Regulation of Intracellular Copper by Induction of Endogenous Metallothioneins Improves the Disease Course in a Mouse Model of Amyotrophic Lateral Sclerosis

Eiichi Tokuda · Shunsuke Watanabe · Eriko Okawa · Shin-ichi Ono

Published online: 12 March 2015

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Abstract Mutations in *SOD1* cause amyotrophic lateral sclerosis (ALS), an incurable motor neuron disease. The pathogenesis of the disease is poorly understood, but intracellular copper dyshomeostasis has been implicated as a key process in the disease. We recently observed that metallothioneins (MTs) are an excellent target for the modification of copper dyshomeostasis in a mouse model of ALS (*SOD1*^{G93A}). Here, we offer a therapeutic strategy designed to increase the level of endogenous MTs. The upregulation of endogenous MTs by dexamethasone, a synthetic glucocorticoid, significantly improved the disease course and rescued motor neurons in *SOD1*^{G93A} mice, even if the induction was initiated when peak body weight had decreased by 10 %. Neuroprotection was associated with the normalization of copper dyshomeostasis, as well as with decreased levels of *SOD1*^{G93A} aggregates. Importantly, these benefits were clearly mediated in a MT-dependent manner, as dexamethasone did

not provide any protection when endogenous MTs were abolished from *SOD1*^{G93A} mice. In conclusion, the upregulation of endogenous MTs represents a promising strategy for the treatment of ALS linked to mutant *SOD1*.

Keywords Amyotrophic lateral sclerosis · Copper dyshomeostasis · Dexamethasone · Metallothioneins · Superoxide dismutase-1

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset form of motor neuron disease. Riluzole is the only available therapeutic option for ALS [1]. However, its therapeutic effect is limited, extending survival by a few months without any improvement in muscle function [1]. Thus, the development of new therapeutic strategies is urgently required. A better understanding of the disease mechanisms of ALS will provide important clues for the development of novel therapeutic targets [2]. Our current knowledge regarding ALS pathogenesis mainly comes from evidence obtained using mouse models of ALS, in which mutated human *SOD1* is overexpressed [3, 4].

Several mechanisms of how superoxide dismutase-1 (*SOD1*) mutants cause ALS have been proposed [5], including dyshomeostasis of intracellular copper. The expression of the copper uptake proteins, STEAP2 and CTR1, is increased, whereas that of the copper efflux protein, ATP7A, is decreased in the spinal cord of mutant *SOD1* mice, regardless of their copper-binding ability [6]. This impairment of the copper trafficking system results in the accumulation of copper ions in the spinal cord [6–10]. The therapeutic effects of copper-

Eiichi Tokuda and Shunsuke Watanabe contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s13311-015-0346-x) contains supplementary material, which is available to authorized users.

E. Tokuda · S. Watanabe · E. Okawa · S.-i. Ono (✉)
Laboratory of Clinical Medicine, School of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan
e-mail: ono.shinichi@nihon-u.ac.jp

E. Tokuda
Department of Medical Biosciences, Clinical Chemistry, Umeå University, Building 6M, 2nd Floor, Umeå 901-85, Sweden

S.-i. Ono
Division of Neurology, Akiru Municipal Medical Center, 78-1 Hikida, Akiru, Tokyo 197-0834, Japan

lowering strategies in mutant SOD1 mice, including pharmacological chelation [6, 11] or the restriction of copper intake [12], support a deleterious role of copper dyshomeostasis in ALS.

In addition to these copper-lowering therapies, metallothioneins (MTs) appear to be an excellent therapeutic target. MTs are a family of small, cysteine-rich proteins that bind copper ions through copper–thiolate clusters [13]. Four MT isoforms exist in mammals, 2 of which, MT-I and MT-II (MT-I/-II), are major isoforms that exert neuroprotection against copper dyshomeostasis [14]. Indeed, the overexpression of MT-I in SOD1^{G93A} mice prolongs survival and restores copper dyshomeostasis [15]. In contrast, the genetic ablation of *Mt-I/-II* in SOD1^{G93A} mice exacerbates disease mortality and accelerates the death of motor neurons [16, 17]. Taken together, the functional potentiation of MT-I/-II represents a proof-of-concept strategy for the normalization of copper dyshomeostasis to improve the course of the disease.

The aim of this study was to investigate whether an alternative strategy focusing on MT-I/-II could ameliorate the disease course in SOD1^{G93A} mice. Here, we show that the induction of MT-I/-II by dexamethasone (Dex), a synthetic glucocorticoid, extended survival and prevented motor neuron death, accompanied by the amelioration of copper dyshomeostasis. These benefits were completely blocked when MT-I/-II was abolished from SOD1^{G93A} mice, indicating that MT-I/-II is required for the therapeutic effects of Dex in SOD1^{G93A} mice.

Methods

Animals

All the animal protocols were approved by the Institutional Animal Care and Use Committee of the School of Pharmacy, Nihon University, and adhered to our institutional animal guidelines. Transgenic mice expressing human SOD1^{G93A} [strain name: B6SJL-Tg(SOD1-G93A)1Gur/J, stock number: 002726] were purchased from the Jackson Laboratory (Bar Harbor, MA, USA) [4]. The transgenic line was maintained through hemizygotes by crossing transgenic males with F₁ nontransgenic females on a B6SJL background.

Generation of SOD1^{G93A} Mice with a Homozygous Deletion of *Mt-I/-II*

Knockout mice with a homozygous deletion of *Mt-I* and *Mt-II* (strain name: 129S7/SvEvBrd-Mt1^{tm1Bri}Mt2^{tm1Bri}/J, stock number: 002211) were purchased from the Jackson Laboratory [18]. In the knockout mice, both the *Mt-I* and *Mt-II* genes are simultaneously disrupted using a vector that inserts

inframe stop codons into the exons of the 2 genes [18]. Homozygous *Mt-I/-II* (*Mt*^{-/-}) knockout mice develop normally and show no apparent phenotypic abnormalities [18]. The knockout mice were maintained as homozygotes on a 129Sv background.

