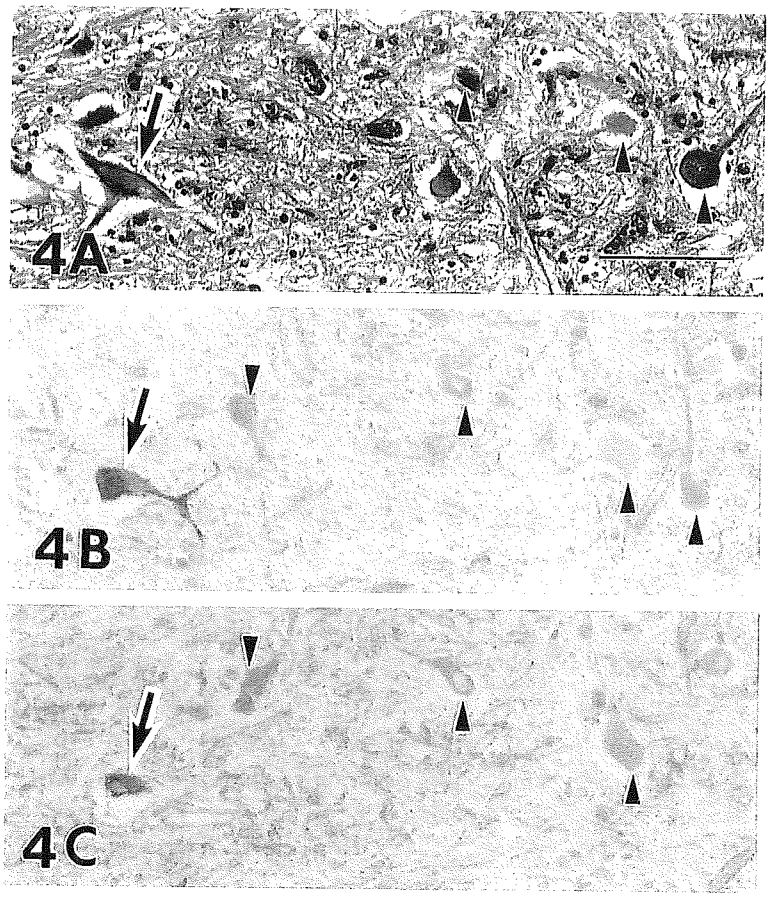

Fig. 4 Serial sections of the spinal anterior horn cells in a patient with SALS after a clinical course of 4 years 8 months (case 33). **A** In the HE preparation, residual motor neurons appear to be atrophic. There is no distinction among these atrophic neurons when observed in the HE preparation alone. **B** Immunostaining for Prxll. The number of residual neurons overexpressing Prxll (*arrow*) is reduced in comparison with that in the SALS patient after a clinical course of 2.5 years (Fig. 3). The number of Prxll-negative neurons is increased (*arrowheads*). **C** Immunostaining for GPxl. Similarly to the Prxll immunostaining, the number of GPxl-overexpressing neurons is diminished (*arrow*). In contrast, the number of GPxl-immunonegative neurons is increased (*arrowheads*). **B, C** No counterstaining. *Bar* **A** (also for **B, C**) 100 μm

difference between the neurons positive for Prxll and GPxl and those negative for both proteins (Figs. 3, 4, 5).

In the five FALS patients with SOD1 mutations, as reported previously [24], neuronal LBHIs in the anterior horn cells showed co-aggregation of Prxll/GPxl with SOD1. Although some SOD1-mutated anterior horn cells in these patients formed neuronal LBHIs, others did not. When we focused on these residual anterior horn cells without inclusions, the stainability and intensity of Prxll and GPxl staining in the residual cells without inclusions in the five FALS patients with SOD1 gene mutations were identical to those of the SALS patients. The numbers of residual anterior horn cells in one member of the Japanese Oki family (case 1, frameshift 126 mutation in SOD1 gene) and three members of the American C family (cases 3–5, A4V substitution in the SOD1 gene), observed within 2 years of the disease onset, were similar, although the loss of the anterior horn cells in the FALS patients was generally more severe in contrast to that seen in the SALS patients with the same disease duration. As in the SALS patients within about 3 years of disease onset, some of the residual anterior horn cells with no LBHIs overexpressed Prxll/GPxl in the four short-term-surviving FALS patients from the two different families and divergent SOD1 gene mutations. In a long-term surviving patient (case 2) with a clinical course of over 10 years, there were a few residual neurons, and most were immunonegative for Prxll/GPxl. However, rare residual neurons expressing Prxll/GPxl were still evident. The findings for the long-term-surviving FALS patient with SOD1 mutation were the same as those of the long-term-surviving SALS patients.

In the ALS animal models with human mutant SOD1, the inclusions exhibited co-aggregation of Prxll/GPxl with SOD1 as reported previously [24]. Noticeably, the Prxll/GPxl-immunoreactive findings in the rat (H46R and G93A rats) and mouse (G1H-G93A and G1L-G93A mice) models were essentially the same throughout the disease course for each ALS animal model. In the preclinical stage, the Prxll/GPxl immunostainability and immunointensity of the anterior horn cells were identical to those in the anterior horn cells of the normal littermates. Although the number of the anterior horn cells in the ALS animal models was decreased after the clinical onset of disease, some of these residual anterior horn cells showed overexpression of Prxll/GPxl, i.e., up-regulation of the redox system. In particular, this redox system up-regulation in the residual motor neurons was prominent at 160 days of age in H46R rats (Fig. 6A, B), at 150 days of age in G93A rats, at 110 days of age in G1H-G93A mice (Fig. 7) and at 215 days of age in G1L-G93A mice. At the end stage of disease in the four different ALS animal models,

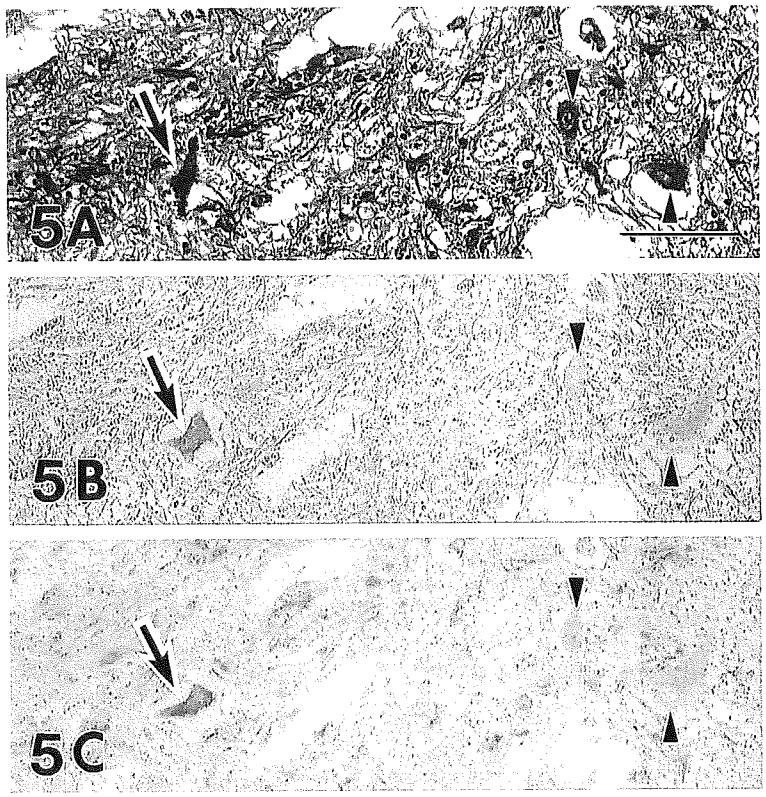

Fig. 5 Serial sections of the spinal anterior horn cells in a patient with SALS after a clinical course of 11 years 5 months (case 40). **A** Light microscopic preparation stained with HE. Small and atrophic motor neurons are seen. **B** Prxll immunoreactivity of the section consecutive to that shown in **A**. Although the residual neuron (*arrow*) appears to be atrophic in the HE preparation, this residual neuron expresses Prxll. **C** GPxl immunoreactivity of the section consecutive to that shown in **B**. The residual neuron that appears to be atrophic in the HE preparation is stained by the anti-GPxl antibody (*arrow*). Although there is no neuron overexpressing Prxll/GPxl, a neuron expressing Prxll/GPxl can be observed (*arrow*). By marked contrast, the number of neurons negative for Prxll/GPxl is increased (*arrowheads*). Observation of the HE-stained section in **A** shows no difference between the atrophic neuron positive for Prxll/GPxl (*arrow*) and the atrophic neurons negative for both proteins (*arrowheads*). **B, C** No counterstaining. *Bar* **A** (also for **B, C**) 100 μm

however, almost all of the residual motor neurons were negative for Prxll/GPxl, in marked contrast to the inclusions positive for PrxII/GPxI (Fig. 6C, D). As seen for the human specimens, no difference between the neurons positive for Prxll/GPxl and those negative for both proteins were observed the HE-stained sections for these animal models (Fig. 7).

