Apoptosis-Inducing Factor and Cyclophilin A Cotranslocate to the Motor Neuronal Nuclei in Amyotrophic Lateral Sclerosis Model Mice

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SUMMARY

Aims: Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron disease whose mechanism is not understood. Recently, it was reported that apoptosis-inducing factor (AIF) was involved in motor neuronal cell death in ALS model mice, and AIF-induced neuronal cell death by interacting with cyclophilin A (CypA). However, it is unknown whether the CypA and AIF-complex induces chromatinolysis in ALS. Therefore, in the present study, we investigated the process of motor neuron degeneration as the disease progresses and to determine whether the CypA-AIF complex would play a role in inducing motor neuronal cell death in mutant superoxide dismutase 1 (SOD1)^{G93A} ALS model mice. **Methodology:** We prepared the nuclear fractions of spinal cords and demonstrated the nuclear translocation of CypA with AIF in SOD1^{G93A} mice by immunoprecipitation. The localization of CypA and AIF in the spinal cords was assessed by immunohistochemistry. **Results:** In the spinal cords of SOD1^{G93A} mice, the expressions of CypA and AIF were detected in the motor neurons, and CypA and AIF cotranslocated to the motor neuronal nuclei with CypA. Furthermore, the expression of CypA was detected in GFAP-positive astrocytes, but not in CD11b-positive microglial cells. On the other hand, these findings were not detected in the spinal cords of wild-type mice. **Conclusions:** From these results, we suggest that CypA and AIF may play cooperative and pivotal roles in motor neuronal death in the murine ALS model.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease, caused by the progressive degeneration of motor neurons in the spinal cord, brainstem, and motor cortex [1]. The majority of cases (90%) have no genetic component (i.e., sporadic ALS). The familial form of ALS accounts for ~10% of cases and is usually transmitted as an autosomal dominant trait [2]. Mutations in the SOD1 protein, a ubiquitously expressed and highly conserved metalloenzyme involved in the detoxification of free radicals, are responsible for ~15% of the familial form of ALS [3–5] and lead to progressive, selective motor neuron degeneration as a result of acquired toxic prop-

erties. However, the exact mechanism responsible for motor neuron degeneration in ALS is not well known [6,7].

Apoptosis-inducing factor (AIF), a key regulator of cell death, has been suggested to control a caspase-independent apoptotic pathway [8,9]. AIF is a flavoprotein with NAD(P)H oxidase and monodehydroascorbate reductase activities localized in the mitochondrial intermembrane space [10]. Under pathological conditions, AIF is released from the mitochondria and translocates into the nuclei of dying neurons, acting together with endonuclease G [10]. Translocation of AIF into the nuclei in dying cells has been observed in many disease models such as ALS [11], brain trauma [12], and cerebral hypoxic ischemia [13].

Cyclophilins constitute a family of phylogenetically conserved proteins found in prokaryotes as well as in humans. Cyclophilin A (CypA), the first described and best characterized member of this family, is primarily localized in cytosol [14]. A key feature of CypA is cis-trans peptidyl prolyl isomerase activity, including accelerated protein folding [15,16]. CypA was also discovered as an intracellular ligand of the immunosuppressive drug cyclosporine A [17]. When the CypA-cyclosporine A complex is formed, it can bind to and inhibit calcineurin (protein phosphatase 2B) [18]. The inhibition of calcineurin activity causes a decrease of the nuclear factor of activated T cells, preventing expression of cytokine interleukin-2 and immune cell activation [19,20].

CypA cooperates with AIF during apoptosis-associated chromatinolysis [21]. Disruption of the CypA homologue *CPR1* in yeast cells abrogated cell death induced by overexpression of AIF [22], and Jurkat cells lacking CypA expression are relatively resistant to AIF-induced cell death [21]. Furthermore, recombinant CypA and AIF proteins have higher DNase activity than either of the proteins alone, and mutant CypA that lacks cis-trans peptidyl prolyl isomerase activity still cooperates with AIF to mediate chromatinolysis. However, the involvement of CypA and AIF in ALS has not been investigated.

Given the background described here, we performed the present study to investigate whether (1) CypA and AIF interact and cotranslocate to the nuclei of motor neurons in a mouse model of ALS and (2) CypA expresses in other cells (reactive astrocytes and microglial cells) to clarify the mechanism of ALS pathogenesis.

