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

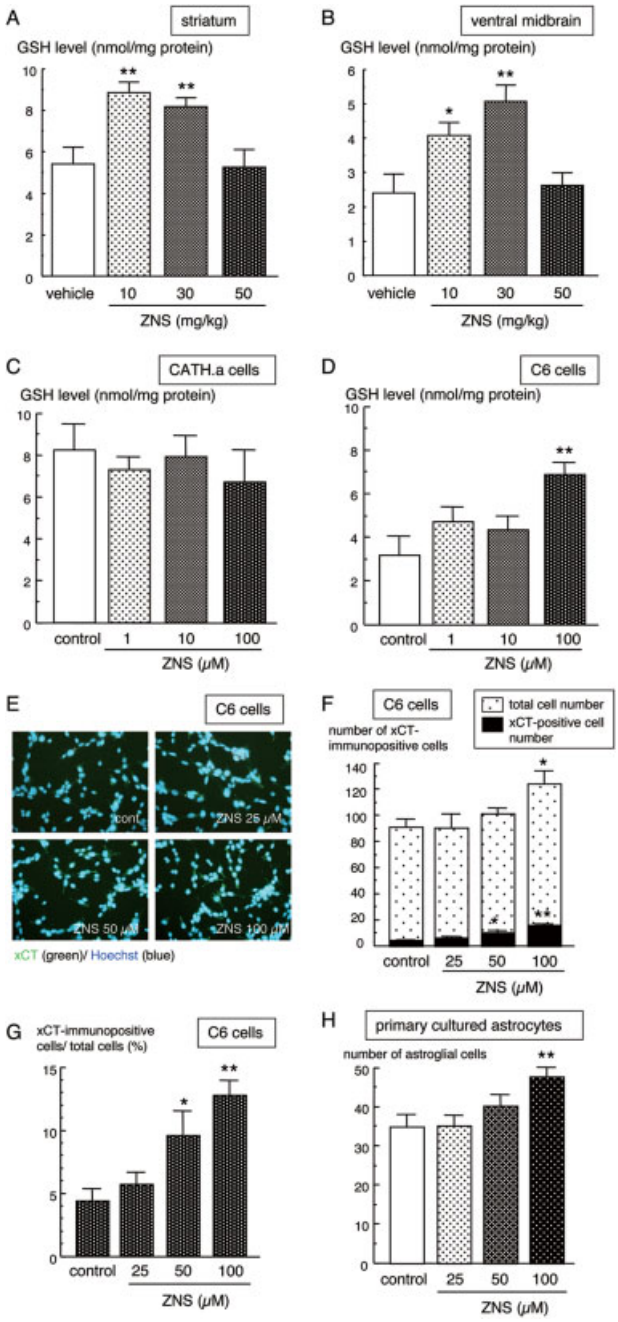

FIGURE 1: Effects of repeated administration of zonisamide (ZNS) on glutathione (GSH) in mouse basal ganglia. Changes in GSH levels in the striatum (A) and the ventral midbrain (B) were measured at 24 hours after the final administration of repeated ZNS administration (10, 30, 50mg/kg/day) for 14 days. 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).