Western blot analysis

When the tissue homogenate of each fresh cervical segment of the human spinal cord was examined by immunoblotting for Prxll, a single band corresponding to approximately 23 kDa was observed, i.e., with the same mobility as human Prxll (Fig. 8A). Immunoblotting showed that the intensity of Prxll immunoreactivity in the SALS patient with a clinical course of 2.5 years (case 19 in Fig. 2) appeared to be almost identical to that in a normal subject. In the SALS patient with a clinical course of 11 years 5 months (case 40 in Fig. 2), Prxll expression was less than that in the normal subject. This observation supported the results of Prxll immunohistochemistry. Immunoblotting for GPxl revealed a single band corresponding to about 22 kDa in two SALS cases and a normal subject (Fig. 8B). This molecular mass was compatible with that of human GPxl. In the SALS case at 2.5 years after disease onset (case 19 in Fig. 2), GPxl was expressed with almost the same intensity as that in the normal subject. However, the level of GPxl expression in the SALS case at 11 years 5 months after onset (case 40 in Fig. 2) decreased below that in the normal subject. This finding reflected the GPxl immunohistochemistry results.

Discussion

Prxll is a novel organ-specific anti-oxidative enzyme that is mainly expressed in mammalian brain [24, 29]. It is a member of the Prx family, the members of which directly regulate the redox system [6, 7, 8, 17, 29]. Prxll is a homodimeric protein composed of two subunits, each with a molecular mass of approximately 23 kDa [15, 16]. Like Prxll, GPxl, one of the major cytosolic isoforms of the GPxl family, also directly controls the redox system [12, 27, 30]. GPxl is a homotetramer consisting of approximately 22-kDa subunits [3]. This is consistent with the results of Western blot analyses, where use of

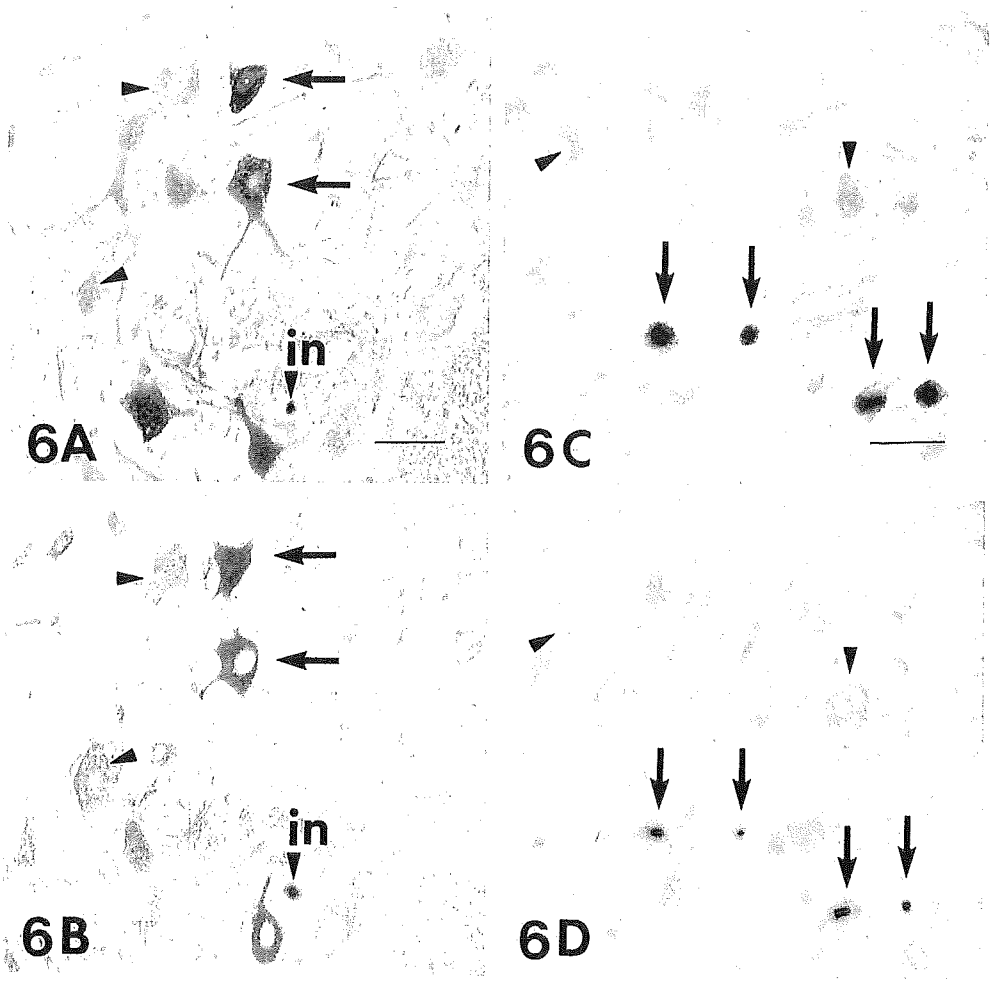

Fig. 6 Expression of Prxll and GPxl detected by immunohistochemistry in the spinal anterior horn in the transgenic rats expressing human SOD1 with an H46R mutation. **A, B** Serial sections of spinal anterior horn cells at 160 days of age in H46R rat immunostained with antibodies against Prxll (**A**) and GPxl (**B**). Residual neurons overexpressing Prxll/GPxl, i.e., showing redox system up-regulation, are observed (*arrows*). An inclusion (*arrowhead* with *in*) in the neuropil is intensely positive for Prxll/GPxl. The other neurons are either faintly stained by both antibodies, or unstained (*arrowheads*). **C, D** Serial sections of spinal anterior horn cells at over 180 days of age in H46R rat immunostained with antibodies against Prxll (**C**) and GPxl (**D**). Round and sausage-like LBHIs are strongly positive for Prxll/GPxl (*arrows*). By marked contrast, the number of neurons negative for Prxll/GPxl is increased (*arrowheads*). **A—D** No counterstaining (*SOD* superoxide dismutase, *LBHI* Lewy body-like hyaline inclusion). *Bar* **A** (also for **B**) 50 μm, **C** (also for **D**) 30 μm

the normal human tissue homogenate yielded a single band of approximately 23 kDa with the two anti-Prxll antibodies and a single band of about 22 kDa with the anti-GPxl antibody.

As expected [24], Prxll/GPxl immunoreactivity in anterior horns of the normal spinal cords of humans, rats and mice was primarily identified in the neurons: cytoplasmic staining was observed in almost all of the anterior horn cells. Intranuclear localization in some neurons was also observed with Prxll immunostaining using the two anti-Prxll antibodies, as previously reported [24]. Considering that endogenous Prxll and GPxl within the neuronal cytoplasm are regulators of the redox system, our finding that almost all of the normal spinal motor neurons coexpressed Prxll/GPxl confirms that these motor neurons maintain themselves utilizing the intracellular Prxll/GPxl system, that is, the redox system.

Corroborating previous findings [24], neuronal LBHIs positive for SOD1, Prxll, and GPxl were observed in both the SOD1-mutated FALS patients and the four transgenic ALS animal models expressing human mutant SOD1. A breakdown of the redox system was seen in the SOD1-mutated motor neurons containing inclusions. It is possible that the intra-inclusional co-aggregation of Prxll/GPxl with SOD1, or the sequestration of Prxll/GPxl with SOD1 into the inclusions causes the intracytoplasmic reduction of Prxll/GPxl, thereby reducing the availability of the redox system [24]. Although inclusions were seen in some motor neurons with the SOD1 gene mutation, other SOD1-mutated motor neurons progressed to the cell death without forming the inclusions.

An interesting feature was the presence of certain residual motor neurons coexpressing Prxll/GPxl throughout the ALS disease course in SALS patients and among SOD1-mutated motor neurons without inclusions. A particularly striking finding was that motor neurons overexpressing Prxll/GPxl, i.e., with redox system up-regulation, were commonly evident during the clinical courses in the divergent disease subtypes: SALS patients, SOD1-mutated FALS patients, and ALS animal models expressing human mutant SOD1.