Methods

Animals

Transgenic mice overexpressing mutated (glycine to alanine in position 93) human SOD1 (G93A) (B6SJL-Tg [SOD1-G93A] 1Gur/J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The hemizygous SOD1^{G93A} mice were maintained by mating transgenic males with wild-type (WT) females. Mouse genotypes were determined by polymerase chain reaction analysis as previously reported [23,24]. WT (non-transgenic) littermates served as controls. All experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

Symptomatic Analysis

The age of death was defined as an animal's inability to right itself after 15 seconds. The date the animal became moribund represented the experimental endpoint,

and the date of death was recorded. In conjunction, mice were tested for their ability to maintain balance on a rotating rod (3 cm diameter) at 5 rpm using a Rota Rod apparatus (Bio Medica Ltd., Osaka, Japan). To adapt the mice to the apparatus, they were allowed to adjust to balancing on the rod as it rotated (5 rpm) for 10 min each time for one week from 63 days of age. After adaptation, locomotor performance was evaluated as the rod rotated at 5 rpm starting at 70 days of age. Each session consisted of three trials (10 min/trial). Performance time for each trial was recorded as the longest time the mice could stay on the rod without falling. We performed measurements two times per week until the animals became moribund.

Tissue Preparation

SOD1^{G93A} and WT mice at 10 (early disease), 14 (decreasing motor function), and 18–20 (endpoint) weeks old ($n = 5$) were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) (Nembutal, Dainippon, Osaka, Japan) and perfused with 2% (w/v) paraformaldehyde solution in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. Spinal cord tissues were removed after a 15-min perfusion at 4°C and immersed in the same fixative solution for 24 h. Each spinal cord included L1, L2, and L3 levels, and was soaked in 25% (w/v) sucrose at 4°C for 1 day, and then frozen in embedding compound (Tissue-Tek, Sakura Finetechnical Co. Ltd., Tokyo, Japan). Embedded tissues were immediately frozen with liquid nitrogen and stored at –80°C. Serial transverse sections were cut on a cryostat to a thickness of 20 μ m at 2-mm intervals (three sections total for each segment) and used for cresyl violet staining or immunohistochemistry.

Immunohistochemistry

The sections were stained with the following antibodies: (1) mouse anti-AIF antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA); (2) rabbit anti-CypA antibody (1:5000; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA, USA); (3) goat anticholine acetyltransferase (ChAT) antibody (1:5000; Millipore, Bedford, MA, USA); (4) mouse anti-GFAP antibody (1:1000; Millipore); (5) mouse anti-CD11b antibody (1:1000; BMA Biomedicals, Augst, Switzerland); and (6) rabbit anti-Iba1 antibody (1:800; Wako Pure Chemical Industries, Ltd., Osaka, Japan) within Can Get Signal immunostain solution A (Toyobo CO., LTD., Osaka, Japan).

Sections were treated with 0.3% H₂O₂ in methanol for 30 min at room temperature and blocked with mouse-on-mouse blocking reagent for 1 h at room temperature. Anti-AIF antibodies were applied to the sections overnight at 4°C. After washing the sections with 0.01 M PBS, sections were incubated with biotinylated

antimouse IgG for 2 h followed by washing, and were incubated with avidin-biotin-peroxidase complex for 30 min at room temperature. The sections were finally visualized using diaminobenzidine (DAB)/H₂O₂ substrate for peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA).

When double-immunostaining for CypA/AIF, CypA/GFAP, CypA/CD11b, or AIF/ChAT were performed, these antibodies were applied to the sections overnight at 4°C after blocking with 0.01 M PBS containing 10% normal goat serum (Vector) or mouse-on-mouse blocking reagent (M.O.M. immunodetection kit, Vector) for 1 h. After washing the sections with 0.01 M PBS, immunoreactivity was visualized by incubating them for 2 h at room temperature with secondary antibodies conjugated with Alexa 488 rabbit anti-mouse, Alexa 546 rabbit anti-goat, Alexa 488 goat anti-rabbit, or Alexa 546 goat anti-mouse (1:1000; Invitrogen Japan K.K., Tokyo, Japan). At the end of immunostaining, Hoechst 33342 (1:1000) was added to the samples for 30 min to visualize the nuclei.

The images of the spinal cord were taken using a microscope (BX50; Olympus, Tokyo, Japan) fitted with $\times 20$ and 40 microscope objective lenses or a confocal microscope (FV10i, Olympus, Tokyo, Japan). The images visualized by DAB were taken using a digital camera (Coolpix 4500, Nikon) and some immunofluorescence images were taken using a charge-coupled device camera (DP30BP; Olympus) at 1360 $\times$ 1024 pixels via Metamorph (Universal Imaging Corp., Downingtown, PA, USA).

Isolation of Spinal Cord Nuclei

Nuclei were prepared from mice spinal cords according to the method of Yeo [11]. Mice nuclei were excised and homogenized in lysis buffer containing 20 mM Hepes buffer solution, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.25 M sucrose, and protease inhibitor cocktail for 10 strokes using a dounce homogenizer at 4°C. The lysates were centrifuged at 700 $\times g$ for 10 min. The pellet was designated as nuclear fraction and the supernatant as microsomal and cytosolic fraction.

