data suggests that caffeine-induced autophagy is necessary for apoptotic cell death. To further confirm this, we compared autophagy-deficient mouse embryonic fibroblasts (MEFs), lacking the *Atg7* gene (*Atg7*$^{-/-}$), without LC3-II expression (**Suppl. Fig. S4E**), and matched wild-type (*Atg7*$^{+/+}$) MEFs, in which autophagy is induced by caffeine in a dose-dependent manner (**Suppl. Fig. S4C and D**). As expected, the level of caffeine-induced cell death (positive to trypan blue staining) in *Atg7*$^{-/-}$ MEFs was less than that in *Atg7*$^{+/+}$ MEFs (**Fig. 7A**). The numbers of early apoptotic cells (annexin V positive, PI negative) were significantly increased in both a time-dependent and dose-dependent manner by caffeine treatment of *Atg7*$^{+/+}$ MEFs compared to *Atg7*$^{-/-}$ MEFs (**Fig. 7B–D**). Also, apoptotic or necrotic cells (annexin V positive) were significantly increased by caffeine treatment of *Atg7*$^{+/+}$ MEFs compared to *Atg7*$^{-/-}$ MEFs (**Suppl. Fig. S6**). Together, these results indicate that caffeine-induced autophagy partly occurs upstream of apoptosis and is not a protective response to caffeine.

In various tumor cell lines, higher concentrations of caffeine alone induce p53-dependent G_1 phase arrest and under certain conditions apoptosis can also occur in a p53-independent manner.[1] Furthermore, disruption at the G_2/M checkpoint by caffeine allows cells time to repair DNA damage by driving them through mitosis, eventually resulting in apoptosis.[36,38,39] Consistent with these reports, the results of our study indicate that increased concentrations of caffeine treatment cause a dose-dependent increase in apoptosis. More recently, autophagy, a process long known to