To generate SOD1^{G93A} mice with a homozygous deletion of *Mt-I/-II* (SOD1^{G93A}/*Mt*^{-/-}), a 2-step breeding strategy was performed to exclude differences caused by the strain background. First, male SOD1^{G93A} mice were crossed with *Mt*^{-/-} females. Next, male SOD1^{G93A}/*Mt*^{+/-} mice were intercrossed with female *Mt*^{+/-} mice to obtain SOD1^{G93A}/*Mt*^{-/-} mice. The SOD1^{G93A}/*Mt*^{-/-} mice on a mixed background (B6SJL × 129Sv) were strictly compared with their simultaneously generated nontransgenic, *Mt*^{-/-}, and SOD1^{G93A} littermates.

To genotype offspring, genomic DNA was extracted from tail biopsies using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Genotypes were determined using polymerase chain reaction with primers according to the recommendations of the Jackson Laboratory.

Time Course of MT Isoform Induction After a Single Injection of Dex

C57BL/6 J mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and maintained in our animal institute. C57BL/6 J mice at 8 weeks of age were intraperitoneally injected with a single dose of Dex (2 mg/kg; Daiichi Sankyo, Tokyo, Japan). At different times after the injection (12–96 h), lumbar spinal cords were dissected from the injected mice (*n*=3 per time point). The temporal expression patterns of Dex-induced MT-I/-II and MT-III were determined using Western blotting.

Preclinical Studies with Dex

The protocol for Dex treatment was designed according to criteria suggested for preclinical studies [19, 20]. The investigators were blind to the mouse genotype and treatment while performing all procedures. The copy number of the human *SOD1* transgene greatly influences the disease course in SOD1^{G93A} mice [21]. Thus, the copy number was determined using a polymerase chain reaction assay according to instructions from the Jackson Laboratory, and mice with approximately 23 copies were selected.

For the therapeutic regimen in SOD1^{G93A} mice on a B6SJL background, mice were randomized into 2 groups to receive intraperitoneal Dex (2 mg/kg; Daiichi Sankyo) or phosphate-buffered saline (PBS) injections. Both groups were balanced with respect to sex and littermates as these factors also affect ALS-like symptoms in this strain [22]. The treatment was initiated at 2 distinct stages of the disease: presymptomatic (30 days of age) or symptomatic (the time when the peak body weight of the mice had decreased by 10 %) [23], and

continued until the clinical end-stage (see details in “Clinical Assessment”). The injections were performed every 48 h to maintain a peak level of MT-I/-II in the lumbar spinal cord (Fig. 1a, b). In the preclinical study using SOD1^{G93A} mice, B6SJL littermates were used as a nontransgenic control.

For the therapeutic regimen in SOD1^{G93A}/Mt^{-/-} mice, we followed the same regimen as that used for the presymptomatic treatment with Dex in SOD1^{G93A} mice. SOD1^{G93A}/Mt^{-/-} or Mt^{-/-} mice were injected with Dex (2 mg/kg) or PBS, starting at the presymptomatic stage (30 days of age) and continuing until the clinical end stage. As a control for the

preclinical study using SOD1^{G93A}/Mt^{-/-} mice, Mt^{-/-} littermates on a mixed background (B6SJL × 129Sv) were used.

Clinical Assessment

All clinical assessments were performed by those who were unaware of the genotype or treatment of the mice. The disease course was evaluated according to criteria based on body weight changes [23]. Disease onset was regarded as the time when each mouse reached its peak weight before its weight began to decline. The end point was defined as the age at

Fig. 1 Dexamethasone (Dex) induces metallothionein (MT)-I/-II protein in the spinal cords of superoxide dismutase-1 (SOD1)^{G93A} mice. (a, b) C57BL/6 J mice were injected intraperitoneally with a single dose of Dex (2 mg/kg). (a) Western blot for MT-I/-II in lumbar spinal cords at different times after a single injection (12–96 h). β -Tubulin was used as an internal marker. (b) The relative expression levels of MT-I/-II after a single injection ($n=3$ per group). $**p<0.01$ vs 0 h. (c–h) SOD1^{G93A} mice were injected intraperitoneally with Dex (2 mg/kg) or phosphate-buffered saline (PBS), starting on the day when peak body weight of the mice had decreased by 10 %. (c) Western blot for MT-I/-II in lumbar spinal cords of mice 20 days after the start of treatment. (d) The relative expression levels of MT-I/-II ($n=3$ per group). $**p<0.01$ vs PBS-treated B6SJL. $^{##}p<0.01$ vs PBS-treated SOD1^{G93A}. (e) Immunohistochemistry for MT-I/-II in lumbar spinal cords of mice treated with Dex or PBS. Scale bar: 100 μ m. (f–h) Confocal microscopy images of lumbar spinal cord sections from SOD1^{G93A} mice treated with Dex or PBS. The sections were double-immunostained with antibodies against MT-I/-II and (f) NeuN, (g) glial fibrillary acidic protein (GFAP), or (h) CD11b. Scale bars: 20 μ m. Data are expressed as mean $\pm$ SD

which a mouse was unable to right itself within 30 s after having been pushed onto its side [19]. The disease duration was regarded as the period from disease onset until the end point. The motor coordination of mice was measured using a rota-rod apparatus (Muromachi Chemicals, Fukuoka, Japan), as described previously [11].

Dissection of Lumbar Spinal Cords

For the analysis of SOD1^{G93A} mice treated with Dex or PBS from the day when the peak body weight of the mice had decreased by 10 %, the lumbar spinal cords were dissected from the injected mice at 120 days of age (20 days after the start of the injections, $n=3$ per group), which is the mean lifespan of PBS-treated SOD1^{G93A} mice. For the analysis of SOD1^{G93A}/*Mt*^{-/-} mice treated with Dex or PBS injections, the lumbar spinal cords were harvested from mice at 100 days of age (70 days after the start of the injections, $n=3$ per group), which is the mean lifespan of PBS-treated SOD1^{G93A}/*Mt*^{-/-} mice. The dissected spinal cords were used for the following biochemical and pathological experiments.