Immunoprecipitation

CypA was immunoprecipitated from several fractions from SOD1^{G93A} and WT mice. Equal amounts of protein were pre-cleaned with normal rabbit IgG (Santa Cruz) and protein G agarose beads. Immunoprecipitation was carried out in lysis buffer with anti-CypA polyclonal antibody for 2 h at 4°C, followed by the incubation in protein G agarose beads for 2 h at 4°C. The resulting immune complexes were pelleted by centrifugation. After wash-

ing in lysis buffer, immunoprecipitated proteins were immunoblotted.

Immunoblotting

Immunoprecipitation lysates were eluted by boiling for 5 min in sodium dodecyl sulfate-sample buffer (Wako). The proteins were separated on 5–20% sodium dodecyl sulfate-polyacrylamide gels and polyvinylidene difluoride membranes (Immunobilon-P; Millipore). After blocking with Block Ace (Snow Brand Milk Products Co. Ltd., Tokyo, Japan) for 30 min, the membranes were incubated with the primary antibodies (mouse anti-AIF antibody [Santa Cruz], rabbit anti-CypA antibody [BIOMOL], or rabbit anti-glyceraldehyde-3-phosphate dehydrogenase [GAPDH] antibody [Cell Signaling Technology, Inc., Danvers, MA, USA]) overnight at 4°C. Subsequently, the membranes were incubated with secondary antibody (horse radish peroxidase [HRP]-conjugated goat anti-rabbit IgG [Pierce Biotechnology, Rockford, IL] or goat anti-mouse IgG [Pierce Biotechnology]). The immunoreactive bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and then measured using LAS-4000 UV mini (FUJIFILM, Tokyo, Japan).

Data Analysis

Motor neurons bearing AIF and/or CypA in laminae VII, VIII, and IX of the ventral horn were counted in one section of L1, L2, and L3, respectively. The numbers of neurons in all segments were summed for each animal. The results were expressed as positive cells in laminae VII, VIII, and IX. Survival analysis was performed by the Kaplan–Meier method. Data were presented as means $\pm$ S.E.M. Statistical comparisons were made using Student's *t*-test, with *P* < 0.05 considered to indicate statistical significance.

Results

Lifespan, Motor Performance, and Weight Loss in SOD1^{G93A} Mice

To clarify the disease progression of SOD1^{G93A} mice in the present study, we investigated lifespan, motor performance, and weight loss in SOD1^{G93A} mice. Average survival time in SOD1^{G93A} mice was 118.9 $\pm$ 3.39 days (Figure S1A), and muscle weakness was observed as the disease progressed, starting at 98–100 days of age (Figure S1B). In addition, the body weight of SOD1^{G93A} mice was significantly decreased at 112–119 days of age compared with WT mice (Figure S1C). These findings were consistent with previous studies [25,26]. In histochemical

analysis, we observed motor neuronal decrease and glial activation as the disease progressed in the spinal cords of SOD1^{G93A} mice (Figure S1D–F). All pictures were taken at the anterior horn of the L1 section in the spinal cords.

AIF and CypA Expression in the Spinal Cord

AIF is synthesized in the cytosol as 67 kDa precursor protein and matures into 57 kDa protein after being transported into the mitochondria [10]. CypA and AIF were expressed in the spinal cords of both SOD1^{G93A} and WT mice (Figure 1). In WT mice, CypA and AIF immunoreactivity was found in large cells with the typical morphology of motor neurons in the anterior horn (laminae VII, VIII, and IX) (Figures 1B and C). In contrast,

the SOD1^{G93A} spinal cords showed CypA and AIF immunoreactivity in shrunken motor neuron-like cells (Figures 1B and C, arrowheads), and CypA immunoreactivity was detected in the cells with the morphology of activated astrocytes (Figure 1C, arrows) in the anterior horn. The number of CypA-immunoreactive-shrunken motor neuron-like cells was significantly increased in the SOD1^{G93A} spinal cords at 14 weeks old compared with age-matched WT mice (Figure 1D).

Expression of AIF in the Motor Neurons

In Figure 1B, the morphology of AIF-immunoreactive cells observed motor-like neurons in SOD1^{G93A} and WT

Figure 1 Time-dependent alteration of CypA and AIF expression in SOD1^{G93A} and wild-type mice. **(A)** Schematic drawing showing the spinal cord regions. The number of neuronal cells that CypA translocated to nuclei was counted in filled-in area (laminae VII, VIII, and IX). Immunohistochemical analysis against **(B)** AIF or **(C)** CypA in the anterior horn of lumbar spinal cords from SOD1^{G93A} and wild-type mice at 10, 14, and

18–20 (endpoint) weeks old. Arrow head, motor neuron-like cells; arrows, astroglial-like cells. Scale bars = 100 μ m **(B)**, 20 μ m **(C)**, or 5 μ m (high-magnification views), respectively. **(D)** Quantitative analysis of neuronal cells that CypA translocated to the nucleus from SOD1^{G93A} and wild-type mice at 14 weeks old. Each value represents the mean $\pm$ S.E.M. ($n = 5$). ** $P < 0.01$ versus wild-type (Student's t -test).