For the SALS patients, although the number of the residual SALS motor neurons decreased with the progression of the clinical disease, some motor neurons overexpressing Prxll/GPxl were present usually up to approximately 3 years after the onset, particularly in the

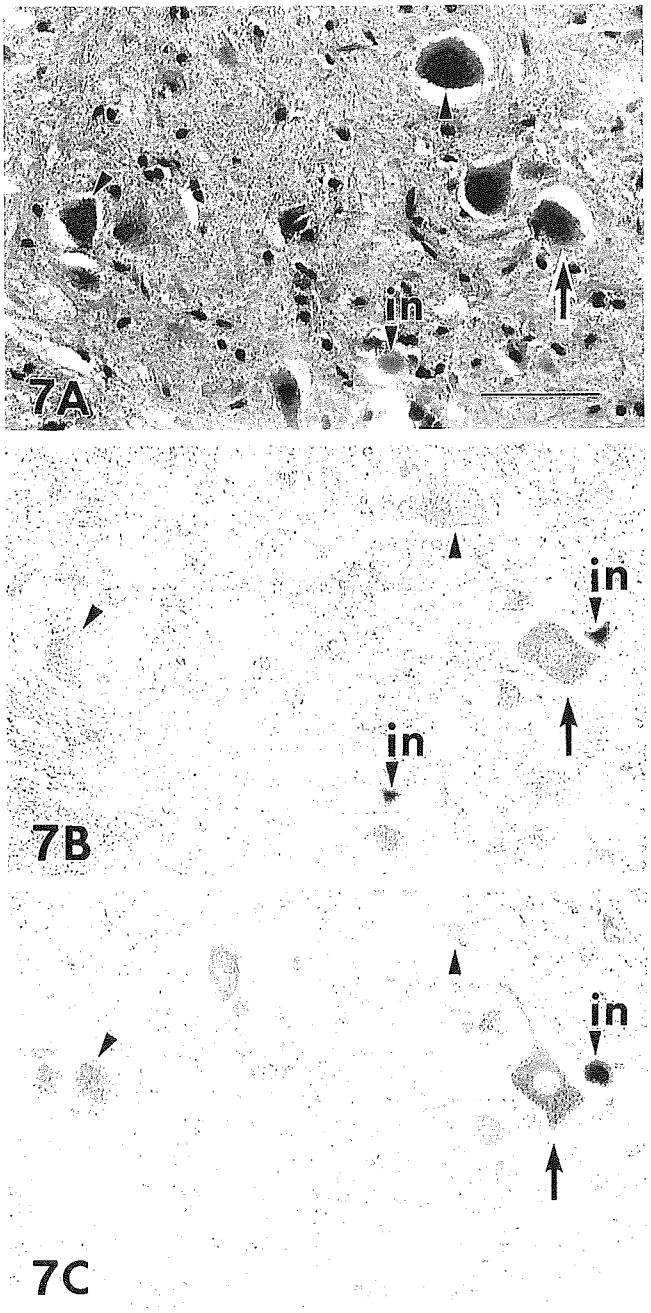

Fig. 7 Expression of Prxll and GPxl detected by immunohistochemistry in the spinal anterior horn in a G1H-G93A transgenic mouse carrying the highly overexpressed human G93A mutant SOD1 gene at 110 days of age. **A** Light microscopic preparation stained with HE. A round LBHI in the neuropil can be seen (*arrowhead* with *in*). **B** Prxll immunoreactivity of the section consecutive to that shown in **A**. **C** GPxl immunoreactivity of the section consecutive to that shown in **B**. Like in human ALS (Figs. 3, 4) and H46R rat (Fig. 6), a residual neuron overexpressing Prxll/GPxl, i.e., showing redox system up-regulation, can be observed (*arrow*). The other neurons are either faintly stained by both antibodies, or unstained (*arrowheads*), which indicates the breakdown of redox system. Round and sausage-like LBHIs in the neuropil are strongly positive for Prxll/GPxl (*arrowhead* with *in*). Observation of the HE-stained section in **A** reveals no difference between the neuron showing the redox system up-regulation (*arrow*) and the neurons exhibiting the redox system breakdown (*arrowheads*). **B, C** No counterstaining. *Bar* **A** (also for **B**, **C**) 50 μm

patients with no respiratory assistance. Thereafter, the number of these overexpressing motor neurons decreased dramatically as disease progressed. The immunohistochemical features are consistent with the Western blot findings that the levels of the Prxll/GPxl in the SALS patient after a clinical course of 2.5 years were almost the same levels as in normal cases in spite of loss of the motor neurons, whereas the levels decreased in the SALS patient after a clinical course of 11 years 5 months.

In the SOD1-mutated FALS patients, as in SALS patients, certain residual motor neurons without LBHIs also overexpressed Prxll/GPxl in the four short-term-surviving patients within 18 months after onset. In the ALS animal models, as in the human diseases, some residual motor neurons showed overexpression of Prxll/GPxl at 160 days of age in H46R rats, at 150 days of age in G93A rats, at 110 days of age in G1H-G93A mice and at 215 days of age in G1L-G93A mice. The presence of this common Prxll/GPxl-overexpression mechanism during the clinical course of ALS suggests that the redox system up-regulation represents one of the endogenous mechanisms that are activated by the ALS stress. At the terminal stage of ALS, however, disruption of this mechanism was observed. Although some residual ALS neurons coexpressed both proteins, while many others were negative, there was no apparent difference among these neurons on HE preparations.

In general, the redox system is one of the most crucial life supporting systems in living cells, serving as an antioxidant enzyme defense system that is synchronously linked to important physiological functions such as cellular differentiation, immune response, growth control, apoptosis, and tumor growth [4, 9, 19, 26, 31,

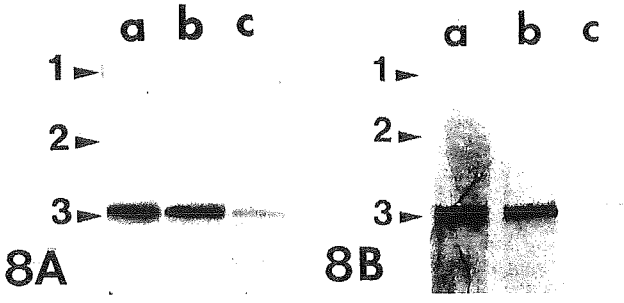

Fig. 8 Western blot analyses using the antibodies against Prxll (**A**) and GPxl (**B**). A 20-μg sample of the soluble protein extract from each sample was applied to each lane. Molecular mass markers: *1* ovalbumin (45 kDa); *2* carbonic anhydrase (31 kDa); *3* trypsin inhibitor (21.5 kDa). **A** *Lane a*: Normal control, *lane b*: an SALS patient with a clinical course of 2.5 years (case 19). *lane c*: an SALS patient with a clinical course of 11 years 5 months (case 40). A single band at approximately 23 kDa is observed in all samples. The intensity of Prxll immunoreactivity in *lane b* (ALS case 19) appears to be almost identical to that in *lane a* (normal). By contrast, Prxll expression in *lane c* (ALS case 40) appears to be lower than that in *lane a* (normal). **B** A single band of about 22 kDa is detected in each sample. Expression of GPxl in *lane b* (ALS case 19) shows almost the same intensity as that in *lane a* (normal). However, the level of GPxl expression in *lane c* (ALS case 40) is decreased below that seen in *lane a* (normal)

34]. Although we cannot exclude the possibility that the cells die as a result of redox stress itself, the residual ALS neurons expressing redox system up-regulation are thought to maintain their viability by protecting themselves from potentially destructive ROSs and by controlling the intraneuronal redox system [1, 5, 10, 14, 34, 39]. A similar up-regulation mechanism for hepatocyte growth factor (HGF, a novel neurotrophic factor) and its receptor (c-Met) also occurs in the SALS and SOD1-mutated FALS patients [22]. Considering that in the animal experiment, overexpression of HGF attenuates motor neuron death and prolongs the life span of G1L-G93A mice [37], motor neurons showing up-regulation of the HGF/c-Met cell-survival system, which is normally present in neurons, are thought to show enhanced cell survival in the presence of ALS stress in humans [22]. Although we cannot readily compare the neurotrophic factor with the redox system, our finding leads us to the conclusion that the residual ALS neurons showing redox system up-regulation would be less susceptible to ALS stress and can protect themselves from ALS neuronal death. Taken together with the fact that Prxll functions not only as a member of the redox system but also as a molecular chaperone [18], the residual ALS neurons overexpresing Prxll/GPxl might have developed to possess simultaneously both the enhanced antioxidant enzyme defense mechanism as a highly-evolved redox system, and the amplified sophisticated system for coping with misfolded proteins, such as mutant SOD1 or unknown pathogenetic proteins leading to SALS. Thus, residual ALS neurons overexpressing redox system-related enzymes could protect themselves from ALS stress. However, it is not thought possible for residual ALS neurons under long-term ALS stress to keep on inducing redox system-related enzymes. Therefore, as ALS progresses, the ability of residual neurons to up-regulate the redox system diminishes, and finally they become even unable to maintain the redox system itself. In other words, ALS neurons showing redox system up-regulation might show enhanced cell survival in the presence of ALS stress. In contrast, breakdown of the redox system in ALS motor neurons that are barely viable would result in cell death, and many residual motor neurons that are unable to coexpress Prxll/GPxl would ultimately become moribund.

