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Brief Reports

Rapid Screening of *ATP13A2* Variant with High-Resolution Melting Analysis

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Abstract: **Several genetic and environmental factors are involved in the pathogenesis of Parkinson's disease (PD). Recently, a novel variant of *ATP13A2* (p.A746T) responsible for *PARK9* was reported as a risk factor for PD in the Han-Chinese population. To investigate the role of this variant in Japanese PD patients, we examined 917 Japanese PD patients (871 index cases) and 190 controls by high-resolution melting curve analysis. We detected heterozygous p.A746T variant in a single patient with sporadic PD and a single control subject. These results suggest that *ATP13A2* p.A746T variant is unlikely to play a role as a common risk factor or a pathogenic mutation for PD at least in Japanese. Our data on Japanese differ from those reported recently on Han-Chinese. Further studies are needed to confirm conclusions on roles of *ATP13A2* variant in Asians or other populations.** © 2010 Movement Disorder Society

Key words: Parkinson's disease; gene risk factor; PARK9; lightscanner

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INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder pathologically characterized by selective dopaminergic neurodegeneration and the presence of Lewy bodies in the midbrain. Clinical features of typical PD are resting tremor, rigidity, bradykinesia, and postural instability. PD is thought to be caused by interaction with multiple genetic and environmental factors; however, the exact etiology of PD remains elusive. To elucidate the mechanisms of nigral degeneration, monogenic forms of PD provide good information to identify the cause of not only monogenic but also sporadic forms of this disease. Case-control studies using genetic analyses are important in identification of susceptibility genes for PD such as *SNCA*, *LRRK2*, and *GBA*.[1–5] In particular, Asian population-specific variants such as *LRRK2* p.G2385R and p.R1628P showed strong association with susceptibility to the pathogenesis of PD.[6,7] Interestingly, the *LRRK2* p.G2385R variant has been associated with PD in Asian population including Japanese and Chinese;[3,6] however, *LRRK2* p.R1628P variant has not been found in Japanese population so far, suggesting some differences in ethnic background based on human migration history among Asians.[7] Furthermore, a novel *ATP13A2* p.A746T variant was reported recently as a risk factor for PD in the Han-Chinese population.[8] Based on the above background, we investigated the role of *ATP13A2* p.A746T variant in Japanese PD patients by examining 917 Japanese PD patients (871 index cases) and 190 normal controls by high-resolution melting curve analysis.

PATIENTS AND METHODS

Subjects

The study subjects were 917 Japanese PD patients (871 index cases) and 190 normal controls. Of these, 61.2% (533/871) were sporadic PD and 38.8% (338/871) had at least one first and/or second-degree relative with parkinsonism. Many had been screened for PD-associated genes and reported previously (screening rates in each gene were as follows; *parkin*: 73.4%, *PINK1*: 79.5%, *DJ-1*: 37.3%, *SNCA* multiplication:

52.5%, and *LRRK2* exon 41: 61.0%).[9–14] Furthermore, 25 out of 917 patients were screened for all exons of *ATP13A2* in our previous study.[14] Patients with pathogenic mutation(s) were not included in this study. Diagnosis of PD was adopted by the participating neurologists based on established criteria.[15] Clinical and demographic data were obtained for 894 patients. The mean age at onset (AAO) was 50.6 ± 14.9 years (±SD, range, 7–88 years). Moreover, 407 out of 917 (44.4%) patients had early-onset PD (onset <50 years). The study included 446 men and 471 women (female:male ratio, 1.06:1). The mean age of the control group at sampling was 58.2 ± 16.5 years (±SD, range, 23–98 years). The study was approved by the ethics committee of Juntendo University School of Medicine. All subjects gave informed and written consent form.

Genetic Analyses

PCR was performed in a 10 μL volume using 10 ng of genomic DNA, 1 μL of LCGreen Plus (Idaho Technology, Salt Lake City, ID), 5 μL of AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA), and 5 pmol of each forward and reverse primer. Mineral oil (15 μL per reaction) was added before starting the PCR. The amplification conditions were as follows: preincubation at 95°C for 9 minutes, 40 cycles of denaturation at 95°C for 20 seconds, annealing and extension at 65°C for 20 seconds, and final step for heteroduplex formation at 94°C for 30 seconds followed by 20°C hold. PCR primers were designed by ExonPrimer (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html), and the sequences of the primers for *ATP13A2* p.A746T detection were as follows: forward; CGCAGACAACGCCAGTTATCC, reverse; GGGCCCCTACATGCCATTG. Following PCR, high-resolution melting analysis was performed using LightScanner (Idaho Technology). Fluorescence was collected from 85 to 95°C at a ramp rate of 0.1°C/s. Melting curve analysis was performed by the method provided with LightScanner Call-It software (version 1.5, Idaho Technology). Samples determined to be variants were sequenced directly using the method reported previously by our group.[14] We used DNA of one patient carrying p.A746T variant (identified in a previous report) as a positive control.[8]

Haplotype analysis was performed in p.A746T carriers using eight microsatellite markers (MSs) and four single nucleotide polymorphisms (SNPs). MSs and SNPs were genotyped by PCR using fluorescence-labeled primers or direct-sequencing, 3130 Genetic Analyzer, and GeneMapper software (Applied Biosystems).

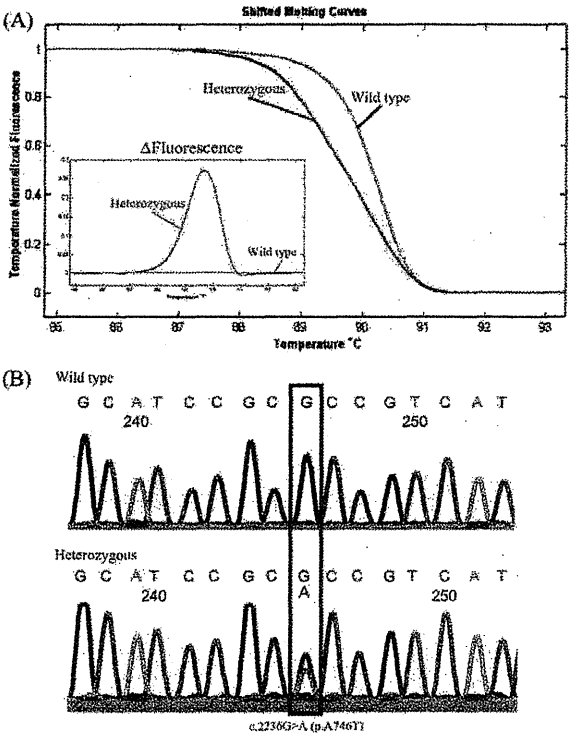

FIG. 1. Genotyping of ATP13A2 p.A746T variant by LightScanner and direct sequencing. The difference between heterozygous p.A746T variant (red) and the reference (black) in shifted melting curve and Δfluorescence was shown (**A**). Chromatograms showing wild type and heterozygous p.A746T variant (**B**). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Sequences of the primers and conditions of PCR are available upon request to the corresponding author.

Statistics Analysis

All data are expressed as mean ± SD. The Fisher's exact test was used to calculate the difference in the allele frequencies. In all statistical analyses, a P value of 0.05 or less was considered statistically significant.

RESULTS

Genotyping was completed showing a high genotyping success rate (98.6%) in high-resolution melting. Heterozygous p.A746T (c.2236G>A) variant was detected in one patient with sporadic PD (1/533, 0.19%) and one control subject (1/190, 0.53%) by high-resolution melting analysis and direct sequencing (Fig. 1 and Table 1). There was no statistical difference in the frequency of p.A746T variant between patients with sporadic PD and control subjects (P = 0.46). None of the patients with family history of PD

TABLE 1. *Comparison of p.A746T frequency among Japanese and Han-Chinese population*

		p.A746T		
		WT	Hetero	Frequency (%)
Japanese	SPD	532	1	0.19
	FPD	384	0	0.00
	Total	916	1	0.11
	Cont	189	1	0.53
Han-Chinese	PD[a]	179	3	1.65

SPD, sporadic PD; FPD, familial PD; Cont, Normal control; WT, wild type; Hetero, heterozygous; Frequency, heterozygous frequency.
[a]Data from Ref. 8.

had p.A746T variant (0/338, 0%). Furthermore, there was no difference in the frequency between the entire PD patients and the control subjects ($P = 0.31$). Whereas the frequency in Japanese patients (1/917, 0.11%) was lower than that in Han-Chinese patients (3/182, 1.65%) (Table 1).[8]

Haplotype analysis of *PARK9* locus revealed that patients carrying the p.A746T variant shared at least one allele of MSs and SNPs (Table 2). The patient with p.A746T variant initially showed left-hand resting tremor at age 46. Subsequently, he developed resting tremor in the right hand, bradykinesia, rigidity, and mild parkinsonian gait without retropulsion (Hoehn and Yahr stage II). He is still doing well with 2 mg of trihexyphenidyl a day 10 years after the onset of the disease. His brain MRI was normal at another hospital and cardiac MIBG uptake was markedly diminished (Heart/Mediastinum ratio 1.17 at early image and 1.14 at delayed image; our normal range is above 1.45 for both imaging). He was taking 2.5 mg of selegiline when the MIBG scintigraphy was done.

DISCUSSION

In this study, we used high-resolution melting analysis for genotyping. This method is a rapid, sensitive, and cost-effective technique for detection of mutation without direct sequencing.[16] Indeed, genotyping p.A746T variant by high-resolution melting analysis was faster, low cost, and associated with a low error rate. Thus, as reported previously, high-resolution melting analysis is useful for primary screening of mutations or SNPs.[16,17]

Based on this sound method, we detected *ATP13A2* p.A746T variant in only one Japanese patient with sporadic PD. The frequency was significantly different from that of the previous report in early-onset PD of Han-Chinese population ($P = 0.016$).[8] These results suggest that *ATP13A2* p.A746T is a rare variant in Japanese population compared with Han-Chinese population. This is similar to the result of *LRRK2* p.R1628P variant in Han-Chinese and Japanese population.[7] Based on these findings, both *ATP13A2* p.A746T and *LRRK2* p.R1628P variants may be more recent mutations than *LRRK2* p.G2385R variant, which is a common variant among Asian population including Han-Chinese and Japanese.[3,6,7] Moreover, the results of haplotype analysis suggest that carriers have inherited the p.A746T variant from a common ancestral founder. However, because we could not determine the haplotype phase, further analyses are needed to verify the founder effect in Asia.