A **B**

Figure 2 Observation of AIF expression in motor neurons. **(A)** Schematic drawing showing the spinal cord regions. Immunofluorescent labeling of AIF (green), anti-choline acetyltransferase (red), and Hoechst 33342 (blue) in the anterior horn (laminae VII, VIII, and IX) of lumbar spinal cords from SOD1^{G93A} and wild-type mice. In the wild-type spinal cords at 14 weeks old, AIF expressed within the large motor neuronal cytoplasm (**2B**, upper panels). In the SOD1^{G93A} spinal cords, AIF was translocated into the motor neuronal nuclei (**2B**, lower panels). Scale bars = 5 μ m.

spinal cords. To clarify this, double immunofluorescence was performed for AIF and ChAT in the spinal cords to visualize motor neurons. As expected, the large cells expressing AIF in SOD1^{G93A} and WT spinal cords at 14 weeks old were motor neurons (Figure 2). Schematic drawing shows the spinal cord regions (Figure 2A). The images of AIF and ChAT-positive cells are showed in the filled-in area (laminae VII, VIII, and IX). In WT mice, AIF was expressed within the large motor neuronal cytoplasm (Figure 2B, upper panels). In addition, most of the shrunken AIF-immunoreactive cells were ChAT-positive motor neurons, and AIF was translocated into the motor neuronal nuclei in the SOD1^{G93A} spinal cords (Figure 2B, lower panels).

AIF and CypA Cotranslocated into the Motor Neuronal Nuclei

Figure 2 confirms that AIF was expressed in the motor neurons, and translocated to the nuclei in the diseased SOD1^{G93A} spinal cords. Then, to investigate whether AIF colocalized with CypA in the motor neuronal nuclei, we performed double immunofluorescence for CypA and

AIF in SOD1^{G93A} and WT spinal cords at 14 weeks old. In WT spinal cords, most of CypA-immunoreactive motor neurons were expressed by AIF within the cytoplasm (Figure 3A, upper and lower left-hand panels). In addition, the complete nucleus translocation of both CypA and AIF was evident in most of the motor neurons in SOD1^{G93A} spinal cords (Figure 3A, upper and lower right-hand panels). Quantitative analysis of motor neuronal nuclei translocation of CypA and AIF is shown in Figure 3C. The number of shrunken motor neuronal nuclei cotranslocated CypA and AIF was significantly increased in SOD1^{G93A} spinal cords at 14 weeks old compared with age-matched WT mice. Furthermore, coimmunoprecipitation from nuclear fractions of SOD1^{G93A} spinal cords showed the association between CypA and AIF, but not in WT mice (Figure 4).

CypA Expression in Activated Astrocytes

To confirm other CypA-immunoreactive cells (except for motor neurons), we double-stained the spinal cord sections with CypA and cell-type markers (i.e., CD11b and GFAP). In SOD1^{G93A} spinal cords, there was strong

Figure 3 Nuclear translocation of CypA and AIF to the motor neuronal nuclei. **(A)** The stained sections were analyzed by confocal microscopy. In the upper panels, immunofluorescent labeling of AIF (red), CypA (green), and Hoechst 33342 (blue) in the anterior horn of lumbar spinal cords from SOD1^{G93A} and wild-type mice at 14 weeks old. Lower panels show the confocal z-sectioning at 0.43 μ m intervals. XZ- and YZ-axis sections are shown in each panel. Scale bars = 5 μ m. **(B)** Schematic drawing showing the spinal

cord regions. The number of CypA and AIF-positive cells is counted in the filled-in area (laminae VII, VIII, and IX). **(C)** Quantitative analysis of motor neurons that AIF and CypA cotranslocated to the nuclei from SOD1^{G93A} and wild-type mice at 14 weeks old. The number of shrunken motor neuronal nuclei cotranslocated CypA and AIF was significantly increased in the SOD1^{G93A} spinal cords compared with wild-type. Each value represents the mean $\pm$ S.E.M. ($n = 5$). ** $P < 0.01$ versus wild-type (Student's *t*-test).

immunoreactivity of CD11b or GFAP (Figures 5A and B). Double immunofluorescence revealed that CypA colocalized GFAP well, but not CD11b, indicating that CypA was partly localized in reactive astrocytes (Figures 5A and