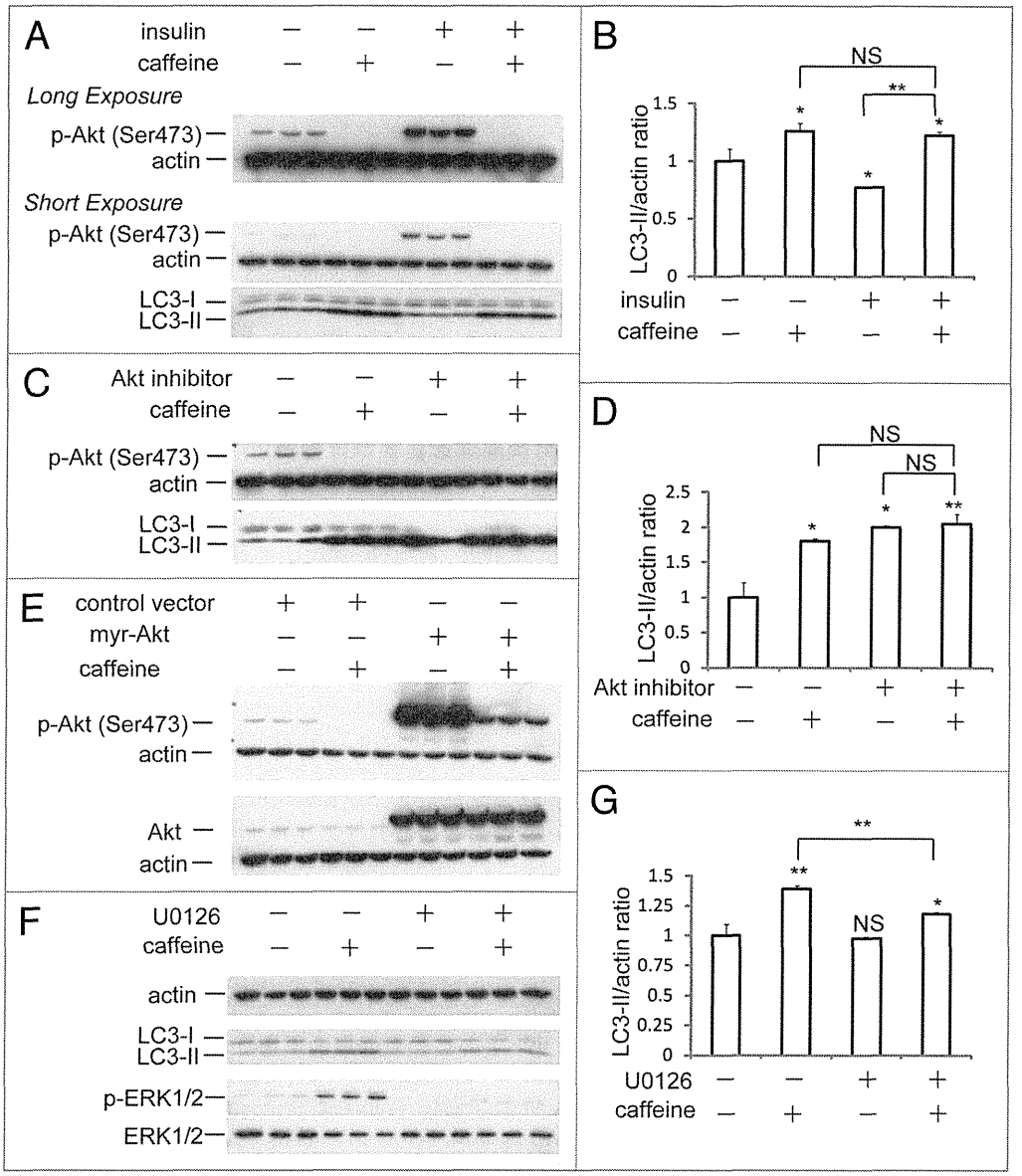


Figure 4. Caffeine-induced autophagy is dependent on PI3K/Akt/mTOR pathway. (A) SH-SY5Y cells treated with 25 mM caffeine for 3 hours followed by treatment with or without 200 nM insulin for 30 minutes were analyzed by immunoblotting. (B) Densitometry analysis of LC3-II levels relative to actin was performed using three independent experiments. (C) SH-SY5Y cells treated with 25 mM caffeine, 50 μM Akt1/2 inhibitors or 25 mM caffeine with 50 μM Akt1/2 inhibitors for 6 hours were analyzed by immunoblotting. (D) Densitometry analysis of LC3-II levels relative to actin was performed using three independent experiments. (E) SH-SY5Y cells were transfected for 24 hours with either a control plasmid DNA (pcDNA3.1) or a plasmid encoding constitutively active Akt (myr-Akt), and then treated with H_2O or 10 mM caffeine for 6 hours. Immunoblotting was performed using antibodies against Akt, p-Akt (Ser 473) and actin. (F) SH-SY5Y cells treated with 25 mM caffeine with or without 20 μM U0126 for 6 hours were analyzed by immunoblotting using antibodies against actin, LC3, p-ERK and ERK. (G) Densitometry analysis was performed using three independent experiments. Error bars, SD; *p < 0.05; **p < 0.01; N.S., not significant.

provide a survival advantage to cells undergoing nutrient deprivation and other stresses, has also been linked to the cell death process.[7] The cross-talk between apoptosis and autophagy is complex and sometimes contradictory; however, it is critical to the overall fate of the cell. In this study, we have shown that autophagy is induced by higher concentrations of caffeine without starvation, mainly via the inhibition of PI3K/Akt/mTOR/p70S6K signaling. Likewise, when caffeine-induced autophagy is blocked by 3-MA treatment or *Atg7* knockout, apoptosis is partially attenuated, suggesting that caffeine-induced autophagy occurs upstream of caffeine-induced apoptosis. It also indicates the involvement of other pathways in caffeine-induced apoptosis. These results provide new insight into the effects of caffeine on cell death and survival and its use as a possible intervention strategy for the upregulation of apoptosis by a harnessing of its autophagic activity in tumor treatment.