Lin et al.[8] did not detect p.A746T variant in any of their 589 control subjects. In comparison, we detected one Japanese control subject carrying heterozygous p.A746T variant, but the frequency was not significantly different compared to PD patients. Thus, although we could not exclude the possibility that our normal subject with p.A746T variant will develop PD in future, our study provided no evidence that *ATP13A2* p.A746T enhances the susceptibility to PD in Japanese. In addition, our analysis did not identify any patient with *ATP13A2* p.A746T and familial parkinsonism despite the large sample size, suggesting that this variant plays no major roles in familial parkinsonism, at least in Japanese. This might be based on the rarity of the p.A746T variant, ethnic differences, and/or human migration history. Also, this finding could be due to phenotypic differences between previously reported atypical parkinsonism patients with

TABLE 2. *Haplotype analysis of PARK9* locus

Marker	PD (JPN)	PD (CHN)	Cont (JPN)
D1S2644	220/232	232/234	232/232
D1S2826	134/134	134/134	126/134
D1S1592	236/240	240/240	244/244
D1S3669	189/189	185/189	193/201
rs2076603 (ex17)	C/T	C/T	C/C
c.2236G>A (ex20, p.A746T)	G/A	G/A	G/A
rs9435662 (ex24)	C/T	C/T	C/C
rs3738815 (ex25)	G/A	G/A	A/A
rs3170740 (ex29)	G/A	G/A	G/G
D1S436	205/205	205/211	205/211
D1S2672	155/155	147/157	155/157
D1S507	187/189	195/201	193/198
D1S228	119/123	123/123	119/123

Genotypes of p.A746T carriers: Japanese [PD (JPN)] and Chinese [PD (CHN)][8] patients and Japanese normal control [Cont (JPN)] were shown.

pathogenic *ATP13A2* mutations and patients with typical parkinsonism.[14,18,19]

In conclusion, based on the present large study, *ATP13A2* p.A746T variant is not a common risk factor or pathogenic mutation for PD at least in Japanese. However, the role of *ATP13A2* variant in PD is worth discussing. Therefore, further analyses should be performed to draw definite conclusions about the role of *ATP13A2* variant in Asians and other populations. Our study indicates that for genotyping or mutation detection, high-resolution melting analysis is a rapid and cost-effective method when the targets are limited.

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Author Roles: Manabu Funayama was involved in conception, organization, and execution of the research project; design, execution, review and critique of the data construction; writing of the first draft, review and critique of the manuscript. Hiroyuki Tomiyama was involved in conception and organization of the research project; design, review and critique of the data construction; review and critique of the manuscript. Ruey-Meei Wu was involved in execution, review and critique of the data construction; review and critique of the manuscript. Kotaro Ogaki was involved in execution of the data construction; writing of the first draft of the manuscript. Hiroyo Yoshino was involved in execution, review and critique of the data construction; review and critique of the manuscript. Yoshikuni Mizuno was involved in execution of the data construction; review and critique of the manuscript. Nobutaka Hattori was involved in organization of the research project, review and critique of the data construction; review and critique of the manuscript.

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Clinical Course of the First Asian Family with Parkinsonism Related to SNCA Triplication

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Abstract: **Triplication of *SNCA* is a rare cause of familial Parkinson's disease compared with duplication. Its clinical course is believed to be more robust than duplication, though it is uncertain. Marked as the first among the Asian population, we identified a Japanese family (paternal grandfather, father, and son) with *SNCA* triplication based on genetic and clinical analyses. The proband had a completely triplicated region including *SNCA*. This allele did not share any common haplotypes with those of previously reported Japanese families with *SNCA* duplication. Clinical analysis indicated early onset, rapidly progressive parkinsonism with mild levodopa response. Further studies are needed to clarify the gene dose effect of *SNCA*.** © 2010 Movement Disorder Society

Key words: SNCA; triplication; duplication; familial Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer disease. About 5% of patients with PD have the familial form, which is caused by a single gene disorder of *SNCA*, *LRRK2*, *UCH-L1*, *PRKN*, *DJ-1*, *PINK1*, or *ATP13A2*. Duplication of the *SNCA* gene is relatively frequent in autosomal dominant PD, and is also seen in sporadic PD due to its low penetrance.[1–5] However, triplication of *SNCA* is rare and to our knowledge, only three families have been described so far.[6–8] The clinical course of triplication is believed to be more robust than duplication due to its gene dose effect,

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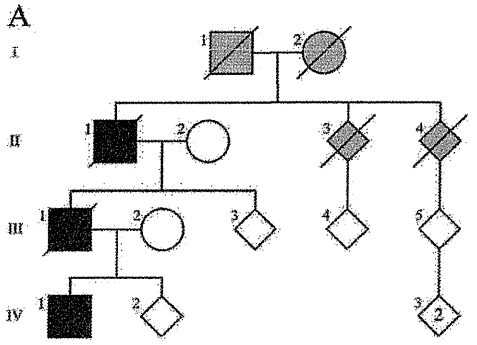

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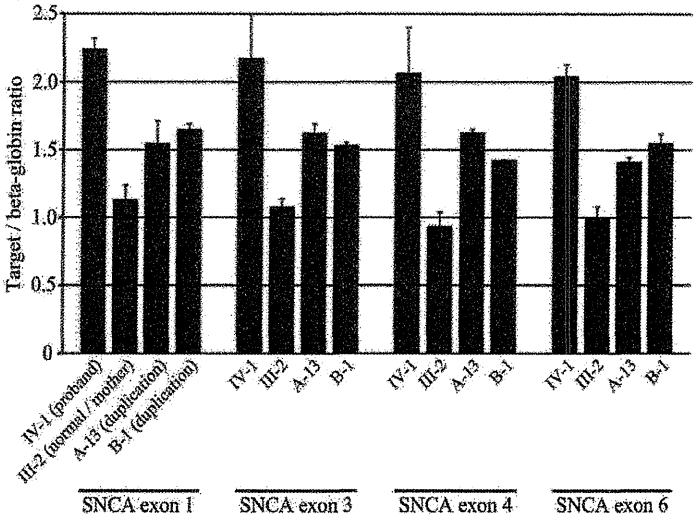

C

	IV-1/control	III-2/control
SNCA Exon 1	2.1	1.0
SNCA Exon 3	2.3	1.1
SNCA Exon 4	1.9	1.2
SNCA Exon 5	1.7	1.0
SNCA Exon 6	2.0	1.1

Gene dosage ratio.

D

	Triplication				Reported Japanese duplication families																	
Microsatellite	IV-1				Family A			Family F			Family B			Family C			Family D			Family G			Family E		
D4S3475	223	219	219	219	221	219	219	219	219	219	219	221	221	221	221	221	221	221	221	219	221	221	221	219	219
D4S3477	261	263	263	263	261	265	265	265	265	265	263	261	261	265	261	261	261	261	261	263	261	261	263	263	263
D4S3480	227	219	219	219	227	236	236	219	236	236	219	228	228	236	228	228	228	228	228	219	228	228	228	219	219
D4S3479	176	178	178	178	176	172	172	178	172	172	178	174	176	172	174	176	176	174	174	178	174	174	174	178	178
D4S3474	211	211	211	211	215	209	209	205	209	209	205	205	213	203	205	213	213	205	205	211	205	205	205	205	205

FIG. 1. Pedigree of SNCA triplication family (**A**). Black symbols: affected patients, gray symbols: unaffected individuals. Gene dosage analyses using real-time PCR (**B**) and MLPA (**C**). Haplotype analysis (**D**). The disease haplotypes are highlighted in gray.

but the precise picture remains to be defined as there is insufficient information about triplication.

Here we report the first Asian family with *SNCA* triplication presented with early-onset and severe clinical features of parkinsonism.

METHODS

Subjects

The pedigree studied is shown in Figure 1A. The DNA was extracted from peripheral blood samples obtained from the proband (IV-1) and his mother (III-2). Approval for the study was obtained from the ethics review committee of Juntendo University. To compare the gene dosages and haplotype of *SNCA* and its flanking region, we also examined patients from Japanese families with duplication of the *SNCA* gene.[1,4]

Genetic Analyses for SNCA

Mutation screening was performed as described previously.[1] Semiquantitative multiplex polymerase chain reaction (PCR) of genomic DNA samples was performed using real-time PCR to detect the dosage of *SNCA* (7500 Fast Real-Time PCR system, Applied Biosystems, Foster City, CA). In the first step, we targeted exon 4 of *SNCA* to screen the gene dosage. The "beta-globin" gene was amplified as an endogenous reference, and patients with *SNCA* duplication, who were confirmed by fluorescence in situ hybridization, were used as positive control (Patients A-13 and B-1).[1] The primer and probe sequences and the methods were described previously.[1] In the second step, we performed semiquantitative analysis on *SNCA* exons 1, 3, 6 and flanking genes (*LOC345278*, *MMRN1*, and *KIAA1680*) for the patients found in the first step to carry multiplication of this gene.

To confirm the gene dosage, we also performed multiplex ligation-dependent probe amplification (MLPA). Employing the SALSA MLPA P051-C1 Parkinson-1 probe mix (MRC Holland, Amsterdam, The Netherlands) using the DNA detection/quantification protocol provided by the manufacturer, products were quantified by the ABI 3130 Genetic Analyzer and Gene Mapper

v3.7 (Applied Biosystems, Foster City, CA). The MLPA data were analyzed as described previously.[9]

To determine whether the same haplotype was shared between our probands with *SNCA* multiplication, we performed haplotype analysis of the proband, mother, and samples of previously described families,[1,4] using five microsatellite including D4S3475, D4S3477, D4S3480, D4S3479, and D4S3474.[4]

RESULTS

Gene Dosage Analysis

Gene dosage analysis, both real-time PCR method and MLPA, revealed that the proband (IV-1) had four *SNCA* alleles (Fig. 1B,C), whereas the mother (III-2) had two normal *SNCA* alleles (Fig. 1B,C). These findings indicated that the proband had triplication of *SNCA*. The range of triplication includes whole exons of *SNCA*, *MMRN1* and exon 1 of *KIAA1680* according to the result of real-time PCR (data not shown).