Western Blot Analysis

Western blot analyses were performed as described previously [9]. For the detection of MT isoforms, protein extracts were chemically modified with 20 mM monobromobimane (Molecular Probes, Eugene, OR, USA) to block the artificial formation of disulfide bonds in MTs during gel electrophoresis [15, 24]. For the detection of MT-I/-II in *Mt*^{-/-} mice, recombinant MT-I protein was used as a positive control (Alexa Biochemicals, Lausen, Switzerland). The following primary antibodies were used: mouse anti-MT-I/-II (1:100; clone E9; Dako, Glostrup, Denmark), and mouse anti-MT-III (1:1,000) [8]. Anti- β -tubulin (1:10,000; clone TUB 2.1; Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control.

Immunohistochemistry

Immunohistochemistry was performed as described previously [15]. Lumbar spinal cord sections (6- μ m thick) were immunostained with a mouse anti-MT-I/-II antibody (1:400; clone E9; Dako). Signals were detected using the Histofine Mouse Stain Kit (Nichirei Biosciences Inc., Tokyo, Japan) and 3,3'-diaminobenzidine (Nichirei Biosciences Inc.) as the chromogen. The sections were mounted in Malinole (Muto Pure Chemicals Co., Tokyo, Japan) and imaged using a light microscope (DP70; Olympus, Tokyo, Japan).

Immunofluorescence

Immunofluorescence was performed as described elsewhere [9]. Lumbar spinal cord sections (6- μ m thick) were

immunostained using the following primary antibodies: mouse anti-MT-I/-II (1:50; clone E9; Dako), mouse anti-NeuN (1:400; clone A60; Chemicon, Temecula, CA, USA), biotin-conjugated anti-NeuN (1:400; clone A60; Chemicon), goat anti-glial fibrillary acidic protein (GFAP) (1:50, sc-6170; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-CD11b (1:50, sc-6614; Santa Cruz Biotechnology), mouse anti-human SOD1 [1:100 (raised against full-length human SOD1) sc-101523; Santa Cruz Biotechnology], goat anti-human SOD1 [1:100 (raised against peptides corresponding to residues surrounding the N-terminus of human SOD1) sc-8636; Santa Cruz Biotechnology], or rabbit antiubiquitin (1:100; Z0458; Dako).

Histopathology

The pathological features of SOD1^{G93A} mice were evaluated as described previously [6]. All measurements were performed by an observer who was blind to the genotype and treatment.

For the quantification of α -motor neurons, every fifth section of the lumbar spinal cord (L4-L5) was immunostained with a mouse anti-NeuN antibody (1:1000; clone A60; Chemicon). α -Motor neurons were molecularly and morphologically identified as described elsewhere [25]. The number of α -motor neurons in the ventral horn was counted in 10 sections per mouse.

For axon counting, the ventral roots were dissected from the lumbar spinal cord (L5) and were immunostained with a mouse anti-SMI-312 antibody (1:2000; ab24574; Abcam, Cambridge, UK). The number of large axons (>4 μ m in diameter) was counted using Image J software (National Institutes of Health, Bethesda, MD, USA).

To quantify fluorescence intensity of astrocytes, microglia, human SOD1, or ubiquitin, lumbar spinal cord sections were immunostained with mouse monoclonal anti-GFAP cocktail (1:200, 556330; BD Biosciences, San Jose, CA, USA), goat polyclonal anti-CD11b (1:50, sc-6614; Santa Cruz Biotechnology), mouse antihuman SOD1 [1:100 (raised against full-length human SOD1) sc-101523; Santa Cruz Biotechnology], or rabbit antiubiquitin (1:100, Z0458; Dako) antibodies, respectively. Fluorescence images were acquired using a confocal microscope (Laser Scanning System Z510; Carl Zeiss, Jena, Germany). The fluorescence intensity in the ventral horn was measured using Image J software (National Institutes of Health).

Quantification of Copper Ions

The total amount of copper ions in lumbar spinal cords was measured using inductively coupled plasma-mass spectrometry (Agilent 7500; Yokogawa Analytical Systems, Tokyo,

USA), as described previously [8]. The values are expressed as $\mu\text{g/g}$ wet tissue.

Measurement of SOD1 Enzymatic Activity

SOD1 enzymatic activity was measured using a SOD1 Assay Kit-WST (Dojindo Molecular Technology Inc., Kumamoto, Japan), as described previously [11]. One unit of SOD1 activity was defined as a 50 % inhibition of the rate of water-soluble tetrazolium salt reduction. The values are presented as units/ μg spinal cord protein.

Measurement of Lipid Peroxides

The concentration of lipid peroxides was determined by measurement of malondialdehyde according to the thiobarbituric acid method as previously described [8]. The malondialdehyde chromophore in lumbar spinal cords was detected using a spectrofluorometer (excitation: 515 nm; emission: 553 nm, FP 6200; JASCO Corporation, Hachioji, Tokyo). The levels of lipid peroxides were estimated using 1, 3,3-tetraethoxypropane as a reference standard. The results are expressed as nanomoles of malondialdehyde reactive substances/g wet lumbar spinal cord.

Measurement of Human SOD1 Protein Level

Human SOD1 protein level was determined as described previously [6]. Briefly, lumbar spinal cords were homogenized in 20 volumes (w/v) of ice-cold lysis buffer containing 1 % (v/v) Nonidet P-40 and cOmplete, EDTA-free (Roche Applied Science, Indianapolis, IN, USA) protease inhibitor in PBS (pH 7.0). The homogenates were sonicated, producing a whole homogenate fraction. To prepare the detergent-insoluble fraction, the sonicated homogenate was centrifuged at $20,000\times g$ for 30 min at 4 °C. The supernatant was collected as the detergent-soluble fraction. The pellets were dissolved in lysis buffer containing 2 % (w/v) sodium dodecyl sulfate. Equal amounts of protein (5 μg) were analyzed by Western blotting with a mouse antihuman SOD1 antibody [1:1000 (raised against full-length human SOD1) sc-101523; Santa Cruz Biotechnology).

