cation of heavy metals like mercury (Hg) and cadmium (Cd), homeostasis of essential metals including copper (Cu) and zinc (Zn), and scavenging reactive oxygen species (ROS) (Aschner et al., 1997; Hidalgo et al., 2001). Four isoforms are identified in mammals, three of which, MT-I, MT-II and MT-III are found in the central nervous system (CNS) (Aschner et al., 1997; Palmiter et al., 1992; Uchida et al., 1991). The localization and induction patterns between the MT-I and MT-II (MT-I/II) isoforms and the MT-III isoform in the CNS appear to be distinct. The expression of MT-I/II is mainly localized in glias (Aschner et al., 1997; Blaauwgeers et al., 1993) and is induced by exposure to metals including Hg, Cd, Cu and Zn, cytokines and ROS (Aschner et al., 1997; Hidalgo et al., 2001). On the other hand, the MT-III isoform is mainly present in neurons (Masters et al., 1994; Uchida et al., 1991), and is not easily induced by exposure to the above agents (Zheng et al., 1995).

Amyotrophic lateral sclerosis (ALS) is a lethal motor neuron disease characterized by selective degeneration of motoneurons, resulting in muscular atrophy including respiratory and bulbar muscles, complete paralysis, and death (Rowland and Shneider, 2001). Approximately 90% of ALS is sporadic and the remaining 10% or so is familial (Cleveland and Rothstein, 2001). Rosen et al. found that about 20% of familial ALS is linked with mutations of the gene encoding Cu/Zn superoxide dismutase (*SOD1*) (Rosen et al., 1993). It has been believed that abnormal SOD1 proteins encoded by mutant *SOD1* do not lose their original enzymatic function but gain a novel cytotoxic function in motoneurons (gain-of-toxic function theory) (Bruijn et al., 2004; Gurney et al., 1994). A presumable mechanism of the gain-of-toxic function is a Cu-mediated oxidative stress. A point mutation in *SOD1* causes chemical structure changes of SOD1 proteins (misfolding proteins), while retaining its original activity and with subsequent clumsy handling of Cu and Zn (Beckman et al., 2001; Bruijn et al., 2004; Valentine and Hart, 2003). As a result of decreased Zn-binding affinity and higher affinity for Cu (Crow et al., 1997; Lyons et al., 1996), Cu-mediated oxidative stress is enhanced, and leads to neuronal death (Said Ahmed et al., 2000; Wiedau-Pazos et al., 1996).

Mouse carrying a human mutant *SOD1* develops an ALS-like disease, and is a good animal model for ALS research. The mice do no exhibit any clinical signs of motor paralysis up to the age of about 12 weeks, and develop paralysis at the age of 14–16 weeks, dying of respiratory failure by 17–18 weeks old (Bruijn et al., 1997; Gurney et al., 1994; Ripps et al., 1995).

In this way, all physiological functions of MT are likely to be associated with the presumable current pathogenesis of ALS. In order to investigate the possible role of the changes in Cu and Zn concentrations and lipid peroxides (LPO) products, we therefore measured temporal changes in the Cu and Zn levels and the amount of LPO together with MT-I/II and MT-III proteins in a rodent model for ALS.

2. Materials and methods

2.1. Chemicals

An enhanced chemiluminescence (ECL) agent, glutathione-sepharose 4B and polyvinylidene difluoride (PVDF) membranes were purchased from Amersham Bioscience (Buckinghamshire, UK). The Protein Assay Rapid Kit$_{WAKO}$ and reduced glutathione buffer were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The pGEM-T Easy vector systems were purchased from Promega K.K. (Tokyo, Japan). Mouse monoclonal anti MT-I/II antibody (clone: E9) was purchased from Dako Cytomation Inc. (Carpinteria, CA, USA). Recombinant MT-I and MT-II proteins, mouse monoclonal anti β-tubulin (clone: TUB 2.1) and mouse monoclonal horseradish conjugated anti IgG antibody were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

We used G93A SOD1 transgenic (SOD1 Tg) mice [B6SJL-Tg (SOD1-G93A)^{dl} 1 Gur/J; 002300] as a model for familial ALS (Gurney et al., 1994). The mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were housed under standard conditions (temperature 22 °C, relative humidity 60%, 12 h light/dark cycle, and free access to food and water) in the animal facility at the College of Pharmacy, Nihon University. The SOD1 Tg male mice were crossed with CF-1 wild-type (WT) female mice. When they were at the age of 8 or 16 weeks, three to five each of male SOD1 Tg and WT mice were killed by decapitation under light anesthesia. The spinal cord (the region responsible for paralysis) and the cerebellum [non-responsible (control) region] were immediately dissected on a glass plate on crushed ice, were frozen in liquid nitrogen, then stored at −80 °C until use. The present study was approved by the ethics committee for laboratory animal use at the College of Pharmacy, Nihon University.