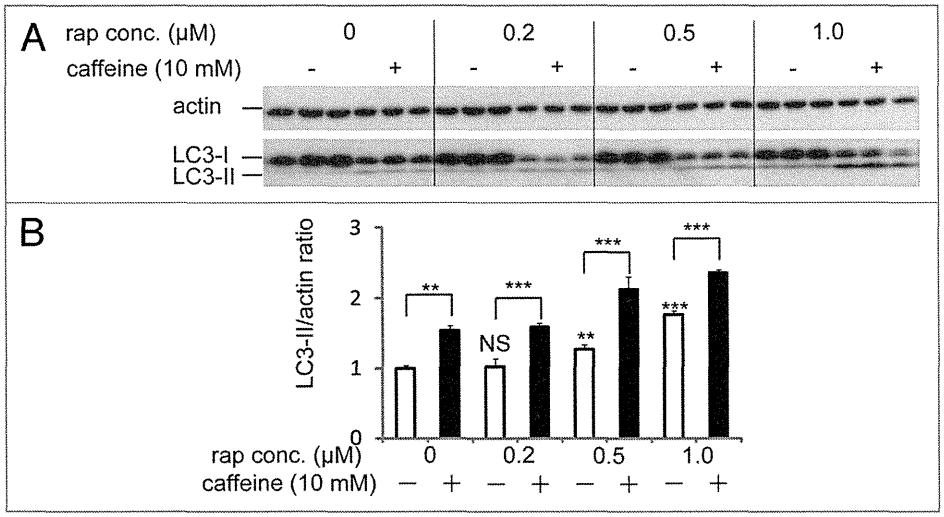


Figure 5. Rapamycin treatment with caffeine has an additive effect on enhancement of autophagy. (A) SH-SY5Y cells treated with various concentrations of rapamycin with or without 10 mM caffeine for 48 hours were analyzed by immunoblotting. (B) Densitometry analysis was performed using three independent 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.

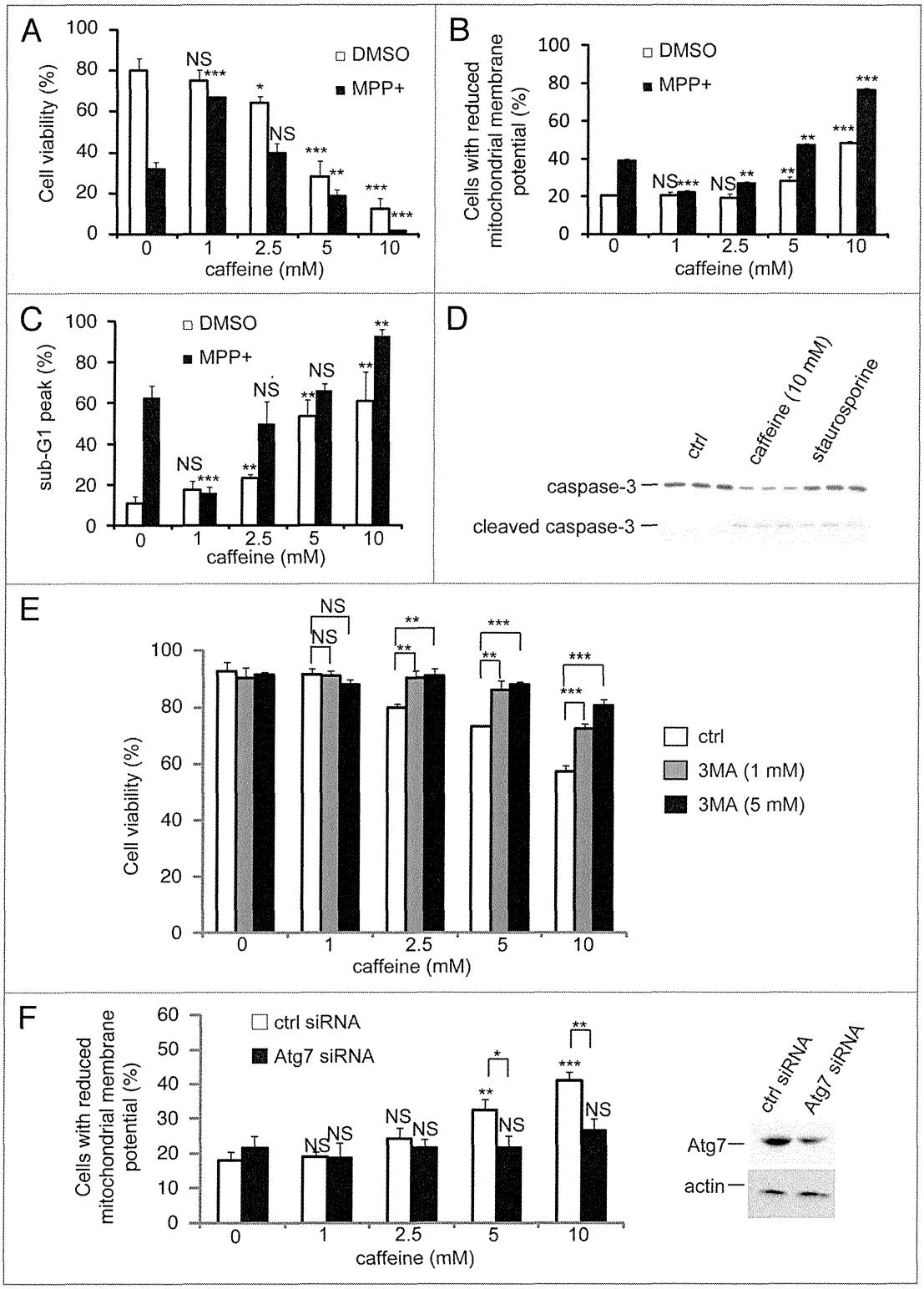
To establish a HeLa GFP-LC3 stable cell line, proliferating HeLa cells were transfected with a GFP-LC3 plasmid.[14] Forty-eight hours post-transfection with Lipofectamine 2000 (Invitrogen), positive stable clones were selected by growing cells with G418 (400 μg/ml) for 2 weeks and maintained in DMEM (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