Statistics

The results are expressed as the mean $\pm$ SD. All statistical tests were performed using Statcel 3 software (OMS Publishing Inc., Tokorozawa, Saitama, Japan). Disease onset and survival were compared using Kaplan–Meier analysis with the log-rank test. Motor coordination was compared using a repeated-measures analysis of variance. After the determination of normality and homoscedasticity, 2 group comparisons were analyzed using a two-tailed Student's *t* test. Multiple group comparisons were

performed using a one-way analysis of variance followed by the Tukey–Kramer post hoc test. Statistical significance was defined as $p<0.05$. The “*n*” values indicate the number of individual animals, but not replicate measures of one sample.

Results

Single Injection of Dex Induces MT-I/-II but not MT-III Protein in the Spinal Cords of Nontransgenic Mice

The peripheral injection of Dex induces MT-I/-II protein in the brains of rats because of the presence of glucocorticoid response elements in *Mt-I/Mt-II* [26, 27]. First, we determined the temporal changes in MT-I/-II protein levels in the lumbar spinal cord after a single injection of Dex. C57BL/6 J mice were intraperitoneally injected with a single dose of Dex (2 mg/kg). At different times after the injection (12–96 h), the level of MT-I/-II in the spinal cord was quantified using Western blotting (Fig. 1a). The induction of MT-I/-II reached a peak level at 24 h after the injection, and this level persisted for a 48-h period (Fig. 1b). The single injection of Dex did not affect the protein level of MT-III, another MT isoform in the central nervous system (Supplementary Fig. 1a, b).

Dex Induces MT-I/-II in Spinal Cord Astrocytes and Microglia in SOD1^{G93A} Mice

We determined whether Dex could induce MT-I/-II in the lumbar spinal cord of SOD1^{G93A} mice harboring a high transgene copy number (Fig. 1c–h). SOD1^{G93A} mice on a B6SJL background and nontransgenic littermates were intraperitoneally injected with Dex (2 mg/kg) or PBS, starting from the time when the peak body weight of the mice had decreased by 10 % [23]. Western blot analyses showed that the level of MT-I/-II in the lumbar spinal cords of PBS-treated SOD1^{G93A} mice at the end stage of the disease (120 days of age) was significantly higher compared with that in PBS-treated nontransgenic B6SJL littermates. Dex induced MT-I/-II protein in SOD1^{G93A} mice at 120 days of age, representing a 20 % increase relative to that in PBS-treated SOD1^{G93A} mice (Fig. 1c, d).

As shown in Fig. 1(e), the distribution pattern of MT-I/-II protein in the lumbar spinal cord of Dex-treated SOD1^{G93A} mice differed considerably from that of PBS-treated SOD1^{G93A} mice. Immunohistochemical studies revealed that MT-I/-II in PBS-treated SOD1^{G93A} mice was distributed not only in the white matter, but also in the entire gray matter, as previously reported by ourselves and others [15, 16, 28]. Notably, Dex predominantly induced MT-I/-II in the ventral horn (Fig. 1e, right), but not the dorsal horn (data not shown), of SOD1^{G93A} mice. To identify the cell types expressing MT-I/-II in the lumbar spinal cord, we performed dual

immunofluorescence using antibodies against NeuN, GFAP, and CD11b, which are markers for neurons, astrocytes, and microglia, respectively (Fig. 1f–h). The cell types expressing MT-I/-II in Dex-treated SOD1^{G93A} mice were similar to those in PBS-treated SOD1^{G93A} mice, demonstrating that Dex-induced MT-I/-II was localized in astrocytes and microglia but not in motor neurons.

We examined the effects of Dex on the level of MT-III in SOD1^{G93A} mice (Supplementary Fig. 1c, d). Consistent with our earlier findings [8], the level of MT-III protein in the lumbar spinal cord of PBS-treated SOD1^{G93A} mice at the end stage of the disease was significantly increased compared

with that in PBS-treated B6SJL mice. However, Dex did not further upregulate MT-III in SOD1^{G93A} mice.

Dex Ameliorates the Disease Course in SOD1^{G93A} Mice, Even When Induction is Started at a Symptomatic Stage

We assessed the therapeutic effects of Dex on the disease course in SOD1^{G93A} mice. Sex-balanced SOD1^{G93A} mice were intraperitoneally injected with Dex (2 mg/kg) or PBS, starting from a presymptomatic stage (30 days of age) until the end stage of the disease (Fig. 2a–c). The time of disease onset in the Dex-treated SOD1^{G93A} mice, regarded as the age when

Fig. 2 Dexamethasone (Dex) ameliorates the disease course of superoxide dismutase-1 (SOD1)^{G93A} mice. Sex-balanced SOD1^{G93A} mice were injected intraperitoneally with Dex or phosphate-buffered saline (PBS) starting at 2 distinct stages of the disease: (a–c) a presymptomatic (30 days of age) or (d–f) a symptomatic stage (the time when peak body weight of the mice had decreased by 10 %). Kaplan–Meier curves for disease onset and survival in SOD1^{G93A} mice treated with Dex or PBS starting at either (a) presymptomatic or (d) symptomatic stage. Diagram showing the disease duration in SOD1^{G93A} mice treated with Dex or PBS starting at either (b) a presymptomatic or (e) a symptomatic stage. Motor coordination of SOD1^{G93A} mice treated with Dex or PBS starting at either (c) a presymptomatic or (f) a symptomatic stage. Results are expressed as mean±SD. ** $p < 0.01$

the mice reached their peak weight, was significantly delayed by 7 days from 87 ± 3.1 to 94 ± 4.9 days (Fig. 2a). Dex markedly prolonged lifespan by 20 days from 122 ± 3.1 to 142 ± 3.3 days (Fig. 2a). Disease duration, regarded as the period between disease onset and the clinical end point, was also significantly extended by 13 days from 35 ± 1.9 to 48 ± 5.8 days (Fig. 2b). Dex-treated SOD1^{G93A} mice exhibited a significant improvement in motor coordination, as measured using a rotarod apparatus (Fig. 2c).

