Eisai, Medtronic, Lundbeck, NeuroMolecular, Novartis, Solvay, Taro, and Teva, speaker's fees or other support from GlaxoSmithKline and UCB, and grant support from Taro; Dr. Ottman, receiving consulting fees from Ortho-McNeil Janssen Scientific Affairs and holding equity in Trigeminal Solutions; Dr. Perera, receiving grant support from Genzyme of Brazil; and Dr. Rozenberg, receiving grant support form Genzyme of Brazil. No other potential conflict of interest relevant to this article was reported.

We thank John Hardy, Ph.D. (from the Institute of Neurology, Queen's Square, London) for helpful discussion; Dora Yearout for technical assistance; John W. Roberts, M.D., for recruitment of subjects at the University of Washington, Seattle; Jae Choi and Julia Fekecs for preparation of drafts of the figures and Chunhui Yu for developing the online survey (all from NHGRI); Neil Oden (from Emmes, Rockville, MD) for statistical assistance; and T. Lumley for maintenance of source codes in R software.

APPENDIX

The full names of the authors, and their degrees, are as follows: Ellen Sidransky, M.D., Michael A. Nalls, Ph.D., Jan O. Aasly, M.D., Ph.D., Judith Aharon-Peretz, M.D., Grazia Annesi, Ph.D., Egberto R. Barbosa, M.D., Anat Bar-Shira, Ph.D., Daniela Berg, M.D., Jose Bras, M.S., Alexis Brice, M.D., Chiung-Mei Chen, M.D., Ph.D., Lorraine N. Clark, Ph.D., Christel Condroyer, B.S., Elvira V. De Marco, Ph.D., Alexandra Dürr, M.D., Ph.D., Michael J. Eblan, B.A., Stanley Fahn, M.D., Matthew J. Farrer, Ph.D., Hon-Chung Fung, M.D., Ph.D., Ziv Gan-Or, B.Med.Sci., Thomas Gasser, M.D., Ruth Gershoni-Baruch, M.D., Nir Giladi, M.D., Alida Griffith, M.D., Tanya Gurevich, M.D., Cristina Januario, M.D., Peter Kropp, Ph.D., Anthony E. Lang, M.D., Guey-Jen Lee-Chen, Ph.D., Suzanne Lesage, Ph.D., Karen Marder, M.D., M.P.H., Ignacio F. Mata, Ph.D., Anat Mirelman, Ph.D., Jun Mitsui, M.D., Ikuko Mizuta, M.D., Ph.D., Giuseppe Nicoletti, M.D., Catarina Oliveira, M.D., Ph.D., Ruth Ottman, Ph.D., Avi Orr-Urtreger, M.D., Ph.D., Lygia V. Pereira, Ph.D., Aldo Quattrone, M.D., Ekaterina Rogaeva, Ph.D., Arndt Rolfs, M.D., Hanna Rosenbaum, M.D., Roberto Rozenberg, Ph.D., Ali Samii, M.D., Ted Samaddar, B.S., Claudia Schulte, Dip.Biol., Manu Sharma, Ph.D., Andrew Singleton, Ph.D., Mariana Spitz, M.D., Eng-King Tan, M.D., Nahid Tayebi, Ph.D., Tatsushi Toda, M.D., Ph.D., André R. Troiano, M.D., Shoji Tsuji, M.D., Ph.D., Matthias Wittstock, M.D., Tyra G. Wolfsberg, Ph.D., Yih-Ru Wu, M.D., Cyrus P. Zabetian, M.D., Yi Zhao, M.D., Ph.D., and Shira G. Ziegler, B.A.

The authors' affiliations are as follows: the Section on Molecular Neurogenetics, Medical Genetics Branch, NHGRI (E.S., M.J.E., T.S., N.T., S.G.Z.), the Laboratory of Neurogenetics, National Institute on Aging (M.A.N., J.B., A.S.), and the Genome Technology Branch, National Human Genome Research Institute (T.G.W.) — all at the National Institutes of Health, Bethesda, MD; the Department of Neurology, St. Olav's Hospital, and the Department of Neuroscience, Norwegian University of Science and Technology — both in Trondheim, Norway (J.O.A.); the Cognitive Neurology and Movement Disorder Unit (J.A.-P.), the Human Genetics Rambam Medical Center (R.G.-B.), the Bruce Rappaport Faculty of Medicine (R.G.-B.), and the Departments of Hematology and Bone Marrow Transplantation, Technion, Israel Institute of Technology (H.R.) — all in Haifa, Israel; the Institute of Neurological Sciences, National Research Council, Cosenza (G.A., E.V.D.M., G.N., A.Q.), and the Institute of Neurology, Department of Medical Sciences, University Magna Graecia, Catanzaro (G.N., A.Q.) — both in Italy; the Movement Disorders Unit, Neurology Department, University of São Paulo Medical School (E.R.B., M.S.), and the Molecular Genetics Lab, Department of Genetics and Evolutionary Biology, Biosciences Institute, University of São Paulo (L.V.P., R.R.) — both in São Paulo; the Genetic Institute (A.B.-S., Z.G.-O., A.O.-U.), the Movement Disorders Unit, Parkinson Center, Department of Neurology, Tel-Aviv Sourasky Medical Center (N.G., T. Gurevich, A.M.), and the Sackler Faculty of Medicine, Tel-Aviv University (Z.G.-O., N.G., T.G., A.O.-U.) — all in Tel Aviv, Israel; the Hertie-Institute for Clinical Brain Research, Department of Neurodegenerative Diseases, University of Tübingen, Tübingen, Germany (D.B., T. Gasser, C.S., M.S.); the Center for Neuroscience and Cell Biology, University of Coimbra (J.B., C.O.), and the Neurology Service, University of Coimbra Hospital (C.J., C.O.) — both in Coimbra, Portugal; INSERM, Centre de Recherche de l'Institut du Cerveau et de la Moëlle Épinière Unité Mixte de Recherche_S975 (formerly Unité Mixte de Recherche_S679), Pierre et Marie Curie University (A.B., C.C., A.D., S.L., A.T.), Assistance Publique–Hôpitaux de Paris, and the Department of Genetics and Cytogenetics, Pitié–Salpêtrière Hospital (A.B., A.D.) — all in Paris; the Department of Neurology, Chang Gung Memorial Hospital, Chang-Gung University College of Medicine, Taipei, Taiwan (C.-M.C., H.-C.F., Y.-R.W.); the Taub Institute for Research on Alzheimer's Disease and the Aging Brain (L.N.C., K.M.), the Center for Human Genetics (L.N.C.), the Departments of Pathology (L.N.C.), Neurology (S.F., K.M., R.O.), and Psychiatry (K.M.), and the Gertrude H. Sergievsky Center (K.M., R.O.), Columbia University College of Physicians and Surgeons; and the Department of Epidemiology, Mailman School of Public Health, Columbia University and New York State Psychiatric Institute (R.O.) — all in New York; the Laboratories of Neurogenetics, Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, FL (M.J.F.); Booth Gardner Parkinson's Care Center, Evergreen Hospital Medical Center, Kirkland, WA (A.G.); the Institute for Medical Psychology (P.K.), the Albrecht-Kossel-Institute for Neuroregeneration (A.R.), and the Department of Neurology (M.W.), University of Rostock, Rostock, Germany; the Department of Medicine, Division of Neurology, Movement Disorders Centre, Toronto Western Hospital (A.E.L.), and the Centre for Research in Neurodegenerative Diseases, Tanz Neuroscience Building, University of Toronto (E.R.) — both in Toronto; the Department of Life Science, National Taiwan Normal University, Taipei, Taiwan (G.-J.L.-C.); the Parkinson's Disease and Geriatric Research Education and Clinical Centers, VA Puget Sound Health Care System, and Department of Neurology, University of Washington — both in Seattle (I.F.M., A.S., C.P.Z.); the Department of Neurology, University of Tokyo Graduate School of Medicine, Tokyo (J.M., S.T.), and the Division of Neurology and Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe (I.M., T.T.) — both in Japan; and Singapore General Hospital, National Neuroscience Institute (E.-K.T., Y.Z.), and Duke–NUS Graduate Medical School (E.-K.T.) — both in Singapore.

REFERENCES

1. Beutler E, Grabowski GA. Gaucher disease. In: Scriver CR, Beaudet AR, Sly W, Valle D, eds. The metabolic and molecular bases of inherited disease. 8th ed. New York: McGraw-Hill, 2001:3635-68.
2. Sidransky E. Gaucher disease: complexity in a "simple" disorder. Mol Genet Metab 2004;83:6-15.
3. Lwin A, Orvisky E, Goker-Alpan O, LaMarca ME, Sidransky E. Glucocerebrosidase mutations in subjects with parkinsonism. Mol Genet Metab 2004;81:70-3.
4. Aharon-Peretz J, Rosenbaum H, Gershoni-Baruch R. Mutations in the glucocerebrosidase gene and Parkinson's disease in Ashkenazi Jews. N Engl J Med 2004; 351:1972-7.
5. Clark LN, Ross BM, Wang Y, et al. Mutations in the glucocerebrosidase gene are associated with early-onset Parkinson disease. Neurology 2007;69:1270-7.
6. Gan-Or Z, Giladi N, Rozovski U, et al. Genotype-phenotype correlations between GBA mutations and Parkinson disease risk and onset. Neurology 2008;70:2277-83.
7. Sato C, Morgan A, Lang AE, et al. Analysis of the glucocerebrosidase gene

in Parkinson's disease. Mov Disord 2005; 20:367-70.

8. Eblan MJ, Nguyen J, Ziegler SG, et al. Glucocerebrosidase mutations are also found in subjects with early-onset parkinsonism from Venezuela. Mov Disord 2006; 21:282-3.

9. Spitz M, Rozenberg R, Pereira Lda V, Reis Barbosa E. Association between Parkinson's disease and glucocerebrosidase mutations in Brazil. Parkinsonism Relat Disord 2008;14:58-62.

10. Bras J, Paisan-Ruiz C, Guerreiro R, et al. Complete screening for glucocerebrosidase mutations in Parkinson disease patients from Portugal. Neurobiol Aging 2009;30:1515-7.

11. Mata IF, Samii A, Schneer SH, et al. Glucocerebrosidase gene mutations: a risk factor for Lewy body disorders. Arch Neurol 2008;65:379-82.

12. Toft M, Pielsticker L, Ross OA, Aasly JO, Farrer MJ. Glucocerebrosidase gene mutations and Parkinson disease in the Norwegian population. Neurology 2006; 66:415-7.

13. De Marco EV, Annesi G, Tarantino P, et al. Glucocerebrosidase gene mutations are associated with Parkinson's disease in southern Italy. Mov Disord 2008;23: 460-3.

14. Ziegler SG, Eblan MJ, Gutti U, et al. Glucocerebrosidase mutations in Chinese subjects from Taiwan with sporadic Parkinson disease. Mol Genet Metab 2007;91: 195-200.

15. Wu YR, Chen CM, Chao CY, et al. Glucocerebrosidase gene mutation is a risk factor for early onset of Parkinson disease among Taiwanese. J Neurol Neurosurg Psychiatry 2007;78:977-9.

16. Tan EK, Tong J, Fook-Chong S, et al. Glucocerebrosidase mutations and risk of Parkinson disease in Chinese patients. Arch Neurol 2007;64:1056-8.

17. Neumann J, Bras J, Deas E, et al. Glucocerebrosidase mutations in clinical and pathologically proven Parkinson's disease. Brain 2009;132:1783-94.

18. Nichols WC, Pankratz N, Marek DK, et al. Mutations in GBA are associated with familial Parkinson disease susceptibility and age at onset. Neurology 2009; 72:310-6.

19. Mitsui J, Mizuta I, Toyoda A, et al. Mutations for Gaucher disease confer high susceptibility to Parkinson disease. Arch Neurol 2009;66:571-6.

20. Kalinderi K, Bostantjopoulou S, Paisan-Ruiz C, Katsarou Z, Hardy J, Fidani L. Complete screening for glucocerebrosidase mutations in Parkinson disease patients from Greece. Neurosci Lett 2009; 452:87-9.

21. The PDGene Database. Alzheimer research forum, 2009. (Accessed September 25, 2009, at http://www.pdgene.org/.)

22. Machaczka M, Rucinska M, Skotnicki AB, Jurczak W. Parkinson's syndrome preceding clinical manifestation of Gaucher's disease. Am J Hematol 1999;61:216-7.

23. Tayebi N, Callahan M, Madike V, et al. Gaucher disease and parkinsonism: a phenotypic and genotypic characterization. Mol Genet Metab 2001;73:313-21.

24. Neudorfer O, Giladi N, Elstein D, et al. Occurrence of Parkinson's syndrome in type I Gaucher disease. QJM 1996;89: 691-4.

25. Tayebi N, Walker J, Stubblefield B, et al. Gaucher disease with parkinsonian manifestations: does glucocerebrosidase deficiency contribute to a vulnerability to parkinsonism? Mol Genet Metab 2003;79: 104-9.

26. Goker-Alpan O, Lopez G, Vithayathil J, Davis J, Hallett M, Sidransky E. The spectrum of parkinsonian manifestations associated with glucocerebrosidase mutations. Arch Neurol 2008;65:1353-7.

27. Goker-Alpan O, Schiffmann R, LaMarca ME, Nussbaum RL, McInerney-Leo A, Sidransky E. Parkinsonism among Gaucher disease carriers. J Med Genet 2004; 41:937-40.

28. Halperin A, Elstein D, Zimran A. Increased incidence of Parkinson disease among relatives of patients with Gaucher disease. Blood Cells Mol Dis 2006;36: 426-8.

29. Hruska KS, LaMarca ME, Scott CR, Sidransky E. Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). Hum Mutat 2008;29:567-83.