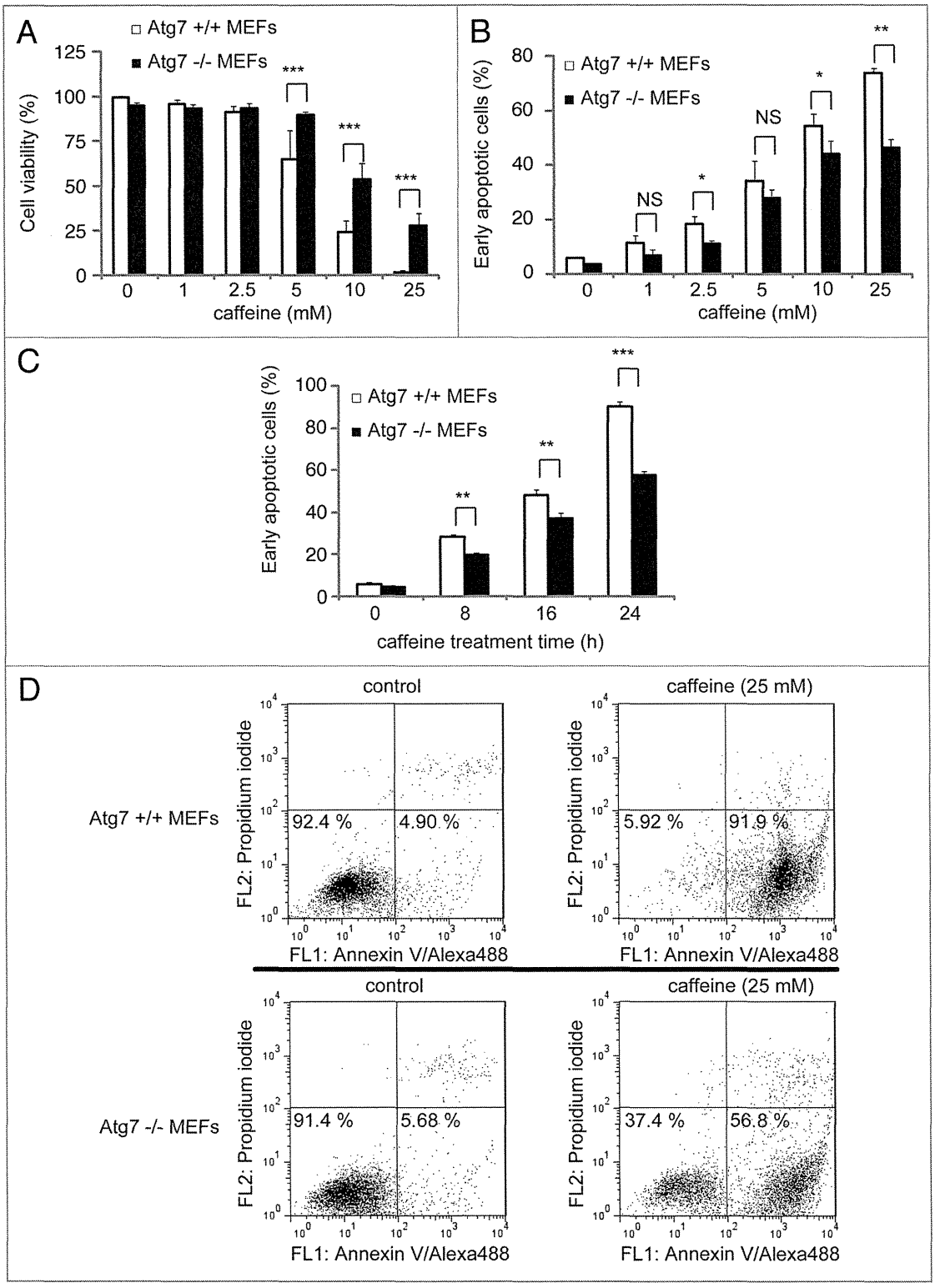


Figure 7. Cells without *Atg7* expression are more resistant to caffeine-induced apoptosis. (A) After *Atg7*$^{+/+}$ or $^{-/-}$ mouse embryonic fibroblasts (MEFs) were treated with 0, 1, 2.5, 5, 10, 25 mM caffeine for 24 hours, the cell viability was measured by trypan blue dye exclusion assay. Data 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).

Acknowledgements

We thank Dr. Takashi Ueno (Department of Biochemistry, Juntendo University) for critical comments and Drs. Masaaki Komatsu and Yu-Shin Sou for providing *Atg7*$^{+/+}$ and $^{-/-}$ MEFs. We are very grateful for a grant from Hayashi Memorial Foundation for Female Natural Scientists (Y.S.), the Grant-in-Aid for Young Scientists (B) (S. Saiki and F. Sato), grants from the All Japan Coffee Association (S. Saiki), the Takeda Scientific Foundation (S. Saiki) and the Nagao Memorial Fund (S. Saiki).

Note

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

References

1. Bode AM, Dong Z. The enigmatic effects of caffeine in cell cycle and cancer. Cancer Lett 2007; 247:26-39.
2. Jang MH, Shin MC, Kang IS, Baik HH, Cho YH, Chu JP, et al. Caffeine induces apoptosis in human neuroblastoma cell line SK-N-MC. J Korean Med Sci 2002; 17:674-8.
3. Gururajanna B, Al-Katib AA, Li YW, Aranha O, Vaitkevicius VK, Sarkar FH. Molecular effects of taxol and caffeine on pancreatic cancer cells. Int J Mol Med 1999; 4:501-7.
4. Qi W, Qiao D, Martinez JD. Caffeine induces TP53-independent G(1)-phase arrest and apoptosis in human lung tumor cells in a dose-dependent manner. Radiat Res 2002; 157:166-74.
5. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature 2008; 451:1069-75.
6. Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature 2006; 443:780-6.
7. Eisenberg-Lerner A, Bialik S, Simon HU, Kimchi A. Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ 2009; 16:966-75.
8. Espert L, Denizot M, Grimaldi M, Robert-Hebmann V, Gay B, Varbanov M, et al. Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4. J Clin Invest 2006; 116:2161-72.