B), and CypA was not translocated to astrocyte nuclei in SOD1^{G93A} spinal cords at 14 weeks old (Figure 5E). These pictures showed GFAP-positive astrocytes and CD11b-positive microglial cells in laminae VII, VIII, and IX

Figure 4 The interaction of CypA and AIF with the nuclei in the motor neurons of SOD1^{G93A} mice. To confirm the nuclear translocation of CypA with AIF in the SOD1^{G93A} spinal cords, we demonstrated coimmunoprecipitation of CypA and AIF in the spinal cords from SOD1^{G93A} and wild-type mice at 14 weeks old. Immunoblotting antibodies are indicated on the right-hand side.

(Figure 5C). The number of CypA-positive astrocytes was significantly increased in SOD1^{G93A} spinal cords at 14 weeks old compared with age-matched WT mice (Figure 5D).

Discussion

The purpose of the present study was to investigate the localization of AIF and/or CypA in motor neurons, astrocytes, or microglial cells in the spinal cords, and also the involvement of the CypA-AIF interaction in the diseased SOD1^{G93A} mice.

In the present study, we confirmed that AIF translocated to the nuclei (Figure 2) and that AIF colocalized with CypA (Figure 3) in the diseased SOD1^{G93A} mice. Recent studies have suggested that activation of caspases might not be sufficient for motor neuron degeneration in ALS [27–29]. A dominant negative inhibitor of the interleukin-1 β -converting enzyme/caspase-1, anti-apoptotic protein Bcl-2, and a broad caspase inhibitor, zVAD-fmk, significantly delay the onset of ALS in mice [30–34]. These results have suggested that the expression of SOD1^{G93A} might induce neurodegeneration through multiple pathways involving multiple caspases and/or caspase-independent pathways, and therefore a caspase-independent cell death pathway might contribute to motor neuronal cell death in ALS pathogenesis as well as the caspase-dependent one. Under pathological conditions, translocation of AIF, a key regulator of caspase-independent cell death, initiates cell apoptosis by cleavage of internucleosomal DNA to relative large fragments. AIF nuclear translocation has been observed in a variety of cell culture systems and animal models subjected to a variety of stress conditions. In some models of apoptosis, AIF has been reported to contribute to neuronal death in the pathogenesis of some acute and chronic (neurodegenerative) diseases [13,35,36]. Furthermore, the expres-

sion levels of AIF were significantly increased in spinal cords with progression of ALS, and AIF translocated into the motor neuronal nuclei in sporadic ALS patients and SOD1^{G93A} mice [11,37]. Facilitating AIF translocation to nuclei consequently led to motor neuron-like cell apoptosis [38]. Therefore, it might be extremely important for the involvement of the caspase-independent cell death pathway in ALS pathogenesis. Especially, AIF nuclear translocation might underlie some of the events linked to ALS disease progression.

We also investigated whether AIF colocalized with CypA in the motor neuronal nuclei in SOD1^{G93A} mice. *In vitro* study, it has been suggested that CypA has a latent nuclease activity [39], and other research [21] recently discovered that AIF interacted with CypA to induce chromatinolysis. Furthermore, AIF was needed for the nuclear translocation of CypA in cerebral hypoxia-ischemia model mice [13]. In SOD1^{G93A} mice, the expression of AIF in the spinal cords increased as the disease developed and AIF translocated to the motor neuronal nuclei. In the present study, the AIF translocation with CypA to the motor neuronal nuclei was confirmed by immunohistological analysis and IP/immunoblotting in the SOD1^{G93A} mice, but not in the WT mice (Figures 3 and 4). These data suggest that AIF translocation to the nuclei is accompanied by CypA in damaged motor neurons of SOD1^{G93A} mice. CypA and AIF bind to each other upon induction of cell-death signaling and mitochondrial membrane permeabilization [13]. Mitochondrial dysfunction and oxidative stress have been involved in ALS pathogenesis [40–42]. Decreased mitochondrial respiratory activity has been noted in spinal cords from SOD1^{G93A} mice [43,44] and in cellular models of the disease [45]. Following mitochondrial damage, AIF is released into cytosol from the mitochondria. It has been suggested that the AIF release was involved in the final step of motor neuron degeneration in ALS pathogenesis. Furthermore, CypA is required for efficient nuclear translocation of AIF in degenerated neurons, and the interaction of CypA and AIF occurs before or when nuclear translocation begins, in the cytosol after cerebral hypoxia-ischemia. Hence, cotranslocation of CypA and AIF in ALS may occur at the late stage of apoptosis cascade. In addition, the inhibition of the interaction between CypA and AIF may delay motor neuronal death and disease progression.