The therapeutic effect of Dex on the disease course was also evident when the treatment was initiated on the day when peak body weight had decreased by 10 % (Fig. 2d–f) [23]. Dex significantly prolonged the lifespan by 15 days from 129 ± 6.7 to 144 ± 6.4 days (Fig. 2d), as well as the disease duration by 16 days from 18 ± 1.9 to 34 ± 5.1 days (Fig. 2e). Interestingly, 1 week after the start of treatment, motor coordination in the SOD1^{G93A} mice was temporally restored, and the decline in motor coordination was significantly delayed (Fig. 2f).

Dex Does Not Provide Therapeutic Benefits to SOD1^{G93A}/Mt^{-/-} Mice

Dex has divergent pharmacological actions other than MT-I/-II induction [29]. To clarify the direct contribution of MT-I/-II to Dex therapy, we generated SOD1^{G93A} mice in which MT-I/-II cannot be induced by Dex. We achieved this by creating

SOD1^{G93A} mice with homozygous deletion of *Mt-I/-II*. As expected, MT-I/-II was not detected in the lumbar spinal cord of SOD1^{G93A}/Mt^{-/-} mice, even after the injection of Dex (Fig. 3a).

Before determining the role of MT-I/-II in Dex treatment, we first characterized how the complete deletion of MT-I/-II influences the disease course in SOD1^{G93A} mice (Supplementary Fig. 2). The deletion of MT-I/-II significantly exacerbated the disease course, including disease onset (88 ± 2.9 days in SOD1^{G93A} vs 73 ± 2.4 days in SOD1^{G93A}/Mt^{-/-}), survival (130 ± 3.2 vs 100 ± 3.0 days), and disease duration (41 ± 0.8 vs 27 ± 1.2 days).

For a preclinical study using SOD1^{G93A}/Mt^{-/-} mice, we followed the same regimen as that used for presymptomatic treatment with Dex in SOD1^{G93A} mice. Sex-balanced SOD1^{G93A}/Mt^{-/-} mice were intraperitoneally injected with Dex (2 mg/kg) or PBS, starting at 30 days of age until the end stage of the disease (Fig. 3b–d). Interestingly, when MT-I/-II was absent from the SOD1^{G93A} mice, Dex did not have any therapeutic effects on the disease course, including disease onset (75 ± 3.9 days in PBS vs 77 ± 4.3 days in Dex), lifespan (102 ± 5.1 days vs 105 ± 3.9 days), and disease duration (27 ± 3.0 days vs 28 ± 3.1 days). Also, Dex did not ameliorate the impaired motor coordination in SOD1^{G93A}/Mt^{-/-} mice. These results strongly indicate that MT-I/-II is required for the therapeutic effects of Dex on SOD1^{G93A} mice.

Fig. 3 Dexamethasone (Dex) does not ameliorate the disease course of superoxide dismutase-1 (SOD1)^{G93A}/Mt^{-/-} mice. Sex-balanced SOD1^{G93A}/Mt^{-/-} mice were injected intraperitoneally with Dex (2 mg/kg) or phosphate-buffered saline (PBS) starting at a presymptomatic stage (30 days of age). (a) Western blot for metallothionein (MT)-I/-II in the lumbar spinal cords of Mt^{-/-} and SOD1^{G93A}/Mt^{-/-} mice treated with Dex or PBS. β -Tubulin was used as an internal marker. For MT-I/-II detection, recombinant MT-I protein was used as a positive control. (b) Kaplan–Meier curves for the disease onset and survival in SOD1^{G93A}/Mt^{-/-} mice treated with Dex or PBS. (c) Diagram showing the disease duration in SOD1^{G93A}/Mt^{-/-} mice treated with Dex or PBS. (d) Motor coordination of SOD1^{G93A}/Mt^{-/-} mice treated with Dex or PBS. Results are expressed as means $\pm$ SD. N.S. = not significant

MT-I/-II is Necessary for the Therapeutic Benefits of Dex on the Pathological Changes Observed in SOD1^{G93A} Mice

The deletion of *MT-I/-II* in SOD1^{G93A} mice accelerated the pathological process (Supplementary Fig. 3); therefore, we studied whether Dex-induced MT-I/-II could prevent pathological abnormalities. SOD1^{G93A} mice were intraperitoneally injected with Dex or PBS, starting at the day when the peak body weight of the mice had decreased by 10 % (Fig. 4). The lumbar spinal cords or ventral roots from the mice were dissected 20 days after the start of the injections. PBS-treated

SOD1^{G93A} mice at the end stage of the disease (120 days of age) exhibited a prominent reduction in the number of α -motor neurons, with survival rates of up to 41 % $\pm$ 3.1 %, compared with PBS-treated nontransgenic mice. In contrast, Dex significantly prevented the loss of α -motor neurons in SOD1^{G93A} mice (72 % $\pm$ 5.6 %; Fig. 4a, e). Dex also conferred protection to the axons in the ventral root. Only 41 % $\pm$ 2.7 % of the axons remained in PBS-treated SOD1^{G93A} mice at the end stage of the disease (120 days), whereas Dex significantly reduced the loss of axons in the ventral roots of age-matched SOD1^{G93A} mice (63 % $\pm$ 3.3 %; Fig. 4b, f). Furthermore, Dex

Fig. 4 Dexamethasone (Dex)-induced metallothionein-I/-II attenuates the pathologic changes in superoxide dismutase-1 (SOD1)^{G93A} mice.

Immunohistochemistry for (a) NeuN in lumbar spinal cords or (b) SMI-312 in ventral roots of SOD1^{G93A} mice treated with Dex (2 mg/kg) or phosphate-buffered saline (PBS), starting on the day when peak body weight of the mice had decreased by 10 %. Confocal microscopy showing lumbar spinal cord sections immunostained with (c) glial fibrillary acidic protein (GFAP) and (d) CD11b. Quantification of (e) α -motor neurons, (f) axons, (g) astrocytes, and (h) microglia ($n=3$ per group). Results are expressed as means $\pm$ SD. ** $p<0.01$ vs PBS-treated B6SJL. ## $p<0.01$ vs PBS-treated SOD1^{G93A}. Scale bars: (a, c, d) 100 μ m; (b) 10 μ m

strikingly inhibited the morphologic activation of astrocytes (Fig. 4c, g) and microglia (Fig. 4d, h).