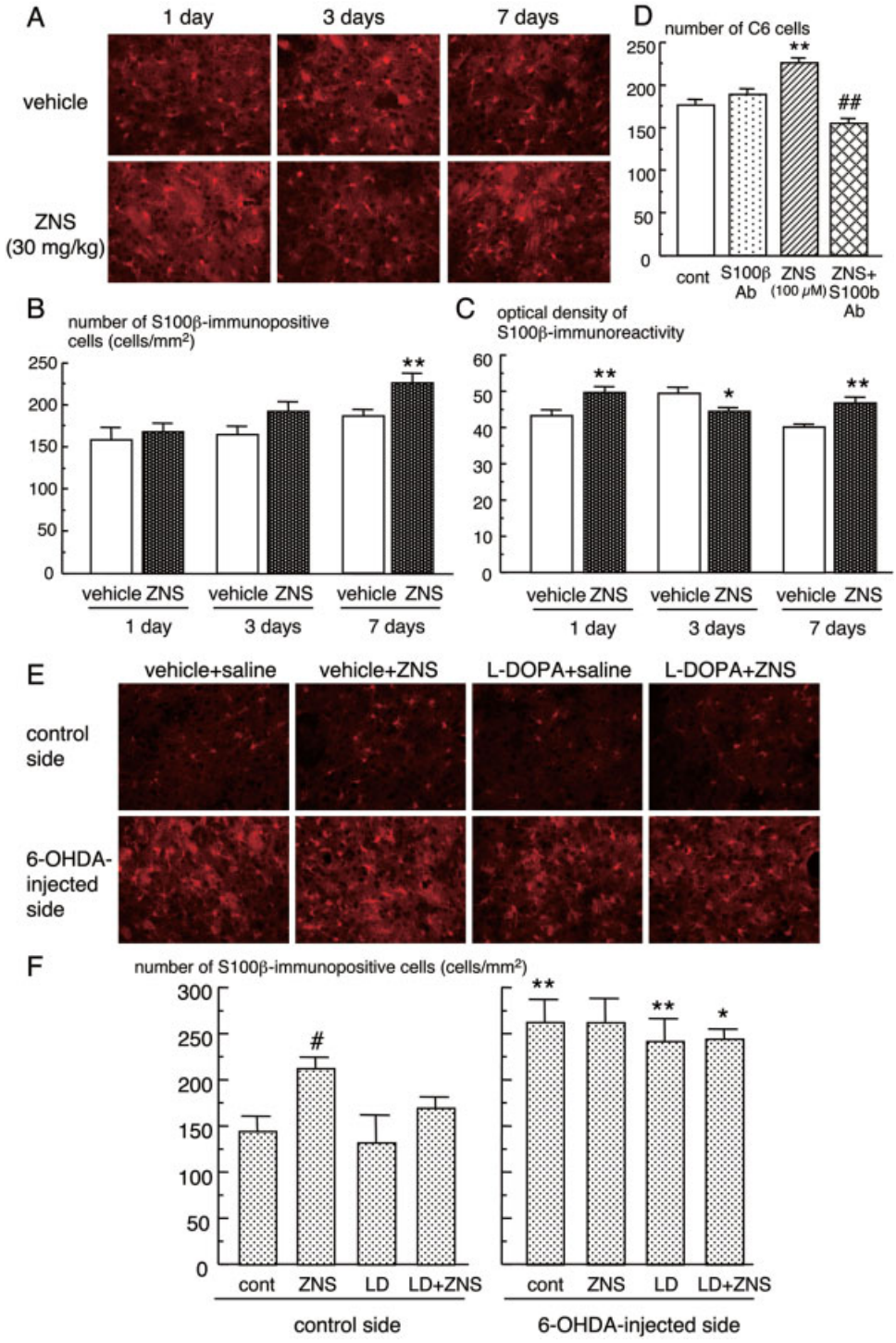

FIGURE 2: Effects of zonisamide (ZNS) administration on astrocytes in the striatum of normal mice and hemi-Parkinson's disease (PD) mice. Representative photos (A), number of immunopositive cells (B), and intracellular and extracellular relative optical density of immunoreactivity (C) in immunostaining 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.