30. Montfort M, Chabás A, Vilageliu L, Grinberg D. Functional analysis of 13 GBA mutant alleles identified in Gaucher disease patients: pathogenic changes and "modifier" polymorphisms. Hum Mutat 2004;23:567-75. [Erratum, Hum Mutat 2005;26:276.]

31. Winfield SL, Tayebi N, Martin BM, Ginns EI, Sidransky E. Identification of three additional genes contiguous to the glucocerebrosidase locus on chromosome 1q21: implications for Gaucher disease. Genome Res 1997;7:1020-6.

32. Tayebi N, Stubblefield BK, Park JK, et al. Reciprocal and nonreciprocal recombination at the glucocerebrosidase gene region: implications for complexity in Gaucher disease. Am J Hum Genet 2003;72: 519-34.

33. Wan L, Hsu CM, Tsai CH, Lee CC, Hwu WL, Tsai FJ. Mutation analysis of Gaucher disease patients in Taiwan: high prevalence of the RecNciI and L444P mutations. Blood Cells Mol Dis 2006;36: 422-5.

34. Gibb WR, Lees AJ. The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. J Neurol Neurosurg Psychiatry 1988;51:745-52.

35. Woolf B. On estimating the relation between blood group and disease. Ann Hum Genet 1955;19:251-3.

36. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing, 2005. (Accessed September 25, 2009, at http://www.R-project.org.)

37. Park JK, Tayebi N, Stubblefield BK, et al. The E326K mutation and Gaucher disease: mutation or polymorphism? Clin Genet 2002;61:32-4.

38. Goker-Alpan O, Giasson BI, Eblan MJ, et al. Glucocerebrosidase mutations are an important risk factor for Lewy body disorders. Neurology 2006;67:908-10.

39. Clark LN, Kartsaklis LA, Wolf Gilbert R, et al. Association of glucocerebrosidase mutations with dementia with Lewy bodies. Arch Neurol 2009;66:578-83.

40. Farrer MJ, Williams LN, Algom AA, et al. Glucosidase-beta variations and Lewy body disorders. Parkinsonism Relat Disord 2009;15:414-6.

41. Segarane B, Li A, Paudel R, et al. Glucocerebrosidase mutations in 108 neuropathologically confirmed cases of multiple system atrophy. Neurology 2009;72:1185-6.

42. DePaolo J, Goker-Alpan O, Samaddar T, Lopez G, Sidransky E. The association between mutations in the lysosomal protein glucocerebrosidase and parkinsonism. Mov Disord 2009 May 7 (Epub ahead of print).

43. Bras J, Singleton A, Cookson MR, Hardy J. Emerging pathways in genetic Parkinson's disease: potential role of ceramide metabolism in Lewy body disease. FEBS J 2008;275:5767-73.

Copyright © 2009 Massachusetts Medical Society.

Analysis of GWAS-linked loci in Parkinson disease reaffirms PARK16 as a susceptibility locus

E.-K. Tan, MD*
H.-K. Kwok, BSc*
L.C. Tan, MD
W.-T. Zhao, MSc
K.M. Prakash, MD
W.-L. Au, MD
R. Pavanni, MD
Y.-Y. Ng, BSc
W. Satake, MD, PhD
Y. Zhao, MD, PhD
T. Toda, MD, PhD
J.-J. Liu, PhD

Address correspondence and reprint requests to Dr. Eng-King Tan, Department of Neurology, Singapore General Hospital, Singapore 169108 gnrtek@sgh.com.sg; or Dr. Jian-Jun Liu, Human Genetics, Genome Institute of Singapore, A*STAR, 60 Biopolis Street, 138672, Singapore liuj3@gis.a-star.edu.sg

ABSTRACT

Objective: A genome-wide association study (GWAS) in the Japanese population identified 2 new Parkinson disease (PD) susceptibility loci on *1q32 (PARK16)* (OMIM 613164) and *BST1* . We analyzed single nucleotide polymorphism (SNPs) located at the GWAS-linked loci (*PARK16, PARK8, PARK1*, and *BST1*) in a Chinese population and also conducted a meta-analysis in Asians by pooling 2 independent replication studies from Japan.

Methods: We conducted an analysis of 13 SNPs associated with PD GWAS-linked loci in 2 case-control cohorts comprised of 1,349 ethnic Chinese subjects.

Results: *PARK16, PARK8*, and *PARK1* loci but not *BST1* were found to be associated with PD. *PARK16* SNPs were associated with a decreased risk while *PARK1* and *PARK8* SNPs were associated with an increased risk of PD. A pooled analysis of our Chinese cohorts and 2 Japanese replication cohorts involving 1,366 subjects with PD and 16,669 controls revealed robust association with these 3 loci and also *BST1*. There was a trend toward a stronger protective effect of SNPs at the *PARK16* locus in sporadic PD compared to familial cases and in older compared to younger subjects.

Conclusions: Our study reaffirms the role of GWAS-linked loci in PD in Asian subjects and the strength of association is similar between Chinese and Japanese subjects. Efforts to elucidate the associated gene within *PARK16* locus are warranted. *Neurology*® **2010;75:508-512**

GLOSSARY

GWAS = genome-wide association study; **PD** = Parkinson disease; **SNP** = single nucleotide polymorphism.

Parkinson disease (PD) (OMIM168600), a neurodegenerative disorder, is characterized by loss of dopaminergic neurons in the pars compacta of the substantia nigra. In recent years, several causative genes have been associated with PD for both familial and sporadic forms of the disease.[1] However, these mutations probably account for a small percentage of PD cases in most populations. Therefore the search for genetic susceptibility risk factors in the vast majority of PD continues to be of scientific interest. Specific to PD, genetic variants involving pathogenic genes (*LRRK2* [*PARK8*], OMIM 607060, *SYN* [*PARK1*], OMIM 168601) and specific candidate genes have been shown to associate with the disease.[2-9] To date, there have been a few PD genome-wide association studies (GWAS) in the Caucasian population.[2,10,11] However, their findings have not been consistently replicated.[12,13] Among the many reasons, sample size and population stratification are some probable limitations. Recently, a GWAS study identified 2 new susceptibility loci on 1q32 (*PARK16*) (OMIM 613164) and *BST1* (bone marrow stromal cell antigen 1) (OMIM 004334) and also associations with known pathogenic genes involved in autosomal dominant forms of parkinsonism (*PARK1* on 4q22 and *PARK8* on 12q12) in the Japanese

Supplemental data at www.neurology.org

*These authors contributed equally to this work.

From the Departments of Neurology (E.-K.T., K.M.P., R.P., Y.-Y.N.) and Clinical Research (E.-K.T., Y.Z.), Singapore General Hospital (E.-K.T., K.M.P., R.P., Y.Z.), Singapore; National Neuroscience Institute (E.-K.T., L.C.T., W.-L.A.), Singapore; Duke-NUS Graduate Medical School (E.-K.T.), Singapore; Division of Neurology/Molecular Brain Science (W.S., T.T.), Kobe University Graduate School of Medicine, Kobe, Japan; and Human Genetics (H.-K.K., W.-T.Z., J.-J.L.), Genome Institute of Singapore, A*STAR, Singapore.

Disclosure: Author disclosures are provided at the end of the article.

Copyright © 2010 by AAN Enterprises, Inc.
Downloaded from www.neurology.org at KOBE UNIVERSITY IGAKUBU BUNKAN on May 5, 2011
Copyright © by AAN Enterprises, Inc. Unauthorized reproduction of this article is prohibited.

population.[14] *PARK1* have also been implicated as a genetic risk factor in another GWAS study using samples from subjects of European ancestry.

Moreover, the disease associations at *PARK16* and *PARK8* were replicated in a Caucasian replication study, using individuals of European ancestry.[15] Since Japanese and Chinese are of close Asian ancestry, we conducted a replication study of the GWAS-linked loci (*PARK16, PARK8, PARK1,* and *BST1*) in a Chinese population and also conducted a meta-analysis in Asian subjects by pooling 2 independent replication studies from Japan.

METHODS Ethnic Han Chinese subjects diagnosed with idiopathic PD by movement disorders neurologists at 2 different centers in Singapore (Singapore General Hospital and National Neuroscience Institute) were included. The diagnosis of PD was based on the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria.[16] Sporadic PD was defined as PD without a family history of disease. Controls of similar race, gender, and age from the same region as the patients with PD were also included. For controls, the age was matched ±5 years to the age at onset of PD cases.

Patient consent. The study received approval from each institutional ethics committee and all the study subjects gave written informed consent for their DNA to be used for genetic research. PD samples that had previously screened positive for pathogenic mutations in *[alpha]-synuclein, Parkin, DJ-1, LRRK2,* and *PINK1* were not included.

Selection of SNPs. Of the 20 SNPs from 4 genes that were reported in the GWAS study,[14] some SNPs are closely correlated (r^2 >0.8). Therefore, we only selected 13 noncorrelated SNPs (r^2 <0.8) (figure) for analysis in this study.

Genetic analysis. Genotyping was carried out with MALDI-TOF mass spectrometry using the Sequenom MassARRAY™ system (San Diego, CA). Multiplex genotyping assays were designed using the Sequenom DESIGNER software (San Diego, CA). PCR (5 ng of genomic DNA) and primer extension reactions were carried out initially according to the Sequenom genotyping assay iPLEX™ protocol. Confirmation of the variants with sequence analysis was carried out for random samples in ABI 3730 automated DNA sequencer (Applied Biosystems).

Statistical analysis. Statistical analyses were performed using *R* 2.10.1 software. χ^2 and Student *t* tests were used for comparing the categorical and continuous variables. We assessed each variant for departure from Hardy-Weinberg equilibrium. The results from pooled datasets were analyzed together and individual and pooled odds ratios and associated 95% confidence intervals were tabulated. We estimated the per-allele odds ratios using logistic regression and used Wald test to test whether the coefficients are significant. To test for association of allele frequency at each SNP with PD, we used χ^2 test with 1 degree of freedom. A multivariate logistic regression analysis adjusted for age and gender was performed. Stratified analysis by family history and age at onset was also carried out. Meta-analysis was performed to combine our Chinese study with 2 Japanese studies. Heterogeneity among sample sets was accessed using Woolf test. The meta-analysis was conducted using the Mantel-Haenszel method. As this is a replication study involving 4 independent loci, we made a modest correction for multiple comparisons with statistical significance defined at $p < 0.01$.

RESULTS Demographics. We studied a total of 1,349 ethnic Chinese subjects comprised of 2 case-control cohorts including a total of 433 patients with PD and 916 controls from 2 independent centers in Singapore. None of the patients with PD were from consanguineous families and about 3% reported a positive family history. The demographics of the study subjects are summarized in table 1.

Genotyping data. Thirteen SNPs located within the *PARK16, PARK8, PARK1,* and *BST1* loci, which were associated with PD in the GWAS study,[14] were analyzed. The 2 case-control cohorts were analyzed together to improve the power of analysis. The frequency of all the SNPs in the studied sample followed Hardy-Weinberg equilibrium, with the

Figure **Selection of genome-wide association study single nucleotide polymorphisms (SNPs)**

Gene	SNP	Position No.	SNPs selected for genotyping	r2
BST1	rs11931532	26	rs11931532_1	>0.8
BST1	rs12645693	37		
BST1	rs4538475	62		>0.8
BST1	rs4698412	59	rs4698412_1	
PARK8	rs1994090	16	rs1994090_1	
PARK8	rs2046932		rs2046932_1	>0.8
PARK8	rs4768212			
PARK8	rs2708453	65		>0.8
PARK8	rs7304279	54	rs7304279_1	
PARK16	rs11240572		rs11240572_1	
PARK16	rs16856139	7	rs16856139_1	
PARK16	rs708730	85		>0.8
PARK16	rs823156	78	rs823156_1	
PARK16	rs823122	46		
PARK16	rs823128	42	rs823128_1	>0.8
PARK16	rs947211	70	rs947211_1	
PARK1	rs11931074	5	rs11931074_1	>0.8
PARK1	rs3857059	25		
PARK1	rs6532194	130	rs6532194_1	
PARK1	rs894278	76	rs894278_1	

Only 1 SNP from each shaded box was selected for analysis as the 2 SNPs in each shaded box are in close linkage disequilibrium (r^2 >0.8).

Downloaded from www.neurology.org at KOBE UNIVERSITY IGAKUBU BUNKAN on May 5, 2011
Copyright © by AAN Enterprises, Inc. Unauthorized reproduction of this article is prohibited.

Table 1	Demographics of subjects with Parkinson disease and controls (total n = 1,349)	
	Parkinson disease	Controls
No.	433	916
Age, median, y	64	56
Age at onset, median, y	60	
Men/women, %	56/44	60/40

exception of rs4698412 (*BST1* locus), which showed a slight deviation ($p = 0.022$).

Ten SNPs belonging to *PARK16, PARK8,* and *PARK1* loci were found to be associated with PD (table 2). *PARK16* SNPs were associated with a decreased risk while *PARK1* and *PARK8* SNPs were associated with an increased risk of PD. A multivariate logistic regression analysis with disease/control group as the outcome measure and adjusting for age and gender revealed significant association with *PARK16, PARK8,* and *PARK1* but not the *BST1* locus (table 3). Stratification by family history revealed a trend toward a stronger protective effect of SNPs at the *PARK16* locus in sporadic PD compared to familial cases and in the older compared to younger subjects (table e-1 on the *Neurology*® Web site at www.neurology.org). At the *PARK8* locus, there was a trend toward a higher risk in familial PD compared to sporadic PD and in the older age group compared to the younger ones (stratified at age at onset <50 years or <55 years) (table e-2, A and B).