We sought to clarify whether the therapeutic benefits of Dex on these pathological changes could be mediated through MT-I/-II (Fig. 5). In the absence of MT-I/-II, Dex did not rescue the pathological events in $SOD1^{G93A}$ mice, including the death of α -motor neurons (22 ± 1.3 % survival in PBS vs 21 ± 2.1 % in Dex), the loss of ventral root axons (29 ± 3.3 % remaining vs 31 ± 3.1 %), astrogliosis, and microgliosis.

Normalization of Copper Dyshomeostasis and Lipid Peroxidation by Dex is Mediated via MT-I/-II

We characterized the mechanisms of action underlying the therapeutic benefits of Dex-induced MT-I/-II. The deletion of *Mt-I/-II* in $SOD1^{G93A}$ mice exacerbated intracellular copper dyshomeostasis and lipid peroxidation (Supplementary Fig. 4) and increased the accumulation of $SOD1^{G93A}$ aggregates (Supplementary Fig. 5) and ubiquitinated proteins in the lumbar spinal cord (see Fig. 9); therefore, we asked whether Dex-

Fig. 5 Dexamethasone (Dex) does not rescue the pathologic changes in superoxide dismutase-1 ($SOD1^{G93A}/Mt^{-/-}$) mice. Immunohistochemistry for (a) NeuN in the lumbar spinal cord or for (b) SMI-312 in the ventral roots from $Mt^{-/-}$ and $SOD1^{G93A}/Mt^{-/-}$ mice at 100 days of age, which were dissected 70 days after the start of the Dex (2 mg/kg) or phosphate-buffered saline (PBS) injections ($n=3$ per group). Confocal microscopic images showing lumbar spinal cord sections immunostained with (c) glial fibrillary acidic protein (GFAP) and (d) CD11b. Quantification of (e) α -motor neurons, (f) axons, (g) astrocytes, and (h) microglia. Results are expressed as means $\pm$ SD. Scale bars: (a, c, d) 100 μ m; (b) 10 μ m. N.S. = not significant

induced MT-I/-II could modify these phenomena. We first determined the effects of Dex-induced MT-I/-II on copper dyshomeostasis (Fig. 6). An inductively coupled plasma-mass spectrometry analysis revealed that Dex significantly decreased the amount of copper ions in SOD1^{G93A} mice and restored the elevated level of copper ions to a normal level (Fig. 6a). This improvement was not related to alteration of the amount of copper that was bound to the SOD1 active site because Dex did not influence SOD1 enzymatic activity (Fig. 6b), which is considered to be a simple indicator of the copper metallation status of SOD1 [30]. We also determined the effects of Dex-induced MT-I/-II on the amount of lipid peroxides. The thiobarbituric acid method revealed that Dex significantly reduced the amount

of lipid peroxides in the spinal cords of SOD1^{G93A} mice (Fig. 6c).

MT-I/-II was also essential for the normalization of copper dyshomeostasis (Fig. 6d) and lipid peroxidation (Fig. 6f). Dex did not restore these alternations in SOD1^{G93A}/Mt^{-/-} mice.

Dex-induced MT-I/-II Decreases the Amount of Insoluble SOD1^{G93A} Aggregates

We examined the effects of Dex-induced MT-I/-II on SOD1 aggregates (Fig. 7), which are a major pathological hallmark of ALS [31]. Using high-speed centrifugation, detergent-insoluble fractions were isolated from the lumbar spinal cords of SOD1^{G93A} mice treated with Dex or PBS, starting at the day when the peak body weight of the mice had decreased by

Fig. 6 Dexamethasone (Dex)-induced metallothionein-I/-II normalizes copper dyshomeostasis and lipid peroxidation of superoxide dismutase (SOD1)^{G93A} mice. Inductively coupled plasma-mass spectrometry analysis showing the total amounts of copper ions in the lumbar spinal cords of either (a) SOD1^{G93A} or (d) SOD1^{G93A}/Mt^{-/-} mice treated with Dex (2 mg/kg) or phosphate-buffered saline (PBS). SOD1 activity in the spinal cords of either (b) SOD1^{G93A} or (e) SOD1^{G93A}/Mt^{-/-} mice treated with Dex or PBS. The concentrations of lipid peroxides (LPO) in the spinal cords of either (c) SOD1^{G93A} or (f) SOD1^{G93A}/Mt^{-/-} mice treated with Dex or PBS. Results are expressed as means±SD. *n*=3 per group. ***p*<0.01 vs PBS-treated B6SJL. ##*p*<0.01 vs PBS-treated SOD1^{G93A}. N.S. = not significant

10 %. The fractions were then analyzed using Western blotting with an antibody that recognizes human SOD1 but not endogenous murine SOD1 (Fig. 7a). Compared with PBS-treated SOD1^{G93A} mice at the end stage of the disease (120 days), Dex-treated SOD1^{G93A} mice at 120 days of age had significantly lower levels of insoluble SOD1, corresponding to a 39 % decrease (Fig. 7b, right). However, Dex did not influence the levels of the total (Fig. 7b, left) or the soluble mutant species (Fig. 7b, middle). Immunofluorescence studies

validated the above biochemical findings, showing that Dex substantially decreased the presence of SOD1 aggregates in the ventral horn of the lumbar spinal cord from SOD1^{G93A} mice (Fig. 7e, f).

MT-I/-II was also required for the modification of insoluble SOD1 aggregates. Dex did not decrease the amount of insoluble mutant SOD1 (Fig. 7c, d) or SOD1 aggregates (Fig. 7g, h) in the lumbar spinal cord of SOD1^{G93A}/Mt^{-/-} mice.