Our data may lead to the development of a new therapy based on redox system up-regulation for the treatment of ALS, which for over 130 years has had an unknown etiology. It remains to be determined whether this redox system up-regulation is a direct or an indirect effect based on the pathogenesis of ALS itself, or whether this redox system up-regulation plays a primary or a secondary role in attenuating ALS-related neuronal death.

Acknowledgements This study was supported in part by a Research Grant on Measures for Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan (S.K. and Y.I.); a Research Grant (2004) from the Faculty of Medicine, Tottori University (S.K.); a Grant-in-Aid for Scientific Research in Priority Area (T.N.) and a Grant-in-Aid for Scientific Research (Y.A.) from the Ministry of Education Culture, Sports, Science and Technology of Japan.

References

1. Andoh T, Chiueh CC, Chock PB (2003) Cyclic GMP-dependent protein kinase regulates the expression of thioredoxin and thioredoxin peroxidase-1 during hormesis in response to oxidative stress-induced apoptosis. J Biol Chem 278:885–890
2. Asayama K, Burr IM (1984) Joint purification of mangano and cuprozinc superoxide dismutases from a single source: a simplified method. Anal Biochem 136:336–339
3. Asayama K, Yokota S, Dobashi K, Hayashibe H, Kawaoi A, Nakazawa S (1994) Purification and immunoelectron microscopic localization of cellular glutathione peroxidase in rat hepatocytes: quantitative analysis by postembedding method. Histochemistry 102:213–219
4. Berggren MI, Husbeck B, Samulitis B, Baker AF, Gallegos A, Powis G (2001) Thioredoxin peroxidase-1 (peroxiredoxin-1) is increased in thioredoxin-1 transfected cells and results in enhanced protection against apoptosis caused by hydrogen peroxide but not by other agents including dexamethasone, etoposide, and doxorubicin. Arch Biochem Biophys 392:103–109
5. Biteau B, Labarre J, Toledano MB (2003) ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. Nature 425:980–984
6. Chae HZ, Kim IH, Kim K, Rhee SG (1993) Cloning, sequencing, and mutation of thiol-specific antioxidant gene of *Saccharomyces cerevisiae*. J Biol Chem 268:16815–16821
7. Chae HZ, Chung SJ, Rhee SG (1994) Thioredoxin-dependent peroxide reductase from yeast. J Biol Chem 269:27670–27678
8. Chae HZ, Robison K, Poole LB, Church G, Storz G, Rhee SG (1994) Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. Proc Natl Acad Sci USA 91:7017–7021
9. Chang TS, Jeong W, Choi SY, Yu S, Kang SW, Rhee SG (2002) Regulation of peroxiredoxin I activity by cdc2-mediated phosphorylation. J Biol Chem 277:25370–25376
10. Chang TS, Jeong W, Woo HA, Lee SM, Park S, Rhee SG (2004) Characterization of mammalian sulfiredoxin and its reactivation of hyperoxidized peroxiredoxin through reduction of cysteine sulfinic acid in the active site to cysteine. J Biol Chem 279:50994–51001
11. Charcot JM, Joffroy A (1869) Deux cas d'atrophie musculaire progressive avec lésions de la substance grise et des faisceaux antéro-latéraux de la moelle épinière. Arch Physiol (Paris) 2:744–760
12. De Haan JB, Bladier C, Griffiths P, Kelner M, O'Shea RD, Cheung NS, Bronson RT, Silvestro MJ, Wild S, Zheng SS, Beart PM, Hertzog PJ, Kola I (1998) Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. J Biol Chem 273:22528–22536
13. Fridovich I (1986) Superoxide dismutases. Adv Enzymol Relat Areas Mol Biol 58:61–97
14. Georgiou G, Masip L (2003) An overoxidation journey with a return ticket. Science 300:592–594
15. Hirotsu S, Abe Y, Nagahara N, Hori H, Nishino T, Okada K, Hakoshima T (1999) Crystallographic characterization of a stress-induced multifunctional protein, rat HBP-23. J Struct Biol 126:80–83
16. Hirotsu S, Abe Y, Okada K, Nagahara N, Hori H, Nishino T, Hakoshima T (1999) Crystal structure of a multifunctional 2-Cys peroxiredoxin heme-binding protein 23 kDa/proliferation-associated gene product. Proc Natl Acad Sci USA 96:12333–12338

17. Ichimiya S, Davis JG, O'Rourke DM, Katsumata M, Greene MI (1997) Murine thioredoxin peroxidase delays neuronal apoptosis and is expressed in areas of the brain most susceptible to hypoxic and ischemic injury. DNA Cell Biol 16:311–321
18. Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, Lee JR, Lee SS, Moon JC, Yun JW, Choi YO, Kim WY, Kang JS, Cheong GW, Yun DJ, Rhee SG, Cho MJ, Lee SY (2004) Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. Cell 117:625–635
19. Jin D-Y, Chae HZ, Rhee SG, Jeang K-T (1997) Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. J Biol Chem 272:30952–30961
20. Kato S, Shimoda M, Watanabe Y, Nakashima K, Takahashi K, Ohama E (1996) Familial amyotrophic lateral sclerosis with a two base pair deletion in superoxide dismutase 1 gene: multisystem degeneration with intracytoplasmic hyaline inclusions in astrocytes. J Neuropathol Exp Neurol 55:1089–1101
21. Kato S, Hayashi H, Nakashima K, Nanba E, Kato M, Hirano A, Nakano I, Asayama K, Ohama E (1997) Pathological characterization of astrocytic hyaline inclusions in familial amyotrophic lateral sclerosis. Am J Pathol 151:611–620
22. Kato S, Funakoshi H, Nakamura T, Kato M, Nakano I, Hirano A, Ohama E (2003) Expression of hepatocyte growth factor and c-Met in the anterior horn cells of the spinal cord in the patients with amyotrophic lateral sclerosis (ALS): immunohistochemical studies on sporadic ALS and familial ALS with superoxide dismutase 1 gene mutation. Acta Neuropathol 106:112–120
23. Kato S, Shaw P, Wood-Allum C, Leigh PN, Show C (2003) Amyotrophic lateral sclerosis. In: Dickson D (ed) Nerodegeneration: the molecular pathology of dementia and movement disorders. ISN Neuropath Press, Basel, pp 350–368
24. Kato S, Saeki Y, Aoki M, Nagai M, Ishigaki A, Itoyama Y, Kato M, Asayama K, Awaya A, Hirano A, Ohama E (2004) Histological evidence of redox system breakdown caused by superoxide dismutase 1 (SOD1) aggregation is common to SOD1-mutated motor neurons in humans and animal models. Acta Neuropathol 107:149–158
25. Kato T, Hirano A, Kurland LT (1987) Asymmetric involvement of the spinal cord involving both large and small anterior horn cells in a case of familial amyotrophic lateral sclerosis. Clin Neuropathol 6:67–70
26. Koo KH, Lee S, Jeong SY, Kim ET, Kim HJ, Kim K, Song K, Chae HZ (2002) Regulation of thioredoxin peroxidase activity by c-terminal truncation. Arch Biochem Biophys 397:312–318
27. Kosower NS, Kosower EM (1978) The glutathione status of cells. Int Rev Cytol 54:109–160
28. Kurland LT, Mulder DW (1955) Epidemiologic investigations of amyotrophic lateral sclerosis. II. Familial aggregations indicative of dominant inheritance. Neurology 5:182–196, 249–268
29. Matsumoto A, Okado A, Fujii T, Fujii J, Egashira M, Niikawa N, Taniguchi N (1999) Cloning of the peroxiredoxin gene family in rats and characterization of the fourth member. FEBS Lett 443:246–250
30. Meister A, Anderson ME (1983) Glutathione. Annu Rev Biochem 52:711–760
31. Mu ZM, Yin XY, Prochownik EV (2002) Pag, a putative tumor suppressor, interacts with the myc box II domain of c-myc and selectively alters its biological function and target gene expression. J Biol Chem 277:43175–43184
32. Nagai M, Aoki M, Miyoshi I, Kato M, Pasinelli P, Kasai N, Brown RH Jr, Itoyama Y (2001) Rats expressing human cytosolic copper-zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. J Neurosci 21:9246–9254
33. Nakano I, Hirano A, Kurland LT, Mulder DW, Holley PW, Saccomanno G (1984) Familial amyotrophic lateral sclerosis. Neuropathology of two brothers in American "C" family. Neurol Med (Tokyo) 20:458–471
34. Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, Van Etten RA (2003) Essential role for the peroxiredoxin prdx1 in erythrocyte antioxidant defence and tumour suppression. Nature 424:561–565
35. Sen CK, Packer L (1996) Antioxidant and redox regulation of gene transcription. FASEB J 10:709–720
36. Shibata N, Hirano A, Kobayashi M, Siddique T, Deng HX, Hung WY, Kato T, Asayama K (1996) Intense superoxide dismutase-1 immunoreactivity in intracytoplasmic hyaline inclusions of familial amyotrophic lateral sclerosis with posterior column involvement. J Neuropathol Exp Neurol 55:481–490
37. Sun W, Funakoshi H, Nakamura T (2002) Overexpression of HGF retards disease progression and prolongs life span in a transgenic mouse model of ALS. J Neurosci 22:6537–6548
38. Takahashi K, Nakamura H, Okada E (1972) Hereditary amyotrophic lateral sclerosis. Histochemical and electron microscopic study of hyaline inclusions in motor neurons. Arch Neurol 27:292–299
39. Wood ZA, Poole LB, Karplus PA (2003) Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science 300:650–653