Pooled analysis of Asian subjects. There was no heterogeneity among the Chinese and Japanese datasets except rs11931532 (*BST1*) (table e-3). As the frequency of the studied SNPs was similar in the Japanese and Chinese control populations, a combined analysis of previously published 2 case-control replication cohorts in Japanese[14] and our cohorts was carried out (1,366 subjects with PD and 16,669 controls). Robust association with *PARK16, PARK8,* and *PARK1* was observed in addition to *BST1* (table e-4).

DISCUSSION A PD GWAS study in an American population identified 11 SNPs using a family-based design in tier 1 and a case-control design in tier 2.[2] A consortium from 14 centers which pooled 5,526 patients with PD and 6,682 controls was unable to replicate any significant association with the PD-associated SNPs.[12]

More recently, in a PD GWAS study conducted in Asia, investigators[14] identified 2 new susceptibility loci (*PARK16, BST1*) and also strong associations at *PARK1* and *PARK8*, 2 known loci implicated in autosomal dominant forms of parkinsonism. In the same study, the findings were replicated in 2 Japanese cohorts. The signal at the *PARK16* locus was less robust in the GWAS study in Caucasians[15] and did not surpass correction for multiple testing. However, the investigators subsequently conducted an analysis in their replication sample and found an association of SNP (rs823128) at the *PARK16* locus. The minor allele frequency (3%–4%) of the implicated *PARK16* SNP in Caucasians is low and this probably accounts for the relatively weaker association.

We conducted a replication study in the Chinese population. In the combined analysis of our 2 cohorts, we were able to independently demonstrate an

Table 2 SNP frequency and summary of analysis in subjects with Parkinson disease and controls

Gene	SNP	Chromosome	Allele (minor/major)	Minor allele frequency (case/control)	p Value[a]	OR	95% CI
BST1	rs11931532	4	C/T	0.49/0.50	0.5719	0.95	0.81-1.12
BST1	rs4698412	4	A/G	0.40/0.39	0.8269	1.02	0.86-1.21
PARK8	rs1994090	12	G/T	0.07/0.04	0.0004	1.92	1.33-2.77
PARK8	rs2046932	12	A/G	0.06/0.03	0.0013	1.85	1.26-2.69
PARK8	rs7304279	12	T/C	0.07/0.03	0.0003	1.95	1.35-2.81
PARK16	rs11240572	1	A/C	0.15/0.18	0.0135	0.75	0.60-0.94
PARK16	rs16856139	1	T/C	0.12/0.14	0.1447	0.83	0.65-1.06
PARK16	rs823156	1	G/A	0.18/0.22	0.0161	0.77	0.63-0.95
PARK16	rs823128	1	G/A	0.12/0.15	0.0316	0.76	0.60-0.98
PARK16	rs947211	1	A/G	0.39/0.42	0.1250	0.88	0.74-1.04
PARK1	rs11931074	4	G/T	0.39/0.45	0.0028	0.78	0.66-0.92
PARK1	rs6532194	4	C/T	0.39/0.45	0.0072	0.80	0.68-0.94
PARK1	rs894278	4	G/T	0.41/0.36	0.0102	1.25	1.05-1.48

Abbreviations: CI = confidence interval; OR = odds ratio; SNP = single nucleotide polymorphism.
[a] Corrected *p* value; significance at <0.01.

Downloaded from www.neurology.org at KOBE UNIVERSITY IGAKUBU BUNKAN on May 5, 2011
Copyright © by AAN Enterprises, Inc. Unauthorized reproduction of this article is prohibited.

Table 3	Multivariate analysis adjusted for age and gender		
SNP	Crude OR (95% CI)	Adjusted OR (95% CI)	p (Wald test)
rs16856139	0.84 (0.62–1.15)	0.84 (0.62–1.15)	0.287
rs823128	0.66 (0.48–0.89)	0.65 (0.48–0.89)	0.006
rs947211	0.7 (0.56–0.87)	0.71 (0.57–0.88)	0.002
rs823156	0.67 (0.52–0.88)	0.68 (0.52–0.89)	0.005
rs11240572	0.63 (0.48–0.84)	0.64 (0.48–0.85)	0.002
rs11931532	1.09 (0.88–1.34)	1.09 (0.88–1.35)	0.423
rs4698412	0.9 (0.72–1.11)	0.89 (0.72–1.11)	0.307
rs11931074	0.72 (0.58–0.88)	0.7 (0.56–0.86)	<0.0001
rs894278	1.27 (1.02–1.58)	1.27 (1.02–1.59)	0.032
rs6532194	0.77 (0.63–0.94)	0.76 (0.62–0.93)	0.008
rs1994090	1.64 (1.01–2.66)	1.67 (1.03–2.72)	0.039
rs7304279	1.64 (1.01–2.66)	1.67 (1.03–2.72)	0.039
rs2046932	1.55 (0.94–2.56)	1.57 (0.95–2.61)	0.078

Abbreviations: CI = confidence interval; OR = odds ratio; SNP = single nucleotide polymorphism.

association of *PARK16*, *PARK8*, and *PARK1* loci, except for BST1. While not all the SNPs located in *PARK 16* reached significant association, the trend and the effect size difference (odds ratio 0.8 to 0.9) between cases and controls was largely similar between our Chinese subjects and the Japanese subjects in the discovery GWAS sample and 2 replication Japanese cohorts. The frequency of the 13 SNPs (10%–50%) was also similar in these 2 Asian races. We observed a more robust association of the SNPs at the *PARK8* and *PARK1* loci. As there was no evidence of genetic heterogeneity between Chinese and Japanese at these GWAS-linked loci, we conducted a pooled evaluation of our cohorts and the 2 replication Japanese cohorts to increase the power of analysis. The pooled analysis further reaffirms the results across each independent dataset and the discovery dataset. As we did not observe any positive trend with the *BST1* locus in our cohort, replication in an independent Chinese cohort is advised. Though the median age of the controls in our study was 4 years younger compared to median age at onset of 60 years in subjects with PD, multivariate analysis after taking into account the effects of age and gender revealed significant associations similar to those observed in the univariate analysis. Furthermore, comparison with a selected set of 428 controls with median age at onset of 60 years showed similar results (data not shown).

Consistent and independent replication of genetic association studies remains the litmus test of the validity of the findings. While identification of susceptibility alleles is an important step, unraveling the biologic basis of their actions, if any, remains a challenging task as many of these variants are not located in the coding regions. Among the identified loci, *PARK16* is interesting as it contains a few candidate genes (*SLC41A1*, *RAB7L1*, *NUCKS1*). rs947211 is associated with transcript level of *NUCKS1*,[14] though Simón-Sánchez et al.[15] did not find any association of the SNPs and expression levels of the genes at *PARK16*, *PARK8*, and *PARK1*. The association of a lower risk with PD suggests that protective gene variants or genes are located within this region or they need interaction with unknown genes to exert their effect. It is possible that these SNPs are not the actual causative variants based on their biologic plausibility and the varied strength of their association. The original GWAS study[14] did not analyze the strength of association between familial and sporadic PD and between older and younger subjects. Despite the limitation of a smaller sample size in the subset analysis, our data revealed a consistent trend toward a stronger protective effect of SNPs at the *PARK16* locus in sporadic PD compared to familial cases and in the older compared to younger subjects (table e-1). The significance of this is unclear, though it suggests that common variants at *PARK16* locus may be more relevant to a general group of patients with PD. There was also a consistent trend toward a higher risk in familial PD compared to sporadic PD and in the older age group compared to the younger ones at the *PARK8* locus (table e-2). As *PARK8* is responsible for autosomal dominant parkinsonism and is associated with late-onset disease, this observation may not be unexpected. Since these SNPs are located from intron 2 of *SLC2A13* to the upstream region of *LRRK2*, our finding strengthens the suggestion that these are likely to be risk variants of the *LRRK2* gene.

While current evidence suggests that common genetic variants play a role in the etiology of typical PD, GWAS studies by their inherent design may not be able to detect rare variants.[9,17] It is also possible that cases selected for GWAS studies may not be particularly enriched with genetic susceptibility alleles, and other compounding factors like reduced penetrance of PD genes and gene-environmental interaction were unaccounted for. Thus multiple approaches including linkage analysis, sequencing, and sibpair analysis would be needed to uncover additional variants/causative genes and susceptibility loci.

ACKNOWLEDGMENT

The authors thank Singapore General Hospital, National Neuroscience Institute, Duke-NUS Graduate Medical School, Singapore Millennium Foundation, National Medical Research Council, and Dr. Yuen Yih for support. The authors also thank Mitsutoshi Yamamoto, Nobutaka Hattori, and Miho Murata for the data published in reference 14.

Downloaded from www.neurology.org at KOBE UNIVERSITY IGAKUBU BUNKAN on May 5, 2011
Copyright © by AAN Enterprises, Inc. Unauthorized reproduction of this article is prohibited.

DISCLOSURE

Dr. E.-K. Tan has received funding or speaker honoraria from Boehringer Ingelheim and Novartis; serves as editor of *Annals, Academy of Medicine, Singapore*, and associate editor of *European Journal of Neurology and Parkinsonism Related Disorders*; and receives research support from Eisai Inc., Schering-Plough Corp., Allergan Inc., the National Medical Research Council, Singapore, and the Singapore Millennium Foundation. H.-K. Kwok reports no disclosures. Dr. L. Tan has received funding for travel or speaker honoraria from Boehringer Ingelheim, Novartis, GlaxoSmith Kline, and Medtronic, Inc.; serves on the editorial board of *Movement Disorders*; and receives research support from Eisai Inc., Schering-Plough Corp., Allergan Inc., the National Medical Research Council, Singapore, and the Singapore Millennium Foundation. W.-T. Zhao, Dr. Kumar, Dr. Au, Dr. Pavanni, Y.-Y. Ng, Dr. Satake, and Dr. Zhao report no disclosures. Dr. Tatsushi serves as an Associate Editor for the *Journal of Human Genetics* and receives research support from the Japan Science and Technology Agency, the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ministry of Health, Labor and Welfare of Japan. Dr. Liu serves as an Associate Editor for *BMC Genetics*.

Received February 10, 2010. Accepted in final form April 26, 2010.

REFERENCES

1. Tan EK, Skipper LM. Pathogenic mutations in Parkinson disease. Hum Mutat 2007;28:641–653.
2. Maraganore DM, de Andrade M, Lesnick TG, et al. High-resolution whole-genome association study of Parkinson disease. Am J Hum Genet 2005;77:685–693.
3. Maraganore DM, de Andrade M, Elbaz A, et al. Collaborative analysis of alpha-synuclein gene promoter variability and Parkinson disease. JAMA 2006;296:661–670.
4. Tan EK, Zhao Y, Skipper L, et al. The LRRK2 Gly2385Arg variant is associated with Parkinson's disease: genetic and functional evidence. Hum Genet 2007;120: 857–863.
5. 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.
6. Skipper L, Li Y, Bonnard C. Comprehensive evaluation of common genetic variation within LRRK2 reveals evidence for association with sporadic Parkinson's disease. Hum Mol Genet 2005;14:3549–3556.
7. Zabetian CP, Hutter CM, Factor SA, et al. Association analysis of MAPT H1 haplotype and subhaplotypes in Parkinson's disease. Ann Neurol 2007;62:137–144.
8. Sidransky E, Nalls MA, Aasly JO, et al. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. N Engl J Med 2009;361:1651–1661.
9. Pankratz N, Wilk JB, Latourelle JC, et al. Genomewide association study for susceptibility genes contributing to familial Parkinson disease. Hum Genet 2009;124:593–605.
10. Fung HC, Scholz S, Matarin M, et al. Genomewide genotyping in Parkinson's disease and neurologically normal controls: first stage analysis and public release of data. Lancet Neurol 2006;5:911–916.
11. Tan EK. The role of common genetic risk variants in Parkinson disease. Clin Genet 2007;72:387–393.
12. Elbaz A, Nelson LM, Payami H, et al. Lack of replication of thirteen single-nucleotide polymorphisms implicated in Parkinson's disease: a large-scale international study. Lancet Neurol 2006;5:917–923.
13. Evangelou E, Maraganore DM, Annesi G, et al. Non-replication of association for six polymorphisms from meta-analysis of genome-wide association studies of Parkinson's disease: large-scale collaborative study. Am J Med Genet B Neuropsychiatr Genet 2010;153B:220–228.
14. 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;41:1303–1307.
15. Simón-Sánchez J, Schulte C, Bras JM, et al. Genome-wide association study reveals genetic risk underlying Parkinson's disease. Nat Genet 2009;41:1308–1312.
16. Hughes AJ, Daniel SE, Kilford L, Lees AJ, Hughes AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 1992;55:181–184.
17. Bodmer W, Bonilla C. Common and rare variants in multifactorial susceptibility to common diseases. Nat Genet 2008;40:695–701.

Neurology® Launches Subspecialty Alerts by E-mail!

Customize your online journal experience by signing up for e-mail alerts related to your subspecialty or area of interest. Access this free service by visiting http://www.neurology.org/cgi/alerts/etoc or click on the "E-mail Alerts" link on the home page. An extensive list of subspecialties, methods, and study design choices will be available for you to choose from—allowing you priority alerts to cutting-edge research in your field!

Downloaded from www.neurology.org at KOBE UNIVERSITY IGAKUBU BUNKAN on May 5, 2011
Copyright © by AAN Enterprises, Inc. Unauthorized reproduction of this article is prohibited.

