Severe Motor Neuron Degeneration in the Spinal Cord of the Tg2576 Mouse Model of Alzheimer Disease

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Abstract. The transgenic mouse Tg2576 is widely used as a murine model of Alzheimer’s disease (AD) and exhibits plaque pathogenesis in the brain and progressive memory impairments. Here we report that Tg2576 mice also have severe spinal cord deficits. At 10 months of age, Tg2576 mice showed a severe defect in the hindlimb extension reflex test and abnormal body trembling and hindlimb tremors when suspended by the tail. The frequency and severity of these abnormalities were overt at 10 months of age and became gradually worsened. On the foot-printing analysis, Tg2576 mice had shorter and narrower strides than the non-transgenic control. Histological analyses showed that neuronal cells including cholinergic neurons in the lumbar cord of Tg2576 mice were severely reduced in number. At 16 months of age, Tg2576 mice showed higher levels of amyloid-\(\beta\) accumulation in the spinal cord. Consistent with this, Tg2576 mice showed that lipid peroxidation levels were increased and mitochondrial metabolic activity were significantly reduced in the spinal cord. Administration of curcumin, a natural compound that has antioxidant properties, notably reversed motor function deficits of Tg2576 mice. The enhanced lipid peroxidation and neuronal loss in the lumbar cord was also partially suppressed by curcumin. Electron microscopic analysis revealed that the sciatic nerve fibers were severely reduced in number and were demyelinated in Tg2576 mice, which were partially rescued by curcumin. These results showed that Tg2576 mice display severe degeneration of motor neurons in the spinal cord and associated motor function deficits.

Keywords: Motor neurons, reactive oxygen species, spinal cord, Tg2576

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INTRODUCTION

The transgenic mouse Tg2576 is widely used as a murine model of Alzheimer’s disease (AD). Tg2576 mice were generated to express human A\(\beta\)PP695 with the Swedish familial AD double mutation K670N-M671L (hA\(\beta\)PPswe) in an attempt to mimic familial cases of AD [1]. Tg2576 mice show AD-like pathological changes, such as amyloid plaque deposits and gliosis in the brain starting at 9–12 months of age [1–3]. Moreover, Tg2576 mice display memory impairments in several behavioral tests, including Morris water maze, Y-maze, passive avoidance, active avoidance, and circular platform [4]. Thus, it has been thought that Tg2576 mice display various neurological and behavioral deficits that resemble those of human AD patients in terms of age-dependent cognitive...
deficits and plaque pathogenesis. More importantly, Tg2576 mice are readily available from a commercial source. Therefore, this model has been used extensively worldwide in the past decade and has contributed to our understanding of AD-like pathophysiology in the brain.

The AD-like pathology displayed by Tg2576 mice is produced by the transgenic expression of a mutant form of human AβPP (hAβPPsw) in the brain under the control of the 6.9-kb hamster prion protein (PrP) promoter. The PrP promoter is able to direct expression of a target gene at high levels in neuronal cells in the central nervous system (CNS). While PrP is pathogenic and causes scrapie in sheep and cow [5], the endogenous cellular prion protein (PrPc) is present in various regions of the body, including the spinal cord, brain stem, and even epithelial cells of the coroid plexus and intestine [6]. Although the ability of the 6.9-Kb PrP promoter has not been characterized in detail, it likely drives expression of the target gene in a distribution pattern similar to that of the endogenous PrPc, i.e., not only in the brain, but also in other regions of the body, including the spinal cord. Recently, it was reported that Tg-AβPP/PS1 AD model mice showed motor function deficits and spinal cord defects [7–9]. It is therefore important to understand whether Tg2576 mice have any spinal cord problems. However, the transgenic effect of the hAβPPsw allele on the spinal cord of Tg2576 mice has not been studied, and AD research has continued to work with this model without knowing the potential pathophysiology in the spinal cord.

In the present study, we demonstrate for the first time that Tg2576 mice exhibit severe motor neuron degeneration in the spinal cord and associated motor dysfunction deficits.

**MATERIALS AND METHODS**

**Animals and curcumin administration**

The transgenic Tg2576 mouse is a murine model of AD generated by overexpressing a mutant form of human AβPP (K670M/N671L) [1]. Tg2576 mice used in this study were purchased mostly from Taconic Farms Inc. (Germantown, NY, USA) or obtained from the breeding with maintaining the genetic background in C57BL6 x SJL F1 hybrid as described previously [1]. After weaning, 2–3 animals were housed per cage in a temperature- and humidity-controlled environment under a 12 h light/dark cycle (lights on at 7 a.m.), and were maintained on a diet of lab chow and water *ad libitum*. Curcumin was purchased from Sigma-Aldrich Inc. (St. Louis, MO), and administrated in a lab chow form (500 ppm) for 6 months beginning at 10 months of age. All animals were handled in accordance with the animal care guidelines of the Ewha Womans University School of Medicine.