Neuropathology 2005; **25**, 365–370

Workshop: Recent Advances in Motor Neuron Disease

Development of a rat model of amyotrophic lateral sclerosis expressing a human *SOD1* transgene

Masashi Aoki,[1] Shinsuke Kato,[2] Makiko Nagai[1] and Yasuto Itoyama[1]

[1]Department of Neurology, Tohoku University School of Medicine, Sendai, and [2]Department of Neuropathology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, Yonago, Japan

Mutations in copper–zinc superoxide dismutase gene (*SOD1*) have been linked to some familial cases of ALS. We report here that rats that express a human *SOD1* transgene with two different ALS-associated mutations (G93A and H46R) develop striking motor neuron degeneration and paralysis. By comparing the two transgenic rats with different *SOD1* mutations, we demonstrate that the time course in these rats was similar to human *SOD1*-mediated familial ALS. As in the human disease and transgenic ALS mice, pathological analysis shows selective loss of motor neurons in the spinal cords of these transgenic rats. In addition, typical neuronal Lewy body-like hyaline inclusions as well as astrocytic hyaline inclusions identical to those in human familial ALS are observed in the spinal cords. The larger size of this rat model as compared with the ALS mice will facilitate studies involving manipulations of spinal fluid (implantation of intrathecal catheters for chronic therapeutic studies; CSF sampling) and spinal cord (e.g., direct administration of viral- and cell-mediated therapies).

Key words: astrocytic hyaline inclusions, amyotrophic lateral sclerosis, Lewy body-like hyaline inclusions, mutation, rat, *SOD1*, transgenic.

INTRODUCTION

ALS is a fatal neurodegenerative disease caused by the selective death of motor neurons.[1] Approximately 10% of the cases of ALS are inherited, usually as an autosomal dominant trait. In 25% of familial cases, the disease is caused by mutations in the gene encoding cytosolic copper–zinc superoxide dismutase (*SOD1*).[2,3] Nearly 100 different mutations in the *SOD1* gene have been identified in familial ALS.[4] Why the mutations cause motor neuron degeneration has not been fully elucidated.

In familial ALS kindred with mutations in the *SOD1* gene, the age of onset of weakness varies greatly but the duration of illness appears to be characteristic to each mutation. For example, in patients with the L84V mutation, the average life expectancy is less than 1.5 years after the onset of symptoms,[5,6] whereas patients harboring the H46R mutation have an average life expectancy of 18 years after the disease onset.[2,7] In view of the evidence supporting the idea that familial ALS variants of *SOD1* enzymes acquire toxic properties, the variations in the duration of illness in different kindred might arise because each mutation imparts different degrees of toxicity to the mutant protein.[8]

To date, several *SOD1* mutants of transgenic mice have been generated.[9–12] These mice exhibit the ALS-like clinical features and have importantly advanced our understanding of the pathogenesis of neuronal cell death induced by mutant SOD1 protein. They have also facilitated therapeutic trials. However, some types of experimental manipulations have been difficult in the ALS mice because of their innate size limitations. It has been almost impossible, for example, to analyze CSF from the ALS mice, even at single time points. It has also been very difficult to use therapies that involve administration of compounds into the CSF. There is only a single report of pump-mediated delivery of therapies to the CSF of the ALS mice, and that approach was intraventricular rather than intrathecal;[13] it is likely that intrathecal administration will produce significantly better therapeutic levels of compounds at the spinal cord level than will the intraventricular approach.[14] It has also been difficult to obtain sufficient tissue to perform extensive biochemical analyzes, such as investigations of post-transcriptional modifications of proteins like SOD1 itself during disease progression. For these reasons and in order

Correspondence: Masashi Aoki MD, PhD, Department of Neurology, Tohoku University School of Medicine, Seiryo-machi, Sendai 980-8574, Japan. Email: aokim@mail.tains.tohoku.ac.jp

Received 1 December 2004; accepted 8 December 2004.

to reproduce the different degrees of toxicity to the mutant protein by mutations, we have developed a rat model of ALS by expressing a human *SOD1* transgene with two ALS-associated mutations: H46R and G93A.[15]

CONSTRUCTION OF TRANSGENIC MICE EXPRESSING MUTANT HUMAN *SOD1*

We elected to make transgenic rats with two mutations in the *SOD1* genes: histidine 46 to arginine (H46R) and glycine 93 to alanine (G93A). In patients we have encountered with these mutations, the phenotypes are quite different. For H46R patients, progression is extremely slow[2,7] whereas patients with the *SOD1*^{G93A} mutation demonstrate a more fulminant, classical clinical course.[16] Moreover, the transgenic ALS mouse with this G93A mutation has been widely distributed and studied throughout the world.[9]

To generate the transgenic rats with the H46R and the G93A mutations, we first obtained human genomic PAC clones encompassing the entire human *SOD1* gene; we then subcloned this gene within an 11.5 kb *Eco*RI–*Bam*HI fragment. Site-directed mutagenesis was used to generate clones with either the H46R or the G93A mutations. The mutated 11.5 kb *Eco*RI–*Bam*HI fragments were microinjected into fertilized eggs from Sprague Dawley (SD) rats (Japan SLC, Hamamatsu, Japan). Twenty-five potential transgenic H46R pups were obtained. From these, five founders with the H46R mutant transgene were identified using PCR and Southern blotting. Fifty-two potential transgenic G93A pups were obtained. From these, seven founders with the G93A mutant transgene were identified. Levels of accumulated mutant *SOD1* were measured for almost all founders by quantitative protein immunoblotting of spinal cord extracts using antibody against a peptide sequence that is identical in human and rat *SOD1*.

The transgenic rats expressing the higher levels of each human *SOD1* mutant (lines G93A-39 and H46R-4) have developed motor neuron disease (Fig. 1). Clinically apparent weakness, denoted by dragging of one hindlimb without limb tremor, was evident somewhat later. The mean age of onset of this clinical weakness for the G93A-39 line was 122.9 days ($n = 14$); for the H46R-4 line, the age of onset was 171.7 days ($n = 11$) (Fig. 1). Simultaneously with the onset of clinical weakness, the affected rats showed prominent weight loss. Although the initial clinical manifestation of weakness was unilateral leg paralysis, this progressed and became bilateral in both lines of rats. In the early stages of the illness, another distinctive abnormality was increased tone in the tail musculature, resulting in an elevated, segmentally spastic tail posture. As the disease progressed, the rats exhibited marked muscle wasting in the hindlimbs, typically dragging themselves about the

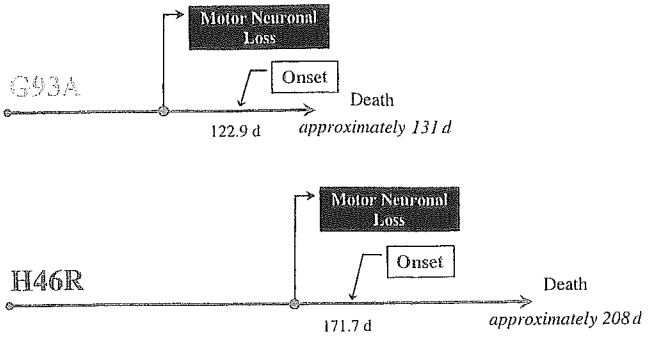

Fig. 1 Progression of mutant superoxide dismutase-mediated disease. From the presymptomatic stage, the anterior horns of the same rats revealed decreased numbers of large, multipolar neuronal cells (motor neurons) with proliferation of small non-neuronal cells with morphological characteristics of astrocytes and microglia. The ages of onset and death for the G93A-39 and H46R-4 rats are indicated.