7. Ford I, Cotter MA, Cameron NE, Greaves M. The effects of treatment with alpha-lipoic acid or evening primrose oil on vascular hemostatic and lipid risk factors, blood flow, and peripheral nerve conduction in the streptozotocin-diabetic rat. Metabolism 2001;50:868–875.
8. Moyad MA. Brewer's/baker's yeast (Saccharomyces cerevisiae) and preventive medicine, part II. Urol Nurs 2008;28: 73–75.
9. Engel WK. Multi-microcramps (MMC) syndrome: a new pathogenic concept of subtle lower motor-neuron (LMN) lability causing very disturbing, continuous muscle pains, sometimes gratifyingly treatable—but often misinterpreted as mysterious "fibromyalgia" or "psychogenic." Neurology 2010;74(suppl 2):A465. Abstract.
10. Chiou LC, Chang CC. Pharmacological relevance of peripheral type benzodiazepine receptors on motor nerve and skeletal muscle. Br J Pharmacol 1994;112: 257–261.
11. Young GL, Jewell D. Interventions for leg cramps in pregnancy. Cochrane Database Syst Rev 2002;(1): CD000121.
12. Roffe C, Sills S, Crome P, Jones P. Randomised, crossover, placebo controlled trial of magnesium citrate in the treatment of chronic persistent leg cramps. Med Sci Monit 2002;8:CR326–CR330.
13. Frusso R, Zarate M, Augustovski F, Rubinstein A. Magnesium for the treatment of nocturnal leg cramps: a crossover randomized trial. J Fam Pract 1999;48:868–871.

CORRECTION

High insulinlike growth factor I is associated with cognitive decline in Huntington disease

In the article "High insulinlike growth factor I is associated with cognitive decline in Huntington disease" by Saleh et al. (*Neurology*® 2010;75:57–63), there were errors in the Acknowledgment and Coinvestigator list. The Acknowledgment should have included the Centre d'Investigation Clinique Groupe Hospitalier Chenevier-Mondor Créteil for assistance in recruiting patients. The Coinvestigator list should have included Graça Morgado (Centre d'Investigation Clinique Groupe Hospitalier Chenevier-Mondor Créteil, Nurse, Patient Recruitment and Investigation). The following members of the Ethical Committee were listed in error as coinvestigators: Franc Bellivier, MD, PhD (Groupe Hospitalier Chenevier-Mondor, Scientific Committee); Catherine Bertrand, PhD (Groupe Hospitalier Chenevier-Mondor, Scientific Committee); Christian Danesi, PhD (UDAF, Patient's Representative); Frédéric Galacteros, MD, PhD (Groupe Hospitalier Chenevier-Mondor, President of the Committee); Hebert Geschwind, MD, PhD (Paris 12 University, Ethical Committee); Isabelle Guigon, PhD (Groupe Hospitalier Chenevier-Mondor, Director of the INSERM); Brigitte Langlois, PhD (Paris 12 University, Ethical Committee); Patrick Pollissard-Pasbecq, PhD (Paris 12 University, Jurist); Aurore Renversade (Groupe Hospitalier Chenevier-Mondor, Nurse); Françoise Roudot-Thoraval, MD, PhD (Groupe Hospitalier Chenevier-Mondor, Scientific Committee); Brigitte Tastet, MD (Paris 12 University, Advisory Committee); and Anick Tibi, PharmD (AGEPS, Scientific Committee).

CORRECTION

Analysis of GWAS-linked loci in Parkinson Disease reaffirms PARK16 as a susceptibility locus

In the article "Analysis of GWAS-linked loci in Parkinson disease reaffirms PARK16 as a susceptibility locus" by E.-K. Tan et al. (*Neurology*® 2010;75:508–512), the joint first author's name should have been listed as H.-H. Kwok, PhD. The authors regret the error.

Downloaded from www.neurology.org at KOBE UNIVERSITY IGAKUBU BUNKAN on May 5, 2011
Copyright © by AAN Enterprises, Inc. Unauthorized reproduction of this article is prohibited.

ELSEVIER

Parkinsonism and Related Disorders 15, Supplement 1 (2009) S17–S20

Parkinsonism & Related Disorders

www.elsevier.com/locate/parkreldis

Levodopa in the early treatment of Parkinson's disease

Miho Murata *

Department of Neurology, National Center Hospital of Neurology & Psychiatry, Kodaira, Japan

Abstract

L-dopa has many advantages as initial therapy for Parkinson's disease (PD). It is safer, more efficacious, associated with fewer adverse effects, few interactions, easier for patients to use and for clinicians to prescribe, and cheaper than dopamine (DA) agonists. Although L-dopa is more likely than DA agonists to introduce motor fluctuations and dyskinesia, L-dopa is also more effective in improving motor function. Furthermore, there is no long-term benefit from delaying L-dopa based on the risk of motor complications or psychiatric symptoms. Many investigations have shown that L-dopa does not accelerate disease progression. Now is the time to re-evaluate L-dopa for initial treatment of PD.
© 2008 Elsevier Ltd. All rights reserved.

Keywords: Parkinson's disease; L-dopa; DA agonist; Motor fluctuation, Dyskinesia

1. Introduction

Although it is recommended that dopamine (DA) agonists should be chosen as initial treatment for Parkinson's disease (PD), it is time to re-evaluate the use of levodopa for initial treatment of PD.

While many dopaminergic drugs have been introduced, L-dopa therapy has remained the gold standard for symptomatic treatment of PD. L-dopa is safer, more efficacious, associated with fewer adverse effects, has few interactions, is easier for patients to use and for clinicians to prescribe, and it is cheaper than DA agonists. Despite these advantages, many previous guidelines (Fig. 1) [1,2] have stated that for early stage patients with PD it is appropriate to start treatment with a DA agonist unless the patient is either older than 75 years or has dementia. The rationale for these recommendations has been that (1) L-dopa may accelerate disease progression, and (2) DA agonists are less likely to induce motor fluctuation.

* Address for correspondence: Miho Murata, MD, PhD. National Center Hospital of Neurology & Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo, Japan 187-8551. Tel.: 81-42-341-2711; Fax: 81-42-346-1735.

E-mail address: mihom@ncnp.go.jp

1353-8020/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.

2. Is L-dopa really neurotoxic?

Concern that exogenous L-dopa may be neurotoxic and contribute to the progression of PD arises from the oxidative stress hypothesis of PD [3]. Many *in vitro* studies have demonstrated that the addition of L-dopa or DA to cultured dopaminergic neurons increased cell death [4,5]. However, these experiments were performed under non-physiologic conditions; the concentrations of L-dopa and DA in these experiments were high (>5 μM) and exceeded what would be expected in the brains of patients treated with therapeutic doses (<2 μM). Furthermore, some *in vitro* studies have shown that L-dopa and DA are neuroprotective when neurons are co-cultured with glial cells [6,7].

The ELLDOPA study, conducted to show whether L-dopa is toxic for PD patients, was a large, double-blind, randomized, controlled clinical trial comparing three different doses (150, 300, 600 mg per day) of L-dopa with placebo in patients with early PD [8]. At the end of the trial (after a 2-week washout period), the mean Unified Parkinson's Disease Rating Scale (UPDRS) score of patients treated with L-dopa was better than that of the placebo group. The mean UPDRS score of the highest dosage group was the best, even after the washout period. These results suggest that L-dopa is not toxic, and may even be neuroprotective. This study also included evaluation with β-CIT SPECT

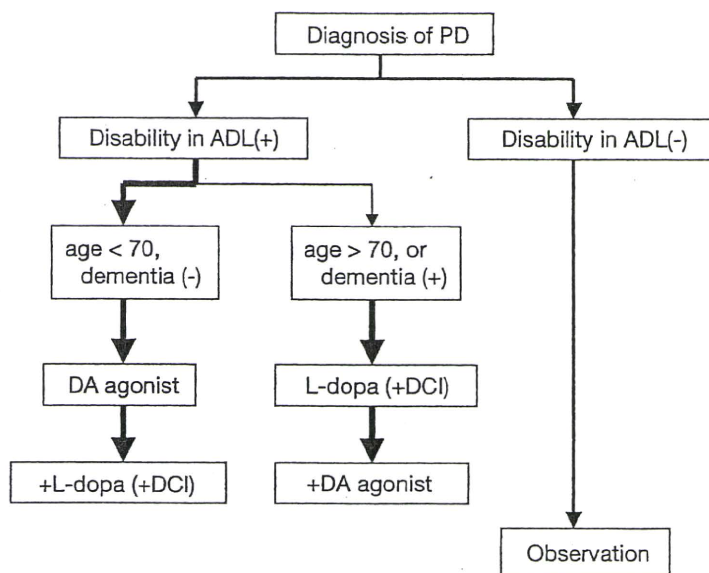


Fig. 1. Guideline for Parkinson's disease (early stage) made by Japanese Society for Neurology, 2002. ADL, activities of daily living; DCI, decarboxylase inhibitor.

imaging, as a marker for intact nigrostriatal dopaminergic neuronal functioning. The imaging studies showed that there was a larger decrease in β-CIT uptake in patients treated with L-dopa in a dose–response manner. These clinical and imaging results suggested that β-CIT SPECT imaging is not appropriate for evaluating the neuroprotective ability of the drugs. Up to that time, several studies had shown that DA agonists are more protective than L-dopa using β-CIT SPECT [9,10] or F-DOPA PET [11]. The results of the ELLDOPA study permitted re-evaluation of the results of these studies, and there is now a consensus that L-dopa does not accelerate disease progression [12].

3. L-dopa is more likely than DA agonists to induce motor fluctuations and dyskinesia

Several clinical studies [9,11] have shown that treatment with DA agonists is less likely to induce motor complications than treatment with L-dopa. Theses studies also showed that L-dopa monotherapy improves activities of daily living and motor function to a greater degree than DA agonists (plus later optional L-dopa). What is of most importance to our patients? The severity of both motor fluctuation and dyskinesia in these studies was low. For example, in the CALM-PD study (4 years) the percentage of disabling dyskinesia was 4.4% in the pramipexole group and 6.9% in the L-dopa group [13]. Furthermore, retrospective investigations failed to show any long-term benefit from delaying L-dopa based on the risk of motor complications, dementia, or psychiatric symptoms [13–16].

Apart from motor complications, the frequency of other common side effects is less with L-dopa than with DA agonists. For example, hallucinations are about three times more likely to occur with ropinirole or pramipexole than with L-dopa [17]. Somnolence, edema, and cardiac valvular fibrosis (pergolide, cabergoline) are also more frequent with DA agonist treatment than with L-dopa [17]. In Japan, those who are taking ropinirole or pramipexole are prohibited from driving because of the risk of sudden onset sleep. Can the incidence of motor fluctuation be reduced only at the expense of improvements in motor function and activities of daily living, and of other side effects such as hallucination, sudden onset sleep, and fibrosis?

4. Mechanism of wearing-off

Disease progression is associated with "wearing off" of therapeutic benefit and the appearance of unpredictable treatment responses, resulting in complex "on-off" response fluctuations. These arise in between doses of L-dopa because the patient no longer has the ability to store dopamine. The other factors driving development of response fluctuations are changes in peripheral L-dopa pharmacokinetics and in post-synaptic function that accompany large-dose and long-term L-dopa therapy [18]. Jenner and colleagues reported on the relation between the amount of lesion and the development of dyskinesia and motor fluctuation by using a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) marmoset model [19]. In primates with 50% lesions (model of early PD), L-dopa produced an antiparkinsonian response with no induction of dyskinesia. In the group with 75% lesions, L-dopa produced an antiparkinsonian response with a gradual development of dyskinesia. In the >90% lesion group, L-dopa produced a pulsatile anitiparkinsonian response and rapid induction of severe dyskinesia.

Pathological examination in patients with PD demonstrates an exponential loss of nigral pigmented neurons [20]. At 5 years from symptom onset, about 50% of the pigmented neurons remain, compared with age-adjusted controls, and at 10 years 30% remain. The presymptomatic phase of PD, dating from the onset of neuronal loss, was estimated to be about 5 years. Therefore, at the initial symptomatic stage

of the disease there is little possibility of developing motor fluctuation and dyskinesia as long as the appropriate dose of L-dopa is used. In fact, the ELLDOPA study showed the incidence of motor complications in the L-dopa 150 mg and 300 mg groups to be almost equal to that in the placebo group [8].

5. The advantage of L-dopa as initial therapy for Parkinson's disease

The initial use of L-dopa for the symptomatic treatment of PD has many advantages over DA agonists: (1) L-dopa has significantly greater efficacy than DA agonists in alleviating the motor symptoms of PD and improving activities of daily living. (2) Titration of L-dopa to therapeutic levels is much easier and faster than that of DA agonists. (3) L-dopa is much less likely to induce hallucinations, somnolence, edema, or constipation compared to DA agonists. (4) There is a clear cost benefit to using L-dopa. (In Japan, L-dopa + decarboxylase inhibitor 300 mg/day costs about 1 US dollar per day; pramipexole 4.5 mg/day about 16 US dollars; and ropinirole 15 mg/day 26 US dollars.) However, late-developing motor fluctuation and dyskinesia deserve consideration, and the potential for other common side effects such as hallucinations and sleep attacks must also be factored into the treatment decision. Furthermore, early in the course of the disease, L-dopa provides an enduring response that can last several days [21].

There is much evidence to show that DA agonists are efficacious in controlling L-dopa motor fluctuations (as later adjunctive therapy). However, another option is to initiate treatment with levodopa, adding a DA agonist after the first sign of developing motor complications has appeared (early combination).