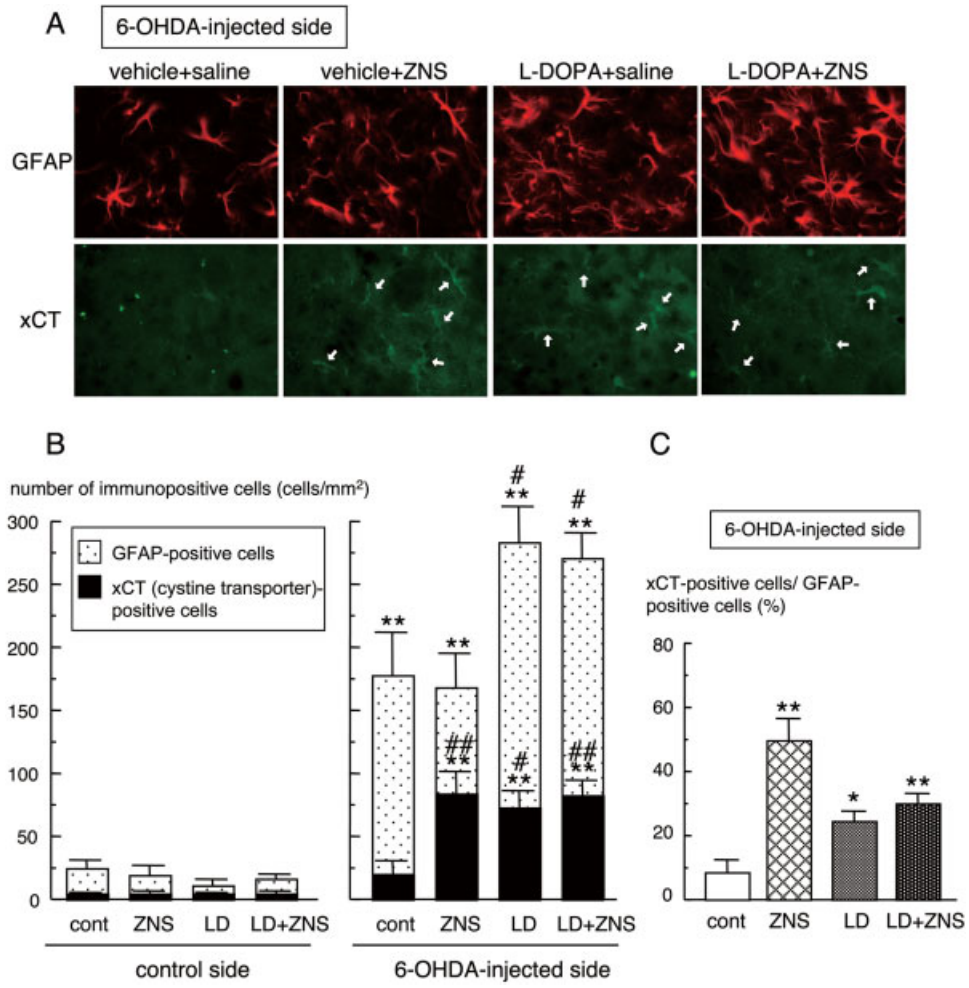

FIGURE 3: Effects of zonisamide (ZNS) administration on cystine transporter in the striatum of hemi-Parkinson's disease (PD) mice. Representative photomicrographs (A), number of immunopositive cells (B), and percentage ratio of cystine/glutamate exchange transporter (xCT)–positive astrocytes to total 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

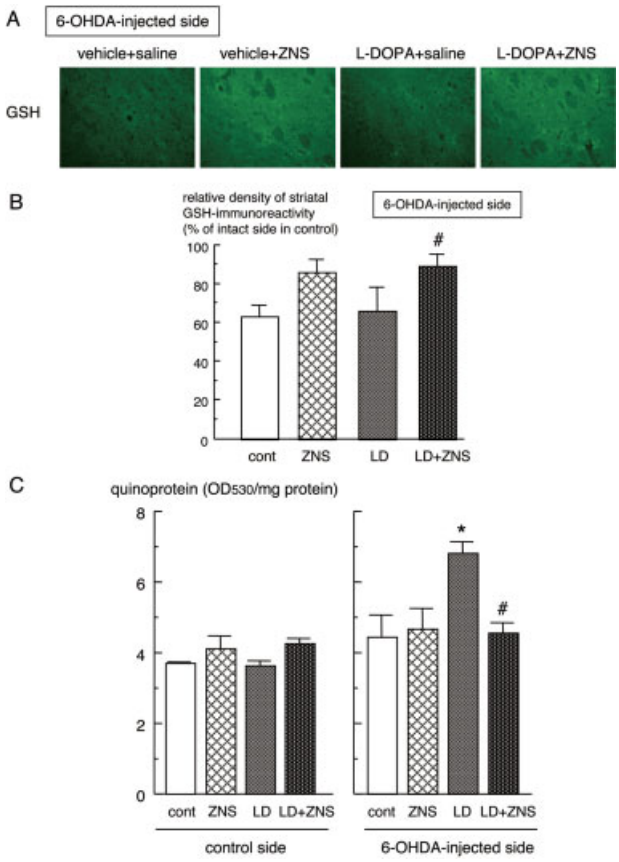

FIGURE 4: Effects of zonisamide (ZNS) administration on glutathione (GSH) and quinone formation in the striatum of hemi-Parkinson's disease (PD) mice. Representative photomicrographs (A), relative density of immunoreactivity (B; n = 4–6) in immunofluorescence staining for GSH on the 6-hydroxydopamine (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-