Fig. 2 An affected transgenic rat from the H46R-4 line demonstrates hindlimb weakness and abnormal posturing with segmental spasticity of the tail.

cage using the forelimbs (Fig. 2). Thereafter, the forelimbs also became weak, in association with further weight loss. At end-stage, the affected rats could not drink water and died. The mean duration of the disease in the G93A-39 and H46R-4 lines were 8.3 days ($n = 14$) and 37.2 days ($n = 11$), respectively. All rats were handled according to approved animal protocols in our institution.

HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYZES

The H46R and G93A transgenic rats exihibit the same histopathological changes as those in human familial ALS patients with *SOD1* gene mutations. Therefore, at present,

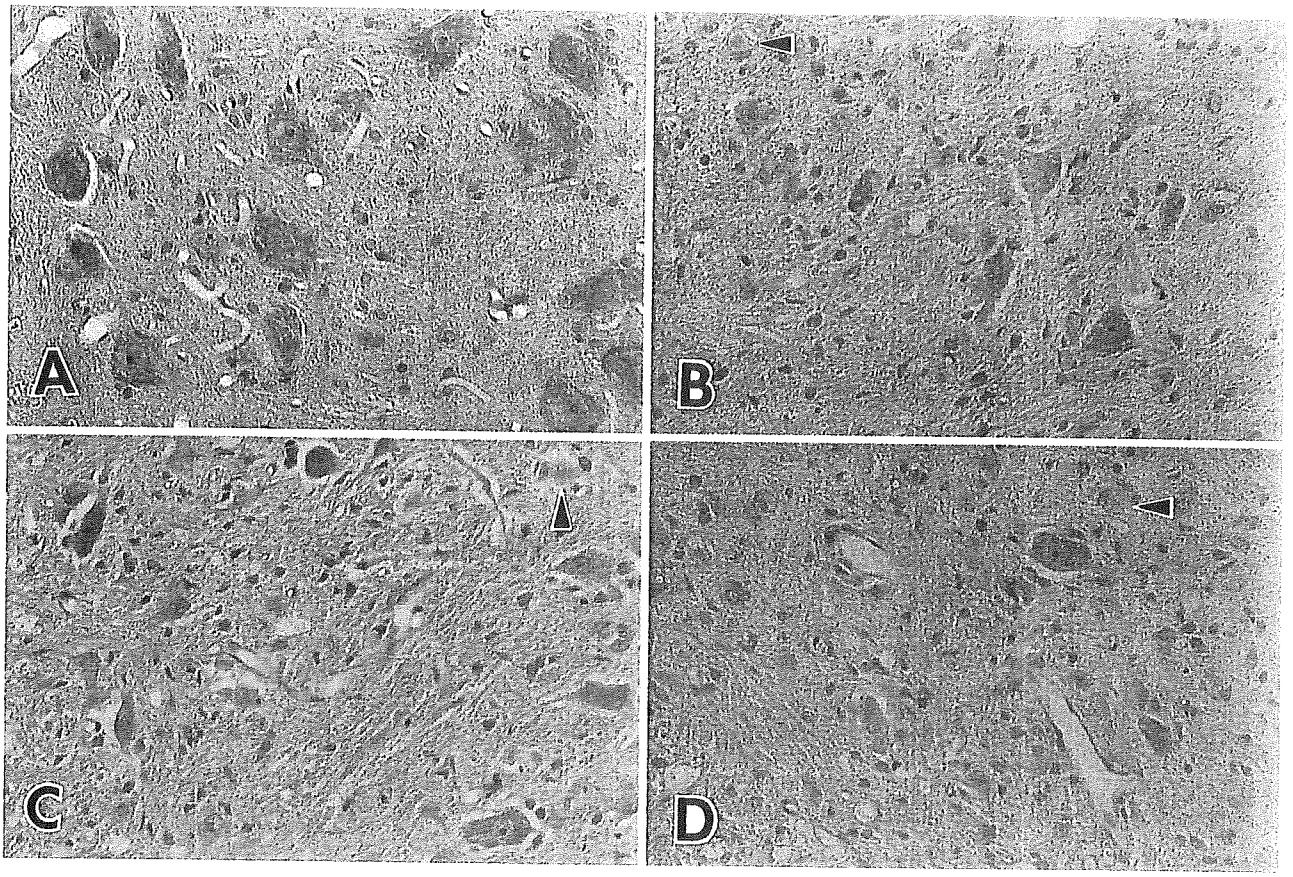

Fig. 3 Histological and histopathological findings in H46R transgenic rats and littermate. (A) The anterior horn of the spinal cord in littermate rat at the age of 160 days: approximately 15 normal anterior horn cells can be observed. (B) The anterior horn of the spinal cord in H46R transgenic rat at the age of 160 days: approximately six anterior horn cells can be counted in the H46R transgenic rat of this age, that is, the number of the anterior horn cells is decreased with astrocytic gliosis, histopathologically, in comparison with the littermate at the same age in (A). A core and halo-type astrocytic hyaline inclusion (Ast-HI) is evident (arrowhead). (C) The anterior horn of the spinal cord in the H46R transgenic rat at the age of 170 days: the histopathological finding of this age reveals loss of the anterior horn cells and gliosis of the spinal cords. A core and halo-type Ast-HI can be observed (arrowhead). (D) The anterior horn of the spinal cord in the H46R transgenic rat at the age of 200 days corresponding to the end-stage: approximately two anterior horn cells can be recognized at this terminal stage; the H46R rats of the terminal stage show severe loss of the anterior horn cells with gliosis of the spinal cords histopathologically compatible with those in ALS patients with clinical courses of over 5 years. A core and halo-type Ast-HI can be seen (arrowhead). As in human ALS patients, small-sized remaining anterior horn cells that appear to be normal are also observed throughout the disease courses in H46R transgenic rats (B–D) ((A–D): HE; magnification: ×400).

we think that the H46R and G93A transgenic rats are neuropathologically most optimal as animal models of familial ALS with the *SOD1* mutations. An essential histopathological finding of the spinal cords in ALS patients is loss of the anterior horn cells.[17] When we focus on the anterior horn cells of the spinal cords in both H46R and G93A transgenic rats, the anterior horn cells of the H46R and G93A transgenic rats are decreased before the development of clinical motor deficits. At the level of cellular pathology, the H46R and G93A transgenic rats develop Lewy body-like hyaline inclusions (LBHI) in neurons and astrocytes, which are morphological hallmarks of certain human familial ALS patients with the *SOD1* gene mutations.[17–20]

With respect to the histopathological aspects of H46R transgenic rats, the number of the anterior horn cells of the 160-day-old H46R rats that exhibit hind limb paresis is decreased with astrocytic gliosis in the spinal cords in comparison with the littermates at the same age (Fig. 3A,B). At 170 days of age when the H46R rats indicate hindlimb paraplegia sometimes associated with forelimb weakness, the histopathological finding of this clinical stage reveals more severe loss of the anterior horn cells and gliosis of the spinal cords in comparison with that of 160 days of age (Fig. 3B,C). At 200 days of age corresponding to the end-stage when the H46R rats clinically display quadriplegia or a moribund state, the H46R rats of this end-stage show severe loss of the anterior horn cells with gliosis of the spi-