6. Continuous stimulation by using L-dopa

Continuous daytime intraintestinal infusion of L-dopa can diminish motor complications [22]. This shows that motor complications can be improved by changing the pharmacokinetics of L-dopa. For example, catechol-*O*-methyltransferase (COMT) inhibitors can extend the half-life of serum L-dopa concentration. The duration of L-dopa efficacy can also be extended by taking L-dopa after a meal (Fig. 2). Long-term L-dopa therapy increases the peak L-dopa concentration (C_{max}) and decreases its half-life ($T_{1/2}$) [23]. C_{max} is decreased and $T_{1/2}$ is increased by taking L-dopa after a meal compared to taking it before meals. Therefore, taking L-dopa after meals not only extends effective time but also decreases dyskinesia. By taking L-dopa after meals, the dose of L-dopa can be increased, but the risk of dyskinesia can be decreased. If needed, a low dose of L-dopa may be taken before meals for immediate improvements, with the remainder of the dose taken after meals.

There is much evidence for the efficacy of DA agonists as adjunctive therapy in controlling L-dopa motor fluctuations and dyskinesia [24]. Monoamine oxidase (MAO) B inhibitors [24] and zonisamide [25] may also be used as adjunctive therapy to improve motor fluctuations.

7. Initial symptomatic treatment for early Parkinson's disease

It is appropriate to start treatment of PD with either L-dopa or DA agonists. As highlighted by the American Academy of Neurology practice parameter [17], the choice of initial treatment depends on the relative importance for the patient of improving motor disability and limiting adverse events versus the possibility of lowering the risk of developing long-term motor complications.

The frequency of the development of wearing-off depends on the age of disease onset. Younger-onset patients (younger than 50 years at onset) are more prone to severe dyskinesia and motor fluctuation, while patients older than 70 years at symptom-onset rarely develop disabling dyskinesia and motor fluctuation. Older patients may be more prone to develop hallucinations and other common adverse effects. Therefore, L-dopa is preferred for elderly patients as initial treatment. In younger patients, DA agonists are preferred, but if the patient is at risk of losing his or her job owing to motor disability, L-dopa should be started. Patients whose age of onset is between 50 and 70 years can be prescribed

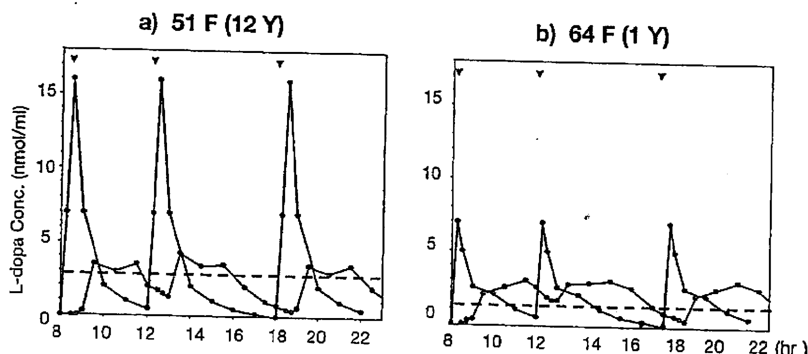


Fig. 2. Effects of a meal on L-dopa pharmacokinetics. (a) 51-year-old woman with 12 years' duration of Parkinson's disease. (b) 64-year-old woman with 1 year duration of Parkinson's disease. A tablet of L-dopa 100 mg + benserazide 20 mg was taken. Black curve: just before meal, red curve: after meal 3 times a day. ▼: meals (at 08.00, 12.00, 18.00 hours). Dashed line: the concentration of effective threshold estimated by clinical symptoms. C_{max} is 2–3 times higher when L-dopa was taken before meals than after meals. L-dopa pharmacokinetics are more stable when taken after meals than before meals.

either L-dopa or a DA agonist. Treatment selection should reflect the patient's needs and under-treatment should be avoided.

Conflict of interest

The author has no conflict of interest to report. No funding applicable.

References

[1] Olanow CW, Koller WC. An algorithm (decision tree) for the management of Parkinson's disease: treatment guidelines. American Academy of Neurology. Neurology 1998;50(Suppl 3):S1–57.
[2] Mizuno Y, Okuma Y, Kikuchi S, Kuno S, Hashimoto T, Hasegawa K, et al. Guidelines for the treatment of Parkinson's disease. Clin Neurol 2002;42:430–94.
[3] Fahn S. Is levodopa toxic? Neurology 1996;47(Suppl 3):S184–95.
[4] Pardo B, Mena MA, Casarejos MJ, Paino CL, De Yebenes JG. Toxic effects of L-DOPA on mesencephalic cell cultures: protection with antioxidants. Brain Res 1995;682:133–43.
[5] Masserano JM, Gong L, Kulaga H, Baker I, Wvatt RJ. Dopamine induces apoptotic cell death of a catecholaminergic cell line derived from the central nervous system. Mol Pharmacol 1996;50:1309–15.
[6] Mena MA, Casarejos MJ, Carazo A, Paino CL, Garcia de Yebenes J. Glia conditioned medium protects fetal rat midbrain neurons in culture from L-DOPA toxicity. Neuroreport 1996;76:441–5.
[7] Mytilineou C, Han SK, Cohen G. Toxic and protective effects of L-dopa on mesencephalic cell cultures. J Neurochem 1993;61:1470–8.
[8] The Parkinson Study Group. Levodopa and the progression of Parkinson's disease. New Engl J Med 2004;351:2498–508.
[9] Parkinson Study Group. Pramipexole vs levodopa as initial treatment for parkinson disease. JAMA 2000;284:1931–8.
[10] Parkinson Study Group. Dopamine transporter brain imaging to assess the effects of pramipexole vs levodopa on Parkinson disease progression. JAMA. 2002;287:1653–61.
[11] Whone AL, Watts RL, Stoessl AJ, Davis M, Reske S, Nahmias C, et al. Slower progression of Parkinson's disease with ropinirole versus levodopa: The REAL-PET study. Ann Neurol 2003;54:93–101.
[12] Suchowersky O, Gronseth G, Perlmutter J, Reich S, Zesiewicz T, Weiner WJ, et al. Practice Parameter: Neuroprotective strategies and alternative therapies for Parkinson disease (an evidence-based review). Neurology 2006;66:976–82.
[13] Halloway RG, Shoulson I, Fahn S, Kiburtz K, Lang A, Marek K, et al. Pramipexole vs levodopa as initial treatment for Parkinson disease: a 4-year randomized controlled trial. Arch Neurol 2004;61:1044–53.
[14] Caracenti T, Scigliano G, Musicco M. The occurrence of motor fluctuations in Parkinsonian patients treated long-term with levodopa: role of early treatment and disease progression. Neurology 1991;41:380–4.
[15] Cedarbaum JM, Gandy SE, McDowell FH. Early initiation of levodopa treatment of motor response fluctuations, dyskinesia or dementia in Parkinson's disease. Neurology 1991;41:622–9.
[16] Lees AJ, Katzenschlager R, Head J, Ben-Shlomo Y. Ten-year follow-up of these different initial treatments in de-novo PD. A randomized trial. Neurology 2001;51:1687–94.
[17] Miyasaki JM, Martin W, Suchowsky O, Weiner WJ, Lang AE. Practice parameter: Initiation of treatment for Parkinson's disease: An evidence-based review. Neurology 2002;58:11–7.
[18] Poewe W, Wenning G. Levodopa in Parkinson's disease: mechanisms of action and pathophysiology of late failure. In: Jankovic JJ, Tolosa E, editors. Parkinson's Disease & Movement Disorders, 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2002. p. 104–15.
[19] Jenner P, Jackson M, Rose S, Smith L. Co-administration of LDOPA/carbidopa with entacapone avoids dyskinesia induction in MPTP-treated primates with full or partial nigral lesions. Mov Disord 2006;(suppl 13):S73.
[20] Fearnley JM, Lees AJ. Ageing and Parkinson's disease: Substantia nigra regional selectivity. Brain 1991;114:2283–301.
[21] Muenter MD, Tyce GM. L-dopa therapy of PD in plasma L-DOPA concentration, therapeutic response, and side effects. Mayo Clin Proc 1971;46:231–9.
[22] Stocchi F, Vacca L, Ruggieri S, Olanow CW. Intermittent vs continuous levodopa administration in patients with advanced Parkinson disease. Arch Neurol 2005;62:905–10.
[23] Murata M, Mizusawa H, Yamanouchi H, Kanazawa I. Chronic levodopa therapy enhances dopa absorption: contribution to wearing-off. J Neural Transm 1996;103:1177–85.
[24] Pahwa R, Factor SA, Lyons KE, Ondo WG, Gronseth G, Bronte-Stewart H, et al. Practice parameter: Treatment of Parkinson disease with motor fluctuations and dyskinesia (an evidence-based review). Neurology 2006;66:983–95.
[25] Murata M, Hasegawa K, Kanazawa I. Zonisamide improves motor function in Parkinson's disease. A randomized, double-blind study. Neurology 2007;68:45–50.

Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Neuroscience Research 60 (2008) 106–113
Neuroscience Research
www.elsevier.com/locate/neures

Preventing effects of a novel anti-parkinsonian agent zonisamide on dopamine quinone formation

Masato Asanuma[a,*], Ikuko Miyazaki[a], Francisco J. Diaz-Corrales[a], Ko Miyoshi[a], Norio Ogawa[a], Miho Murata[b]

[a] *Department of Brain Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikatacho, Okayama 700-8558, Japan*
[b] *Department of Neurology, Musashi Hospital, National Center of Neurology and Psychiatry, Tokyo, Japan*

Received 10 August 2007; accepted 3 October 2007
Available online 10 October 2007

Abstract

The neurotoxicity of dopamine (DA) quinones as dopaminergic neuron-specific oxidative stress is considered to play a role in the pathogenesis and/or progression of Parkinson's disease (PD), since DA quinones conjugate with several key PD pathogenic molecules (*e.g.*, tyrosine hydroxylase, α-synuclein and parkin) to form protein-bound quinone (quinoprotein) and consequently inhibit their functions. Zonisamide (ZNS) is used as an anti-epileptic agent but also improved the cardinal symptoms of PD in recent clinical trials in Japan. To evaluate the effects of ZNS on excess cytosolic free DA-induced quinone toxicity, we examined changes in DA quinone-related indices after ZNS treatment both in *in vitro* cell-free system and in cultured cells. Co-incubation of DA and ZNS in a cell-free system caused conversion of DA to stable melanin via formation of DA-semiquinone radicals and DA chrome. Long-term (5 days) treatment with ZNS decreased quinoprotein and increased DA/DOPA chromes in dopaminergic CATH.a cells. ZNS significantly inhibited quinoprotein formation induced by treatment with tetrahydrobiopterin and ketanserin that elevate cytosolic free DA in the cells. Our results suggest that the novel anti-parkinsonian agent ZNS possesses preventing effects against DA quinone formation induced by excess amount of cytosolic DA outside the synaptic vesicles.
© 2007 Elsevier Ireland Ltd and the Japan Neuroscience Society. All rights reserved.

Keywords: Zonisamide; Dopamine chrome; Dopamine quinone; Quinoprotein; Parkinson's disease

1. Introduction

Under normal conditions, dopamine (DA) is stable in the synaptic vesicle. However, when levodopa is administered to the damaged dopaminergic neuronal system of Parkinson's disease (PD), a large amount of DA remains in the cytosol outside the synaptic vesicle, since the damaged dopaminergic system has too small DA pool to store DA (Sulzer et al., 2000; Sulzer and Zecca, 2000; Asanuma et al., 2003; Ogawa et al., 2005). The toxicity of excess levodopa and/or DA has been well documented in many *in vitro* and *in vivo* animal studies using parkinsonian models (Ogawa et al., 1993; Basma et al., 1995; Walkinshaw and Waters, 1995; Hastings et al., 1996; Asanuma et al., 2003), despite its marked beneficial effects. Free excess DA is easily metabolized via monoamine oxidase (MAO)-B or by auto-oxidation to produce cytotoxic reactive oxygen species (ROS), and then forms neuromelanin (Sulzer et al., 2000; Sulzer and Zecca, 2000). In the oxidation of DA by MAO, DA is converted to dihydroxyphenylacetic acid (DOPAC) to generate general ROS hydrogen peroxide. On the other hand, non-enzymatic and spontaneous auto-oxidation of DA and L-DOPA produces superoxide and reactive quinones such as DA quinones and DOPA quinones (Tse et al., 1976; Graham, 1978). DA quinones are also generated in the enzymatic oxidation of DA by prostaglandin H synthase (cyclooxygenase-2), lipoxygenase, tyrosinase and xanthine oxidase (Korytowski et al., 1987; Rosei et al., 1994; Hastings, 1995; Foppoli et al., 1997; Chae et al., 2007). These quinones are oxidized to the cyclized aminochromes: DA chrome (aminochrome) and DOPA chrome, and then are finally polymerized to form melanin. Although ROS generation by the auto-oxidation of DA may explain widespread

* Corresponding author. Tel.: +81 86 235 7408; fax: +81 86 235 7412.
E-mail address: asachan@cc.okayama-u.ac.jp (M. Asanuma).

0168-0102/$ – see front matter © 2007 Elsevier Ireland Ltd and the Japan Neuroscience Society. All rights reserved.
doi:10.1016/j.neures.2007.10.002

toxicity but not specific damage of DA neurons, the highly reactive DA quinone or DOPA quinone itself exerts predominant cytotoxicity in DA neurons and surrounding neural cells, since these quinones are generated from free cytosolic DA outside the synaptic vesicle or from L-DOPA (Sulzer et al., 2000).