**Assessment of Amyloid-β levels**

Aβ1−40 and Aβ1−42 ELISA assays were performed as described previously [10,11]. The L3-5 spinal cord was individually homogenized in Tris-buffered saline (20 mM Tris and 137 mM NaCl, [pH 7.6]) supplemented with protease inhibitor mixtures (Complete Mini; Roche, IN, USA). Homogenates were centrifuged at 100,000 g for 1 h at 4°C, and the supernatant was used to measure soluble forms of Aβ1−40 and Aβ1−42. The pellet was sonicated in 70% formic acid and centrifuged as above, and the supernatant was collected. The formic acid extract was then neutralized with 1 M Tris buffer (pH 11) by 1:20 dilution, and the diluted formic acid extract were used for each assay. Human Amyloid Aβ1−40 and Aβ1−42 colorimetric sandwich ELISA kits (BioSource, Camarillo, CA, USA) were used according to the manufacturer’s instructions.

**Measurement of lipid peroxidation by malondialdehyde (MDA) assay**

Lipid peroxidation was assessed by measuring MDA levels using the Bioxytech MDA-586 kit (Oxis Research, Portland, OR, USA) as described previously [10,12]. Briefly, spinal cords (L3-5) were homogenized in 4 vol. of ice-cold 20 mM PBS (phosphate buffered saline; pH 7.4) containing 5 mM butylated hydroxytoluene. Homogenates were centrifuged at 3,000 g for 10 min at 4°C, and the supernatant was used for each assay. The protein concentration of the supernatant was determined by the Bradford method (Bio-Rad Laboratories, USA). For each reaction, 200 µl of the supernatant was mixed with 10 µl of probucol, 640 µl of diluted R1 reagent (1:3 of methanol:N-methyl-2-phenylindole), and 150 µl of 12 N HCl. Each reaction was incubated at 45°C for 60 min and centrifuged at 10,000 g for 10 min. The absorbance of the supernatant was then measured at 586 nm. MDA data were normalized to the protein concentration and expressed as a percentage of the sham control value. Protein-bound MDA content was obtained by subtraction.
Mitochondria preparation

Mitochondria in the lumbar cord were isolated using a QProteome Mitochondria Isolation Kit (QIAGEN, Hilden, Germany) by following the manufacturer’s instructions. Briefly, the lumbar cord was homogenized in 500 µl ice – cold lysis buffer containing protease inhibitors. Homogenates were centrifuged at 1,000 g for 10 min at 4°C, and the pellets were collected and resuspended in 1.5 ml lysis buffer. After passing through a syringe 10 times, they were then centrifuged at 1,000 g for 10 min at 4°C, and the supernatants containing the mitochondria were collected and recentrifuged at 6,000 g for 10 min at 4°C. The pellets were resuspended in 1 ml of 10 mM Tris buffer (pH 7.6). After assay of protein concentration, they were subjected to mitochondria activity assay.

Assessments of mitochondria metabolic function

Metabolic activities of mitochondrial complex I, II, or IV was measured by following the procedures described previously [13,14], with some modifications. For complex I assay, 1 µg of the mitochondria preparation was preincubated at 37°C in 240 µL of the incubation mixture (5 mM potassium phosphate, 70 g/L BSA, 60 mM DCIP, 17.5 mM deacylubiquinone, and 1.0 mM antimycin-A, pH 7.8). After 3 min, 5 µL of 160 mM NADH was added and the absorbance was measured at 600 nm using Spectra 190 max UV detector (Molecular devices, Sunnydale, CA, USA) at every 10-s intervals for 5 min at 37°C. After 5 min, 2.5 µL of 100 µM rotenone in DMSO was added and the absorbance was measured again at 10-s intervals for 5 min. For Complex II assay, the incubation mixture contained 5 mM potassium phosphate, 70 g/L BSA, 0.25 mM EDTA, 0.1 mM ATP, 1 M succinate, 0.1 M potassium cyanide (KCN), 30 mM DCIP, 17.5 mM decylubiquinone, 1 mM antimycin-A, and 3 mM rotenone, pH 7.8. One µg of the mitochondria fraction was preincubated at 37°C in 240 µL of the incubation mixture without KCN and succinate. After 10 min, KCN and succinate were added and the absorbance was measured at 600 nm at 10-sec intervals for 5 min at 37°C. Blank absorbance was measured in the presence of 500 mM malonate.