2.3. Motor function testing (infrared beam test)

The motor performance was assessed by the infrared beam test with Digiscan (Omnitech Electronics Inc., Columbus, OH, USA). Briefly, two sets of infrared beams placed at a distance of 5 cm from the transparent acrylic box floor, traversed the area in perpendicular directions. The beam obstructions served as indicators of the horizontal activities of the mice. An additional set of beams located at a distance of 10 cm from the box floor was used to determine the vertical activities (mice standing on two hind paws). The motor performance was measured weekly

from 4 to 17 weeks of age. The mice were first allowed to acclimatize themselves to the observation situation in a box with infrared sensors prior to the measurements of the motor performance. They were then individually placed in the box, and their motor performance was measured every minute for 5 min. The activity of the mice was expressed as the average count per min (counts/min).

2.4. PCR analysis of the genotype for G93A SOD1 transgenic mice

All offspring were genotyped using the polymerase chain reaction (PCR) process on genomic DNA isolated from their tails, which were digested overnight with proteinase K at 50 °C. The PCR primers to detect mutant *SOD1* transgene were selected according to the recommendations of The Jackson Laboratory: sense, 5′-CAT CAG CCC TAA TCC ATC TGA-3′, and antisense, 5′-CGC GAC TAA CAA TCA AAG TGA-3′. PCR sessions were performed for 30 cycles under the following conditions: denaturation at 94 °C for 1 min, annealing at 55 °C for 45 s and extension at 72 °C for 1 min 20 s. The primers amplified a 236 bp DNA region from mice carrying the human mutant *SOD1* gene (Gong and Elliott, 2000).

2.5. Preparation of the mouse monoclonal antibody specific for MT-III

MT-III cDNA of the entire coding regions was amplified by RT-PCR. The PCR products were ligated into the pGEM-T Easy expression vector as previously described (Hanahan, 1983). MT-III-glutathione-*S*-transferase (MT-III-GST) fusion protein was expressed in *E. coli* and purified on an affinity column with immobilized glutathione-sepharose 4B. BALB/c male mice were immunized with MT-III-GST fusion protein. The fusion protocol, mouse myeloma with immunized mouse spleen cells, was described previously (Köhler and Milstein, 1975). The power and specificity of the antibody were tested using Western blot analysis. The anti MT-III antibody recognized only the recombinant MT-III protein at the expected position (16.8 kDa), but neither the MT-I nor MT-II protein (Fig. 1).

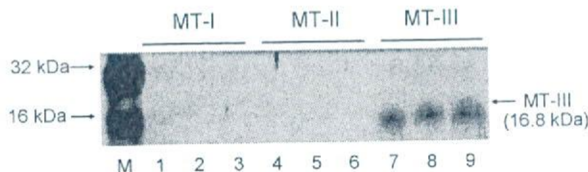

Fig. 1. Specific reactivity of prepared anti metallothionein III antibody. Two micrograms each of metallothionein (MT)-I, MT-II, and MT-III recombinant proteins were electrophoresed on an 18% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes and immunoreacted with the mouse monoclonal anti MT-III antibody. M: molecular marker, MT-I, MT-II and MT-III denote recombinant MT-I, MT-II and MT-III proteins, respectively (three lanes each).

2.6. Protein preparation and iodoacetamide treatment

Spinal cord and cerebellum tissues obtained from both SOD1 Tg and WT mice at 8- and 16-week-old were homogenized in a lysated buffer containing 10 mM Tris–HCl (pH 7), 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 137 mM sodium chloride, 1% NP-40 and 10% glycerine. After treatment with sonication for 20 s for three times, the homogenates were centrifuged at $9000 \times g$ for 30 min, and the supernatants were saved. The protein extracts were treated with 10% iodoacetamide and 10% tributyl phosphine/isopropanol. The mixtures were incubated in a water bath at 60 °C for 50 min. After cooling on ice, the samples treated with iodoacetamide were centrifuged at $800 \times g$ for 5 min, and then the supernatants were collected.