The generated DA quinones covalently conjugate with the sulfhydryl group of cysteine on functional proteins, resulting predominantly in the formation of 5-cysteinyl-DA (Graham, 1978; Fornstedt et al., 1986). DA quinones conjugate with cysteine residues of various functional proteins including several key molecules involved in the pathogenesis of PD (*e.g.*, tyrosine hydroxylase, DA transporter and parkin) to form protein-bound quinones (quinoproteins), and inhibit the function of these proteins to cause DA neuron-specific cytotoxicity (Xu et al., 1998; Kuhn et al., 1999; Whitehead et al., 2001; LaVoie et al., 2005; Machida et al., 2005). We reported previously that repeated levodopa administration elevated striatal DA turnover and formation of quinoproteins specifically in the parkinsonian side, but not in the control side, of hemi-parkinsonian models (Ogawa et al., 2000; Asanuma et al., 2005; Miyazaki et al., 2005). Therefore, the excess amount of cytosolic DA outside the synaptic vesicles after levodopa treatment may exert neurodegenerative effects through quinone generation, at least in the damaged dopaminergic nerve terminals. The DA-induced formation of DA quinones and the consequent dopaminergic cell damage *in vitro* and *in vivo* could be prevented by treatment with superoxide dismutase, glutathione, and certain thiol reagents through their quinone-quenching activities (Offen et al., 1996; Lai and Yu, 1997; Kuhn et al., 1999; Haque et al., 2003). We also demonstrated recently that DA agonists pergolide and pramipexole exhibit quenching properties against *in vitro* generated DA-semiquinone radicals (Asanuma et al., 2005; Miyazaki et al., 2005), and that pergolide effectively prevented repeated levodopa-induced elevation of striatal quinoprotein specifically in parkinsonian models (Miyazaki et al., 2005). Thus, DA quinones act as neurotoxic compounds by eliciting dopaminergic neuron-specific oxidative stress and thus play a role in the pathogenesis and/or progression of PD and neurotoxin-induced parkinsonism (Choi et al., 2003, 2005; Asanuma et al., 2004; LaVoie et al., 2005; Machida et al., 2005; Ogawa et al., 2005; Chae et al., 2007).

Zonisamide (1,2-benzisoxazole-3-methaesulfonamide: ZNS), which was originally synthesized in Japan, has been used as an anti-epileptic agent in Japan, South Korea, USA and Europe. An open trial of ZNS (50–200 mg/day) administered in conjunction with anti-PD drugs showed lessening of symptoms, especially wearing-off (Murata et al., 2001), and induced more than 30% improvement of UPDRS total score up to 3 years (Murata, 2004). The addition of ZNS to levodopa treatment of patients experiencing 'wearing-off' fluctuations resulted in lessening of motor fluctuation and significant improvement of the duration, severity and activities of daily living in 'off' time and score of motor examination. Furthermore, a recent nationwide double-blind controlled study in Japan reported that an adjunctive treatment with lower dose of ZNS (25–100 mg/day) to levodopa improved all the cardinal symptoms of PD (Murata, 2004; Murata et al., 2007).

Several pharmacological effects of ZNS have been proposed to be related to its beneficial effects on PD. ZNS is a specific T-type Ca^{++} channel blocker (Suzuki et al., 1992; Kito et al., 1996), which increases burst firing of dopaminergic neurons in the substantia nigra. A single dose of ZNS increased intracellular and extracellular DOPA, DA and homovanillic acid (HVA) levels and decreased DOPAC level in the rat striatum presumably through its moderate MAO-inhibiting effect (Okada et al., 1992, 1995). Long-term administration of ZNS increased activity and protein level of tyrosine hydroxylase in the rat striatum (Murata, 2004), and thus enhanced DA synthesis. However, these effects cannot fully explain the mechanism of its therapeutic effects on levodopa-induced adverse effects.

To evaluate the effects of ZNS on excess cytosolic free DA-induced quinone toxicity, we examined changes in DA quinone-related indices after ZNS treatment both in *in vitro* cell-free DA-semiquinone generating system and in cultured dopaminergic neuronal cells.

2. Materials and methods

2.1. Materials

DA hydrochloride and L-DOPA were purchased from Wako Pure Chemical (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. ZNS and its sodium salt were provided by Dainippon Sumitomo Pharma (Osaka, Japan).

2.2. ESR spectrometry of generated DA-semiquinone radicals

The spectra of semiquinone radicals generated from DA in a cell-free system were recorded with an electron spin resonance (ESR) spectrometer (JES-FR30, JEOL Co., Tokyo) using a flat quartz cuvette as reported previously (Korytowski et al., 1987; Haque et al., 2003). DA or L-DOPA was dissolved in 10 mM sodium phosphate buffer (PB: pH 7.4), and the pH was adjusted to 7.0 by adding 0.1 M NaOH at 4 °C. For the experiment on time-dependency, the pH-adjusted DA or L-DOPA (final concentration 1 mM) was immediately incubated with ZNS sodium salt dissolved in 10 mM PB (final concentration 8 mM, pH 10.8) for 1–60 min at 37 °C, and the spectra for these combinations were analyzed. As a positive control to generate DA-semiquinone, tyrosinase (final concentration 12.5 μg/ml) was incubated instead of ZNS. Furthermore, 0.1 N NaOH (pH 10.9) or pH-adjusted 10 mM PB (pH 10.8) was used instead of ZNS as a negative control. For the experiment on dose-dependency, the pH-adjusted DA (final concentration 1 mM, pH 7.0), with or without various concentrations (ranging from 2 to 8 mM) of ZNS sodium salt dissolved in 10 mM PB (pH 10.8), was immediately incubated for 1 min at 37 °C, and the spectra for these combinations were analyzed. The pH of each final incubation mixture was approximately 8.0. The signal intensity was evaluated by the relative peak height of the second signal of the semiquinone radical spin adduct (the peak height of the second signal is higher than the others and is directly proportional to double integration of the spectra) to the intensity of the Mn^{2+} signal, which was used as the internal standard to correct for measurement error. The conditions of the ESR spectrometer to estimate the semiquinone radical, including magnetic field, power, modulation frequency, modulation amplitude, response time, temperature, amplitude, and sweep time were 335 ± 5 mT, 4 mW, 9.41 GHz, 79 μT, 0.1 s, 25 °C, 1 × 1000 and 1 min, respectively. Furthermore, the levels of DA and its metabolites in the reaction mixture were measured by high-performance liquid chromatography (HPLC) as described previously (Ogawa et al., 2000; Asanuma et al., 2005).

2.3. Effects of ZNS on generation of DA chrome

To examine the effects of ZNS on generation of DA chrome in a cell-free system, pH-adjusted 1 mM DA in 10 mM PB (pH 6.8) and 0.2% Triton X-100 solution were incubated with or without 200 μM ZNS dissolved in 10 mM PB (pH 6.8) for 1 min to 3 h at 37 °C. The level of DA chrome in the final mixture (pH 6.8) was estimated by measuring absorbance of incubation mixture at 475 nm.

2.4. Cell culture and drug treatment

Dopaminergic CATH.a cells (ATCC; #CRL-11179), derived from mouse DA-containing neurons, were cultured at 37 °C in 5% CO_2 in RPMI 1640 culture medium (Invitrogen, San Diego, CA) supplemented with 4% fetal bovine serum, 8% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) for the extraction of cell lysates used for the measurement of protein-bound quinone and DA/DOPA chrome at a density of 1.0×10^5 cells/cm^2. After 24 h, CATH.a cells were exposed to 1–100 μM ZNS diluted in phosphate buffered saline (PBS) for 5 days for the measurements of quinoprotein and DA/DOPA chrome. To examine the effects of ZNS on excess cytosolic free DA-induced quinone elevation, CATH.a cells were exposed simultaneously to 1–100 μM ZNS with 100 μM tetrahydrobiopterin (BH_4) and 10 μM ketanserin, which enhance DA synthesis and blocks vesicle monoamine transporter, respectively (Choi et al., 2005), for 3 h before extraction of total cell lysates for quinoprotein measurement.

2.5. Protein-bound quinone; quinoprotein measurement

Total cell lysates from drug-treated CATH.a cells were prepared with 10 μg/ml phenylmethylsulfonyl fluoride (Sigma) in ice cold-RIPA buffer [PBS (pH 7.4), 1% nonidet P-40 (NP-40), 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate]. The nitrobluetetrazolium (NBT)/glycinate colorimetric assay was performed to detect protein-bound quinones (quinoprotein) (Paz et al., 1991). The cell lysate was added to 500 μl of NBT reagent (0.24 mM NBT in 2 M potassium glycinate, pH 10.0) followed by incubation in the dark for 2 h under constant shaking. The absorbance of blue-purple color developed in the reaction mixture was measured at 530 nm.

2.6. Measurement of DA/DOPA chrome in CATH.a cells

For the measurement of DA/DOPA chrome, cells were solubilized in 500 μl of 1% Triton X-100 solution for 2 h and then centrifuged at 20,000 × g for 30 min at 4 °C. The supernatant was used as cell extract and incubated for 3 min at room temperature. The level of DA/DOPA chrome was calculated by measuring absorbance of incubated cell extract at 475 nm. Absorbance values in the *in vitro* incubation of DA (0–500 μM) with tyrosinase (10 μg/ml) for 30 min were used as a standard to calculate the concentration of DA/DOPA chrome in the cell extract.

2.7. Protein measurement

The protein concentration was determined using the Bio-Rad protein assay kit or Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA), based on the method of Bradford and Lowry, respectively, using bovine serum albumin as a standard.

2.8. Statistical analysis

Results are expressed as mean ± S.E.M. values. Statistical analysis of the data was performed using one-way ANOVA followed by *post hoc* Fisher's PLSD test. A *p*-value less than 0.05 denoted the presence of a statistically significant difference.

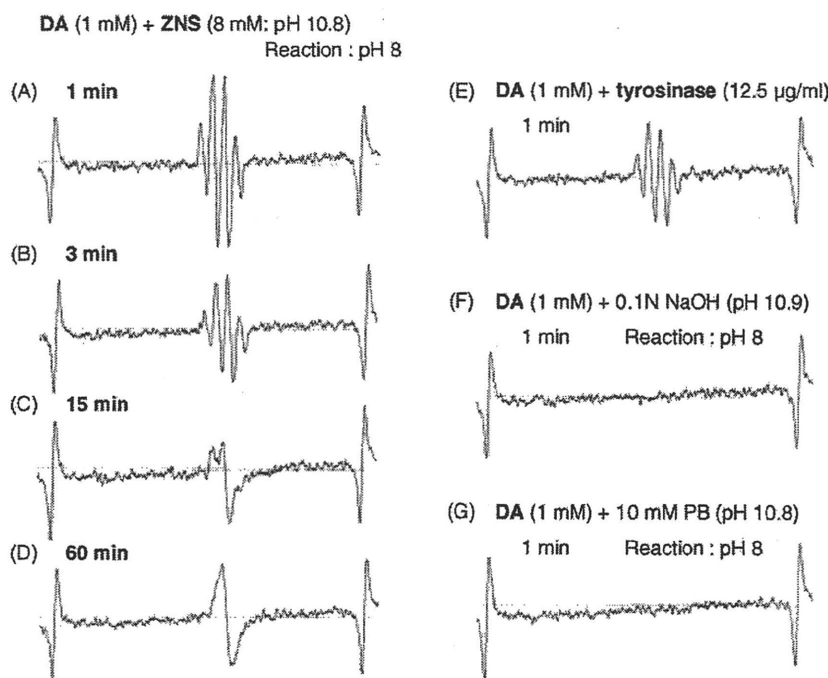


Fig. 1. Effects of ZNS on DA-semiquinone radicals generated from DA in a cell-free system. (A–D) Representative time-course changes in ESR spectra of DA-semiquinone radicals (A–B) to melanin (D) in the incubation of pH-adjusted 1 mM DA (pH 7.0) with 8 mM ZNS (sodium salt) in 10 mM PB (pH 10.8) for 1–60 min at 37 °C at pH 8.0 (incubation mixture). (E) Formation of DA-semiquinone radicals via tyrosinase (12.5 μg/ml)-catalyzed oxidation of 1 mM DA. (F and G) No signals for radical formation at pH 8.0 (incubation mixture) when pH-adjusted 1 mM DA (pH 7.0) was incubated for 1 min at 37 °C with 0.1 N NaOH (pH 10.9) (F) or pH-adjusted 10 mM PB (pH 10.8) (G). Each experiment was performed as triplicate assays.

3. Results

3.1. Effects of ZNS on generated DA-semiquinone radicals in a cell-free system

When a high dose of DA (5 mM) was incubated at 37 °C at neutral pH 7–8, the formation of DA-semiquinone radicals started immediately within 1 min, peaked at around 1 min, then gradually decreased and continued for 10 min, as shown in a previous study (Haque et al., 2003). In the present ESR study, however, no signals for radical formation were detected at pH 8 (incubation mixture) when a lower dose of pH-adjusted 1 mM DA (pH 7.0) was incubated for 1 min at 37 °C with 0.1 N NaOH (pH 10.9) or pH-adjusted 10 mM PB (pH 10.8) (Fig. 1F and G). Interestingly, when pH-adjusted 1 mM DA (pH 7.0) was incubated at 37 °C with 8 mM ZNS (sodium salt) in 10 mM PB (pH 10.8), the formation of DA-semiquinone radicals, which was identified by four waves in ESR spectrometry, started immediately within 1 min and peaked at around 1 min, at pH 8 (incubation mixture) (Fig. 1A and B), as well as formation of DA-semiquinone radicals via tyrosinase-catalyzed oxidation of DA (Fig. 1E). Then, the DA-semiquinone radical induced by incubation of DA and ZNS converted to melanin, which was recognized by a wide single wave, at 15–60 min (Fig. 1C and D). The incubation of pH-adjusted 1 mM DA (pH 7.0) and 2–8 mM ZNS (sodium salt, pH 10.8) at pH 8 (incubation mixture) resulted in DA-semiquinone radical formation at 1 min (Fig. 2A–D) and subsequent melanin formation at 60 min (data not shown) in a ZNS concentration-dependent manner. Furthermore, incubation of pH-adjusted 1 mM L-DOPA (pH 7.0) with 8 mM ZNS (sodium salt, pH 10.8) at 37 °C at pH 8 (incubation mixture) resulted in immediate generation of DOPA-semiquinone radicals within 1 min, with a peak at around 1 min, and conversion to melanin within up to 20 min (Fig. 2E–G). DOPAC, 3-methoxy tyramine and HVA, which are metabolites of DA via MAO and/or catecholamine *o*-methyltransferase, were not detected in any incubation mixture of pH-adjusted DA and ZNS (sodium salt) at either dose of ZNS or incubation time by HPLC (data not shown).