estingly, ZNS administration increased both intracellular and extracellular S100β-positive signals in and around astrocytes. S100β is secreted primarily by astrocytes, and extracellular S100β exerts autocrine effects to promote astrocyte proliferation.[19,20] In the C6 cells, anti-S100β antibody completely neutralized the astrocyte-proliferating effects of ZNS, indicating that ZNS might promote proliferation via its enhancing effect on S100β production or secretion in/from astrocytes. Taken together with the effects of ZNS on xCT expression in activated astrocytes, these data suggest that ZNS-induced increase in GSH is mediated through enhancing effects on astroglial cyst(e)ine transport system and/or on astroglial proliferation via S100β production or secretion. Especially in the lesioned side of the striatum of hemi-PD mice, ZNS enhanced xCT expression in activated astrocytes but not further astroglial proliferation. Thus, the former enhancing effects on astroglial cyct(e)ine transport system appear to mainly contribute to the GSH-increasing effects of ZNS in the dopaminergic neurodegenerative state in PD models.

The reduction of nigrostriatal DA neurons in the lesioned side of hemiparkinsonian mice was significantly prevented by repeated 1-week injections of ZNS, starting at 3 weeks after the 6-OHDA lesioning. This neuroprotective effect might reflect the strong antioxidative properties of ZNS via GSH, whereby it protects against sustained oxidative stress caused by mitochondrial dysfunction, among other causes, but not against free radical or quinone generation from 6-OHDA itself in the short term. It is therefore possible that the GSH-increasing effect of ZNS contributes to the neuroprotective effect of the drug against dopaminergic neurodegeneration.

The large amount of free cytosolic DA outside the synaptic vesicle is spontaneously oxidized to produce reactive quinones such as DA quinones or DOPA quinones.[23–28] The generated DA quinones covalently conjugate with the sulfhydryl group of cysteine on various functional proteins[24,29] including several key molecules, for example, TH, DAT, and parkin, which are involved