For Complex IV assay, the incubation mixture contained cytochrome c solution (17 µM, cytochrome c, 0.03 M potassium phosphate (pH 7.4), and 1.2 M sodium hydrosulfite. One µg of the mitochondria fraction was preincubated at 30°C in 300 µL of the incubation mixture. After 3 min, KCN was added to the final concentration of 0.1 M and the absorbance was measured at 550 nm at 10-sec intervals for 5 min at 30°C.

Citrate synthase (CS) activity was measured by following the procedure described previously [15]. Briefly, the incubation mixture contained 50 mM Tris- HCl (pH 7.5), 10 mM Acetyl CoA, and 10 mM DNTB. One µg of the mitochondria fraction was preincubated at 30°C in 240 µL of the incubation mixture without KCN. After 10 min, KCN was added and the absorbance was measured at 410 nm at 10-sec intervals for 5 min at 30°C.

Hindlimb extension reflexes

Hindlimb extension reflexes were evaluated according to the procedure and scoring system described by [16,17]. Briefly, mice were suspended by the tail, and the degree of motor deficit was scored on a 0 to 2 scale: a normal extension reflex in both hindlimbs was scored as 2; imbalanced extension in the hindlimbs as 1.5; extension reflex in only one hindlimb as 1.0; the absence of any hindlimb extension as 0.5; and total paralysis as 0.

Body trembling and hindlimb tremor assessments

Body trembling and hindlimb tremors were evaluated according to the following procedure and scoring system. Briefly, mice were suspended by the tail, and the degree of body trembling and hindlimb tremors was scored using a 0–4 rating scale: 0, normal in both hindlimbs and body; 1, weak tremors (1–5 frequency/10 sec) of the hindlimbs and body; 2, intermediate tremors (6–10 frequency/10 sec) of the hindlimbs and body; 3, severe tremors (11–15 frequency/10 sec) of the hindlimbs and body; and 4, total paralysis as 4.

Footprint analysis

Footprint analysis was performed as described previously [18] with a minor modification. Briefly, mice were trained to cross an illuminated alley (10 cm wide and 33 cm long) and to go straight to the opposite side located at the end of the alley. Their hindlimbs were then coated with Chinese ink, and the mice were allowed to walk along the corridor, the floor of which was covered with a sheet of an oriental white paper. This task was recorded in 1–2 trials until 10 clearly visible footprints per animal were obtained. The footprints were then scanned and the stride intervals were evaluated using TOMORO ScopeEye 3.6 program (Techsan Community, Seoul, Korea).
Cresyl violet staining was performed as previously described [19]. Mice were sacrificed and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The spinal cord was removed and post-fixed in the same fixative at 4°C overnight. Lumbar cord (L3-L5) was coronally cut into 40-µm-thick sections with a vibratome (Leica VT 1000S; Leica Instruments, Nussloch, Germany). Spinal cord sections were stained with 0.5% cresyl violet.

For immunohistochemistry, free-floating sections were incubated with 4% bovine serum albumin in PBST (PBS containing 0.1% Triton x-100; pH 7.4) for 1 h, and then with anti-ChAT (Chemicon, Temecula, CA, USA), monoclonal anti-COX (cytochrome c oxidase) complex IV subunit I (Mitosciences Inc, Eugene, OR), or polyclonal anti-HNE (Alpha Diagnostic Intl. Inc, Texas, USA) at 4°C overnight. The sections were washed with PBST and were reacted with biotinylated secondary antibodies diluted at 1:200 in PBST and visualized by using the ABC Elite kit (Vector Laboratories; Burligame, CA, USA). Stained images were analyzed using Olympus BX 51 microscope equipped with a DP71 camera and DP-B software (Olympus Co., Tokyo, Japan). Cell counts were performed in a blind manner without knowledge of genotypes. Cell density of cresyl violet-stained cells or ChAT-positive cells in the lumbar cord was counted using TOMORO Scope-Eye 3.6 program (Techsan Community, Seoul, Korea) after converting captured images of stained areas into digital images.

Resin histology and electron microscopy

Mice were transcardially perfused with PBS, pH 7.4, containing 4% paraformaldehyde and 2.5% glutaraldehyde. After washing in PBS three times, the sciatic nerve and the ventral root at L5 were carefully dissected and post-fixed at 4°C for 1 h with 1% osmium tetroxide (0.1 M PBS, pH 7.4). After washing with fresh 1% osmium tetroxide in PBS, the tissue samples were stored at 4°C until they were embedded in Epon 812 (Polysciences, USA). Semi-thin (1-µm thick) sections were cut from each block using an ultramicrotome (Reichert-Jung, USA), then stained with 1% alkaline toluidine blue, and examined by light microscopy (Olympus BX51; Tokyo, Japan). Ultra-thin (70-nm thick) sections were prepared, treated with lead citrate and uranyl acetate to enhance contrast, and examined using a Hitachi H-7650 (Hitachi Co., Tokyo, Japan).