3.2. Effects of ZNS on generation of DA chrome in a cell-free system

Because a high dose of ZNS was required to detect the conversion effects from DA to melanin in ESR spectrometry, we used sodium salt of ZNS, which is highly soluble in 10 mM PB, at a dose of 2–8 mM. However, the pH value of the mixture of ESR examination was slightly alkaline at 8.0 because of high alkalinity of ZNS sodium salt solution (pH 10.8) in the cell-free system. To examine the effects of relatively low dose of ZNS on conversion of DA to melanin at neutral pH, we evaluated generation of DA chrome, which is an intermediate in conversion of DA quinone to melanin, using 200 μM ZNS at pH 6.8, but not its sodium salt (Fig. 3). Although the incubation of pH-adjusted 1 mM DA (pH 6.8) alone at 37 °C showed time-dependent but not significant increases in DA

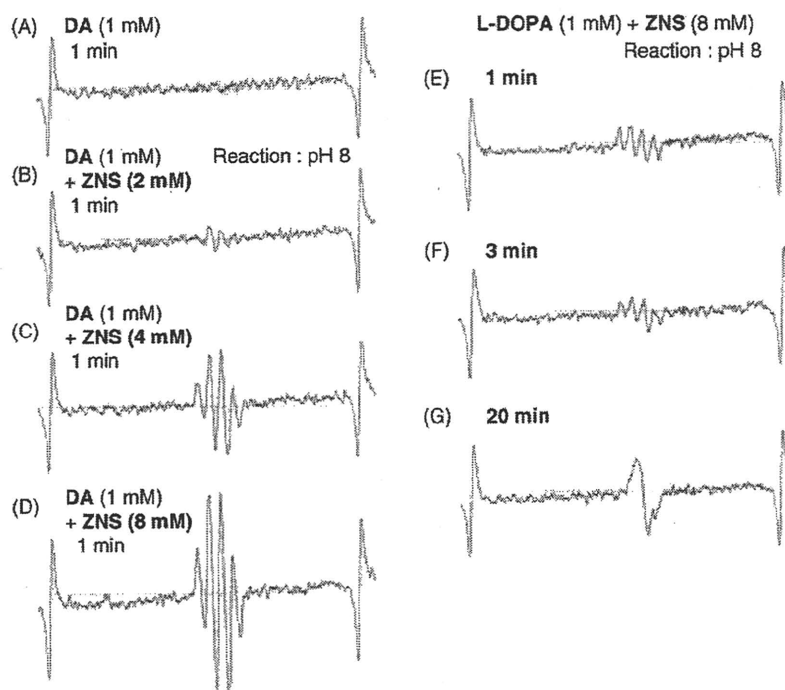


Fig. 2. Effects of ZNS on semiquinone radicals generated from DA or L-DOPA. (A–D) Dose-dependent effects of ZNS on DA-semiquinone radicals generated from DA in a cell-free system. The pH-adjusted 1 mM DA (pH 7.0) was simultaneously incubated with 2–8 mM ZNS (pH 10.8) at pH 8.0 (incubation mixture) for 1 min at 37 °C, and then the relative signal intensity of DA-semiquinone radicals was measured by ESR spectrometry. (E–G) Representative time-course changes in ESR spectra of DOPA-semiquinone radicals (E and F) to melanin (G) in the incubation of pH-adjusted L-DOPA (1 mM, pH 7.0) with 8 mM ZNS (sodium salt) in 10 mM PB (pH 10.8) for 1–20 min at 37 °C at pH 8.0 (incubation mixture). Three independent assays were performed in each experiment.

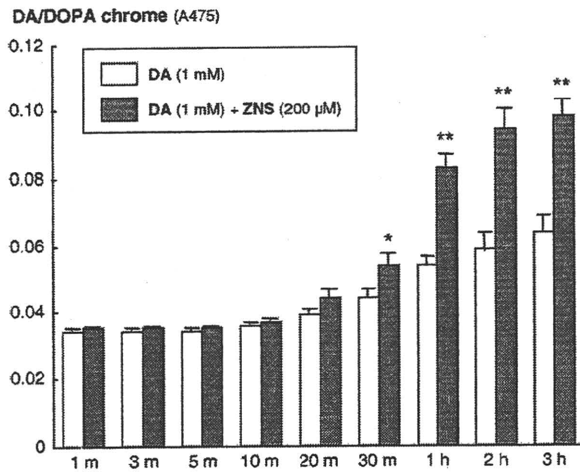


Fig. 3. Effects of ZNS on time-course changes in DA/DOPA chrome generated in a cell-free system. Levels of generated DA/DOPA chrome were measured after incubation of pH-adjusted 1 mM DA (pH 6.8) with/without 200 μM ZNS (pH 6.8) at 37 °C for 1 min to 3 h. Co-incubation of pH-adjusted DA (1 mM) and ZNS (200 μM) at neutral pH significantly increased DA/DOPA chrome at 30 min to 3 h, compared with time-matched pH-adjusted DA alone. Each value is the mean of absorbance at 475 nm ± S.E.M. ($n = 5$). *$p < 0.05$, **$p < 0.001$ compared with time-matched group treated with DA alone.

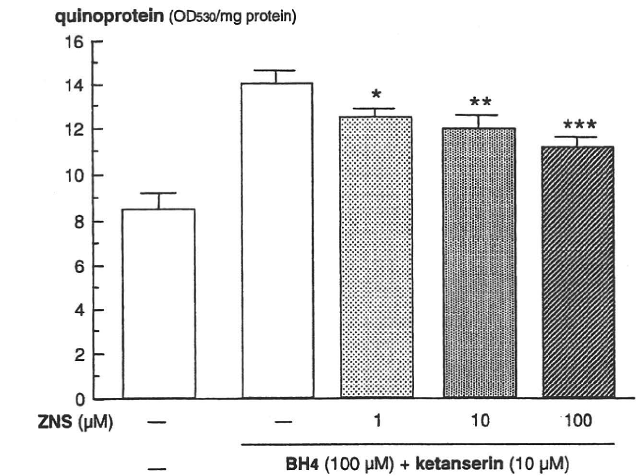


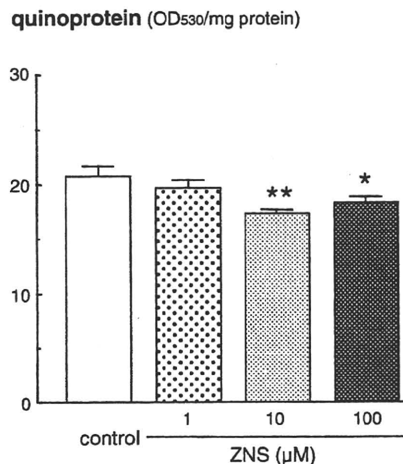
Fig. 5. Effects of ZNS treatment on excess cytosolic free DA-induced quinoprotein formation in dopaminergic CATH.a cells. Quinoprotein level in CATH.a cells were measured after treatment with BH_4 (100 μM) and ketanserin (10 μM), with or without ZNS (1–100 μM) for 3 h. The simultaneous treatment of ZNS (1–100 μM) significantly and dose-dependently inhibited BH_4 plus ketanserin-induced quinoprotein formation. Data are mean ± S.E.M. ($n = 8$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with cells treated with BH_4 plus ketanserin without ZNS.

chrome level, co-incubation of pH-adjusted 1 mM DA (pH 6.8) and 200 μM ZNS (pH 6.8) at 37 °C significantly increased DA chrome at 30 min to 3 h, compared with time-matched pH-adjusted DA alone.

3.3. *Effects of ZNS on DA quinone formation in CATH.a cells*

We examined changes in DA, its metabolites, quinoprotein and DA/DOPA chrome using *in vitro* dopaminergic CATH.a cells after 5-day treatment of ZNS. Quinoprotein level was significantly decreased after 5-day treatment of ZNS (10–100 μM) (Fig. 4) with reduction of DOPAC level (data not shown). On the other hand, 5-day treatment of ZNS (1–100 μM) significantly increased DA/DOPA chrome level in CATH.a cells (Fig. 4).

Finally, we examined the effects of ZNS on excess cytosolic free DA-induced quinone formation by measuring quinoprotein levels in CATH.a cells after treatment with BH_4 (100 μM) and ketanserin (10 μM) with/without ZNS (1–100 μM) for 3 h. The quinoprotein levels in CATH.a cells co-treated with BH_4 and ketanserin for 3 h (which increase cytosolic free DA) were relatively higher than that in control at day 1 (Fig. 5), in agreement with a previous report (Choi et al., 2005). The simultaneous treatment of ZNS (1–100 μM) significantly and dose-dependently inhibited BH_4- and ketanserin-induced quinoprotein formation in the cells (Fig. 5).



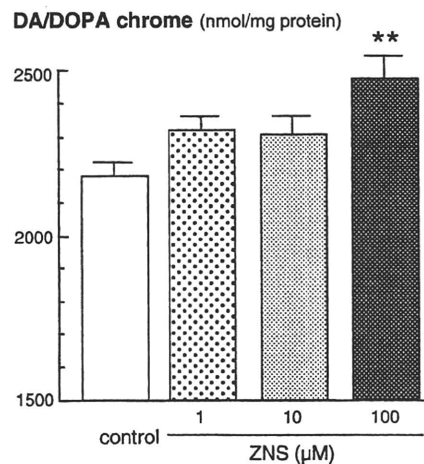


Fig. 4. Effects of long-term treatment of ZNS on quinoprotein and DA/DOPA chrome in dopaminergic CATH.a cells. After treatment with ZNS (1–100 μM) for 5 days, levels of quinoprotein ($n = 5$) and DA/DOPA chrome ($n = 6$) were measured in dopaminergic CATH.a cells, as indicated in Section 2. Data are mean ± S.E.M. *$p < 0.05$, **$p < 0.01$ compared with the control group.

4. Discussion

The main findings of this study are: (1) co-incubation of DA and ZNS in a cell-free system caused conversion of DA to stable melanin via formation of DA-semiquinone radicals and DA chrome; (2) long-term treatment with ZNS for 5 days decreased quinoprotein and increased DA/DOPA chromes in dopaminergic CATH.a cells; and (3) ZNS significantly inhibited quinoprotein formation induced by BH_4 and ketanserin that increase cytosolic free DA in the cells.

Long-term treatment of patients with PD by levodopa frequently causes various adverse effects including the wearing-off phenomenon, dyskinesia and psychiatric symptoms (Ahlskog and Muenter, 2001; Ogawa et al., 2005). However, long-term levodopa treatment-induced adverse effects that might be based on permanent neuronal network remodeling were seen specifically in PD patients but not in normal subjects or neurological diseases other than PD (Ogawa et al., 2005). Since the striatal damaged nerve terminal have too small DA pool to store DA at advanced stage of PD, repeated intermittent pulsatile levodopa stimulation results in free DA excess in the cytosol outside the synaptic vesicle (Sulzer et al., 2000; Sulzer and Zecca, 2000; Asanuma et al., 2003; Ogawa et al., 2005). The previous study revealed that repeated levodopa administration elevated striatal quinoprotein levels specifically on the parkinsonian side, not on the control side, of hemi-parkinsonian mice (Miyazaki et al., 2005). Therefore, the parkinsonian side-specific elevation of quinone generation may be due to excess amount of cytosolic DA outside the synaptic vesicles, which is easily oxidized to DA quinones in damaged dopaminergic nerve terminals after repeated levodopa treatment. In cultured dopaminergic cells, simultaneous treatment with ZNS dose-dependently suppressed BH_4- and ketanserin-induced quinoprotein formation via elevation of cytosolic free DA (Fig. 5), suggesting that ZNS has potent neuroprotective effects against neurotoxicity of DA quinone induced by excess amount of cytosolic DA outside the synaptic vesicles.

The protective effects of ZNS against levodopa-induced DA quinone toxicity in parkinsonian models may be based, in part, on its stabilizing effects against free DA and cytotoxic DA quinone; ZNS can convert free DA to melanin via the formation of DA-semiquinone radicals (Figs. 1 and 2) and subsequent intermediate DA chrome in the cell-free system (Fig. 3). This possible mechanism is also supported by the present results of long-term treatment with ZNS in cultured dopaminergic CATH.a cells. Long-term ZNS treatment in CATH.a cells for 5 days significantly decreased quinoprotein and increased DA/DOPA chromes (Fig. 4) with reduction of DOPAC (data not shown), suggesting that continuous ZNS exposure to DA-rich CATH.a cells promotes conversion of free DA and DA quinone to DA chrome, then to melanin. However, these effects do not seem to be exerted in a dose-dependent manner. Likewise relatively low doses of ZNS (25, 50 mg/day) rather than 100 mg/day significantly improved UPDRS Part III total score in the clinical trial (Murata et al., 2007), there may be optimal concentration range of ZNS to exert its stabilizing effects against DA quinones.