Haplotype Analysis

Haplotype analysis indicated that the haplotype of the proband was different from that of Japanese families with *SNCA* duplication, as reported by our group previously (Fig. 1D).[4]

Case Records

II–1

This is the grandfather of the proband. At 49 years of age, he developed bradykinesia, slowness of speech, and sialorrhea. Hyposmia was not obvious. At age 50, he noted resting tremor in the left hand and foot. In addition, he became akinetic and depressed. At age 51, he was diagnosed with PD and treated with 600 mg/day levodopa (L-dopa). The treatment alleviated parkinsonism, but at age 52, he developed frequent drop attacks due to severe orthostatic hypotension. Another autonomic nervous system-related symptom was severe constipation. After several drop attacks, he suffered head contusion, which was followed by progressive deterioration of cognitive function until age 54. The parkinsonism was Hoehn & Yahr stage V at age 55. He died during an attack of pneumonia at age 57.

III–1

This is the father of the proband. At age 33 years, he developed masked face, resting tremor, bradykinesia, and antecollis. However, he did not complain of hyposmia. He was diagnosed the same year with PD and treated with L-dopa, which resulted in alleviation of symptoms. At age 34, he became apathic and developed dizziness due to orthostatic hypotension. At age 35, he became a pathological gambler and suffered from insomnia. At age 36, he was arrested for stealing. Subsequently, he was admitted to the hospital for further evaluation and treatment. On examination, the clinical symptoms included bradykinesia, masked face, muscle rigidity, antecollis and disturbed postural reflex. However, the gait showed no remarkable disturbance. He also had orthostatic hypotension, impotence, and constipation. In addition to the motor and autonomic symptoms, he had neurosis, insomnia, and suicide intent. This symptom complex was collectively diagnosed as depression. The parkinsonism gradually worsened after discharge from the hospital and was staged as Hoehn & Yahr stage V at age 37. He died at age 40.

IV–1

The proband was a 31-year-old man with familial parkinsonism of three generation. At age 28 years, he developed tremor and rigidity in the left hand and foot. No complaint of hyposmia was reported. One year later, he developed bradykinesia, orthostatic hypotension, and mild decline of intellectual activity. At age 30, he was admitted to the hospital and diagnosed with Parkinson disease, Hoehn & Yahr stage III. He had resting tremor, muscle rigidity, bradykinesia (masked face and gait disturbance), and disturbed postural reflex. Remarkable orthostatic hypotension was noted (systolic blood pressure in supine position 120 mm Hg, falling to 80 mm Hg on standing). Constipation was not prominent. No pyramidal signs were noted. As for intellectual activity, the WAIS-R score was 75 (verbal IQ 78 and motor IQ 76), although the mini mental state examination score was 29/30. There was no history of hallucination, nightmare, or REM sleep behavior disorder. Magnetic resonance imaging of the brain did not showed any remarkable findings. Single photon emission computed tomography of cerebral blood flow showed reduced blood flow in the parieto-occipital area. Myocardial scintigraphy of ^{123}I-metaiodobenzylguanidine showed markedly low heart-to-mediastinum ratio of 1.27 in the early stage and 1.23 in the late stage.

Treatment of parkinsonism consisted of 150 mg/day of L-dopa to alleviate symptoms. The dose was later increased to 300 mg/day of L-dopa, and combined with pramipexol (3 mg/day), selegiline (2.5 mg/day), and droxydopa (800 mg/day). Doubling the selegirin dose resulted in a transient episode of drug hypersensitivity,

but the symptoms were almost relieved and the Hoehn & Yahr stage improved to III.

DISCUSSION

This is the first case report of parkinsonism with *SNCA* triplication in an Asian family. The paternal grandfather, father and son of one family developed parkinsonism with ages at onset (AAO) under 50, suggesting autosomal dominant hereditary pattern and high penetrance. Based on the quantitative PCR study, we confirmed that this family have triplicated genetic region, which includes the *SNCA* gene.

To clarify the mechanism of copy number variation of the *SNCA* gene, we performed haplotype analysis of this triplication family and other unrelated Japanese family members with *SNCA* duplication. If the triplicated allele were generated from a duplicated allele, the proband of triplication might share a common haplotype with other families. However, the result of the analysis indicated that the triplication family had a different allele. Thus, the mechanism of *SNCA* triplication, whether it is self-duplicated by two-step or generated from recombination process, remains an important issue to be analyzed in future studies.

Limited to the three consecutive generations (proband, farther, and paternal grandfather), the penetrance is high. However, only three individuals were affected among 15 members of the family, and incomplete penetrance is the more favorable explanation. On the other hand, the AAO of the three affected members were under 50 years, which is younger than AAO of patients with *SNCA* duplication (mean ± SD: 48.5 ± 11.2 years)[4].

The AAO was markedly different between the generations. The paternal grandfather (II-1) developed parkinsonism at about 50 years of age, which is almost similar to *SNCA* duplication. On the other hand, both the proband and his father developed parkinsonism around 30 years of age. This phenomenon might imply anticipation between II-1 and III-1. In the case of a Swedish family, the ancestors of the family members with triplication were considered to have carried duplication.[8,10] Alternatively, it could reflect insufficient information about parkinsonism in the grandfather due to diagnostic difficulties in the past.

The clinical features of this family included early-onset parkinsonism with cognitive or mental disorders, and an aggressive disease course within a period of about 10 years. Treatment with L-dopa was effective at least in the early stage, though it did not improve the outcome. The two features; early AAO and cognitive or mental disturbances, are explainable by the dosage effect of α-synuclein; a higher production of the protein is associated with earlier AAO. Furthermore, a larger amount of the product results in a wider pathological picture. The clinical course of PD is mainly influenced by the AAO in general, but the mechanism of early ineffectiveness of L-dopa remains elusive. A more generalized pathology, which commonly involves the autonomic nervous system, manifested by orthostatic hypotension, is one possible explanation of the poor prognosis.

Finally, *SNCA* triplication is a rare event compared to the frequency of duplication. The clinical phenotype of triplication is severe among the Japanese population. In other words, the copy number of *SNCA* could determine the severity of the PD phenotype similar to the age at onset in the Asian population.[1,4] Further research on *SNCA* triplication is needed including its mechanism and differences in the clinical features between duplication and triplication.

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Research project: Conception, Organization; Manuscript: Review and Critique.

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Spinocerebellar Ataxia Type 10: Frequency of Epilepsy in a Large Sample of Brazilian Patients

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Abstract: **Spinocerebellar ataxia type 10 (SCA10) is an autosomal dominant disorder caused by an ATTCT repeat intronic expansion in the SCA10 gene. SCA 10 has been reported in Mexican, Brazilian, Argentinean and Venezuelan families. Its phenotype is overall characterized by cerebellar ataxia and epilepsy. Interestingly, Brazilian patients reported so far showed pure cerebellar ataxia, without epilepsy. Here, authors provide a systematic analysis of the presence, frequency and electroencephalographic presentation of epilepsy among 80 SCA10 patients from 10 Brazilian families. Overall, the frequency of epilepsy was considered rare, been found in 3.75 % of the cases while this finding in populations from other geographic areas reaches 60% of SCA10 cases.** © 2010 Movement Disorder Society

Key words: spinocerebellar ataxia type 10; SCA; autosomal dominant cerebellar ataxia; epilepsy

Spinocerebellar ataxia type 10 (SCA10) is an autosomal dominant disorder caused by a large expansion of a pentanucleotide (ATTCT) repeat in the intron 9 of the SCA10 gene on chromosome 22.[1–5] SCA10 is the only neurodegenerative disease caused by an expansion of a pentanucleotide repeat. Pathogenic alleles range from 800 to 4500 ATTCTs (normal 10 to 29).[1,3] SCA10 has previously been reported in Mexican families, in which the disease presented with a unique combination of pure cerebellar ataxia, epilepsy and, at times, polyneuropathy, pyramidal signs, and cognitive dysfunction.[1–5] In 2004, we described the clinical phenotype of five Brazilian families with SCA10 presenting with pure cerebellar ataxia but no associated epilepsy.[6]

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Zonisamide reduces cell death in SH-SY5Y cells via an anti-apoptotic effect and by upregulating MnSOD

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ABSTRACT

Zonisamide, originally known as an antiepileptic drug, has been approved in Japan as adjunctive therapy with levodopa for the treatment of Parkinson's disease (PD). Although zonisamide reduces neurotoxicity, the precise mechanism of this action is not known. Here, we show that zonisamide increases cell viability in SH-SY5Y cells via an anti-apoptotic effect and by upregulating levels of manganese superoxide dismutase (MnSOD). These results would give us novel evidences of PD treatment.

Parkinson's disease (PD) is the second most common neurodegenerative disease characterized by pronounced loss of dopaminergic neurons in the substantia nigra pars compacta. At present, there is no known treatment to suppress the progression of this cell death and the goal of current therapies is only to alleviate symptoms. New therapies are, therefore, required to delay disease progression and to improve the long-term prognosis of PD. Mitochondrial dysfunction due to oxidative stress has been proposed as a major factor in the pathogenesis of PD [3,22] and reduced activity in mitochondrial complex I is associated with PD [13,22]. After complex I blockade with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice, there is a time-dependent and region-specific activation of mitochondrial cytochrome c, which activates caspase-9 and -3, resulting in apoptosis [20].