Protein concentration was determined with the pyrogallol red method according to the manufacturer's directions (Protein Assay Rapid Kit_{wako}, Wako Pure Chemicals, Osaka, Japan). Briefly, homogenized samples were mixed with the pyrogallol red-molybdate agent in microplate wells. The mixtures in the wells were left for 20 min at room temperature and then measured by a microplate reader (3550, Bio-Rad, Hercules, CA, USA) furnished with a 600 nm wavelength filter.

2.7. SDS-PAGE and Western blotting

An aliquot of 20 μg protein treated with iodoacetamide was separated on an 18% polyacrylamide gel with Laemmli system (Laemmli, 1970) at 100 V until the tracking dye, containing 10 mM Tris–HCl (pH 7.5), 10 mM EDTA, 20% glycerol, 1% SDS, 0.025% bromophenol blue, 100 mM DTT and 0.7 M 2-mercaptoethanol, reached the bottom of the gel. Electrophoresed proteins were transferred to PVDF membranes at 100 V for 3 h. After transferring, the membranes were blocked in the solutions containing 3% BSA, 1 M Tris–HCl (pH 7.5) and 40 mM sodium chloride for 1 h at room temperature and immunoblotted overnight at 4 °C with the primary antibodies used against mouse monoclonal anti MT-I/II antibody (dilution at 1:100), mouse monoclonal anti MT-III antibody and mouse monoclonal anti β-tubulin (dilution at 1:10,000). After washing with TBS containing 0.1% Tween-20 solution, the membranes were incubated with a mouse monoclonal horseradish conjugated anti IgG antibody (dilution at 1:10,000) for 1 h at room temperature. Immunoreaction was visualized using the ECL agent. After exposure, films were scanned by an image analyzer (LAS1000 plus lumino-image analyzer, Fuji Film, Tokyo, Japan). Band intensities were quantified with NIH images (National Institutes of Health, Bethesda, MD, USA). The expression of MT-I/II or MT-III protein was determined as a ratio of MT-I/II or MT-III to β-tubulin.

2.8. Analysis of copper and zinc concentrations

Spinal cord and cerebellum samples were digested in concentrated nitric acid (65%, v/v) overnight at room temperature

until no visual resides remained. The tissues were incubated in a boiling water bath for 1 h to facilitate digestion. Digested tissues were diluted in ultra-pure water. Copper and zinc concentrations in each sample tissue were measured by inductively coupled plasma mass spectrometry (Agilent 7500, Yokogawa Analytical Systems, Tokyo, Japan). Blanked samples were processed in the same ways. Metal concentrations were reported in micrograms per gram of wet tissue weight.

2.9. Measurement of lipid peroxides

The LPO concentration was determined using the thiobarbituric acid (TBA) method as described elsewhere (Ono et al., 1998). Briefly, tissues were homogenized in ice-cold PBS. All subsequent steps were performed using amber-colored tubes. The homogenized samples were added to 40 mM sulfuric acid and 10% phosphotungstic acid, and then were centrifuged at $1500 \times g$ for 10 min. A TBA agent containing 8.8 M acetic acid and 20 mM TBA was added. Samples were incubated in a boiling bath for 1 h. After cooling with tap water for 5 min, *n*-butanol was added in order to extract the malondialdehyde, and the mixture was shaken vigorously by hand. After centrifugation at $1500 \times g$ for 10 min, the *n*-butanol phase was saved. The absorbance of the supernatant (*n*-butanol phase) was measured with a spectofluorometer (FP 6200, Nihon Bunko, Tokyo, Japan) at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. The LPO level was estimated using 1,3,3-tetraetoxypropane as a reference standard. Results were expressed as nanomoles of malondialdehyde reactive substances per gram of wet tissue weight.

2.10. Statistical analysis

All the data on the motor performance were represented as mean ± S.E.M. We used repeated-measures ANOVA to compare the motor performance of the SOD1 Tg with that of the WT mice. All other statistical analyses were carried out using Student's *t*-test, including comparison of the values between the SOD1 Tg and WT mice and between the 8- and 16-week-old mice. *P* values <0.05 were considered to be statistically significant.

3. Results

3.1. Activity of the G93A SOD1 transgenic mouse

The vertical activities of the mice are shown in Fig. 2. The SOD1 Tg mice showed no signs of paralysis up to the age of 12 weeks, and thereafter began to exhibit a week-dependent decrease in activity, showing the development of motor paralysis. At 16 weeks of age, an evident reduction in vertical activity was observed. The horizontal activities of the mice showed the same pattern (data not shown).