9. Foukas LC, Daniele N, Ktori C, Anderson KE, Jensen J, Shepherd PR. Direct effects of caffeine and theophylline on p110delta and other phosphoinositide 3-kinases. Differential effects on lipid kinase and protein kinase activities. J Biol Chem 2002; 277:37124-30.
10. Kudchodkar SB, Yu Y, Maguire TG, Alwine JC. Human cytomegalovirus infection alters the substrate specificities and rapamycin sensitivities of raptor- and rictor-containing complexes. Proc Natl Acad Sci USA 2006; 103:14182-7.
11. Winter G, Hazan R, Bakalinsky AT, Abeliovich H. Caffeine induces macroautophagy and confers a cytocidal effect on food spoilage yeast in combination with benzoic acid. Autophagy 2008; 4:28-36.
12. Rubinsztein DC, Cuervo AM, Ravikumar B, Sarkar S, Korolchuk V, Kaushik S, Klionsky DJ. In search of an "autophagomometer". Autophagy 2009; 5:585-9.
13. Tanida I, Ueno T, Kominami E. LC3 and Autophagy. Methods Mol Biol 2008; 445:77-88.
14. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000; 19:5720-8.
15. Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell Struct Funct 1998; 23:33-42.
16. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell 140:313-26.
17. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100:57-70.
18. Ikenoue T, Hong S, Inoki K. Monitoring mammalian target of rapamycin (mTOR) activity. Methods Enzymol 2009; 452:165-80.
19. Sinn B, Tallen G, Schroeder G, Grassl B, Schulze J, Budach V, Tinhofer I. Caffeine confers radiosensitization of PTEN-deficient malignant glioma cells by enhancing ionizing radiation-induced G_1 arrest and negatively regulating Akt phosphorylation. Mol Cancer Ther 9:480-8.
20. Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, Abraham RT. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 1999; 59:4375-82.
21. Inoki K, Li Y, Xu T, Guan KL. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev 2003; 17:1829-34.
22. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol 2002; 4:648-57.
23. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, et al. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell 2003; 11:1457-66.
24. Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis PN, Rosen N. Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol-3-kinase/Akt-dependent pathway. J Biol Chem 1998; 273:29864-72.