Fig. 7 Dexamethasone (Dex)-induced metallothionein-I/-II decreases the amount of insoluble superoxide dismutase (SOD1)^{G93A} aggregates. Western blots for human SOD1 (hSOD1) in whole homogenates, soluble, and insoluble fractions from the lumbar spinal cords of either (a) SOD1^{G93A} or (c) SOD1^{G93A}/Mt^{-/-} mice treated with Dex (2 mg/kg) or PBS. β -Tubulin in the whole homogenate was used as a loading control. The relative expression levels of hSOD1 in either (b) SOD1^{G93A} or (d) SOD1^{G93A}/Mt^{-/-} mice treated with Dex or phosphate-buffered saline (PBS). Confocal images of the lumbar spinal cord sections from either (e) SOD1^{G93A} or (g) SOD1^{G93A}/Mt^{-/-} mice treated with Dex or PBS. The sections were immunostained with hSOD1. Scale bars: 100 μ m. Quantification of fluorescence intensity for hSOD1 in (f) SOD1^{G93A} or (h) SOD1^{G93A}/Mt^{-/-} mice treated with Dex or PBS. Values are represented as means $\pm$ SD. $n=3$ per group. ** $p<0.01$. N.S. = not significant

Dex-induced MT-I/-II Effectively Interacts with SOD1^{G93A} Aggregates Within Astrocytes and Microglia

We explored the molecular relationship between MT-I/-II and SOD1^{G93A} aggregates (Fig. 8). Lumbar spinal cord sections from Dex- or PBS-treated SOD1^{G93A} mice were double-immunostained with antibodies against MT-I/-II and human SOD1. MT-I/-II was co-localized with SOD1^{G93A} aggregates in PBS-treated SOD1^{G93A} mice at the end stage of the disease. Dex-induced MT-I/-II was also co-localized with the SOD1^{G93A} aggregates and effectively interacted with the aggregates (Fig. 8a).

Given that Dex-induced MT-I/-II was distributed in the astrocytes and microglia of SOD1^{G93A} mice (Fig. 1g, h), we hypothesized that Dex-induced MT-I/-II may contribute to the reduction in SOD1^{G93A} aggregates within these cells. Double immunofluorescence showed that Dex-induced MT-I/-II remarkably decreased the number of SOD1^{G93A} aggregates in astrocytes (Fig. 8b) and microglia (data not shown) compared with PBS-treated SOD1^{G93A} mice.

Once again, MT-I/-II appears to be a key molecule in the reduction of SOD1^{G93A} aggregates during Dex treatment. Dex did not influence the amount of SOD1 aggregates within astrocytes (Fig. 8c) and microglia (data not shown) in SOD1^{G93A}/*Mt*^{-/-} mice.

MT-I/-II Interacts with Ubiquitinated Protein aggregates and Dex-induced MT-I/-II Decreases the Amount of These Aggregates

In addition to SOD1 aggregates, ubiquitinated protein aggregates are also a pathological feature of ALS [32]. We therefore examined the effects of Dex-induced MT-I/-II on ubiquitinated aggregates (Fig. 9). Lumbar spinal cord sections from Dex- or PBS-treated SOD1^{G93A} mice were double-immunostained with antibodies against MT-I/-II and ubiquitin (Fig. 9a). Confocal microscopy revealed that MT-I/-II was co-localized with ubiquitinated protein aggregates in PBS-treated SOD1^{G93A} mice, and that Dex-induced MT-I/-II effectively interacted with these aggregates. Furthermore, Dex-induced MT-I/-II resulted in a remarkable reduction in the number of aggregates (Fig. 9b, c). Dex did not decrease the amount of ubiquitinated protein aggregates in the spinal cords of SOD1^{G93A}/*Mt*^{-/-} mice (Fig. 9d, e).

Discussion

We have shown that the induction of MT-I/-II by Dex restored copper dyshomeostasis and modified the disease course observed in SOD1^{G93A} mice. We have also shown that MT-I/-II was essential for these effects because Dex did not rescue the ALS-like phenotype of SOD1^{G93A}/*Mt*^{-/-} mice. These results

Fig. 8 Dexamethasone (Dex)-induced metallothionein (MT)-I/-II efficiently interacts with superoxide dismutase-1 (SOD1)^{G93A} aggregates and decreases the amount of astrocytic SOD1^{G93A} aggregates. Confocal images showing lumbar spinal cord sections from either (a, b) SOD1^{G93A} or (c) SOD1^{G93A}/*Mt*^{-/-} mice treated with Dex (2 mg/kg) or phosphate-buffered saline (PBS). The sections were double immunostained with human SOD1 and either (a) MT-I/-II or (b, c) glial fibrillary acidic protein (GFAP). The arrows in (a) indicate co-aggregates of MT-I/-II and SOD1^{G93A}. Scale bars: 20 μ m. *n*=3 per group

are consistent with our recent findings, which demonstrated that overexpression of MT-I in SOD1^{G93A} mice ameliorates the disease course through the normalization of copper dyshomeostasis [15]. Thus, the functional potentiation of MT-I/-II is a promising strategy for the treatment of mutant SOD1-linked ALS.

What are the therapeutic advantages of MT-I/-II induction? Three main benefits may exist. First, despite the striking induction of MT-I/-II during the disease process [8, 15, 28], MT-I/-II was further induced by Dex in the spinal cords of SOD1^{G93A} mice. This highly inducible property may be advantageous for its clinical use in ALS, even though MT-I/-II levels are already increased in the spinal cords of patients with sporadic ALS [33]. Second, the therapeutic benefits of MT-I/-II required only a modest increase in the protein level, equivalent to a 20 % increase. The degree of MT-I/-II induction observed in Dex-treated SOD1^{G93A} mice corresponded to that observed in MT-I overexpressing SOD1^{G93A} mice [15]. Thus, MT-I/-II exerted neuroprotection against SOD1^{G93A} toxicity, even though the induction was largely restricted. Finally, MT-I/-II induction significantly prolonged the survival and prevented the death of motor neurons, even when the induction was started on the day when peak body weight had decreased by 10 %, which is probably equivalent to an advanced stage in a clinical setting.