A large proportion of nonneuronal cells in the ventral horn of the spinal cord are astrocytes. Aside from the dramatic loss of motor neurons, spinal cord specimens from both human ALS cases and SOD1^{G93A} mice exhibited a strong glial activation [46–48]. In addition, astrocytes isolated from SOD1^{G93A} rats [49] or mice [50,51] are toxic to cocultured motor neurons. Because glial cells can produce potent proapoptotic molecules,

Figure 5 Other cells that express CypA in the lumbar spinal cords of SOD1^{G93A} mice. The lumbar spinal cords from SOD1^{G93A} and wild-type mice at 14 weeks old were double-immunostained with antibodies against (A) CD11b (red) and cyclophilin A (CypA, green) and (B) glial fibrillary acid protein (GFAP, red) and CypA (green) to confirm the types of CypA-expressing cells. Scale bars = 20 μ m. (C) Schematic drawing showing the spinal cord regions. All images of glial cells are shown in the filled-in area (laminae VII, VIII, and IX) in the anterior horn of the spinal cords. (D) Quan-

titative analysis of CypA-positive astrocytes from SOD1^{G93A} and wild-type mice at 14 weeks old. The number of CypA-positive astrocytes was significantly increased in the SOD1^{G93A} spinal cords compared with wild-type. Each value represents the mean $\pm$ S.E.M. ($n = 5$). ** $P < 0.01$ versus wild-type (Student's t -test). (E) Immunofluorescent labeling of GFAP (red), CypA (green), and Hoechst 33342 (blue) in the anterior horn of lumbar spinal cords from SOD1^{G93A} mice at 14 weeks old. Scale bars = 10 μ m.

including tumor necrosis factor- α and interleukin-1 β , it is possible that glial activation triggers or enhances the apoptotic cell death of motor neurons in ALS. Therefore, functional alterations in activated astrocytes can shape the interaction with surrounding cells such as damaged neurons, microglia, and immune cells, and consequently can modulate motor neuron survival [52–55]. It is reported that AIF expressed in GFAP-positive astrocytes of the spinal cords of SOD1^{G93A} mice [11] and, in the present study, CypA expressed in GFAP-positive astrocytes, but

not in activated microglial cells in the spinal cords of SOD1^{G93A} mice. Furthermore, unlike the motor neurons, CypA was not translocated to the astrocyte nuclei in the spinal cords of SOD1^{G93A} mice. These findings indicate that CypA and AIF expression in activated astrocytes may partly contribute to ALS pathogenesis, and CypA translocation to the nuclei may involve the cell death pathway in ALS. Further studies will be needed to clarify the precise roles performed by CypA and AIF in the spinal cords of SOD1^{G93A} mice.

In conclusion, our findings indicate that CypA and/or AIF involve motor neuronal degeneration in ALS SOD1^{G93A} mice, providing useful information to clarify the mechanism of motor neuronal death in ALS.

Conflict of Interest

The authors have no conflict of interest.