25. Degtyarev M, De Maziere A, Orr C, Lin J, Lee BB, Tien JY, et al. Akt inhibition promotes autophagy and sensitizes PTEN-null tumors to lysosomotropic agents. J Cell Biol 2008; 183:101-16.
26. Wan X, Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. Oncogene 2007; 26:1932-40.
27. Sun SY, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, Khuri FR. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. Cancer Res 2005; 65:7052-8.
28. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 2006; 66:1500-8.
29. Cirstea D, Hideshima T, Rodig S, Santo L, Pozzi S, Vallet S, et al. Dual inhibition of akt/mammalian target of rapamycin pathway by nanoparticle albumin-bound-rapamycin and perifosine induces antitumor activity in multiple myeloma. Mol Cancer Ther 2010; 9:963-75.
30. Aoki H, Takada Y, Kondo S, Sawaya R, Aggarwal BB, Kondo Y. Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. Mol Pharmacol 2007; 72:29-39.
31. Ellington AA, Berhow MA, Singletary KW. Inhibition of Akt signaling and enhanced ERK1/2 activity are involved in induction of macroautophagy by triterpenoid B-group soyasaponins in colon cancer cells. Carcinogenesis 2006; 27:298-306.
32. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Potential therapeutic applications of autophagy. Nat Rev Drug Discov 2007; 6:304-12.
33. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz LG, et al. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet 2004; 36:585-95.
34. Kotake Y, Ohta S. MPP^+ analogs acting on mitochondria and inducing neuro-degeneration. Curr Med Chem 2003; 10:2507-16.
35. Hagan MP, Hopcia KL, Sylvester FC, Held KD. Caffeine-induced apoptosis reveals a persistent lesion after treatment with bromodeoxyuridine and ultraviolet-B light. Radiat Res 1997; 147:674-9.
36. Efferth T, Fabry U, Glatte P, Osieka R. Expression of apoptosis-related oncoproteins and modulation of apoptosis by caffeine in human leukemic cells. J Cancer Res Clin Oncol 1995; 121:648-56.
37. Shinomiya N, Takemura T, Iwamoto K, Rokutanda M. Caffeine induces S-phase apoptosis in cis-diamminedichloroplatinum-treated cells, whereas cis-diamminedichloroplatinum induces a block in G_2/M. Cytometry 1997; 27:365-73.
38. Lau CC, Pardee AB. Mechanism by which caffeine potentiates lethality of nitrogen mustard. Proc Natl Acad Sci USA 1982; 79:2942-6.
39. Takagi M, Shigeta T, Asada M, Iwata S, Nakazawa S, Kanke Y, et al. DNA damage-associated cell cycle and cell death control is differentially modulated by caffeine in clones with p53 mutations. Leukemia 1999; 13:70-7.
40. Ormerod MG, Collins MK, Rodriguez-Tarduchy G, Robertson D. Apoptosis in interleukin-3-dependent haemopoietic cells. Quantification by two flow cytometric methods. J Immunol Methods 1992; 153:57-65.
41. Kawatani M, Uchi M, Simizu S, Osada H, Imoto M. Transmembrane domain of Bcl-2 is required for inhibition of ceramide synthesis, but not cytochrome *c* release in the pathway of inostamycin-induced apoptosis. Exp Cell Res 2003; 286:57-66.
42. Kawajiri S, Saiki S, Sato S, Sato F, Hatano T, Eguchi H, Hattori N. PINK1 is recruited to mitochondria with parkin and associates with LC3 in mitophagy. FEBS Lett 2010; 584:1073-9.
43. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J Biol Chem 2007; 282:5641-52.