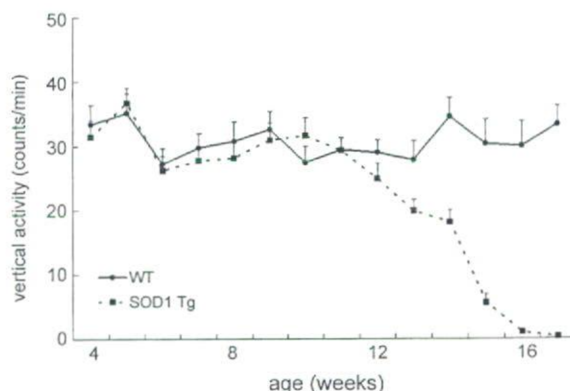

Fig. 2. Time-course of changes in the motor performance in the G93A SOD1 transgenic mice. The motor performance of the mice was measured by the infrared beam test. Significant difference in the vertical activities was found between the G93A SOD1 transgenic (SOD1 Tg) mice and the wild-type (WT) mice ($p = 1.38 \times 10^{-6}$; repeated-measures ANOVA). The motor performance in the SOD1 Tg mice showed a sharp week-dependent decrease after 12 weeks of age, suggestive of development and progression of paralysis ($p = 5.38 \times 10^{-22}$; repeated-measures ANOVA).

3.2. MT-I/II protein expression

The expression of MT-I/II protein in the spinal cord, the region responsible for motor paralysis, is shown in Fig. 3A and B. The protein level had already significantly increased in SOD1 Tg mice at 8 weeks of age, at which time the mice had not yet developed paralysis (Fig. 3A and B). At 16 weeks of age, when paralysis became apparent, the increase in the MT-I/II levels was maintained, with levels significantly higher than that at 8 weeks of age (Fig. 3A and B). On the other hand, the levels of MT-I/II protein in the cerebellum, which is not responsible for paralysis, did not show any differences between SOD1 Tg and WT mice at either 8 or 16 weeks of age (Fig. 3C and D).

3.3. MT-III protein expression

In 8-week-old SOD1 Tg mice, the MT-III levels in the spinal cord remained the same as those of the WT mice (Fig. 4A and B). However, at 16 weeks of age, when SOD Tg mice exhibited motor paresis, the MT-III level had significantly increased (Fig. 4A and B). On the other hand, in the cerebellum, no significant difference was observed between SOD1 Tg and WT mice at either 8 or 16 weeks of age (Fig. 4C and D).

3.4. Copper and zinc concentrations

Cu concentrations in the SOD1 Tg mouse spinal cord showed a significant increase compared with those of