RESULTS

Transgenic Tg2576 mice show visible plaque deposits in the brain, starting at 9 months of age: after this age plaque deposition and Aβ levels are enhanced slowly and exponentially with age [1,10,20]. While handing Tg2576 mice in their home cages, we observed that these mice displayed abnormal motor function phenotypes at 10 months of age. It is somewhat surprising that motor function phenotypes in this model have not been described thus far, given the fact that Tg2576 mice are so popular and have been widely used in various behavioral studies. We observed that, while being suspended by the tail, Tg2576 mice held and retracted the hindlimbs in the inward direction; in starkly contrast to the stretching of the hindlimbs in the outward direction in control mice (Fig. 1A; Supplemental Fig. 1, available online: http://www.j-alz.com/issues/21/vol21-1.html#supplementarydata). Thus, the hindlimb extension reflex response in Tg2576 mice was severely defective, and these neurologic deficits were more severe at 16 months of age than that at 10 months of age (Fig. 1C). Tg2576 mice at 10–16 months of age also displayed severe body trembling and hindlimb tremors when suspended by the tail (Fig. 1B). The frequency and severity of the motor function abnormalities progressively worsened after 10 months of age in both female and male Tg2576 mice (Fig. 1D).

The distinct impaired motor function phenotypes of Tg2576 mice prompted us to investigate potential histological defects in the spinal cord. Cresyl violet staining showed that the numbers of cresyl violet-stained cells in the ventral horn of the Tg2576 mice at 10 months of age were reduced compared with that in the non-transgenic control mice and further reduced to approximately 52.5% of the non-transgenic control mice at 16 months of age (Figs 2A–G). Anti-ChAT-positive neurons in the lumbar cord of Tg2576 mice were also gradually reduced in number with aging, and remained...
neurons at 16 months of age expressed ChAT at much lower levels (Figs 2H–N), indicating that cholinergic neurons in the spinal cord of Tg2576 mice were affected. Consistent with these findings, histological analysis of toluidine blue stained ventral root at the level of L5 showed that the nerve fibers in Tg2576 mice had a defect in myelination and were reduced in number at 10 months of age (Supplemental Fig. 2).

Immunohistological analysis with anti-Aβ1–42 antibody (Bam10) showed no evidence of plaque deposition in the lumbar cord of Tg2576 mice at 16 months of age. However, ELISA assay revealed that the levels of Tris-buffer soluble Aβ1–42 and Aβ1–40 were markedly enhanced in the lumbar cord (L3-L5) in an age dependent manner (Figs 3A and B). Similarly, formic acid-extracted insoluble forms of Aβ1–42 and Aβ1–40 levels were increased (Figs 3C and D). Because high levels of Aβ1–42 result in strong oxidative stress in the brains of Tg2576 mice [10,11], we investigated whether Tg2576 mice have enhanced oxidative stress in the spinal cord. Analysis of malondialdehyde (MDA) levels revealed that lipid peroxidation levels in the lumbar cord (L3-L5) of Tg2576 mice at 6–14.5 months of age were markedly enhanced compared to that in the non-transgenic control, and the increase was age-dependent (Fig. 4A). Consistent with this data, immunohistochemistry performed with anti-HNE (4-hydroxy-2-nonenal) staining revealed that the levels of HNE, which is an oxidized by-product of lipid peroxidation, were notably enhanced in the spinal cord of Tg2576 mice (Fig. 4B).
The high oxidative stress levels in the spinal cord of Tg2576 mice led us to test whether antioxidants can afford a therapeutic effect against motor function deficits of Tg2576 mice. To address this, we chose curcumin, because it has a strong antioxidant property [21] and confers anti-AD-like effects on the brains of Tg2576 mice [22]. Administration of curcumin (500 ppm) to Tg2576 mice starting at 10 months of age partially suppressed the enhanced HNE levels in the spinal cord of Tg2576 mice at 18 months of age (Figs 4B–E). These results suggest that the high oxidative stress pressure correlated with the severity of motor function defects and neuronal loss in the spinal cord of Tg2576 mice.