REVIEW

Molecular pathogenesis of Parkinson's disease: update

Shinji Saiki, Shigeto Sato, Nobutaka Hattori

Department of Neurology, Juntendo University School of Medicine, Bunkyo, Tokyo, Japan

Correspondence to
Professor N Hattori, Department of Neurology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan; nhattori@juntendo.ac.jp

Received 16 August 2011
Revised 6 November 2011
Accepted 9 November 2011
Published Online First 3 December 2011

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

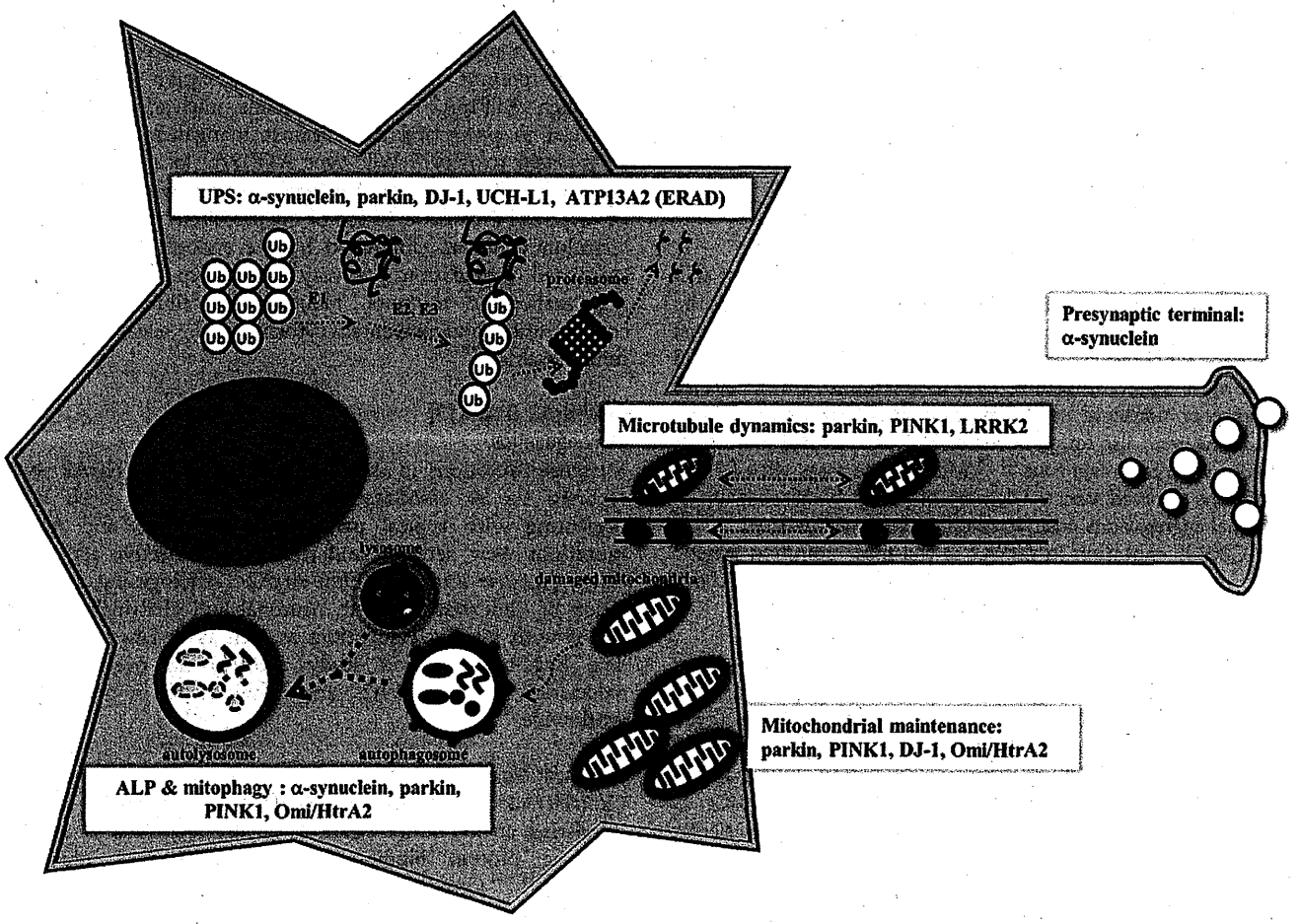


Figure 1 Schematic representation of the possible pathogenesis in hereditary Parkinson's disease. ALP, autophagy–lysosome pathway; ERAD, endoplasmic reticulum associated degradation; Ub, ubiquitin; UPS, ubiquitin proteasome system.