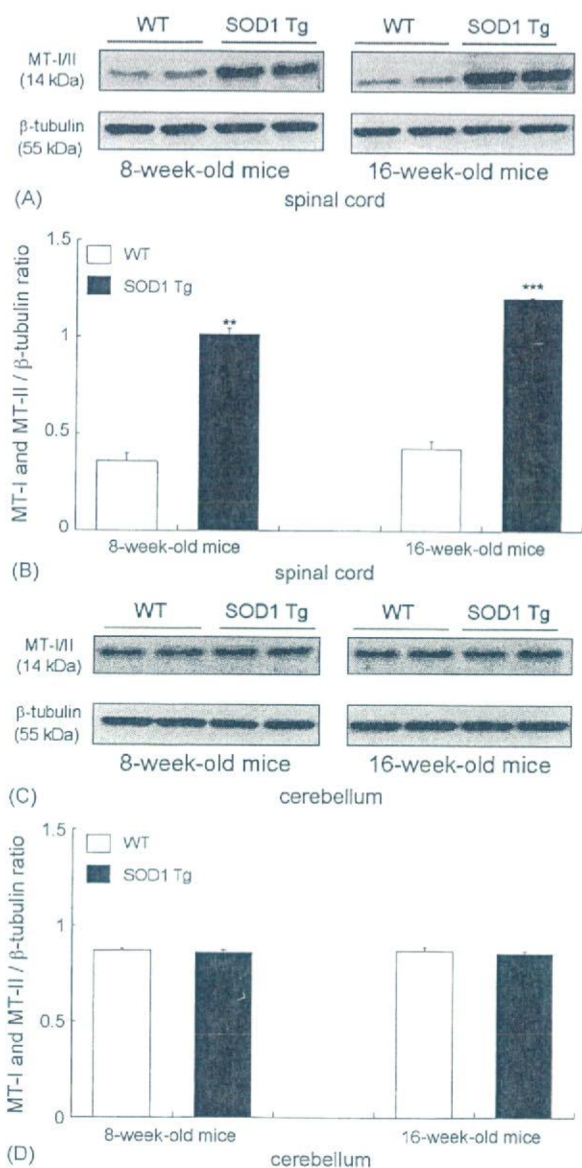

Fig. 3. Changes in metallothionein I and II proteins in G93A SOD1 transgenic mice. The spinal cord is the region responsible for motor paralysis in G93A SOD1 transgenic (SOD1 Tg) mice. Metallothionein-I and II (MT-I/II) proteins were significantly increased in the spinal cord of 8-week-old SOD1 Tg mice [$p = 1.9 \times 10^{-4}$ vs. 8-week-old wild-type (WT) mice], when motor paralysis was not yet apparent. At 16 weeks of age, when the SOD1 Tg mice had developed evident paralysis, MT-I/II proteins showed a further increase ($p = 5.8 \times 10^{-5}$ vs. 8-week-old WT and $p = 0.019$ vs. 8-week-old SOD1 Tg mice) (panels A and B). The cerebellum is not a region responsible for motor paralysis. Cerebellar MT-I/II levels showed no differences between SOD1 Tg and WT mice at either 8 or 16 weeks of age (panels C and D). Data from three to five mice each are shown as mean ± S.E.M. (panels B and D). Western blot analyses from each mouse (SOD1 Tg and WT mice) are shown in panels A and C. $^{**}p < 0.01$; $^{***}p < 0.001$ by Student's t-test.

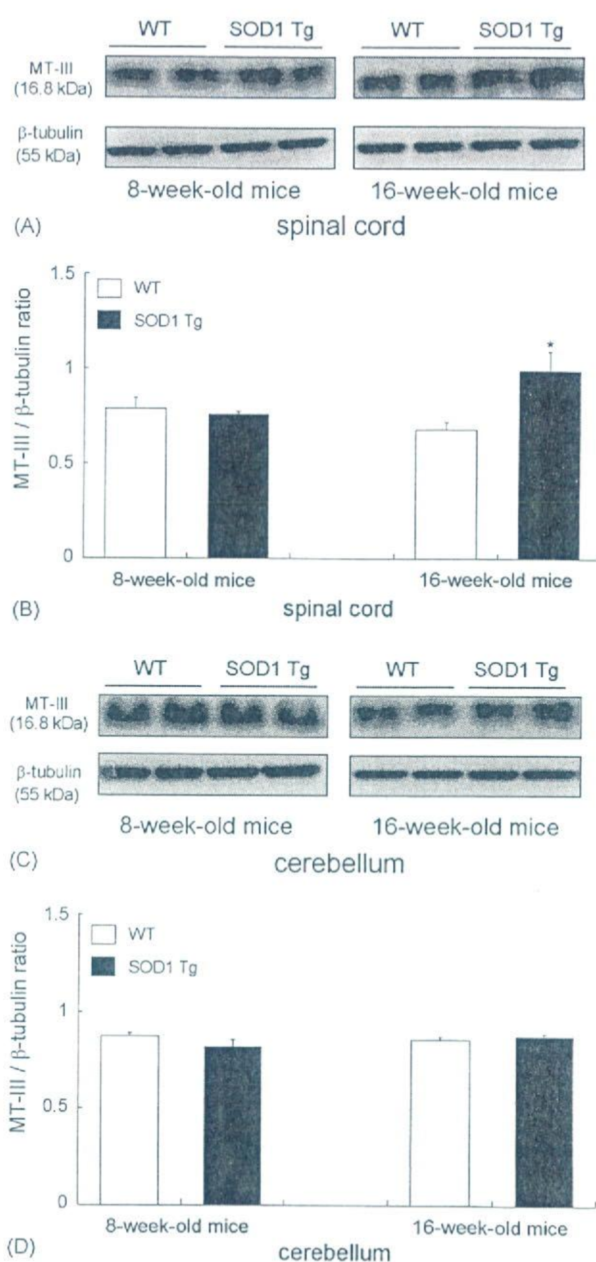

Fig. 4. Changes in metallothionein III protein in G93A SOD1 transgenic mice. In 8-week-old G93A SOD1 transgenic (SOD1 Tg) mice, when the mice were still asymptomatic, no change in MT-III protein level was observed in the spinal cord. However at 16 weeks of age, when SOD1 Tg mice were clearly symptomatic, MT-III level was significantly increased [$p = 0.04$ vs. wild-type (WT) mice at the same age, and $p = 0.02$ vs. 8-week-old SOD1 Tg mice] (panels A and B). No change in MT-III level was observed in the cerebellum at any age (panels C and D). Data from three to five mice each are shown as mean ± S.E.M. (panels B and D). Western blot analyses from each mouse (SOD1 Tg and WT mice) are shown in panels A and C. $^{*}p < 0.05$ by Student's t-test.