Zonisamide, originally known as an anticonvulsant agent, has been approved in Japan as adjunctive therapy with levodopa for patients with PD [15,16]. The mechanism of zonisamide to improve Parkinsonism has been reported to modify the turnover and synthesis of dopamine (DA) [18,28]. Moreover, it has been recently reported that zonisamide attenuates MPTP-induced and 6-hydroxydopamine (6-OHDA)-induced neurotoxicity and dopaminergic neuron-specific oxidative stress in mice [1,2,29,30]. Although zonisamide is likely to delay the progress of PD, the exact mechanism of its action remains unclear.

In this study, we show that zonisamide increases the viability of differentiated SH-SY5Y cells by inhibiting activation of proapoptotic molecules and upregulating levels of manganese superoxide dismutase (MnSOD), also known as superoxide dismutase-2 (SOD2).

The following antibodies were used in this study: anti-caspase-3 antibody (rabbit), anti-caspase-9 antibody (rabbit), and anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody (rabbit) were obtained from Cell Signaling Technology. Anti-actin antibody (mouse) was from Millipore. Anti-MnSOD antibody (rabbit) was from Upstate.

SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% penicillin–streptomycin (Invitrogen-GIBCO) at 37 °C and 5% CO_2. To induce cell differentiation, SH-SY5Y cells were incubated in complete medium plus 10 μM *all-trans* retinoic acid (Sigma) for 72 h. For pharmacological studies, zonisamide (Dainippon Sumitomo Pharma Co.), staurosporine (Cell Signaling Technology), 1-methyl-4-phenyl-pyridium ion (MPP^+) (Sigma), and dopamine hydrochloride (Sigma) were added at indicated times and concentrations.

Cell viability was assessed by the WST assay; the ability of mitochondrial activity to reduce 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) to formazan using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions.

TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining was performed using the *In situ* Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's instructions. Cells were then mounted with Vectashield containing DAPI (Vector Lab-

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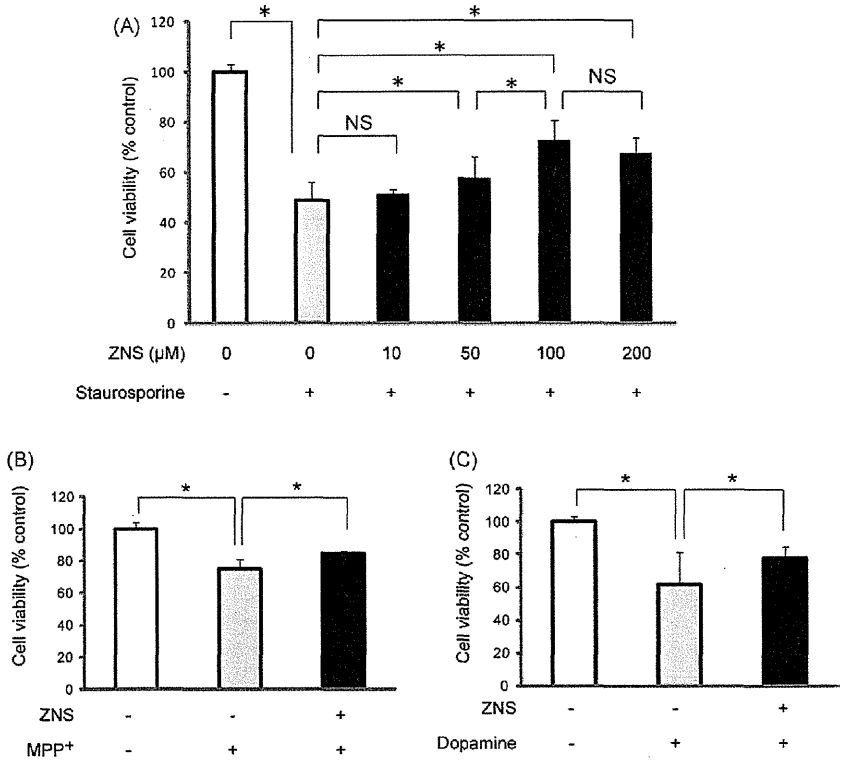

Fig. 1. Zonisamide increases cell viability. (A) Differentiated SH-SY5Y cells were treated with staurosporine (0.2 μM) for 5 h before the WST assay was performed, with or without 24 h treatment with various concentrations of zonisamide. Absorbance at 450 nm was measured. (B) and (C) MPP^+ (500 μM) treatment for 24 h (B), or dopamine (50 μM) treatment for 24 h (C). WST assay was performed with or without 24 h treatment with zonisamide (100 μM). ZNS, zonisamide. *$p < 0.05$. NS, non-significant. Error bars indicate standard deviation of at least five experiments.

oratories, Burlingame, CA, USA) and examined using a fluorescence microscope (BZ-9000; KEYENCE, Japan). Image processing was performed using Adobe Photoshop CS3.

For immunoblot analysis, cells were lysed on ice in lysis buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease inhibitors (complete, Mini, EDTA-free, Roche Applied Science)]. Cell lysates were mixed with NuPAGE 4× LDS sample buffer (Invitrogen). The samples were separated by SDS-PAGE, and proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked with 5% non-fat milk (BD Difco) in Tris-buffered saline containing 0.05% Tween20 (TBS-T) and then incubated overnight at 4 °C with the primary antibody. The membranes were incubated for 1 hour at room temperature with the secondary antibody and visualized using ECL plus reagent (GE Health Care Bio-Sciences) or with Western Lightning (Perkin Elmer-Cetus). After scanning the images, the intensity of each immunoreactive band was estimated by densitometric quantification using ImageJ 1.42 software.

We first examined the effects of zonisamide on the viability of differentiated human neuroblastoma cells (SH-SY5Y cells), which are dopaminergic and can differentiate into neuronal-like phenotypes when treated for 3–5 days with retinoic acid (10 μM). Differentiation is accompanied by the arrest of cell proliferation and increased dopamine metabolism [17,24]. We assessed cell viability using the WST-8 assay [11,25]. In this assay, the tetrazolium salt, WST-8, is cleaved to formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is mostly dependent on the production of glycolytic NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells. For staurosporine-treated cells, treatment with zonisamide at 50 μM or over induced an increase of cell viability, with the greatest effect being at 100 μM (Fig. 1A). Subsequent experiments were, therefore, performed using 100 μM zonisamide. Various PD-cellular models have been established using neurotoxins that cause neurotoxicity towards dopaminergic neurons, such as MPTP, DA, rotenone and paraquat [4,5,7,10,23]. Therefore, we next performed similar experiments in the presence of MPP^+ and DA. Zonisamide treatment also prevented subsequence of cell death following MPP^+-treatment (Fig. 1B) and DA-treatment (Fig. 1C). These results indicate that zonisamide has neuroprotective effects in PD-cellular models.

Apoptosis is a major cell death pathway in PD and other neurodegenerative diseases [19]. To investigate the mechanisms by which zonisamide exerts its cytoprotective effects, we examined whether the zonisamide-induced increase of cell viability occurs via an anti-apoptotic effect. We assessed apoptosis in differentiated, staurosporine-treated SH-SY5Y cells using TUNEL staining. As expected, the proportion of TUNEL-positive cells was reduced by zonisamide treatment compared with untreated cells (Fig. 2A and B). To confirm the anti-apoptotic effect of zonisamide, we also examined proapoptotic molecules [caspase-9, -3, and phospho-c-Jun N-terminal kinase (p-JNK)] in differentiated SH-SY5Y cells. Zonisamide treatment reduced the levels of cleaved caspase-9, -3, and p-JNK (Fig. 2C and D). Cleaved caspase-9 is produced in response to mitochondrial damage and the production of cleaved caspase-3 is downstream of cleaved caspase-9. Activation of JNK by reactive oxygen species (ROS) is critical for the induction of apoptosis in neuronal cells [21,27]. Our results suggest that zonisamide blocks the activation of proapoptotic molecules in differentiated SH-SY5Y cells. Also, although PD pathogenesis has been associated with both an excess and a deficiency of autophagic activity [9,26], zonisamide had no effect on autophagic activity because no alteration in the LC3-II/actin ratio, an indicator of autophagic activity, was observed (data not shown).

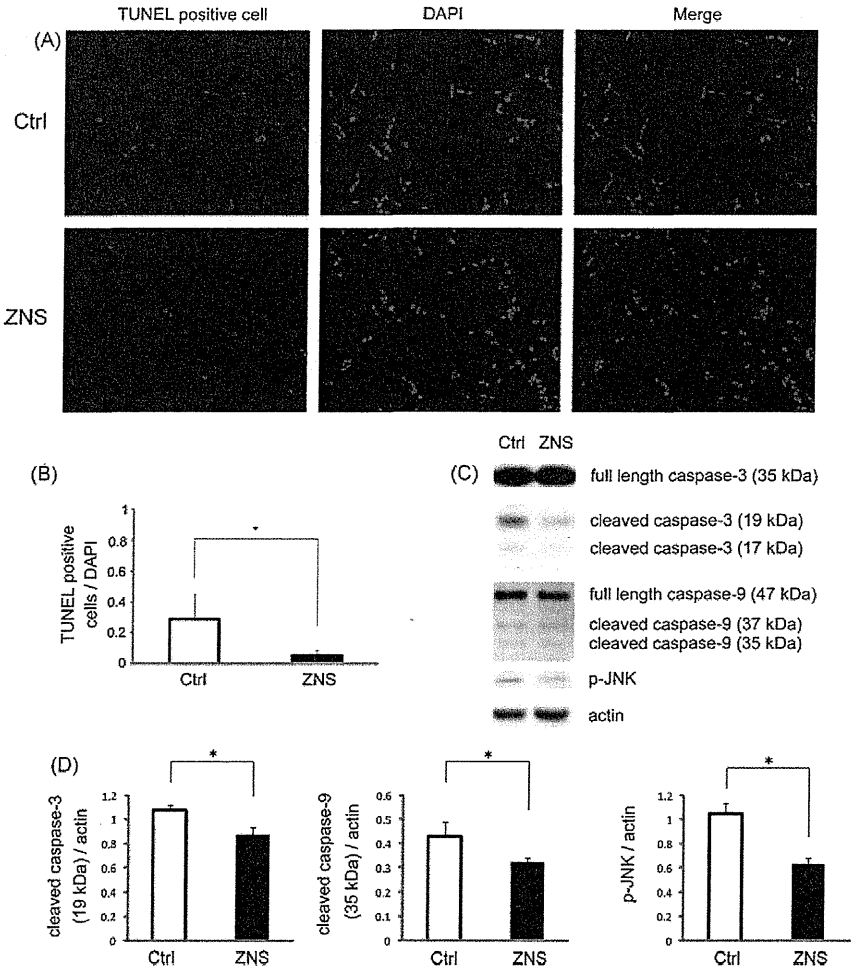

Fig. 2. Zonisamide has anti-apoptotic effects. Differentiated SH-SY5Y cells were treated with staurosporine (0.2 μM) for 3 h, with or without of 24 h treatment with zonisamide (100 μM). (A) TUNEL staining. TUNEL-positive cells are in red. DAPI stained nuclei are in blue. (B) Quantification of TUNEL staining: ratio of TUNEL-positive cells to DAPI stained nuclei is shown. (C) Full length caspase-3, -9, Cleaved caspase-3, -9, and p-JNK levels were analyzed by western blotting. The bottom panel shows actin as a loading control. (D) Quantification of (C): ratios of cleaved caspase-3, -9, and p-JNK to actin are shown. $^*p < 0.05$. Error bars indicate standard deviation of at least three independent experiments. Ctrl, control. ZNS, zonisamide.