Next, we examined whether the mitochondria respiratory systems are affected in Tg2576 mice. The mitochondria respiratory complex I activity in the lumbar cord of Tg2576 mice was comparable to that in non-transgenic control. In contrast, the activities of the complex II and complex IV were both reduced to 62.2% and 52.7%, respectively, of the non-transgenic control (Figs 5A–C). The activity of citrate synthase was reduced to 68.8% of the non-transgenic control, implicating that mitochondrial metabolic activity in the glucose metabolism is also impaired (Fig. 5D). Consistent with this, an immunohistochemical study using anti-COX (complex IV) subunit 1 against histological sections of the spinal cord revealed that COX subunit 1 level was enhanced in Tg2576 mice, while it was partially reduced in curcumin-treated mice. These results support that Tg2576 mice showed impaired mitochondrial metabolic activity in the spinal cord and curcumin partially suppressed the mitochondrial impairment.

Concerning behavioral phenotypes, administration of curcumin starting at 10 months of age markedly
Fig. 3. Accumulation of Aβ1-42 and Aβ1-40 levels in the lumbar cord of Tg2576 mice. A-D) Amounts of Tris-buffer soluble (A, B) or formic acid-extracted (C, D) Aβ1-40 (A, C) and Aβ1-42 (B, D) in the lumbar cord of non-transgenic control mice (Non-Tg, 16 months of age) and Tg2576 mice (Tg) at 6, 10, or 16 months of age were measured by ELISA. Each group contained 4–6 animals and the ELISA was performed in duplicate. * and ** indicate a difference between groups at p < 0.05 and p < 0.01, respectively (one-way ANOVA with Newman-Keuls multiple range test).

Improved hindlimb extension reflex defects (Fig. 6A) and hindlimb tremors (Fig. 6B) as examined at 10–14.5 months of age. Tg2576 mice at 16 months of age were not able to walk normally on the floor. To quantitatively assess their neurological deficits, we analyzed the footprint patterns of the mice when they were forced to walk along a narrow corridor (Figs 7A and B). In the footprinting test, the stride length of Tg2576 mice was shorter than that of the non-transgenic control, while the curcumin-fed group displayed no such phenotype (Fig. 7C). Similarly, Tg2576 mice tended to show a slightly narrower stride width than the non-transgenic control, while curcumin administration prevented this phenotype (Fig. 7D). These results suggest that Tg2576 mice have abnormal gaits, which were significantly improved by curcumin.

Next, we examined whether the therapeutic effects of curcumin on the neurological deficits of Tg2576 mice were produced via a neuroprotective mechanism. Cresyl violet staining of the lumbar cord L3-L5 showed that cresyl violet-positive cells in the ventral horn of the Tg2576 mice at 16 months of age were reduced in number compared with those in the non-transgenic control mice, and this reduction was inhibited by curcumin (Figs 8A–C,G). Anti-ChAT staining revealed that cholinergic neurons in the lumbar cord of Tg2576 mice at 16 months of age were enhanced in number after curcumin treatment (Figs 8D–F,H).

We continued to explore the nature of the neuropathologic defects in the sciatic nerves of Tg2576 mice and their pathology change with curcumin. Toluidine blue staining of semi-thin-sectioned sciatic nerves showed that the number of nerve fibers in the sciatic nerves of Tg2576 mice at 16 months of age was reduced to 61.3% of that in the non-transgenic control, while
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Fig. 4. Enhanced lipid peroxidation in the spinal cord of Tg2576 mice. A) MDA (malondialdehyde) levels in the lumbar cord of non-transgenic control mice (Non-Tg) at 14.5 months of age and Tg2576 mice at 6, 10 and 14.5 months of age are presented. B-E) Quantification of (B), and representative photomicrographs (C-E) showing, anti-HNE immunoreactivity in the ventral horn of the lumbar L3-5 of non-transgenic control (nonTg; C), Tg2576 mice (Tg; D) and Tg2576 mice treated with curcumin (Tg+Cur; E), all at 16 months of age. The levels of anti-HNE immunoreactivity within the circled area (600 µm in diameter) in Fig. 4C were quantified. Curcumin (500 ppm) was administered to Tg2576 mice starting at 10 months of age to 16 months of age. Both MDA and HNE are oxidized by-products of lipid peroxidation. Each group contained 4–6 animals. * and ** denote differences between the indicated groups at p < 0.05 and p < 0.01, respectively (one-way ANOVA with Newman-Keuls multiple range test).

Curcumin treatment reversed the reduction to 90% of the non-transgenic control. Moreover, severe demyelination phenotypes were evident in the nerve fibers of Tg2576 mice and their deficits were partially reversed by curcumin (Figs 9A–C; Supplemental Fig. 3). Consistent with this, an EM study revealed that the extent of demyelination was severe in Tg2576 mice at 16 months of age, and these defects were partially suppressed in Tg2576 mice fed curcumin (Figs 9D–I). Severe demyelination in Tg2576 mice is consistent with the neurological phenotypes of hindlimb tremors and body trembling.