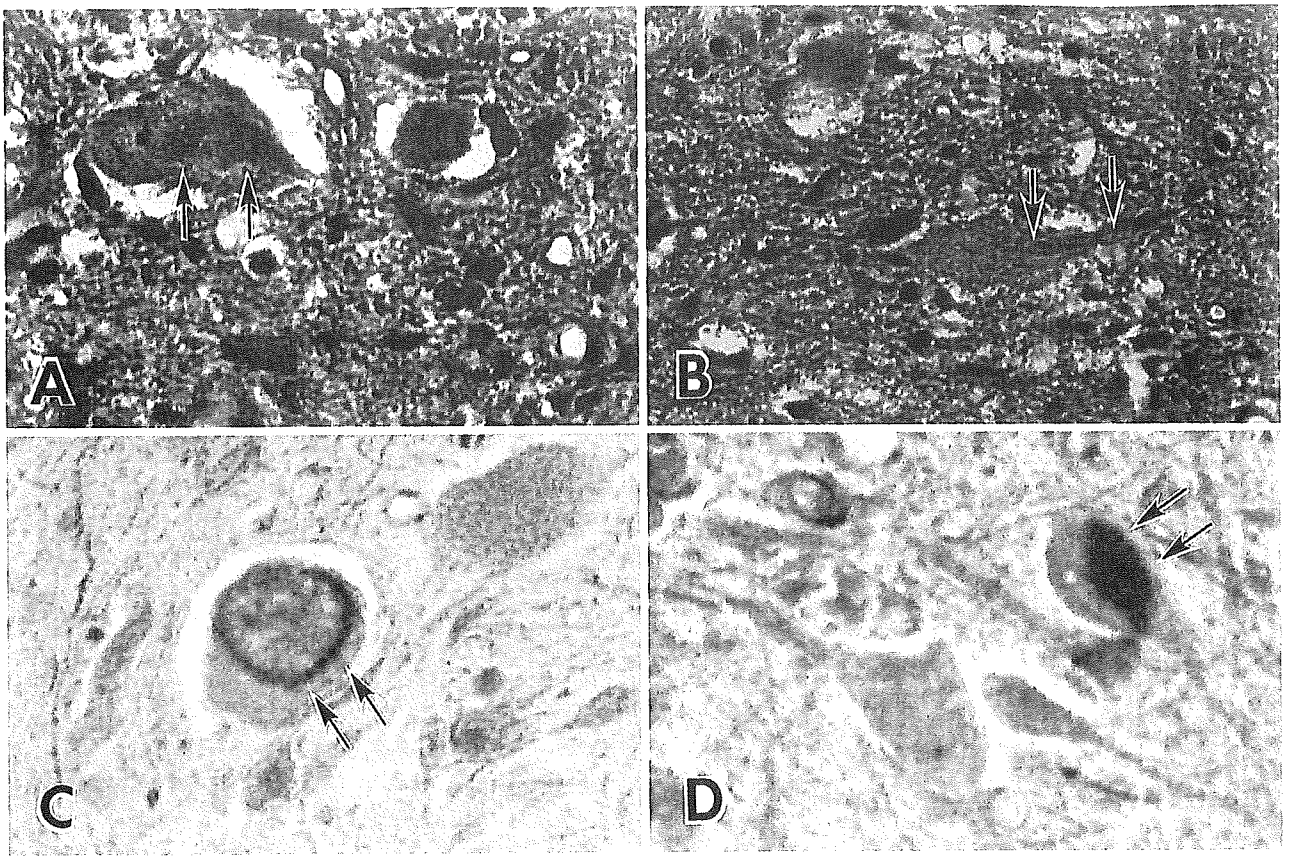

Fig. 4 Neuronal Lewy body-like hyaline inclusions (LBHI) in H46R transgenic rats. (A) A typical intracytoplasmic neuronal LBHI with a core and halo is indicated by double arrows (HE; magnification: ×820). (B) A neuronal LBHI with a core and halo is located from the cytoplasm to the dendrite (double arrows) (HE; magnification: ×820). (C) An intracytoplasmic LBHI is positive for SOD1; only the periphery of the neuronal LBHI is strongly immunostained (double arrows) (Immunostaining for SOD1; magnification: ×820). (D) An intracytoplasmic LBHI is diffusely immunostained (double arrows) (Immunostaining for SOD1; magnification: ×820). (Figure 4C is from Kato *et al.*[18] and reproduced with permission from *Acta Neuropathol*).

nal cords histopathologically compatible with those in ALS patients with clinical courses of over 5 years (Fig. 3D). As in human ALS patients, small-sized remaining anterior horn cells that appear to be normal in HE preparations are also observed throughout the disease courses in H46R and G93A transgenic rats (Fig. 3A–D).

As for the cell-pathological and immunohistochemical aspects, the rodent familial ALS model of these rats has the other important cellular pathological finding compatible with that in human familial ALS patients with the *SOD1* gene mutations; typical intracytoplasmic neuronal LBHI are observed in the remaining anterior horn cells of the spinal cords in the rat model of familial ALS. Cell-pathologically, the intracytoplasmic neuronal LBHI in the rat model of ALS are identical to those in human familial ALS. In both the rat model and human familial ALS, neuronal LBHI are formed not only in the cytoplasm but also in dendrites (Fig. 4A,B). Immunohistochemically, as in human mutant *SOD1*-mediated familial ALS,[17,19,20] the neuronal LBHI in the rat model of ALS with the H46R and G93A are positive for SOD1 (Fig. 4C,D). The reaction product deposits of the antibody against SOD1 are generally restricted to the periphery of the LBHI that show eosinophilic cores with palor peripheral halos in HE preparations (Fig. 4C). The immunostaining in intracytoplasmic and intradendtritic ill-defind LBHI is distributed throughout each of the inclusions (Fig. 4D). The rat model of ALS with the H46R and G93A also develops astrocytic hyaline inclusions (Ast-HI) that are identical structures observed in human long-term surviving familial ALS patients with the *SOD1* gene mutation.[17,19,20] In HE preparations, similarly in neuronal LBHI, Ast-HI are eosinophilic (Fig. 5A) or slightly pale inclusions and sometimes show an eosinophilic core with palor peripheral halos (Fig. 3B–D). The Ast-HI are generally round to oval and sometimes sausage-like in shape. As in neuronal LBHI, immunohistochemically, Ast-HI are intensely immunostained by the antibody against SOD1 (Fig. 5B).

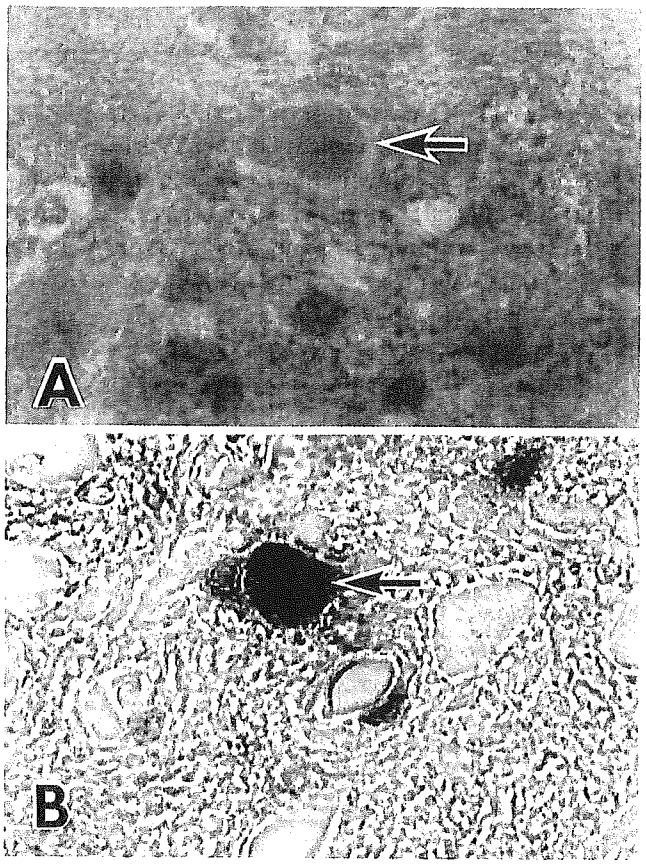

Fig. 5 Astrocytic hyaline inclusions (Ast-HI) in H46R transgenic rats. (A) Typical eosinophilic Ast-HI can be seen (arrow) (HE; magnification: ×1200). (B) An Ast-HI is strongly positive for SOD1 (arrow) (Immunostaining for SOD1; magnification: ×1200).

DISCUSSION

We have established lines of rats that express transgenes for mutant SOD1 protein with two different ALS-associated mutations: H46R and G93A. Rats with the highest transgene copy numbers and levels of expression of the mutant protein develop a paralytic disorder characterized by fulminant motor neuron death accompanied by astrogliosis and microgliosis. Particularly striking in our data is not only the earlier onset of the G93A disease but also the much more rapid course in the G93A-39 (8 days) as compared to the H46R-4 (37 days) rats. We do not understand the basis for this difference in rate of disease progression, but we note those factors determining the time course in these rats are likely to be relevant to human mutant *SOD1*-mediated familial ALS. The human H46R cases also progress very slowly, with a mean survival of 16.8 ± 6.8 years.[2,7] By contrast, the mean survival of the G93A cases in one report was 2.2 ± 1.5 years.[16] Although it is tempting to speculate that this shorter disease duration is a consequence of the higher retained dismutation activity in the G93A-39 line, we cannot firmly conclude this.