References

- Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. *N Engl J Med* 2001;**344**:1688–1700.
- Mulder DW, Kurland LT, Offord KP, Beard CM. Familial adult motor neuron disease: Amyotrophic lateral sclerosis. *Neurology* 1986;**36**:511–517.
- Rosen DR, Siddique T, Patterson D, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 1993;**362**:59–62.
- Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 1995;**64**:97–112.
- Pramatarova A, Figlewicz DA, Krizus A, et al. Identification of new mutations in the Cu/Zn superoxide dismutase gene of patients with familial amyotrophic lateral sclerosis. *Am J Hum Genet* 1995;**56**:592–596.
- Pasinelli P, Brown RH. Molecular biology of amyotrophic lateral sclerosis: Insights from genetics. *Nat Rev Neurosci* 2006;**7**:710–723.
- Boillee S, Vande VC, Cleveland DW. ALS: A disease of motor neurons and their nonneuronal neighbors. *Neuron* 2006;**52**:39–59.
- Susin SA, Lorenzo HK, Zamzami N, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999;**397**:441–446.
- Cregan SP, Fortin A, MacLaurin JG, et al. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J Cell Biol* 2002;**158**:507–517.
- Cande C, Cecconi F, Dessen P, Kroemer G. Apoptosis-inducing factor (AIF): Key to the conserved caspase-independent pathways of cell death? *J Cell Sci* 2002;**115**:4727–4734.
- Oh YK, Shin KS, Kang SJ. AIF translocates to the nucleus in the spinal motor neurons in a mouse model of ALS. *Neurosci Lett* 2006;**406**:205–210.
- Zhang X, Chen J, Graham SH, et al. Intranuclear localization of apoptosis-inducing factor (AIF) and large scale DNA fragmentation after traumatic brain injury in rats and in neuronal cultures exposed to peroxynitrite. *J Neurochem* 2002;**82**:181–191.
- Zhu C, Wang X, Deinum J, et al. Cyclophilin A participates in the nuclear translocation of apoptosis-inducing factor in neurons after cerebral hypoxia-ischemia. *J Exp Med* 2007;**204**:1741–1748.
- Wang P, Heitman J. The cyclophilins. *Genome Biol* 2005;**6**:226.1–226.6.
- Kern D, Kern G, Scherer G, Fischer G, Drakenberg T. Kinetic analysis of cyclophilin-catalyzed prolyl cis/trans isomerization by dynamic NMR spectroscopy. *Biochemistry* 1995;**34**:13594–13602.
- Ou WB, Luo W, Park YD, Zhou HM. Chaperone-like activity of peptidyl-prolyl cis-trans isomerase during creatine kinase refolding. *Protein Sci* 2001;**10**:2346–2353.
- Handschumacher RE, Harding MW, Rice J, Drugge RJ, Speicher DW. Cyclophilin: A specific cytosolic binding protein for cyclosporin A. *Science* 1984;**226**:544–547.
- Liu J, Farmer JD, Jr., Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 1991;**66**:807–815.
- Jain J, McCaffrey PG, Miner Z, et al. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. *Nature* 1993;**365**:352–355.
- McCaffrey PG, Perrino BA, Soderling TR, Rao A. NF-ATp, a T lymphocyte DNA-binding protein that is a target for calcineurin and immunosuppressive drugs. *J Biol Chem* 1993;**268**:3747–3752.
- Cande C, Vahsen N, Kouranti I, et al. AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. *Oncogene* 2004;**23**:1514–1521.
- Wissing S, Ludovico P, Herker E, et al. An AIF orthologue regulates apoptosis in yeast. *J Cell Biol* 2004;**166**:969–974.
- Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 1994;**264**:1772–1775.
- Sun W, Funakoshi H, Nakamura T. Overexpression of HGF retards disease progression and prolongs life span in a transgenic mouse model of ALS. *J Neurosci* 2002;**22**:6537–6548.
- Klivenyi P, Kiaei M, Gardian G, Calingasan NY, Beal MF. Additive neuroprotective effects of creatine and cyclooxygenase 2 inhibitors in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurochem* 2004;**88**:576–582.
- Van Den BL, Tilkin P, Lemmens G, Robberecht W. Minocycline delays disease onset and mortality in a transgenic model of ALS. *Neuroreport* 2002;**13**:1067–1070.
- Migheli A, Atzori C, Piva R, et al. Lack of apoptosis in mice with ALS. *Nat Med* 1999;**5**:966–967.
- Tomik B, Adamek D, Pierzchalski P, et al. Does apoptosis occur in amyotrophic lateral sclerosis? TUNEL experience from human amyotrophic lateral sclerosis (ALS) tissues. *Folia Neuropathol* 2005;**43**:75–80.
- Kang SJ, Sanchez I, Jing N, Yuan J. Dissociation between neurodegeneration and caspase-11-mediated activation of caspase-1 and caspase-3 in a mouse model of