WT mice at both 8 and 16 weeks of age (Fig. 5A). In SOD1 Tg mice, further increases in the Cu level at 16 weeks of age were seen than that in 8-week-old mice (Fig. 5A). In the cerebellum, however, no alteration in the Cu level was either the SOD1 Tg or WT mice at any time (Fig. 5B). On the contrary, significant decreases in Zn levels were noted in the SOD1 Tg mouse spinal cord at both 8 and 16 weeks of age (Fig. 5C). No change in the Zn level was observed in the cerebellum at any time (Fig. 5D).

3.5. Amounts of lipid peroxides

Significant increases were already seen in the LPO concentration in 8-week-old SOD1 Tg mice spinal cords (Fig. 6A), which had further increased at the age of 16 weeks (Fig. 6A). However, the LPO level in cerebellum showed no change between SOD1 Tg and WT mice either at 8 or 16 weeks of age (Fig. 6B).

4. Discussion

We found that MT-I/II protein expression significantly increased in spinal cord of SOD1 Tg mice (Fig. 3A and B). The increase was observed as early as 8 weeks of age, when the SOD1 Tg mice had not yet exhibited any symptoms of motor paralysis. In 8-week-old SOD1 Tg mice, the Cu concentration had also significantly increased, with an inverse decrease in Zn levels and a concomitant elevation of LPO amounts in the spinal cord (Figs. 5A, C, and 6A). The increase in the MT-I/II and Cu levels increased in an age-dependent manner, being higher at 16 than at 8 weeks of age. Based on these results, we propose that these conditions were strongly implicated in the pathogenesis of motor neuron death in SOD1 Tg mice, since these observations exclusively occurred in the spinal cord, the region responsible for paralysis, but not in cerebellum, a region not responsible for paralysis (Figs. 3C, D, 5B, D, and 6B).