DISCUSSION

The present study demonstrates that Tg2576 mice have various motor function defects, including hindlimb extension reflex impairment, body trembling, hindlimb tremors, and abnormal gaits. Moreover, Tg2576 mice show severe neuronal loss in the spinal cord and axonal degeneration of the sciatic nerves. Such spinal cord phenotypes in Tg2576 mice raise some important issues for AD-related behavioral studies and AD-related pathology.

Our results suggest that behavioral analyses of Tg2576 mice should be evaluated considering that this model has spinal cord deficits. AD models have been used to search for the mechanisms underlying pathological changes, such as plaque pathology and behavioral changes, such as memory impairment. Many behavioral studies of cognition are carried out under the assumption that subject mice show normal movement behaviors because cognitive functions of animals are deduced from behavioral performance in behavioral tests. For example, learning and memory measured by
Fig. 5. Mitochondria function was defective in the spinal cord of Tg2576 mice. A-D) Mitochondrial metabolic activities of the complex I (A), complex II (B), complex IV (C), and citrate synthase (D) in the lumbar cord of Tg2576 mice at 16 months of age. * denotes differences between the indicated groups at \( p < 0.05 \) (Student’s t-test). E-J) Representative photomicrographs showing anti-COX (complex IV) subunit 1 immunoreactivity in the ventral horn of the lumbar L3-5 of non-transgenic control (non-Tg; E and H), Tg2576 mice (Tg; F and I) and Tg2576 mice treated with curcumin (Tg+Cur; G and J), all at 16 months of age. Photomicrographs of H-J represent a high magnification of the ventral horn. K) Quantification of anti-COX (complex IV) subunit 1 immunoreactivity within the circled area (600 \( \mu \)m in diameter) in Fig. 4E in the ventral horn of non-transgenic control (E), Tg2576 mice (F) and Tg2576 mice treated with curcumin (G). * denotes differences between the indicated groups at \( p < 0.05 \) (one-way ANOVA with Newman-Keuls multiple range test).

Fig. 6. Curcumin notably rescued motor extension reflex and body trembling phenotypes of Tg2576 mice. A, B) Abnormalities of tail suspension-induced hindlimb extension reflex (A) and body trembling and hindlimb tremor phenotypes (B) of Tg2576 mice were rescued by curcumin. Curcumin (500 ppm) was administered starting at 10 months of age and behavioral performance was assessed at 16 months of age. Hindlimb extension reflex and body trembling and hindlimb tremor phenotypes were scored as described in Fig. 1 legend and Materials and Methods. Data are presented as means ± SEM (\( n = 5–8 \)). * and ** denote a significant difference between groups at \( p < 0.05 \) and \( p < 0.01 \), respectively (One-way ANOVA with Newman-Keuls multiple range test).
Fig. 7. Footprint analysis of Tg2576 mice and improvement of motor function deficits by curcumin. A) Experimental set-up of footprint analysis (left panel). Stride length and stride width were defined as indicated (right panel). B) Representative footprints of each group. Individual mice with hindlimbs coated with Chinese ink were allowed to walk on a sheet of oriental white paper. Non-Tg, non-transgenic control; Tg, Tg2576; Tg-Cur, Tg2576 mice fed curcumin. C, D) Tg2576 mice had shorter (C) and wider (D) strides than non-transgenic control. Data are presented as means ± SEM (n = 5–8). ** denotes a significant difference between groups at p < 0.01. One-way ANOVA with Newman-Keuls multiple range test was applied.

The Morris water maze test are expressed as a function of a distance swum in search of a hidden platform in the water maze. Poor swimming performance in the test is regarded as an indication of cognitive defects. However, any defects or potential weakness in the spinal cord might influence the performance of the task in such tests that require normal motor function. The Morris water maze test is based on extensive repeated swimming tests. Previous studies have intended to provide evidence of normal motor function in that Tg2576 and control non-transgenic mice show comparable swimming velocities. However, knowing that Tg2576 mice have spinal cord defects, it is now obvious that more rigorous investigations of Tg2576 mice are needed. Tg2576 mice show abnormal locomotor activity in the open field test. Because that reduced locomotor activity can be caused by problems in emotion [4,23], motor function-driven behavioral tests need to be interpreted with additional care.