- amyotrophic lateral sclerosis. *J Neurosci* 2003;**23**:5455–5460.
30. Friedlander RM, Brown RH, Gagliardini V, Wang J, Yuan J. Inhibition of ICE slows ALS in mice. *Nature* 1997;**388**:31.
 31. Kostic V, Jackson-Lewis V, de Bilbao F, Dubois-Dauphin M, Przedborski S. Bcl-2: Prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* 1997;**277**:559–562.
 32. Pasinelli P, Houseweart MK, Brown RH, Jr., Cleveland DW. Caspase-1 and -3 are sequentially activated in motor neuron death in Cu,Zn superoxide dismutase-mediated familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 2000;**97**:13901–13906.
 33. Li M, Ona VO, Guegan C, et al. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science* 2000;**288**:335–339.
 34. Guegan C, Vila M, Rosoklija G, Hays AP, Przedborski S. Recruitment of the mitochondrial-dependent apoptotic pathway in amyotrophic lateral sclerosis. *J Neurosci* 2001;**21**:6569–6576.
 35. Hisatomi T, Sakamoto T, Murata T, et al. Relocalization of apoptosis-inducing factor in photoreceptor apoptosis induced by retinal detachment in vivo. *Am J Pathol* 2001;**158**:1271–1278.
 36. Ferri KF, Jacotot E, Blanco J, et al. Apoptosis control in syncytia induced by the HIV type 1-envelope glycoprotein complex: Role of mitochondria and caspases. *J Exp Med* 2000;**192**:1081–1092.
 37. Shibata N, Kakita A, Takahashi H, et al. Persistent cleavage and nuclear translocation of apoptosis-inducing factor in motor neurons in the spinal cord of sporadic amyotrophic lateral sclerosis patients. *Acta Neuropathol* 2009;**118**:755–762.
 38. Chi L, Ke Y, Luo C, Gozal D, Liu R. Depletion of reduced glutathione enhances motor neuron degeneration in vitro and in vivo. *Neuroscience* 2007;**144**:991–1003.
 39. Montague JW, Hughes FM, Jr., Cidlowski JA. Native recombinant cyclophilins A, B, and C degrade DNA independently of peptidylprolyl cis-trans-isomerase activity. Potential roles of cyclophilins in apoptosis. *J Biol Chem* 1997;**272**:6677–6684.
 40. Bruijn LI, Miller TM, Cleveland DW. Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Annu Rev Neurosci* 2004;**27**:723–749.
 41. Manfredi G, Xu Z. Mitochondrial dysfunction and its role in motor neuron degeneration in ALS. *Mitochondrion* 2005;**5**:77–87.
 42. Dupuis L, Gonzalez de Aguilar JL, Oudart H, de Tapia M, Barbeito L, Loeffler JP. Mitochondria in amyotrophic lateral sclerosis: A trigger and a target. *Neurodegener Dis* 2004;**1**:245–254.
 43. Mattiazzi M, D'Aurelio M, Gajewski CD, et al. Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *J Biol Chem* 2002;**277**:29626–29633.
 44. Jung C, Higgins CM, Xu Z. A quantitative histochemical assay for activities of mitochondrial electron transport chain complexes in mouse spinal cord sections. *J Neurosci Methods* 2002;**114**:165–172.
 45. Menzies FM, Ince PG, Shaw PJ. Mitochondrial involvement in amyotrophic lateral sclerosis. *Neurochem Int* 2002;**40**:543–551.
 46. Brock TO, McIlwain DL. Astrocytic proteins in the dorsal and ventral roots in amyotrophic lateral sclerosis and Werdnig-Hoffmann disease. *J Neuropathol Exp Neurol* 1984;**43**:609–619.
 47. Almer G, Vukosavic S, Romero N, Przedborski S. Inducible nitric oxide synthase up-regulation in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J Neurochem* 1999;**72**:2415–2425.
 48. Levine JB, Kong J, Nadler M, Xu Z. Astrocytes interact intimately with degenerating motor neurons in mouse amyotrophic lateral sclerosis (ALS). *Glia* 1999;**28**:215–224.
 49. Vargas MR, Pehar M, Cassina P, Beckman JS, Barbeito L. Increased glutathione biosynthesis by Nrf2 activation in astrocytes prevents p75NTR-dependent motor neuron apoptosis. *J Neurochem* 2006;**97**:687–696.
 50. Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci* 2007;**10**:608–614.
 51. Nagai M, Re DB, Nagata T, et al. Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci* 2007;**10**:615–622.
 52. Barbeito LH, Pehar M, Cassina P, et al. A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis. *Brain Res Brain Res Rev* 2004;**47**:263–274.
 53. Pehar M, Cassina P, Vargas MR, et al. Astrocytic production of nerve growth factor in motor neuron apoptosis: Implications for amyotrophic lateral sclerosis. *J Neurochem* 2004;**89**:464–473.
 54. Cassina P, Pehar M, Vargas MR, et al. Astrocyte activation by fibroblast growth factor-1 and motor neuron apoptosis: Implications for amyotrophic lateral sclerosis. *J Neurochem* 2005;**93**:38–46.
 55. Yamanaka K, Chun SJ, Boillee S, et al. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci* 2008;**11**:251–253.

Supporting Information

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

Figure S1. Disease progression in superoxide dismutase 1 (SOD1)G93A mice monitored by three effective

measures. (A) Life span. Average survival time is 118.9 ± 3.39 days in SOD1^{G93A} mice. (B) Rota Rod test. The locomotor performance of SOD1^{G93A} mice was measured using a Rota Rod. Muscle weakness was observed at 98–100 days of age. (C) The body mass curves from SOD1^{G93A} mice during disease progression. Body weight of SOD1^{G93A} mice was significantly decreased at 112–119 days of age compared with wild-type (WT) ones. Histochemistry in the spinal cords of SOD1^{G93A} and wild-type mice; (D) cresyl violet staining, (E) glial fibrillary acid protein- and (F) Iba-1-immunostaining. The spinal cords

of SOD1^{G93A} mice were time-dependently damaged during disease progression. All pictures were taken at the anterior horn of the L1 sections in the spinal cords. Scale bars = 50 μ m. Each value represents the mean $\pm$ S.E.M. (n = 10). ** $P < 0.01$ versus wild-type (Student's *t*-test).

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