Treatment involving MT-I/-II induction might also be capable of influencing the pathogenesis of ALS by suppressing the expression of SOD1 mutants and their pathogenetic effects. We consider there are 4 rationales for suggesting MT-I/-II as a treatment target. First, MT-I/-II directly interacted with SOD1^{G93A} aggregates in the spinal cord [15], and Dex-induced MT-I/-II effectively recognized these aggregates. Second, the genetic or pharmacological induction of MT-I/-II significantly decreased the amount of insoluble SOD1^{G93A} [15]. Third, the ablation of *Mt*-I/-II enhanced the accumulation of insoluble SOD1^{G93A}. Finally, MT-I/-II induction significantly decreased SOD1^{G93A} expression in astrocytes and microglia. The expression of SOD1 mutants in astrocytes and microglia is a well-known determinant of the disease duration [23, 34–36]. This is consistent with MT-I/-II induction having the ability to significantly prolong survival even when induction was initiated on the day when peak body weight of the mice had decreased by 10 %.

Fig. 9 Metallothionein (MT)-I/-II is a key regulator for ubiquitinated protein aggregates in superoxide dismutase (SOD1)^{G93A} mice. (a) Confocal images showing lumbar spinal cord sections from SOD1^{G93A} mice treated with dexamethasone (Dex) (2 mg/kg) or phosphate-buffered saline (PBS), starting on the day when peak body weight of the mice had decreased by 10 %. The sections were double-immunostained with MT-I/-II and ubiquitin. The arrows indicate co-aggregates of MT-I/-II and ubiquitinated proteins. Confocal microscopy images showing lumbar spinal cord sections immunostained with ubiquitin in either (b) SOD1^{G93A} or (d) SOD1^{G93A}/*Mt*^{-/-} mice treated with Dex or PBS. Scale bars: (a) 20 μ m; (b, c) 100 μ m. Quantification of fluorescence intensity of ubiquitinated proteins in (c) SOD1^{G93A} or (e) SOD1^{G93A}/*Mt*^{-/-} mice treated with Dex or PBS. Values are represented as means $\pm$ SD. $n=3$ per group. ** $p<0.01$. N.S. = not significant

Given the function of MT-I/-II as a copper regulator, our data strongly suggest that MT-I/-II modifies SOD1 aggregates through the normalization of copper dyshomeostasis. How does MT-I/-II modulate SOD1 aggregates? We speculate that MT-I/-II attenuates the impairment of the ubiquitin-proteasome system, which is probably triggered by copper dyshomeostasis, promoting the degradation of SOD1 aggregates by the system. This speculation is supported by the following three observations. First, copper ions inhibited the proteolytic activities of the proteasome in the bovine central nervous system [37]. Second, the coordination of copper ions to ubiquitin compromised the structural stability of the protein, leading to the aggregation of ubiquitin [38]. Finally, increased levels of copper ions in the spinal cords of SOD1^{G93A} rats were concomitant with the inhibition of proteasome activity and significantly shortened survival [39]. Thus, MT-I/-II may remove aberrant copper ions from ubiquitinated proteins through a direct interaction with ubiquitin, facilitating proteolytic activity of the proteasome.

Apart from the normalization of intracellular copper dyshomeostasis, another possible mechanism by which Dex-

induced MT-I/-II ameliorates the disease course should be discussed. MT-I/-II is a well-known antioxidant, and its neuroprotective effects may be mediated through the suppression of oxidative stress [13]. Oxidative stress has been implicated in the pathogenesis of mutant SOD1-linked ALS [40], and is exacerbated by intracellular copper dyshomeostasis. In the present study, we showed that the endogenous MT-I/-II level was correlated with oxidative damage in the spinal cords of SOD1^{G93A} mice. These observations indicate that the upregulation of MT-I/-II by Dex treatment significantly decreased the amount of lipid peroxides in SOD1^{G93A} mice, whereas the absence of *Mt-I/-II* in SOD1^{G93A} mice resulted in a further increase in the level of lipid peroxides.

The therapeutic benefits observed in the SOD1^{G93A} mice in the present study might be partly mediated through Dex itself. A Dex dose of 2 mg/kg in mice corresponds to a dose of 500 mg/kg in humans, which approximates the dose used in pulsed steroid therapy (three consecutive administrations of methyl prednisolone at a dose of 500–1000 mg/kg). We suspect that the effects of adrenal glucocorticoids were negligible in the present study because the dose of Dex was sufficient to

suppress the effects of endogenous adrenal glucocorticoids. Nevertheless, our observation that treatment with Dex in SOD1^{G93A}/*Mt*^{-/-} mice did not result in a good outcome means that the possible therapeutic contribution of glucocorticoids cannot be ruled out. The possible cooperation of intrinsic glucocorticoids with or without MT-I/-II should be thoroughly considered. In fact, glucocorticoid levels increase constantly in patients with ALS, whereas their levels exhibit a circadian rhythm in healthy humans [41]. Moreover, the serum glucocorticoid levels were significantly higher in SOD1^{G93A} mice at a terminal stage of illness and were negatively correlated with both disease onset and survival [42]. Additionally, wobbler mice, another rodent model for ALS [43], become vulnerable after adrenalectomy (surgical removal of the adrenal glands, which produce intrinsic glucocorticoids) [44]. Although these changes might be secondary rather than primary effects, further studies investigating how alterations in endogenous glucocorticoids after adrenalectomy influence the disease course in SOD1^{G93A} mice with or without *Mt-I/-II* are needed.

Acknowledgments This work was supported by a Grant-in-Aid for a research fellowship of the Japan Society for the Promotion of Science (JSPS) for Young Scientists from JSPS (08J10542 to E.T.), by a Grant-in-Aid for Exploratory Research from JSPS (21659222 to S.O.), and by an Academic Frontier Project for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology of Japan (years 2007–2009 to S.O.). We thank Drs. Shin-ichi Miyairi (Laboratory of Organic Chemistry, School of Pharmacy, Nihon University, Funabashi, Chiba, Japan) and Akira Naganuma (Laboratory of Molecular and Biochemical Toxicology, School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi, Japan) for kindly providing the anti-MT-III antibody.

Conflict of Interest The authors declare that they have no conflict of interest.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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