Tg2576 mice are an excellent model of plaque pathology in the AD brain, but do not clearly show neurodegeneration in the brain, a true signature in the brain with human AD pathology. Meanwhile, cognitive impairments and psychiatric deficits are important symptoms of AD, while motor function deficits are also frequently observed in patients with AD [24]. In this sense, Tg2576 mice may serve as an AD model to explore Aβ-induced neurodegeneration in the CNS, albeit in the spinal cord. In the past years, most studies with murine models of AD have focused on functions and dysfunctions of the AD brain, and consequently less attention was paid to the spinal cord. The Tg2576 mice are very popular in AD research, and a phenotype of Aβ-induced neurodegeneration in the spinal cord might extend the usefulness of this model. Moreover,
Fig. 8. Curcumin suppressed neuronal loss in the spinal cord of Tg2576 mice. A-F) Representative photomicrographs showing cresyl violet (A-C) or anti-ChAT (D-F) stained lumbar L3-5 spinal cord of non-transgenic control (non-Tg; A and D), Tg2576 mice (Tg; B and E), and Tg2576 mice fed curcumin (Tg-Cur; C and F) at 16 months of age. G, H) Quantification of the numbers of cresyl violet (G) or anti-ChAT (H) stained cells in the ventral horn of the three mouse groups described above. Cells larger than 5 µm in diameter found within the circled area (600 µm in diameter) in Fig. 8A in both ventral horns for each animal were counted using TOMORO ScopeEye 3.6 program. Counting of cells with larger than 15 µm within in the circled area in Fig. 8A showed similar results (data not shown). Data represent means ± SEM (the numbers of animals included in each group were 4–8, and 2–3 sections for each animal were examined). ** indicates a difference at the p < 0.01 level in each group (One-way ANOVA with Newman-Keuls multiple range test).

since human AD patients often show motor function deficits [25–27], Tg2576 mice will be a useful model to study how Aβ-induced neurodegeneration causes the spinal cord pathology. Recently, it was reported that transgenic Tg-AβPP/PS1 AD model mice showed motor function defects and/or spinal cord deficits [7–9]. These lines showed also axonal pathology in the spinal cord, as Tg2576 mice displayed. Tg-AβPP/PS1 mice also showed hyperphosphorylation of tau proteins, enhanced activity of GSK-3 in the spinal cord, and impairments in axonal transport in sciatic nerves [7]. Although the mechanisms for the spinal cord defects in Tg-AβPP/PS1 and Tg2576 mice may not be identical, the results of these studies, including ours, suggest that a common mechanism, such as a mechanism requiring Aβ-induced reactive oxygen species (ROS) accumulation, is likely involved in the pathogenesis of spinal cord deficits of Tg-AβPP/PS1 and Tg2576 mice.

We provide evidence that high ROS levels are related to neuronal injury in the spinal cord of Tg2576 mice. Because Tg2576 mice are transgenic animals expressing a mutant form of human AβPP under the control of the PrP promoter [1] and Aβ levels are enhanced in the spinal cord of Tg2576 mice (Fig. 4), age-dependent ROS accumulation in Tg2576 mice is likely caused by Aβ (Fig. 3). In fact, several lines of evidence support that Aβ1-42 is neurotoxic, because it can induce oxidative stress and increase lipid peroxidation in vitro and in vivo [28–30]. Tg2576 mice at 14.5 months of age show a significant level of visible plaques and at 16 months of age they show more plaques, but no evidence for neuronal loss, in the brain ([10] unpublished observa-
Fig. 9. Sciatic nerve degeneration of Tg2576 mice and partial suppression by curcumin. A-C) Transverse sections of toluidine blue-stained sciatic nerves of non-transgenic control mice (non-Tg; A), Tg2576 mice (Tg; B), and Tg2576 mice fed curcumin (Tg+Cur; C) at 16 months of age. Neuronal fibers in Tg2576 mice had a defect in myelination and a vacuole developed within the myelination sheath, resulting in ring-like shapes in their transverse sections (inset in B). Administration of curcumin partially suppressed the demyelination and vacuolization (C).Insets are high magnification. Scale bars in Fig. 9C; 50 µm. D-I) Electron micrographs of transverse sections of the sciatic nerves of non-transgenic control mice (D, G), Tg2576 mice (E, H) and Tg2576 mice fed curcumin (F, I) at 16 months of age. Low (D-F) and high (G-I) magnifications are shown. Scale bars are indicated (F, I).