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.

Funding The authors are very grateful for the CREST Grant from the Japan Science and Technology Agency (NH), grants from the Ministry of Health, Labour and Welfare (NH) and the Ministry of Education, Culture, Sports, Science and Technology (NH), Grant-in-Aid for Young Scientists (A) (S Saiki), a promoted grant from Juntendo University (S Saiki) and grants from the Takeda Scientific Foundation (S Sato, S Saiki) and the Life Science Foundation (S Saiki).

Competing interests None.

Contributors All authors contributed to this work, including interpretation of the references and manuscript writing.

Provenance and peer review Commissioned; externally peer reviewed.

REFERENCES

1. **Hatano T,** Kubo S, Sato S, *et al*. Pathogenesis of familial Parkinson's disease: new insights based on monogenic forms of Parkinson's disease. *J Neurochem* 2009;**111**:1075–93.
2. **Nuytemans K,** Theuns J, Cruts M, *et al*: Genetic etiology of Parkinson disease associated with mutations in the SNCA, PARK2, PINK1, PARK7, and LRRK2 genes: a mutation update. *Hum Mutat* 2010;**31**:763–80.
3. **Gasser T.** Molecular pathogenesis of Parkinson disease: insights from genetic studies. *Expert Rev Mol Med* 2009;**11**:e22.
4. **Polymeropoulos MH,** Lavedan C, Leroy E, *et al*. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 1997;**276**:2045–7.
5. **Venda LL,** Cragg SJ, Buchman VL, *et al*. α-Synuclein and dopamine at the crossroads of Parkinson's disease. *Trends Neurosci* 2010;**33**:559–68.
6. **Simon-Sanchez J,** Schulte C, Bras JM, *et al*. Genome-wide association study reveals genetic risk underlying Parkinson's disease. *Nat Genet* 2009;**41**:1308–12.
7. **Satake W,** Nakabayashi Y, Mizuta I, *et al*. Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. *Nat Genet* 2009;**41**:1303–7.
8. **Farrer M,** Maraganore DM, Lockhart P, *et al*. α-Synuclein gene haplotypes are associated with Parkinson's disease. *Hum Mol Genet* 2001;**10**:1847–51.
9. **Maraganore DM,** de Andrade M, Elbaz A, *et al*. Collaborative analysis of alpha-synuclein gene promoter variability and Parkinson disease. *JAMA* 2006;**296**:661–70.
10. **Giasson BI,** Duda JE, Murray IV, *et al*. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 2000;**290**:985–9.
11. **Kruger R,** Eberhardt O, Riess O, *et al*. Parkinson's disease: one biochemical pathway to fit all genes? *Trends Mol Med* 2002;**8**:236–40.
12. **Masliah E,** Rockenstein E, Veinbergs I, *et al*. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 2000;**287**:1265–9.
13. **Kahle PJ,** Neumann M, Ozmen L, *et al*. Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brain. *J Neurosci* 2000;**20**:6365–73.
14. **Giasson BI,** Duda JE, Quinn SM, *et al*. Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. *Neuron* 2002;**34**:521–33.
15. **Cookson MR,** van der Brug M. Cell systems and the toxic mechanism(s) of alpha-synuclein. *Exp Neurol* 2008;**209**:5–11.
16. **Falsone SF,** Kungl AJ, Rek A, *et al*. The molecular chaperone Hsp90 modulates intermediate steps of amyloid assembly of the Parkinson-related protein alpha-synuclein. *J Biol Chem* 2009;**284**:31190–9.
17. **Webb JL,** Ravikumar B, Atkins J, *et al*. Alpha-synuclein is degraded by both autophagy and the proteasome. *J Biol Chem* 2003;**278**:25009–13.
18. **Cuervo AM,** Stefanis L, Fredenburg R, *et al*. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science* 2004;**305**:1292–5.
19. **Martinez-Vicente M,** Talloczy Z, Kaushik S, *et al*. Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. *J Clin Invest* 2008;**118**:777–88.
20. **Winslow AR,** Chen CW, Corrochano S, *et al*. α-Synuclein impairs macroautophagy: implications for Parkinson's disease. *J Cell Biol* 2010;**190**:1023–37.
21. **Brundin P,** Li JY, Holton JL, *et al*. Research in motion: the enigma of Parkinson's disease pathology spread. *Nat Rev Neurosci* 2008;**9**:741–5.