MnSOD over-expression attenuates MPTP toxicity and protects cells from apoptosis [12,31], while transgenic mice over-expressing MnSOD are more resistant to MPTP toxicity [8]. Based on the results presented in Figs. 1 and 2, we examined whether zonisamide treatment regulates MnSOD activity. We assayed the effects of zonisamide treatment on MnSOD levels in differentiated, staurosporine-treated SH-SY5Y cells. Zonisamide treatment induced an increase in MnSOD levels (Fig. 3A and B). MnSOD is an antioxidant localized in mitochondria and represents a major defense against superoxide free radicals produced in mitochondria. Zonisamide is also known to scavenge hydroxyl radicals and nitric oxide radicals in cell-free systems, which is consistent with our data [2,14]. Although NF-κB is known to be upstream of MnSOD [6], zonisamide treatment did not affect NF-κB levels (data not shown). We did not investigate the zonisamide-induced mechanism of MnSOD upregulation.

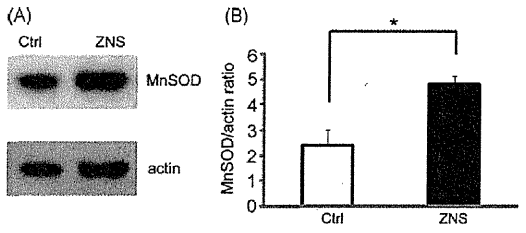

Fig. 3. Zonisamide upregulates levels of MnSOD. (A) Manganese superoxidase dismutase (MnSOD) levels in differentiated SH-SY5Y cells treated with staurosporine (0.2 μM) for 3 h with or without 24 h treatment with zonisamide (100 μM) were analyzed by western blotting. The bottom panel shows actin as a loading control. (B) Quantification of MnSOD western blot: ratio of MnSOD to actin is shown; $^*p < 0.05$. Error bars indicate standard deviation of at least three independent experiments. Ctrl, control. ZNS, zonisamide.

In the present study, we provide several lines of evidence showing that zonisamide increased neuronal cell viability via effects on apoptosis and oxidative stress. This interpretation is based on: (1) zonisamide increased cell viability of cells treated with staurosporine and of cells in PD-cellular models. (2) Zonisamide reduced the number of TUNEL-positive cells and the levels of proapoptotic molecules. (3) Zonisamide upregulated levels of MnSOD.

Previous studies have shown that zonisamide significantly attenuates MPTP-induced neurotoxicity by inhibiting microglial activation or 6-OHDA-induced neurotoxicity by increasing glutathione (GSH) via enhancing astroglial cysteine transport system and astroglial proliferation [1,29,30]. However, the direct pharmacological effects on neurons has not been fully investigated. Consistent with previous reports that have investigated cytoprotective effects in PD-cellular models, we demonstrated that

zonisamide inhibited activation of proapoptotic molecules and upregulated levels of MnSOD in neuronal cells without glial cells. Therefore, we assume that zonisamide has direct and indirect effects on neurons, leading to the neuroprotection. Higher levels of MnSOD might be associated with enhanced mitochondrial maintenance and might contribute to reduce apoptosis that has been induced by mitochondrial damage in PD.

In conclusion, our results demonstrate that zonisamide could reduce neuronal cell death via an anti-apoptotic effect and by upregulating MnSOD levels. Although a more detailed elucidation of the action of zonisamide is needed, these results suggest that zonisamide might provide a new approach for the therapy of PD and other progressive neurodegenerative diseases.

Competing financial interest

The authors declare that they have no competing financial interests.

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ORIGINAL ARTICLE

Neuroprotective Effects of Zonisamide Target Astrocyte

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Objective: Recent double-blind, controlled trials in Japan showed that the antiepileptic agent zonisamide (ZNS) improves the cardinal symptoms of Parkinson's disease. Glutathione (GSH) exerts antioxidative activity through quenching reactive oxygen species and dopamine quinone. GSH depletion within dopaminergic neurons impairs mitochondrial complex I activity, followed by age-dependent nigrostriatal neurodegeneration. This study examined changes in GSH and GSH synthesis-related molecules, and the neuroprotective effects of ZNS on dopaminergic neurodegeneration using 6-hydroxydopamine–injected hemiparkinsonian mice brain and cultured neurons or astrocytes.

Methods and Results: ZNS increased both the cell number and GSH levels in astroglial C6 cells, but not in dopaminergic neuronal CATH.a cells. Repeated injections of ZNS (30mg/kg intraperitoneally) for 14 days also significantly increased GSH levels and S100β-positive astrocytes in mouse basal ganglia. Repeated ZNS injections (30mg/kg) for 7 days in the hemiparkinsonian mice increased the expression of cystine/glutamate exchange transporter xCT in activated astrocytes, which supply cysteine to neurons for GSH synthesis. Treatment of these mice with ZNS also increased GSH levels and completely suppressed striatal levodopa–induced quinone formation. Reduction of nigrostriatal dopamine neurons in the lesioned side of hemiparkinsonian mice was significantly abrogated by repeated injections of ZNS with or without adjunctive levodopa starting 3 weeks after 6-hydroxydopamine lesioning.

Interpretation: These results provide new pharmacological evidence for the effects of ZNS. ZNS markedly increased GSH levels by enhancing the astroglial cystine transport system and/or astroglial proliferation via S100β production or secretion. ZNS acts as a neuroprotectant against oxidative stress and progressive dopaminergic neurodegeneration.

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Zonisamide (1,2-benzisoxazole-3-methaesulfonamide: ZNS) was originally synthesized in Japan and is currently used as an antiepileptic agent in Japan, South Korea, the United States, and Europe. An open trial of ZNS (50–200mg/day) treatment showed lessening of symptoms, especially wearing off of Parkinson's disease (PD), when ZNS is used in conjunction with anti-PD drugs,[1] and more than 30% improvement of total score of the Unified Parkinson's Disease Rating Score up to 3 years.[2] The addition of ZNS to L-dopa treatment in 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 the combination of low-dose ZNS (25–100mg/day) and L-dopa improved all cardinal symptoms of PD.[2,3] In Japan, ZNS was released in March 2009 as a novel antiparkinsonian agent.

Several pharmacological effects of ZNS are consid-

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Additional Supporting Information may be found in the online version of this article.

ered related to its beneficial effects on PD. ZNS is a specific T-type Ca^{++} channel blocker,[4,5] which increases burst firing of dopamine (DA) neurons in the substantia nigra. Acute administration of ZNS increased intracellular and extracellular DOPA, DA, and homovanillic acid levels, and decreased 3, 4-dihydroxyphenylacetic acid (DOPAC) level in the rat striatum presumably by its moderate monoamine oxidase–inhibiting effect or enhancement of DA synthesis.[6,7] However, the lack of change in the efficacy of ZNS when coadministered with a monoamine oxidase B inhibitor[3] suggests that monoamine oxidase inhibition is not the main mechanism of its antiparkinsonism effect. It has been reported that ZNS increases DA release in the striatum.[7–9] Long-term administration of ZNS increased the activity and protein level of tyrosine hydroxylase (TH) in the rat striatum,[2] and thus enhanced DA synthesis. These effects of ZNS on DA release and DA synthesis could explain its therapeutic efficacy against motor symptoms of PD. However, these effects cannot fully explain the mechanism of its long-term therapeutic efficacy, especially on L-dopa–induced adverse effects.

Glutathione (GSH) acts as antioxidant against reactive oxygen species–induced neurodegeneration. Astrocytes but not neurons express cystine/glutamate exchange transporter (xCT), which takes up cystine, reduce it to cysteine, and consequently supply cysteine, the substrate for GSH synthesis in neurons. Therefore, GSH synthesis in neurons is dependent on the expression of xCT on astrocytes.[10,11] The experimental findings in recent studies[12–14] suggest that GSH and its synthesis-related molecules in astrocytes provide protection against age-dependent nigrostriatal dopaminergic neurodegeneration. To characterize the neuroprotective profile of ZNS in more detail, we examined in this study the effects of ZNS on changes in GSH, its synthesis-related molecules, and on degeneration of DA neurons, using hemiparkinsonian mice brain and cultured neurons or astrocytes.

Materials and Methods

Materials and Animals

The chemicals and animals purchased are listed in the Supplementary text (see Materials and Animals section). ZNS and its sodium salt were provided by Dainippon Sumitomo Pharma (Osaka, Japan). All animal procedures described in this study were in strict accordance with the Guidelines for Animal Experiments at Okayama University Medical School.