tion), whereas in the spinal cord of Tg2576 mice, visible plaques were not deposited, but neuronal loss occurred. Our analysis suggests that the level of Aβ1−42 in the spinal cord of Tg2576 mice at 16 months of age is similar to the level detected in the brain of Tg2576 mice at 10 months of age and approximately 10% of that in the brain of Tg2576 mice at 14.5 months of age ([10] unpublished observation). The lipid peroxidation level in the spinal cord was similar to that in the brain of Tg2576 mice at 14.5 months of age (this study) [10]. Because soluble or oligomeric Aβ is pathogenic [31–33], we speculate that Aβ itself, or ROS produced by Aβ, might cause neuronal injury in the spinal cord of Tg2576 mice. It is also possible that antioxidant systems in the spinal cord are not as powerfully fortified as in the brain of Tg2576 mice at 14.5 months of age, and so neuronal loss occurs in the spinal cord but is not in the brain in the similar condition. In addition, the spinal cord of Tg2576 mice at 16 months has deficits in mitochondrial function (Fig. 5). Mitochondria are a major source of free radical production and mitochondrial metabolism impairment is a prominent feature of neurodegenerative diseases [34]. Recent studies suggest that Aβ peptides cause mitochondrial respiratory dysfunctions [35,36]. Together, these results suggest that accumulated Aβ in the spinal cord of Tg2576 mice might cause mitochondrial dysfunctions and neurotoxicity.

It may be worth noting that both neuronal loss and sciatic nerve degeneration of Tg2576 mice are reminiscent of the pathology displayed by mouse models of amyotrophic lateral sclerosis (ALS) in several respects. ALS shows progressive neurodegeneration of motor neurons in the primary motor cortex, brainstem, and spinal cord and produces motor function defects [37]. Mutations in the gene for SOD1 can cause familial ALS [38,39]. Moreover, transgenic G93A-SOD1 mice overexpressing a mutant form of human SOD1 show
spinal cord phenotypes, including high ROS levels and neural loss in the spinal cord, such as in Tg2576 mice. Age-dependent severe neuronal loss occurs in the spinal cord of Tg2576 mice and G93A-SOD1 mice. These results support the notion that high ROS levels may cause neuronal loss in the spinal cords of both Tg2576 and Tg-G93A-SOD1 mice.

Our results suggest that the severities of motor function defects of Tg2576 mice, including hindlimb extension reflex impairment, body trembling, hindlimb tremors, and abnormal gaits, were not linearly proportional to the decrease in the number of ChAT-positive neurons or cresyl violet-stained cells in the ventral horn. Non-transgenic mice showed a detectable decrease over ages in the numbers of ChAT-positive neurons in the ventral horn at 16 months of age compared with those at 6 months of age, but did not show severe phenotypes at 16 months of age (Figs 1 and 2). In addition, our results showed that motor function defects of Tg2576 mice appear to be expressed at a significant level when the numbers of cresyl violet-stained cells in the ventral horn were declined below a certain level (Figs 1 and 2). Moreover, the nerve fibers in the ventral root had a defect in myelination and were reduced in number (Supplemental Fig. 3), suggesting the possibility that muscle denervation might occur in Tg2576 mice. It may be worthy to also note that the behavioral data we analyzed were about overtly expressed motor function phenotypes, rather than healthy state of motor function or motor function competence. We do not exclude the possibility that motor function defects of Tg2576 mice are produced in part by sciatic nerve degeneration, although the present study does not explore the time course of its progression.

Administration of curcumin ameliorates the neurological phenotypes of Tg2576 mice. The therapeutic effects of curcumin were so strong that both the neuronal loss and the behavioral abnormalities appeared to be rescued by treatment with this natural compound, as it suppressed plaque pathology in the brain of Tg2576 mice [22]. While the therapeutic effects of curcumin on neuronal loss and behavioral deficits were dramatic for the early stages of pathological expressions, the effects were detectably diminished at 15–16 months of age. EM study revealed that the sciatic nerves were impaired in Tg2576 mice. Though the present study did not provide the mechanism underlying the sciatic nerve degeneration, curcumin appears to be beneficial by delaying the pathogenesis of nerve degeneration, possibly due to its antioxidant effect [40] or its capability to reduce Aβ levels [18,41]. However, our EM study of the sciatic nerves showed that the therapeutic effects of curcumin practically vanished in Tg2576 mice at 16 months of age, though the visible cell bodies in the ventral horn of the lumbar cord remained detectably higher than in untreated Tg2576 mice. We speculate that catastrophic pressures in Tg2576 mice at 15–16 months of age, which might be caused by multiple mechanisms, override the therapeutic effects of curcumin. Considering these results and the fact that Tg2576 mice at 10–13 months of age showed severe body trembling and hindlimb tremors (Fig. 1D), it is possible that defects in axonal fibers, including demyelination, in Tg2576 mice are responsible, at least in part, for the pathological expression.

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