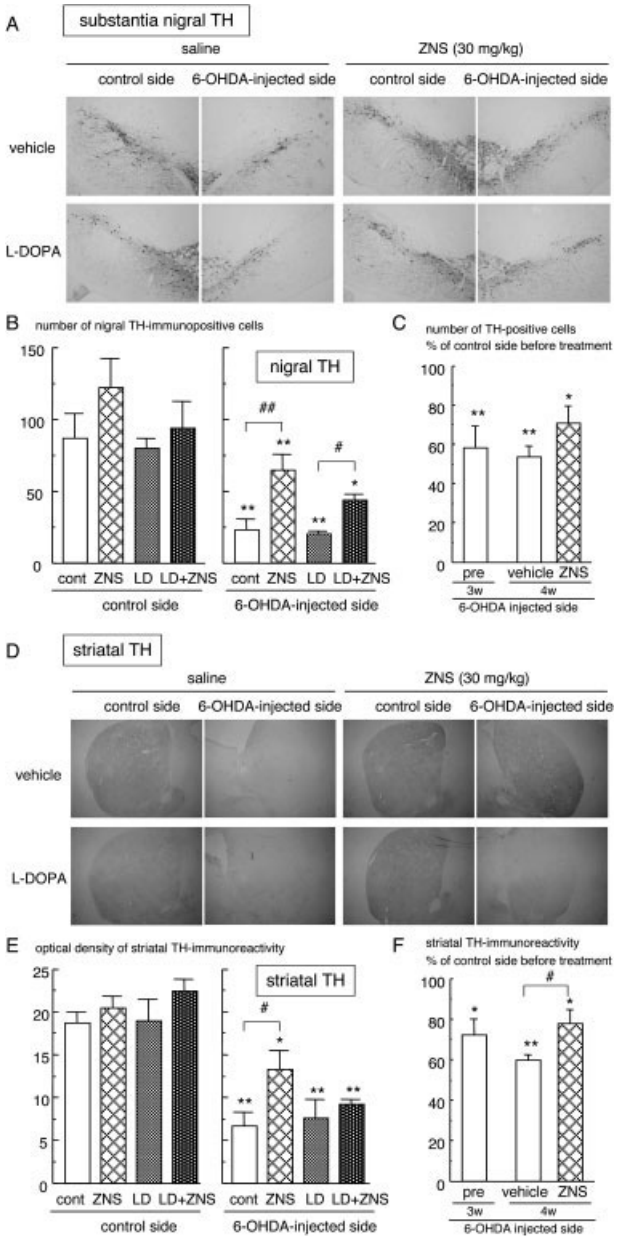

FIGURE 5: Effects of in vivo zonisamide (ZNS) administration on reduction of dopaminergic neuronal damage in the basal ganglia of hemi-Parkinson's disease (PD) mice. Representative photomicrographs of immunohistochemistry for tyrosine hydroxylase (TH) in the substantia nigra (A) and in the midstriatum (D) of hemi-PD mice after the 1-week administration with L-dopa and/or ZNS (at 4 weeks after the 6-hydroxydopamine [6-OHDA] lesioning). Changes in the number of nigral TH-positive dopaminergic neurons (B) and the striatal immunoreactivity of TH (E) of hemi-PD mice at 4 weeks after the 6-OHDA lesioning, and effects of administration with L-dopa/carbidopa (50/5mg/kg/day) and/or ZNS (30mg/kg/day) for 7 days starting at 3 weeks after the lesioning. LD = L-dopa–treated group; LD+ZNS = L-dopa+ZNS-treated group. Data are means ± standard error of the mean (SEM) (n = 5–6). *$p < 0.05$, **$p < 0.01$ compared with the control side of each drug-treated group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the two groups indicated. Time-dependent changes in the number of TH-positive neurons in the substantia nigra (C) and signal intensity of striatal TH immunoreactivity (F) in the lesioned side at 3 weeks (pre) and 4 weeks after 6-OHDA lesioning, and effects of the repeated 1-week administration of ZNS starting 3 weeks after the lesioning. Data are means ± SEM presented as percentage of mean value on the control side in the pretreatment group (n = 6). *$p < 0.05$, **$p < 0.01$ compared with the control side in the pretreatment group at 3 weeks after 6-OHDA lesioning; $^{\#}p < 0.05$ compared with the two groups indicated.

in the pathogenesis of PD, to form protein-bound quinones (quinoproteins), and cause the dysfunction of these proteins.[30–34] We reported previously that repeated administration of L-dopa increased striatal DA turnover and formation of quinoproteins specifically in the parkinsonian side, but not in the control side, of hemiparkinsonian animal models.[16,35] The sulfhydryl group of free cysteine in GSH and thiol reagents compete with the sulfhydryl group on cysteine in functional proteins bound by DA quinones.[15,23,30,36,37] Therefore, GSH acts as an antioxidant through its quenching properties not only for general reactive oxygen species but also DA quinones.[38] That ZNS dramatically increased GSH levels in the striatum suggests ZNS treatment can suppress in vivo quinone formation by increasing GSH. Therefore, we also examined effects of ZNS in 6-OHDA–lesioned hemi-PD mice that received adjunct L-dopa treatment. As expected, repeated ZNS injections resulted in complete suppression of striatal quinone formation in L-dopa–treated PD models. With regard to the effects of L-dopa treatment on neurodegeneration, repeated L-dopa injections did not aggravate neurodegeneration of nigrostriatal DA neurons in the lesioned side of parkinsonian models, in agreement with the results of a clinical Earlier vs. Later Levodopa (ELLDOPA) study that showed no aggravation of clinical PD symptoms and less deterioration of neurodegeneration after long-term L-dopa treatment.[39] The protective effects of ZNS treatment against reduction of nigral DA neurons were observed in both the ZNS alone–treated group and the L-dopa+ZNS–treated group. These findings suggest that the neuroprotective effects of ZNS against progressive nigral neuronal loss are mediated through its antioxidative properties via GSH against predominantly general reactive oxygen species rather than quinone formation.

Furthermore, recent studies emphasized the involvement of GSH or astroglial dysfunction in the pathogenesis of PD. Chinta and colleagues[12,13] showed that depletion of GSH levels in dopaminergic neurons of GCL-knockdown transgenic mice caused impairment of mitochondrial complex I activity and consequent age-dependent nigrostriatal neurodegeneration. Furthermore, Solano and colleagues[14] demonstrated astroglial dysfunction in parkin null mice; glia-conditioned medium of midbrain astrocytes from aged parkin knock-out mice contained low levels of GSH, rendered DA neurons less susceptible to oxidative stress, and the vulnerability was corrected by supplementation with GSH. These findings suggested that GSH or its synthesis-related molecules in astrocytes, or both, play an important role in protecting against age-dependent nigrostriatal dopaminergic neurodegeneration.