Cell Culture and Drug Treatment

Dopaminergic CATH.a cells, C6 glioma cells, or primary striatal glial-rich cells were plated onto culture plates or chamber glass culture slides (see Supplementary text Cell Culture Procedure section). After 24 hours, CATH.a cells, C6 cells, or primary cultured astrocytes were exposed to 1 to 100μM ZNS diluted in phosphate-buffered saline for 24 hours or shorter periods where indicated. For neutralization assay, C6 cells were incubated with ZNS or anti-S100β antibody (diluted 1:2,000, final concentration 19μg/ml; Sigma-Aldrich, St. Louis, MO), or both, for 24 hours.

Treatment of Mice with Zonisamide

Healthy male ICR mice weighing 28 to 32gm (7 weeks old; Charles River Japan Inc., Yokohama, Japan) were used in these experiments. For GSH measurements and Western blot analyses, mice were injected intraperitoneally (IP) with ZNS sodium salt (10, 30, or 50mg/kg) dissolved in saline every day for 14 days. One day after the final administration of the drug, mice were transcardially perfused with ice-cold saline under sodium pentobarbital anesthesia (70mg/kg IP), and striatal or ventral midbrain tissue was dissected out immediately. For time-course immunohistochemical analysis, mice were injected IP with ZNS sodium salt (30mg/kg/day) for 1, 3, or 7 days. One day after the final ZNS treatment, mice were transcardially perfused with saline followed by a fixative.

6-Hydroxydopamine–Lesioned Hemiparkinsonian Mice and Zonisamide Treatment

Male ICR mice weighing 30 to 35gm (8 weeks old) were prepared as a hemiparkinsonian model. Unilateral striatal lesions were induced by intrastriatal injections of 6-hydroxydopamine (6-OHDA) using the method described previously (see Supplementary text 6-OHDA-Lesioned Hemiparkinsonian Mice section). Two weeks after 6-OHDA injection, mice that developed hemiparkinsonism were selected by apomorphine injection (see Supplementary text 6-OHDA-Lesioned Hemiparkinsonian Mice section). One week after the apomorphine test (ie, 3 weeks after 6-OHDA injection), hemiparkinsonian mice were injected IP with ZNS sodium salt (30mg/kg/day), L-dopa/carbidopa (50/5mg/kg/day) suspended in 0.5% methylcellulose, or a combination of ZNS and L-dopa/carbidopa once a day for 7 days. At 1 day after the final injection of the drugs (ie, 4 weeks after 6-OHDA injection), mice were perfused transcardially with ice-cold saline or saline followed by a fixative under pentobarbital anesthesia (70mg/kg IP) for quinoprotein measurement or immunohistochemistry, respectively. Brains were collected from hemiparkinsonian mice at 3 weeks after 6-OHDA injection (pretreatment) for immunohistochemistry.

Determination of Total Glutathione

GSH levels in cultured cells or brain tissues were determined using the enzymatic recycling method[15] (see Supplementary text Determination of Total GSH section).

Protein-Bound Quinone: Quinoprotein Measurement

Levels of protein-bound quinones (quinoprotein) in the striatal tissue were measured using the nitrobluetetrazolium/glycinate

colorimetric assay with minor modifications[16] (see Supplementary text Quinoprotein Measurement section).

Western Blot Analysis

Western blot analysis was performed as described previously[17] using lysates from cells or brain tissue. The detailed experimental procedures concerning extraction of protein lysates, reagents and antibodies used, and Western blot analysis are described in the Supplementary text (see Western Blot Analysis section).

Immunohistochemistry

C6 cells or primary cultured astrocytes on the chamber slides were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). Mice were perfused with saline followed by a fixative containing 4% paraformaldehyde and 0.35% glutaraldehyde in 0.1M PB (pH 7.4) under anesthesia. Brain sections were prepared by cutting brain coronally on a cryostat at levels containing the midstriatum (+0.6 to +1.0mm from the bregma) and the substantia nigra pars compacta (−2.8 to −3.0mm from bregma) (see details in Supplementary text Immunohistochemistry section).

The detailed experimental methods concerning reagents and antibodies used, immunofluorescence staining, and standard free-floating immunohistochemistry are described in the Supplementary text (see Immunohistochemistry section).

Statistical Analysis

Values were expressed as mean ± standard error of the mean. Differences between groups were examined for statistical significance using one-way analysis of variance followed by post hoc Fisher's partial least squares difference test. A p value less than 0.05 denoted the presence of a statistically significant difference.

Results

Effects of Zonisamide on Glutathione and Its Synthesis-Related Molecules in Mouse Basal Ganglia

Changes in GSH levels were examined in the mouse striatal or ventral midbrain tissue after administration of ZNS. Repeated administration of ZNS (10, 30mg/kg/day IP) for 14 days significantly increased GSH levels in the striatum (Fig 1A) and the ventral midbrain (see Fig 1B) (approximately 1.64-fold and approximately 2.11-fold vs vehicle-treated control, respectively). To determine the mechanism of increase in GSH levels, we examined changes in GSH synthesis and quinone formation-related molecules in mice brain. ZNS administration for 14 days had no effects on glutamate cysteine ligase (GCL), a GSH-synthesizing enzyme, glutamyl-*S*-transferase (GST), a GSH-conjugating enzyme, or quinone reductase-1 in the striatum (see Supplementary Figs 1A–C).

Effects of Zonisamide on Glutathione and Its Synthesis-Related Molecules in Neuronal and Astroglial Cells

Next, we assessed whether ZNS increases GSH in neurons or glial cells using in vitro cultured cells. Incubation with ZNS for 24 hours significantly increased GSH levels in astroglial C6 cells (see Fig 1D) but not in dopaminergic neuronal CATH.a cells (see Fig 1C). As described earlier, because GSH synthesis in neurons is dependent on the cyst(e)ine transport system of xCT on astrocytes,[10,11] we examined changes in GSH synthesis-related molecules, astroglial cystine transport, and antioxidative system after incubation of dopaminergic neuronal CATH.a cells and astroglial C6 cells with ZNS. Immunostaining showed that ZNS (100μM, 24 hours) increased the number of GCL-immunopositive C6 cells (see Supplementary Figs 2B, C), but not the ratio of GCL-positive cells to total C6 cells (see Supplementary Fig 2D). Furthermore, Western blot analysis showed that ZNS had no effects on GCL protein levels in neuronal and astroglial cells (see Supplementary Figs 2A, E).

Interestingly, ZNS (50 and 100μM, 24 hours) dose-dependently increased the number of xCT-immunopositive astroglial C6 cells (see Figs 1E, F) and the ratio of xCT-positive cells to total C6 cells (see Fig 1G). However, 24-hour incubation with ZNS showed no effects on nuclear immunoreactivity in C6 cells (see Supplementary Fig 3D) or protein levels in nuclear extracts (see Supplementary Fig 3C) of nuclear factor erythroid 2-related factor (Nrf2), which promotes gene expression of various antioxidant molecules including GCL, GST, superoxide dismutase, quinone reductase-1, and xCT.[18] Incubation of cells with ZNS for shorter periods (6 or 12 hours) did not affect nuclear Nrf2 protein levels (see Supplementary Figs 3A, B).

Furthermore, ZNS (100μM, 24 hours) significantly increased the number of astroglial C6 cells and primary cultured astroglial cells (see Figs 1F, 1H, and Supplementary Fig 2C). A similar, though less marked increase in the cell number was noted at 50μM (see Fig 1H).

Effects of Zonisamide Administration on Astrocytes in Striata of Normal Mice and Hemiparkinsonian Mice

Next, we examined the effects of ZNS administration on the number of astrocytes in the striatum. Repeated administration of ZNS (30mg/kg/day IP) for 7 days increased the number of S100β-positive astrocytes in the mouse striatum (Figs 2A, B). However, few glial fibrillary acidic protein (GFAP)–positive activated astrocytes were seen in the striatum with or without ZNS administration (data not shown). ZNS also increased the total relative

intensity of S100β immunoreactivity including intracellular S100β-positive signals and diffuse extracellular S100β-positive reactivity around astrocytes (see Figs 2A, C). S100β is a small calcium-binding protein found primarily in astrocytes, and extracellular S100β is known to exert autocrine effects to promote astrocyte proliferation.[19,20] To determine the role of S100β in the astrocyte proliferating effect of ZNS, we used anti-S100β antibody to neutralize the protein in ZNS-treated astroglial C6 cells. Coincubation with anti-S100β antibody completely inhibited ZNS-induced increase in the number of C6 cells (see Fig 2D). Furthermore, we examined the effects of repeated ZNS injections on S100β-positive astrocytes in the striatum of 6-OHDA–lesioned hemiparkinsonian mice. The number of S100β-positive astrocytes was significantly and markedly increased on the lesioned side in the striatum of hemiparkinsonian mice 4 weeks after 6-OHDA injection (see Figs 2E, F). Although repeated injections of ZNS sodium salt (30mg/kg/day), L-dopa/carbidopa (50/5mg/kg/day), or a combination of ZNS and L-dopa/carbidopa for 7 days did not modulate astrocyte proliferation in the lesioned striatum, a 7-day treatment with ZNS alone significantly increased the number of S100β-positive astrocytes in the intact side of the striatum (see Figs 2E, F), coinciding with the astrocyte proliferating effects of the drug in the normal mouse striatum (see Figs 2A, B).