Several neuroprotective strategies have been proposed against DA quinone-induced cytotoxicity: (1) quenching excess free DA and DA quinone, (2) inhibiting quinone formation, and (3) enhancing intrinsic antioxidative system against DA quinone toxicity (Asanuma et al., 2004). Regarding quenching DA quinone, DA quinone can be scavenged by direct conjugation with some drugs, *e.g.*, thiol-containing compounds (*N*-acetylcyteine and dithiothreitol) (Offen et al., 1996) and DA agonists (pergolide and pramipexole) (Asanuma et al., 2005; Miyazaki et al., 2005). Furthermore, another possible method to quench DA quinone-induced cytotoxicity is the conversion of free DA and DA quinone to stable melanin. The final oxidized form of DA quinone, neuromelanin, exerts cytoprotective effects through its binding capacity to toxic metals (Zecca et al., 2003). Although large amount of neuromelanin with iron is reported to be potentially cytotoxic, physiological amount of neuromelanin is not toxic and rather cytoprotective with its high storage capacity for toxic metals in the substantia nigra (Gerlach et al., 2003; Zecca et al., 2003). This cytoprotective potency by stabilization of DA quinone has been clarified by our previous report that melanin-synthesizing enzyme tyrosinase ameliorates methamphetamine-induced neurotoxicity and quinoprotein formation *in vitro* and *in vivo* by its rapid conversion of DA quinone to melanin (Miyazaki et al., 2006). Also in the present study, we showed that ZNS can convert free DA to melanin via the formation of DA quinone and the intermediate DA chrome in the cell-free system. Therefore, these stabilizing effects of ZNS on free DA and DA quinone by the conversion to melanin may be one of the plausible mechanisms of its prevention against DA quinone-induced cytotoxicity.

ZNS is also known to scavenge hydroxyl radicals and nitric oxide radicals in a cell-free system (Mori et al., 1998) and inhibits lipid peroxidation and oxidative DNA damage in the rat brain (Komatsu et al., 2000). The general ROS such as hydroxyl radicals and nitric oxide radicals show widespread toxicity not only in DA neurons but also in other regions. Since the DA quinone is specifically generated from free cytosolic DA (Sulzer et al., 2000), the stabilizing effects of ZNS on free DA and cytotoxic DA quinone as dopaminergic neuron-specific oxidative stress may play a role in its preventing property against DA or levodopa-induced DA quinone toxicity, in addition to its scavenging activity against general ROS.

In conclusion, ZNS suppressed excess cytosolic free DA-induced quinone generation in dopaminergic cells. Furthermore, ZNS stabilized free DA and DA quinone as dopaminergic neuron-specific oxidative stress by the conversion to melanin. The stabilizing effects of ZNS against cytotoxic DA quinones may play a role in the efficacy of its adjunctive treatment to levodopa in parkinsonian patients.

Acknowledgments

This work was supported in part by Health and Labour Sciences Research Grants for Research on Measures for Intractable Diseases, for Research on Psychiatric and Neuro-

logical Diseases and Mental Health, and for Comprehensive Research on Aging and Health from the Japanese Ministry of Health, Labour and Welfare, and by Grants-in-Aid for Young Scientists (B) and for Scientific Research (C) from the Japanese Ministry of Education, Culture, Sports, Science and Technology. We would like to thank Mr. Yusuke Urakubo, Mr. Yasutsugu Tsutsui, and Mr. Masashi Yoshimoto for their technical assistance.

References

Ahlskog, J.E., Muenter, M.D., 2001. Frequency of levodopa-related dyskinesias and motor fluctuations as estimated from the cumulative literature. Mov. Disord. 16, 448–458.

Asanuma, M., Miyazaki, I., Diaz-Corrales, F.J., Ogawa, N., 2004. Quinone formation as dopaminergic neuron-specific oxidative stress in pathogenesis of sporadic Parkinson's disease and neurotoxin-induced parkinsonism. Acta Med. Okayama 58, 221–233.

Asanuma, M., Miyazaki, I., Diaz-Corrales, F.J., Shimizu, M., Tanaka, K., Ogawa, N., 2005. Pramipexole has ameliorating effects on levodopa-induced abnormal dopamine turnover in parkinsonian striatum and quenching effects on dopamine-semiquinone generated *in vitro*. Neurol. Res. 27, 533–539.

Asanuma, M., Miyazaki, I., Ogawa, N., 2003. Dopamine- or L-DOPA-induced neurotoxicity: the role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. Neurotox. Res. 5, 165–176.

Basma, A.N., Morris, E.J., Nicklas, W.J., Geller, H.M., 1995. L-dopa cytotoxicity to PC12 cells in culture is via its autoxidation. J. Neurochem. 64, 825–832.

Chae, S.W., Bang, Y.J., Kim, K.M., Lee, K.Y., Kang, B.Y., Kim, E.M., Inoue, H., Hwang, O., Choi, H.J., 2007. Role of cyclooxygenase-2 in tetrahydrobiopterin-induced dopamine oxidation. Biochem. Biophys. Res. Commun. 359, 735–741.

Choi, H.J., Kim, S.W., Lee, S.Y., Hwang, O., 2003. Dopamine-dependent cytotoxicity of tetrahydrobiopterin: a possible mechanism for selective neurodegeneration in Parkinson's disease. J. Neurochem. 86, 143–152.

Choi, H.J., Lee, S.Y., Cho, Y., Hwang, O., 2005. Inhibition of vesicular monoamine transporter enhances vulnerability of dopaminergic cells: relevance to Parkinson's disease. Neurochem. Int. 46, 329–335.

Foppoli. C., Coccia, R., Cini, C., Rosei, M.A., 1997. Catecholamines oxidation by xanthine oxidase. Biochim. Biophys. Acta 1334, 200–206.

Fornstedt, B., Rosengren, E., Carlsson, A., 1986. Occurrence and distribution of 5-*S*-cysteinyl derivatives of dopamine, dopa and dopac in the brains of eight mammalian species. Neuropharmacology 25, 451–454.

Gerlach, M., Double, K.L., Ben-Shachar, D., Zecca, L., Youdim, M.B., Riederer, P., 2003. Neuromelanin and its interaction with iron as a potential risk factor for dopaminergic neurodegeneration underlying Parkinson's disease. Neurotox. Res. 5, 35–44.

Graham, D.G., 1978. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Mol. Pharmacol. 14, 633–643.

Haque, M.E., Asanuma, M., Higashi, Y., Miyazaki, I., Tanaka, K., Ogawa, N., 2003. Apoptosis-inducing neurotoxicity of dopamine and its metabolites via reactive quinone generation in neuroblastoma cells. Biochim. Biophys. Acta 1619, 39–52.

Hastings, T.G., 1995. Enzymatic oxidation of dopamine: the role of prostaglandin H synthase. J. Neurochem. 64, 919–924.

Hastings, T.G., Lewis, D.A., Zigmond, M.J., 1996. Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. Proc. Natl. Acad. Sci. U.S.A. 93, 1956–1961.

Kito, M., Maehara, M., Watanabe, K., 1996. Mechanisms of T-type calcium channel blockade by zonisamide. Seizure 5, 115–119.

Komatsu, M., Hiramatsu, M., Willmore, L.J., 2000. Zonisamide reduces the increase in 8-hydroxy-2'-deoxyguanosine levels formed during iron-induced epileptogenesis in the brains of rats. Epilepsia 41, 1091–1094.

Korytowski, W., Sarna, T., Kalyanaraman, B., Sealy, R.C., 1987. Tyrosinase-catalyzed oxidation of dopa and related catechol(amine)s: a kinetic electron spin resonance investigation using spin-stabilization and spin label oximetry. Biochim. Biophys. Acta 924, 383–392.

Kuhn, D.M., Arthur Jr., R.E., Thomas, D.M., Elferink, L.A., 1999. Tyrosine hydroxylase is inactivated by catechol-quinones and converted to a redox-cycling quinoprotein: possible relevance to Parkinson's disease. J. Neurochem. 73, 1309–1317.

Lai, C.T., Yu, P.H., 1997. Dopamine- and L-β-3,4-dihydroxyphenylalanine hydrochloride (L-Dopa)-induced cytotoxicity towards catecholaminergic neuroblastoma SH-SY5Y cells. Effects of oxidative stress and antioxidative factors. Biochem. Pharmacol. 53, 363–372.

LaVoie, M.J., Ostaszewski, B.L., Weihofen, A., Schlossmacher, M.G., Selkoe, D.J., 2005. Dopamine covalently modifies and functionally inactivates parkin. Nat. Med. 11, 1214–1221.

Machida, Y., Chiba, T., Takayanagi, A., Tanaka, Y., Asanuma, M., Ogawa, N., Koyama, A., Iwatsubo, T., Ito, S., Jansen, P.H., Shimizu, N., Tanaka, K., Mizuno, Y., Hattori, N., 2005. Common anti-apoptotic roles of parkin and alpha-synuclein in human dopaminergic cells. Biochem. Biophys. Res. Commun. 332, 233–240.

Miyazaki, I., Asanuma, M., Diaz-Corrales, F.J., Fukuda, M., Kitaichi, K., Miyoshi, K., Ogawa, N., 2006. Methamphetamine-induced dopaminergic neurotoxicity is regulated by quinone formation-related molecules. FASEB J. 20, 571–573.

Miyazaki, I., Asanuma, M., Diaz-Corrales, F.J., Miyoshi, K., Ogawa, N., 2005. Dopamine agonist pergolide prevents levodopa-induced quinoprotein formation in parkinsonian striatum and shows quenching effects on dopamine-semiquinone generated *in vitro*. Clin. Neuropharmacol. 28, 155–160.

Mori, A., Noda, Y., Packer, L., 1998. The anticonvulsant zonisamide scavenges free radicals. Epilepsy Res. 30, 153–158.

Murata, M., 2004. Novel therapeutic effects of the anti-convulsant, zonisamide, on Parkinson's disease. Curr. Pharm. Des. 10, 687–693.

Murata, M., et al., 2007. Zonisamide improves motor function in Parkinson disease: a randomized, double-blind study. Neurology 68, 45–50.

Murata, M., Horiuchi, E., Kanazawa, I., 2001. Zonisamide has beneficial effects on Parkinson's disease patients. Neurosci. Res. 41, 397–399.

Offen, D., Ziv, I., Sternin, H., Melamed, E., Hochman, A., 1996. Prevention of dopamine-induced cell death by thiol antioxidants: possible implications for treatment of Parkinson's disease. Exp. Neurol. 141, 32–39.

Ogawa, N., Asanuma, M., Miyazaki, I., Diaz-Corrales, F.J., Miyoshi, K., 2005. L-DOPA treatment from the viewpoint of neuroprotection: possible mechanism of specific and progressive dopaminergic neuronal death in Parkinson's disease. J. Neurol. 252 (Suppl. 4), iv23–iv31.

Ogawa, N., Edamatsu, R., Mizukawa, K., Asanuma, M., Kohno, M., Mori, A., 1993. Degeneration of dopaminergic neurons and free radicals. Possible participation of levodopa. Adv. Neurol. 60, 242–250.

Ogawa, N., Tanaka, K., Asanuma, M., 2000. Bromocriptine markedly suppress levodopa-induced abnormal increase of dopamine turnover in the parkinsonian striatum. Neurochem. Res. 25, 755–758.

Okada, M., Kaneko, S., Hirano, T., Ishida, M., Kondo, T., Otani, K., Fukushima, Y., 1992. Effects of zonisamide on extracellular levels of monoamine and its metabolite, and on Ca^{2+} dependent dopamine release. Epilepsy Res. 13, 113–119.

Okada, M., Kaneko, S., Hirano, T., Mizuno, K., Kondo, T., Otani, K., Fukushima, Y., 1995. Effects of zonisamide on dopaminergic system. Epilepsy Res. 22, 193–205.

Paz, M.A., Fluckiger, R., Boak, A., Kagan, H.M., Gallop, P.M., 1991. Specific detection of quinoproteins by redox-cycling staining. J. Biol. Chem. 266, 689–692.

Rosei, M.A., Blarzino, C., Foppoli, C., Mosca, L., Coccia, R., 1994. Lipoxygenase-catalyzed oxidation of catecholamines. Biochem. Biophys. Res. Commun. 200, 344–350.

Sulzer, D., Bogulavsky, J., Larsen, K.E., Behr, G., Karatekin, E., Kleinman, M.H., Turro, N., Krantz, D., Edwards, R.H., Greene, L.A., Zecca, L., 2000. Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. Proc. Natl. Acad. Sci. U.S.A. 97, 11869–11874.

Sulzer, D., Zecca, L., 2000. Intraneuronal dopamine-quinone synthesis: a review. Neurotox. Res. 1, 181–195.
Suzuki, S., Kawakami, K., Nishimura, S., Watanabe, Y., Yagi, K., Seino, M., Miyamoto, K., 1992. Zonisamide blocks T-type calcium channel in cultured neurons of rat cerebral cortex. Epilepsy Res. 12, 21–27.
Tse, D.C., McCreery, R.L., Adams, R.N., 1976. Potential oxidative pathways of brain catecholamines. J. Med. Chem. 19, 37–40.
Walkinshaw, G., Waters, C.M., 1995. Induction of apoptosis in catecholaminergic PC12 cells by L-DOPA. Implications for the treatment of Parkinson's disease. J. Clin. Invest. 95, 2458–2464.
Whitehead, R.E., Ferrer, J.V., Javitch, J.A., Justice, J.B., 2001. Reaction of oxidized dopamine with endogenous cysteine residues in the human dopamine transporter. J. Neurochem. 76, 1242–1251.
Xu, Y., Stokes, A.H., Roskoski Jr., R., Vrana, K.E., 1998. Dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. J. Neurosci. Res. 54, 691–697.
Zecca, L., Zucca, F.A., Wilms, H., Sulzer, D., 2003. Neuromelanin of the substantia nigra: a neuronal black hole with protective and toxic characteristics. Trends Neurosci. 26, 578–580.