The clinical use of GSH in PD was reported in a small pilot study.[40] In that case, intravenous administration of 600mg GSH twice daily for 30 days significantly improved disability in PD patients. More recently, a small, double-blind, clinical trial also showed mild but not significant improvements in the Unified Parkinson's Disease Rating Scale activities of daily living plus motor score of PD patients treated with 1,400mg GSH intravenously three times a week over 4 weeks.[41] However, large amounts of these molecules were required to achieve efficacy because of their low transport capacity across the blood–brain barrier and their peripheral metabolism.[42] Furthermore, the clinical nosotropic effects of GSH on motor symptoms of PD remain difficult to explain biologically to date because there is no evidence to show how GSH modulates PD motor symptoms. Therefore, the increasing GSH levels in the brain by ZNS treatment might prove clinically useful through its antioxidant effects to diminish oxidative stress, and hence potentially prevent further pathological progression and enhancement of intrinsic neuroprotective mechanisms in PD patients rather than through improvement of motor symptoms.

About the concentration of ZNS, the peak of ZNS concentration in mouse plasma was seen several hours after single or repeated daily administration. The plasma concentration of ZNS was 3.5 to 5.0μg/ml or 13.5 to 14.9μg/ml at 2 hours after the cessation of repeated ZNS administration (10 or 30mg/kg/day) for 7 or 14 days (see Supplementary Table 1). The previous study also showed similar peak plasma concentration of ZNS in rats.[43] The maximum concentration (C_{max}) in rat plasma at 3 hours after the single ZNS treatment (20mg/kg orally) is 14.1μg/ml (prescribing information, Dainippon Sumitomo Pharma). In humans, the average plasma concentration of ZNS at steady state is 3.5μg/ml after daily ZNS treatment at a dose of 50mg/day orally (prescribing information). The dosage of ZNS (30mg/kg/day), which increased GSH levels in this study, appears to be greater than the clinically effective dosage against PD symptoms (25–50mg/day). Therefore, a relatively higher dosage of ZNS, compared with the clinical dosage used for PD motor symptoms, might be required to induce the neuroprotective GSH-increasing effects in PD patients. However, plasma concentration of ZNS rapidly declines to less than 1μg/ml at 24 hours after single or repeated ZNS treatment (10 or 30mg/kg/day) for 7 or 14 days in mice (see Supplementary Table 1) because of its quite shorter half-life time in rodents ($T_{1/2}$ = 8 hours) than in humans ($T_{1/2}$ = 62 hours). In the repeated treatment of ZNS (20mg/kg/day orally) for 14 days in rats, plasma concentration of ZNS settles down at 24 hours after every treat-

ment to approximate 2 to 2.4μg/ml (prescribing information, Dainippon Sumitomo Pharma). The level of plasma ZNS 24 hours after ZNS treatment in rodents is close to or rather lower than the average plasma concentration of 3.5μg/ml at steady state after repeated ZNS treatment (50mg/day) in PD patients. Furthermore, the increase of DA and DOPA contents and TH activity in rat striatum after repeated treatment of ZNS for 2 or 3 weeks was most prominent at a dose of 50mg/kg.[2,7] The marked increases in striatal GSH levels were detected at 24 hours after the cessation of the repeated administration of ZNS (10, 30mg/kg) for 14 days. Taken together with these findings, it is possible that the clinically effective dosage against PD symptoms might exert similar neuroprotective GSH-increasing effects in PD patients. Because the pharmacokinetics of ZNS in humans is different from that in rodents, further clinical studies will be needed to clarify the potentially neuroprotective dosage of the drug to increase GSH levels in PD patients.

In conclusion, the novel antiparkinsonian agent ZNS has unique astrocyte-targeted pharmacological properties that result in a marked increase in GSH levels in the basal ganglia. ZNS might elicit this effect by enhancing astroglial cyst(e)ine transport and/or astroglial proliferation via S100β production or secretion. Furthermore, ZNS appears to act as a neuroprotectant against oxidative stress and dopaminergic neurodegeneration. Further examination of this unique astrocyte-targeting agent may ultimately provide neuroprotection in PD.

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