Effects of Zonisamide Administration on Glutathione and a Cystine Transporter in the Striatum of Hemiparkinsonian Mice

Because ZNS treatment dose-dependently increased xCT expression on the surface of cultured astroglial C6 cells (see Fig 1G), we examined xCT expression in the striatum of hemiparkinsonian mice by immunohistochemistry. In the hemiparkinsonian mice, repeated administration with vehicle and saline markedly activated the GFAP-positive astrocytes with lower xCT expression in the striatum on the 6-OHDA–lesioned side (Figs 3A, B). The number of xCT/GFAP-positive astrocytes was significantly increased on the parkinsonian side after the repeated L-dopa/carbidopa injections (50/5mg/kg/day) with or without coadministration of ZNS (see Figs 3A–C). However, repeated injections with ZNS alone (30mg/kg/day) significantly increased xCT expression on activated

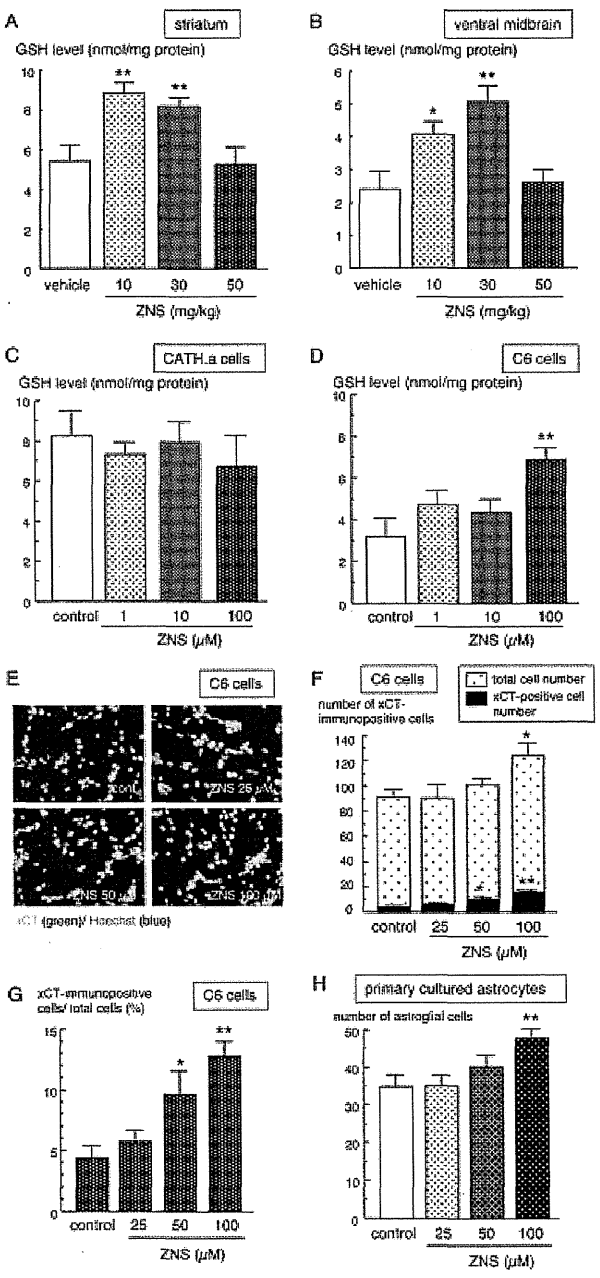

FIGURE 1: Effects of repeated administration of zonisamide (ZNS) on glutathione (GSH) in mouse basal ganglia. Changes in GSH levels in the striatum (A) and the ventral midbrain (B) were measured at 24 hours after the final administration of repeated ZNS administration (10, 30, 50mg/kg/day) for 14 days. Data are mean ± standard error of the mean (SEM) values of six animals. *$p < 0.05$, **$p < 0.01$ compared with the vehicle-treated control group (A, B). Effects of ZNS on GSH and cystine/glutamate exchange transporter (xCT) in neuronal or astroglial cells. GSH levels were measured in dopaminergic CATH.a cells (C) and astroglial C6 cells (D) after the treatment with ZNS (1–100μM) for 24 hours (n = 5–6). Representative photos of xCT immunohistochemistry (E), changes in the immunopositive cell number (F), and ratio of immunopositive cells to total cells (G) in astroglial C6 cells after the treatment with ZNS (25–100μM) for 24 hours (n = 9–11). Changes in cell number of primary cultured astroglial cells after the treatment with ZNS (25–100μM) for 24 hours (H) (n = 8). Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$ compared with the control group (D, G, H). *$p < 0.01$, **$p < 0.001$ compared with the control group (F).

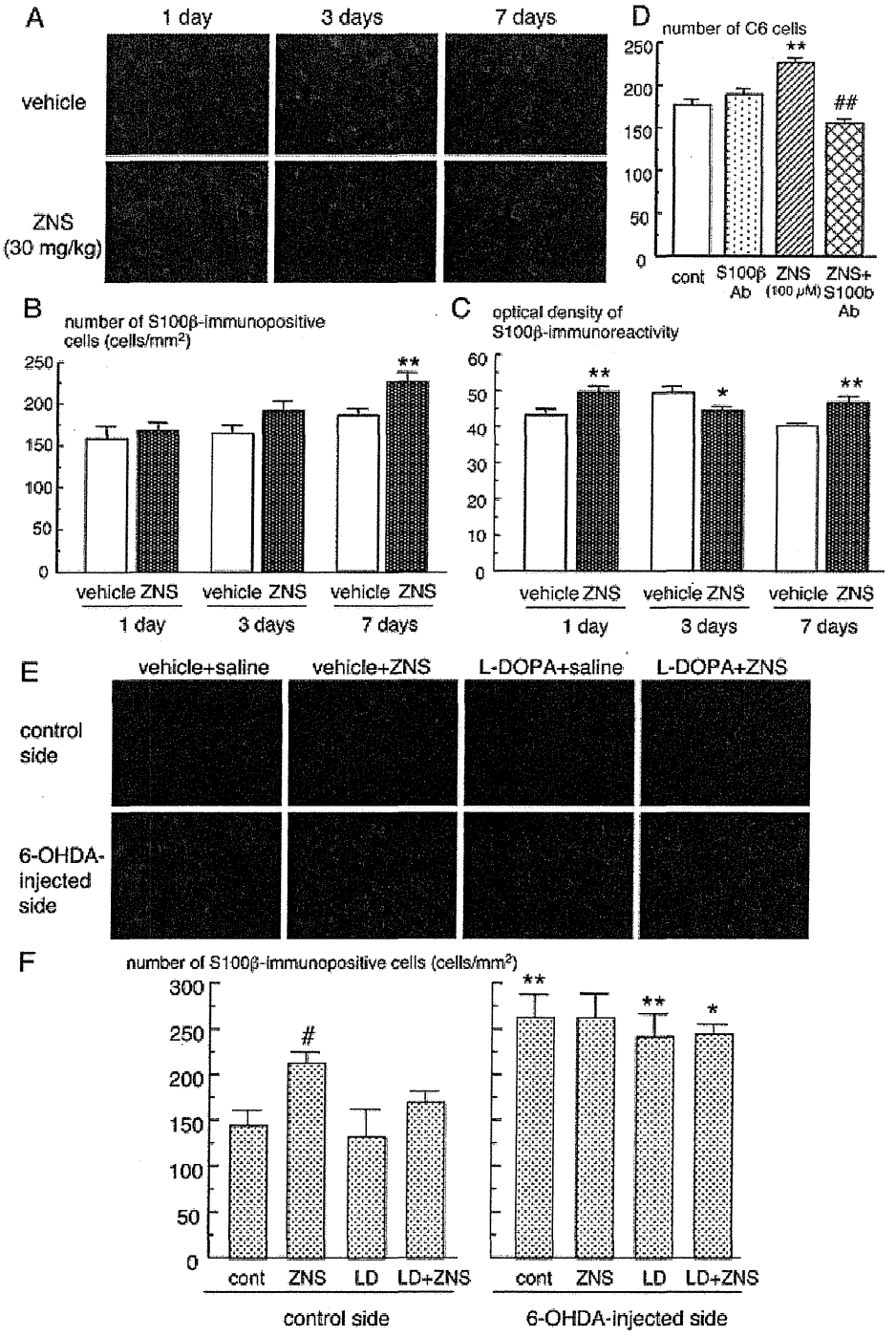

FIGURE 2: Effects of zonisamide (ZNS) administration on astrocytes in the striatum of normal mice and hemi-Parkinson's disease (PD) mice. Representative photos (A), number of immunopositive cells (B), and intracellular and extracellular relative optical density of immunoreactivity (C) in immunostaining for S100β in the midstriatal brain slices after administration of ZNS (30mg/kg/day) for 1, 3, or 7 days. Data are means ± standard error of the mean (SEM) of 10 animals. *$p < 0.05$, **$p < 0.01$ compared with time-matched, vehicle-injected control group. Effects of neutralization using anti-S100β antibody (Ab) on the increase in the number of astroglial C6 cells induced by ZNS treatment (100μM) for 24 hours (D; n = 8). **$p < 0.001$ compared with the control group. ##$p < 0.001$ compared with the cells treated with ZNS alone. Representative photos (E) and number of immunopositive cells (F) of S100β staining at 4 weeks after the lesioning in the midstriatal brain slices of hemi-PD mice administered with L-dopa/carbidopa (50/5mg/kg/day) and/or ZNS (30mg/kg/day) for 7 days (starting at 3 weeks after the lesioning). cont = vehicle-treated group; LD = L-dopa–treated group; LD+ZNS = L-dopa+ZNS–treated group. Data are mean ± SEM of five to six animals. *$p < 0.01$, **$p < 0.001$ compared with the control side of each drug-treated group. #$p < 0.05$ compared with side-matched, vehicle-treated control group.