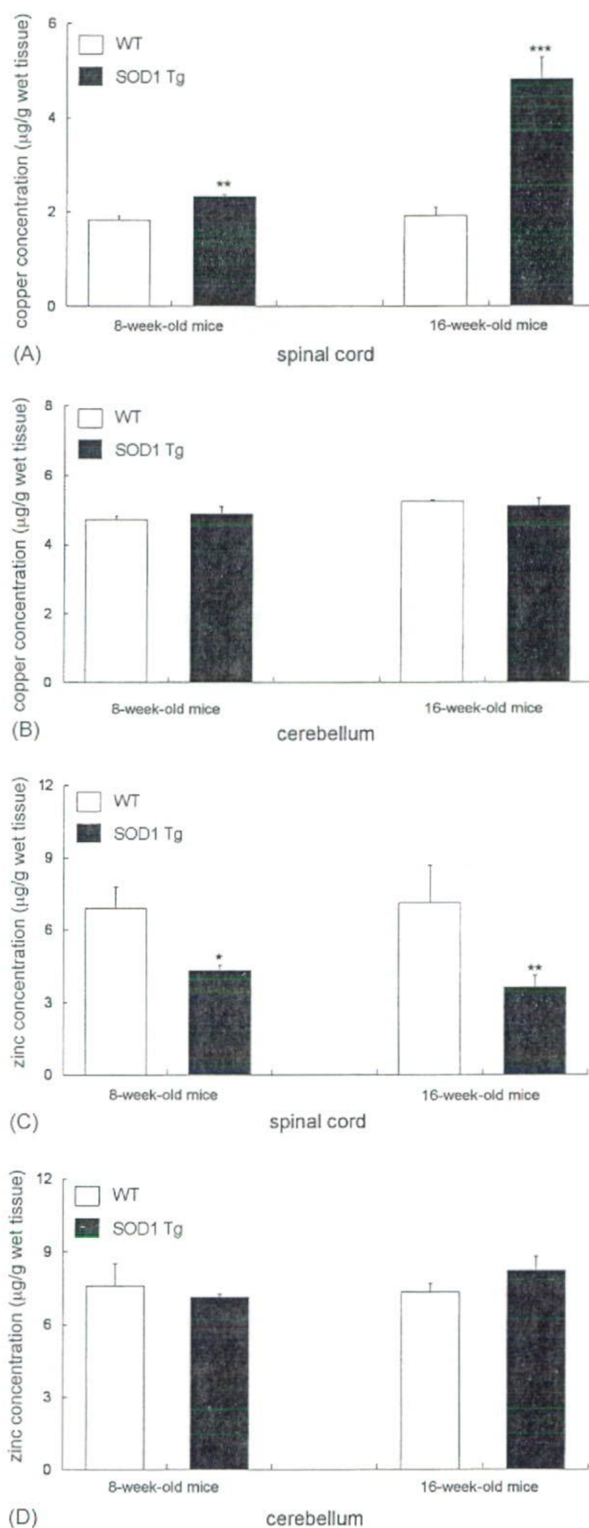

Fig. 5. Copper and zinc concentrations in G93A SOD1 transgenic mice. A significant increase in copper concentration was observed in the spinal cord of the as-yet-asymptomatic 8-week-old G93A SOD1 transgenic (SOD1 Tg) mice [$p = 1.9 \times 10^{-3}$ vs. wild-type (WT) mice at the same age], with a further increase at 16 weeks of age ($p = 3.0 \times 10^{-4}$ vs. WT at the same age, and $p = 6.0 \times 10^{-4}$ vs. 8-week-old SOD1 Tg mice) (panel A). An inverse decrease in zinc level was observed in both 8- and 16-week-old SOD1 Tg mice ($p = 0.02$ vs. 8-week-old WT mice, and $p = 9.2 \times 10^{-4}$ vs. 16-week-old WT (panel C). In the cerebellum, no alternation was found either in copper or zinc level (panels B and D). Data from three to five mice each are shown as mean ± S.E.M. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by Student's t-test.

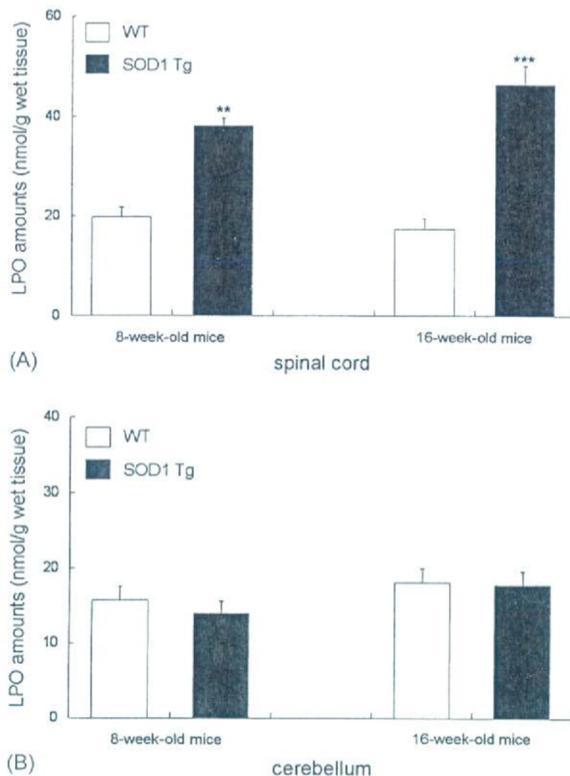

Fig. 6. Lipid peroxides in G93A SOD1 Transgenic mice. Lipid peroxides (LPO) levels were significantly increased in the spinal cord of both 8-week-old G93ASOD1 transgenic (SOD1 Tg) mice and 16-week-old SOD1 Tg mice [$p = 2.0 \times 10^{-3}$ vs. 8-week-old wild-type (WT) mice and $p = 3.1 \times 10^{-5}$ vs.16-week-old WT mice] (panel A). In the cerebellum, no significant change was found in the LPO level at any age (panel B). $^{**}p < 0.01$; $^{***}p < 0.001$ by Student's t-test.

The present findings are partially in agreement with previous reports. An increase in the spinal MT-I/II isoform was reported in this line of transgenic mouse (Gong and Elliott, 2000; Nagano et al., 2001; Olsen et al., 2001) or in patients with ALS (Blaauwgeers et al., 1996; Sillevis Smitt et al., 1994). We reported, on the basis of the mRNA findings, that *MT-I* up-regulation was taking place as early as 8 weeks of age in the same line of transgenic mouse spinal cord, but not in the cerebellum (Ono et al., 2006). Although an elevation of Cu concentration was previously reported in the same line of SOD1 Tg mouse (Bush, 2002), no study has previously revealed an increase in Cu and a concomitant inverse decrease in Zn levels in the spinal cord of the same SOD1 Tg mouse at as early as 8 weeks of age.

It is currently accepted that mutant SOD1 is implicated in the pathogenesis of familial ALS. The mutant SOD1 is believed to gain a novel cytotoxic function, while retaining its original enzymatic activity (gain-of-toxic function theory) (Bruijn et al., 2004; Gurney et al., 1994). The mutant SOD1 is characterized by clumsy handling of Cu and Zn (Beckman et al., 2001; Bruijn et al., 2004; Valentine and Hart, 2003), and subsequent alternations in Cu and Zn levels likely play an important role in the gain-of-toxic function (Borchelt et al., 1994). Some evidence exists to support the idea; First, Cu-chelating agents such as D-penicillamine and trientine extend survival in SOD1 Tg mice (Andreassen et al., 2001; Hottinger et al., 1997; Nagano et al., 2003). Second, *atp7b*, encoding Cu-transporting ATPase, is down-regulated in the spinal cord of SOD1 Tg mice (Olsen et al., 2001). Third, crossing an SOD1 Tg mouse with a mouse model for Menkes' disease, which causes congenital Cu deficiency, extend the clinical duration of survival (Kiaei et al., 2004). Fourth, a Zn decrease in mutant SOD1 causes peroxynitrate-mediated tyrosine nitration (Beckman et al., 1993; Estévez et al., 1999) and an increase in biological 3-nitrotyrosin, a hallmark of protein nitration, in the spinal cord in patients with ALS (Beal et al., 1997). Liu et al. demonstrated that ROS production increased with aging, measured by using a spin trap, azulenyl nitrone, in the spinal cord of the SOD1 Tg mouse (Liu et al., 1998). The present study revealed that in 8-week-old SOD1 Tg mice, LPO amounts, measured as malondialdehyde reactive substances, exclusively accumulated in the region responsible for motor paralysis, namely the spinal cord, before the onset of paralysis (Fig. 6A and B). Based on these findings, LPO accumulation is a probable result of Cu and Zn changes in the spinal cord, presumably due to Cu-mediated oxidative stress. Taking the classic functions of the MT-I/II isoform into account, it is reasonable to postulate that the spinal MT-I/II protein increase observed in the present study is a compensatory induction in response to Cu-mediated oxidative stress.

We also found that MT-III protein levels significantly increased in the SOD1 Tg mouse spinal cord (Fig. 4A and B), which was exclusively observed in the paralysis-responsible spinal cord, as was the case with the MT-I/II isoforms. Although *MT-III* mRNA changes have previously been reported (Gong and Elliott, 2000; Olsen et al., 2001), no MT-III protein study has been shown in this line of transgenic mouse. It should be emphasized that unlike MT-I/II, the MT-III protein remained unchanged in 8-week-old SOD1 Tg mice and finally increased at 16 weeks of age, at the end stage of the disease (Fig. 4A and B). The spinal Cu level in 16-week-old SOD1 Tg mice was further increased compared with 8-week-old mice (Fig. 5A), suggesting further LPO production. Nevertheless, the concentration of spinal LPO at 16 weeks of age was not higher than that in 8-week-old mice (Fig. 6A). However, as MT-III protein synthesis induction occurred

at an advanced stage of the disease, MT-III would also appear to have some counteractivity against LPO production. It should be pointed out that crossing SOD1 Tg mice with either MT-I/II or MT-III knock-out mice markedly accelerated the expression of paralysis and shortened survival (Puttaparthi et al., 2002). Different temporal induction patterns in MT isoforms could point to another property of MT-III that the MT-I/II isoform does not have. Hozumi et al. showed that MT-III isoform synthesis was induced after stab wounds in rats (Hozumi et al., 1995). In the same line of SOD1 Tg mice, we reported *MT-III* mRNA up-regulation, although not statistically significant, at an advanced stage of the disease (Ono et al., 2006). The MT-III protein seems to be induced in response to critical tissue damage in the CNS. It is reasonable and acceptable that the MT-III isoform in addition to the MT-I/II isoforms have a potential application in the modification of the disease expression and/or progression.

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