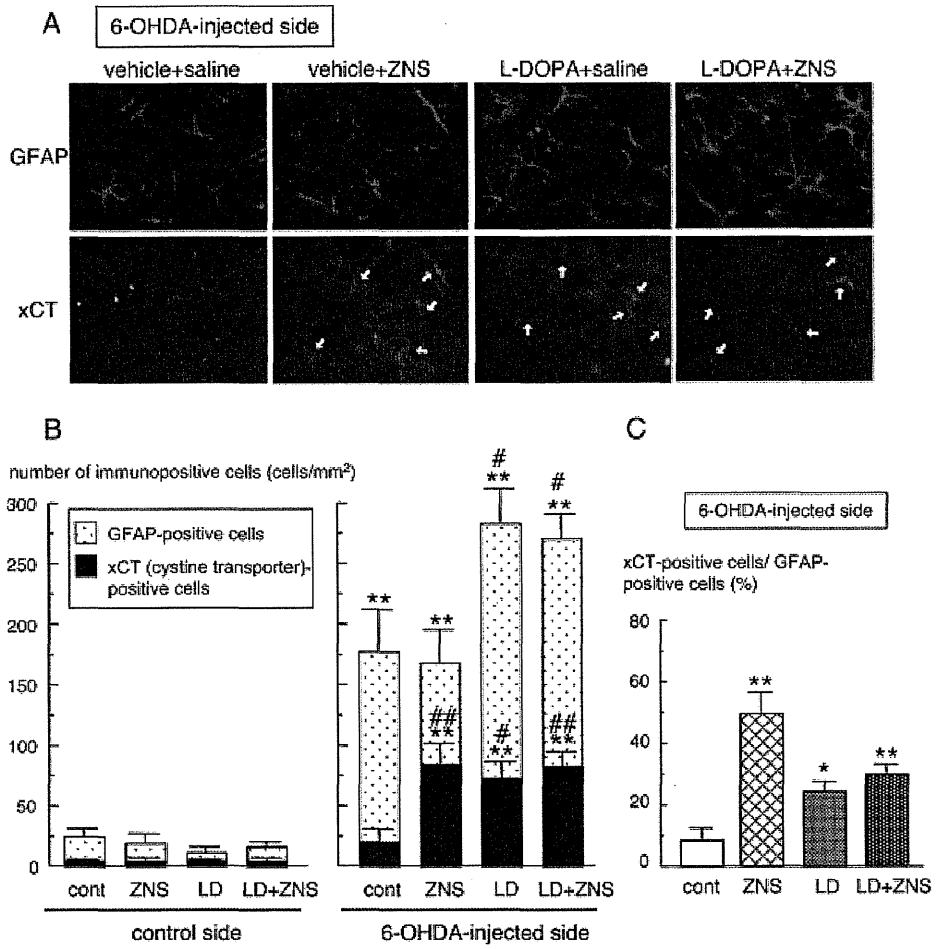

FIGURE 3: Effects of zonisamide (ZNS) administration on cystine transporter in the striatum of hemi-Parkinson's disease (PD) mice. Representative photomicrographs (A), number of immunopositive cells (B), and percentage ratio of cystine/glutamate exchange transporter (xCT)–positive astrocytes to total glial fibrillary acidic protein (GFAP)–positive astrocytes (C) of double-immunofluorescence staining for GFAP and xCT in the midstriatum of hemiparkinsonian mice that were administered with L-dopa/carbidopa (50/5mg/kg/day) and/or ZNS (30mg/kg/day) for 7 days (starting at 3 weeks after the lesioning). cont = vehicle-treated group; LD = L-dopa–treated groups; LD+ZNS = L-dopa+ZNS–treated group; 6-OHDA = 6-hydroxydopamine. Data are means ± standard error of the mean of five to six animals. **$p < 0.001$ compared with the control side of each drug-treated group; #$p < 0.01$, ##$p < 0.001$ compared with side-matched, vehicle-treated control group (B). *$p < 0.05$, **$p < 0.01$ compared with the parkinsonian side of the vehicle-treated control group (C).

striatal astrocytes without increasing the number of GFAP-positive astrocytes (see Figs 3A–C).

On the lesioned side of the striatum in the hemiparkinsonian mice, striatal GSH immunoreactivity was reduced to 62.6% of that on the intact side (Figs 4A, B). Striatal GSH immunoreactivity in neuronal nuclei–positive neurons was increased, although not significantly, by the repeated ZNS administration alone (30mg/kg/day). However, the immunoreactivity was significantly increased in the group cotreated with ZNS and L-dopa compared with the group treated with L-dopa alone (see Figs 4A, B).

The level of striatal quinoprotein was significantly increased specifically on the parkinsonian side but not on the control side after repeated L-dopa/carbidopa injections (50/5mg/kg/day) (see Fig 4C), coinciding with our previous report.[16] As expected, repeated administration of ZNS (30mg/kg/day) completely suppressed L-dopa–induced quinoprotein formation in the striatum on the lesioned side (see Fig 4C).

Effects of Zonisamide Administration on Dopaminergic Neuronal Damage in Hemiparkinsonian Mice

Finally, we examined the proposed neuroprotective effects of in vivo ZNS treatment on degeneration of DA neurons in 6-OHDA–lesioned hemiparkinsonian mice. At 4 weeks after the 6-OHDA treatment, the number of nigral TH-positive dopaminergic neurons and the striatal TH or DA transporter (DAT) immunoreactivity were both markedly

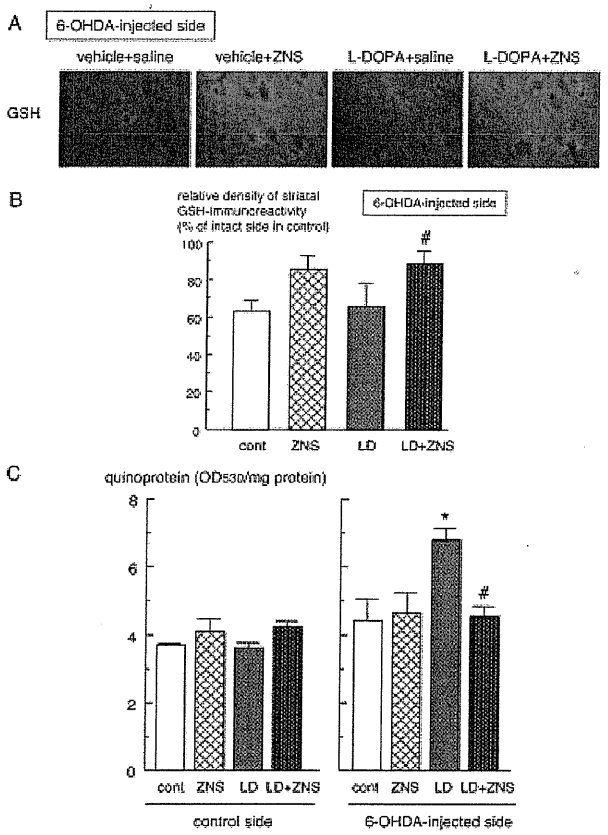

FIGURE 4: Effects of zonisamide (ZNS) administration on glutathione (GSH) and quinone formation in the striatum of hemi-Parkinson's disease (PD) mice. Representative photomicrographs (A), relative density of immunoreactivity (B; n = 4–6) in immunofluorescence staining for GSH on the 6-hydroxydopamine (6-OHDA)–injected side of the mid-striatal brain slices, and the levels of quinoprotein formation in the striatal tissue (C; n = 6–8) of hemi-PD mice that were administered with L-dopa/carbidopa (50/5mg/kg/day) and/or ZNS (30mg/kg/day) for 7 days (starting at 3 weeks after the lesioning). cont = vehicle-treated group; LD = L-dopa–treated group; LD+ZNS = L-dopa+ZNS-treated group. Data are means ± standard error of the mean. $^{\#}p < 0.05$ compared with L-dopa–treated group (B). $^{*}p < 0.001$ compared with side-matched, vehicle-treated control group; $^{\#}p < 0.001$ compared with the parkinsonian side of the L-dopa–treated group (C).

decreased in vehicle-treated animals with or without L-dopa (Figs 5A, 5B, 5D, 5E, and Supplementary Figs 4A, B). Repeated injections of L-dopa showed no significant changes in the reduction of these dopaminergic indices (see Fig 5 and Supplementary Fig 4). This reduction of TH-positive DA neurons in the lesioned side of the substantia nigra in hemiparkinsonian mice was significantly ameliorated by repeated injections of ZNS (30mg/kg IP, for 1 week starting 3 weeks after the 6-OHDA treatment) with or without adjunctive treatment with L-dopa (see Figs 5A, B). Furthermore, ZNS alone for 1 week also inhibited the reduction of striatal TH-positive fibers and DAT immunoreactivity as a marker of dopaminergic nerve terminal activity in the parkinsonian side (see Figs 5D, 5E, and Supplementary Figs 4A and 4B). In another experiment, we examined time-dependent changes in the number of nigral TH-positive neurons and in the intensity of striatal TH- or DAT-positive signals in the lesioned side at 3 and 4 weeks after 6-OHDA treatment. Both the number of nigral TH-positive neurons and the striatal TH or DAT immunoreactivity were slightly or significantly lower on the parkinsonian side at 4 weeks after the 6-OHDA injection, compared with mice at 3 weeks after the 6-OHDA treatment as the pretreatment group. These reductions in nigrostriatal DA neurons in the lesioned side of hemiparkinsonian mice were prevented by repeated 1-week administration of ZNS starting 3 weeks after 6-OHDA injection (see Figs 5C, 5F, and Supplementary Fig 4C).

Discussion

This study showed that repeated injections of ZNS resulted in a marked increase of GSH in the mouse basal ganglia. Furthermore, ZNS treatment increased GSH levels in astroglial cells, but not in dopaminergic neuronal cells. However, ZNS treatment did not affect the levels of GCL and GST in cultured cells and mouse striatum, in agreement with a previous report on ineffectiveness of ZNS on GST activity.[21] Because astrocytes predominantly express the cystine transporter xCT, which takes up cystine and consequently supplies cysteine to neurons as a substrate, GSH synthesis in neurons is dependent on xCT expression on astrocytes.[10,11] Repeated injections of ZNS increased the expression of xCT and GSH levels in the activated striatal astrocytes, and completely suppressed L-dopa–induced quinone formation in hemiparkinsonian mice. These results indicate that ZNS enhances the supply of the substrate for GSH synthesis from astrocytes to neurons. Although ZNS treatment dose-dependently and markedly increased the proportion of xCT-positive cells relative to total number of astroglial cells, it did not change the nuclear levels of master transcription factor Nrf2, which promotes gene expression of various antioxidant molecules including GCL and xCT,[18,22] in astroglial C6 cells. The mechanism by which ZNS increases the expression of xCT on activated astrocytes thus remains obscure, and further experiments on other transcription factors that regulate xCT expression are needed.

This study also showed that ZNS increased the proliferation of astroglial cell lines and primary astroglial-enriched cell cultures. The repeated administration of ZNS increased S100β-positive astrocytes, but not GFAP-positive activated astrocytes, in the mouse striatum. Inter-