A transgenic rat model of human ALS will offer several advantages with respect to the existing transgenic mouse ALS models.[15,21] Given its larger size, it will facilitate all studies that entail CSF analysis and, in particular, those that entail multiple, serial manipulations of CSF in the same animal. Thus, it will be possible in this model to obtain adequate CSF for conventional biochemical studies as well as analyzes of small molecules and even DNA/RNA species that may distinguish the ALS from the wild-type CSF. Moreover, this model should be ideal for administration of therapies via chronic intrathecal pumps, a strategy that has been employed recently in human ALS clinical trials.[22] Another advantage of the ALS rats is that they can tolerate some forms of immunosuppressive therapy that are problematic in mice, such as cyclosporine A. This point arises in the context of an emerging interest in possible strategies to use implanted neural stem cells as therapy in ALS. It should now be possible to achieve appropriate immunosuppression in the ALS rats to allow survival of implanted cells and hence determine the efficacy of this approach. As a corollary, we also note that the larger size of the rat spinal cord will facilitate delivery of cells to the target spinal cord regions.

CONCLUSIONS

We have established lines of rats that express transgenes for mutant SOD1 protein with two different ALS-associated mutations: H46R and G93A. As in the human disease and transgenic ALS mice, pathological analysis demonstrates selective loss of motor neurons in the spinal cords of these transgenic rats. In addition, typical neuronal LBHI as well as Ast-HI identical to those in human familial ALS are observed in the spinal cords of the rats. Therefore, at present, we think that the H46R and G93A transgenic rats are neuropathologically most optimal as animal models of familial ALS with the *SOD1*-mutations.

ACKNOWLEDGMENTS

This work was supported by Grant-in-Aid from the Ministry of Health, Labour and Welfare (MA, SK, YI) and Haruki ALS Research Foundation (MA, YI).

REFERENCES

1. Brown RH Jr. Superoxide dismutase in familial amyotrophic lateral sclerosis models for gain of function. *Curr Opin Neurobiol* 1995; **5**: 841–846.
2. Aoki M, Ogasawara M, Matsubara Y *et al.* Mild ALS in Japan associated with novel SOD mutation. *Nat Genet* 1993; **5**: 323–324.

3. 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.
4. Andersen PM, Sims KB, Xin WW *et al.* Sixteen novel mutations in the Cu/Zn superoxide dismutase gene in amyotrophic lateral sclerosis: a decade of discoveries, defects and disputes. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2003; **4**: 62–73.
5. Aoki M, Abe K, Houi K *et al.* Variance of age at onset in a Japanese family with amyotrophic lateral sclerosis associated with a novel Cu/Zn superoxide dismutase mutation. *Ann Neurol* 1995; **37**: 676–679.
6. Deng HX, Tainer JA, Mitsumoto H *et al.* Two novel SOD1 mutations in patients with familial amyotrophic lateral sclerosis. *Hum Mol Genet* 1995; **4**: 1113–1116.
7. Aoki M, Ogasawara M, Matsubara Y *et al.* Familial amyotrophic lateral sclerosis (ALS) in Japan associated with H46R mutation in Cu/Zn superoxide dismutase gene: a possible new subtype of familial ALS. *J Neurol Sci* 1994; **126**: 77–83.
8. Aoki M, Abe K, Itoyama Y. Molecular analyses of the Cu/Zn superoxide dismutase gene in patients with familial amyotrophic lateral sclerosis (ALS) in Japan. *Cell Mol Neurobiol* 1998; **18**: 639–647.
9. 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.
10. Wong PC, Pardo CA, Borchelt DR *et al.* An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 1995; **14**: 1105–1116.
11. Bruijn LI, Becher MW, Lee MK *et al.* ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 1997; **18**: 327–338.
12. Wang J, Xu G, Gonzales V *et al.* Fibrillar inclusions and motor neuron degeneration in transgenic mice expressing superoxide dismutase 1 with a disrupted copper-binding site. *Neurobiol Dis* 2002; **10**: 128–138.
13. 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.
14. Gurney ME, Tomasselli AG, Heinrikson RL. Stay the executioner's hand. *Science* 2000; **288**: 283–284.
15. Nagai M, Aoki M, Miyoshi I *et al.* Rats expressing human cytosolic copper-zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. *J Neurosci* 2001; **21**: 9246–9254.
16. Cudkowicz M, McKenna-Yasek D, Sapp P *et al.* Epidemiology of SOD1mutations in amyotrophic lateral sclerosis. *Ann Neurol* 1997; **41**: 210–212.
17. Kato S, Shaw P, Wood-Allum C, Leigh PN, Show C. Amyotrophic lateral sclerosis. In: Dickson D (ed.) *Nerodegeneration: the Molecular Pathology of Dementia and Movement Disorders*. Basel: ISN Neuropath Press, 2003; 350–368.
18. Kato S, Saeki Y, Aoki M *et al.* Histological evidence of redox system breakdown caused by superoxide dismutase 1 (SOD1) aggregation is common to SOD1-mutated motor neurons in humans and animal models. *Acta Neuropathol* 2004; **107**: 149–158.
19. Kato S, Saito M, Hirano A, Ohama E. Recent advances in research on neuropathological aspects of familial amyotrophic lateral sclerosis with superoxide dismutase 1 gene mutations: neuronal Lewy body-like hyaline inclusions and astrocytic hyaline inclusions. *Histol Histopathol* 1999; **14**: 973–989.
20. Kato S, Takikawa M, Nakashima K *et al.* New consensus research on neuropathological aspects of familial amyotrophic lateral sclerosis with superoxide dismutase 1 (SOD1) gene mutations: inclusions containing SOD1 in neurons and astrocytes. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2000; **1**: 163–184.
21. Howland DS, Liu J, She Y *et al.* Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci USA* 2002; **99**: 1604–1609.
22. Mitsumoto H, Gordon P, Kaufmann P, Gooch CL, Przedborski S, Rowland LP. Randomized control trials in ALS: lessons learned. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2004; **5** (Suppl. 1): 8–13.

Journal of Neuroscience Research 82:63–70 (2005)

Motoneuron Degeneration After Facial Nerve Avulsion Is Exacerbated in Presymptomatic Transgenic Rats Expressing Human Mutant Cu/Zn Superoxide Dismutase

Ken Ikeda,[1,2] Masashi Aoki,[3] Yoko Kawazoe,[1] Tsuyoshi Sakamoto,[1] Yuichi Hayashi,[1] Aya Ishigaki,[3] Makiko Nagai,[3] Rieko Kamii,[3] Shinsuke Kato,[4] Yasuto Itoyama,[3] and Kazuhiko Watabe[1]*

[1]Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan
[2]Department of Neurology, PL Tokyo Health Care Center, Tokyo, Japan
[3]Department of Neurology, Tohoku University Graduate School of Medicine, Sendai, Japan
[4]Department of Neuropathology, Institute of Neurological Sciences, Faculty of Medicine, Tottori University, Yonago, Japan

We investigated motoneuron degeneration after proximal nerve injury in presymptomatic transgenic (tg) rats expressing human mutant Cu/Zn superoxide dismutase (SOD1). The right facial nerves of presymptomatic tg rats expressing human H46R or G93A SOD1 and their non-tg littermates were avulsed, and facial nuclei were examined at 2 weeks postoperation. Nissl-stained cell counts revealed that facial motoneuron loss after avulsion was exacerbated in H46R- and G93A-tg rats compared with their non-tg littermates. The loss of motoneurons in G93A-tg rats after avulsion was significantly greater than that in H46R-tg rats. Intense cytoplasmic immunolabeling for SOD1 in injured motoneurons after avulsion was demonstrated in H46R- and G93A-tg rats but not in their littermates. Facial axotomy did not induce significant motoneuron loss nor enhance SOD1 immunoreactivity in these tg rats and non-tg littermates at 2 weeks postoperation, although both axotomy and avulsion elicited intense immunolabeling for activating transcription factor-3, phosphorylated c-Jun, and phosphorylated heat shock protein 27 in injured motoneurons of all these animals. The present data indicate the increased vulnerability of injured motoneurons after avulsion in the presymptomatic mutant SOD1-tg rats.

Key words: axotomy; facial nerve; amyotrophic lateral sclerosis; ALS; mutant Cu/Zn superoxide dismutase; SOD1; transgenic rat

Since the discovery of the mutation of Cu/Zn superoxide dismutase (SOD1) in patients with familial amyotrophic lateral sclerosis (ALS) and the development of transgenic (tg) mice and rats expressing human mutant SOD1 that show clinicopathological characteristics comparable to human familial ALS, the mutant SOD1-tg animals have been the most widely used experimental models for elucidating the pathomechanism of and the therapeutic approach for familial ALS as well as sporadic ALS (Cleveland and Rothstein, 2001). Although the precise mechanism of motoneuron degeneration in mutant SOD1-tg animals is largely unknown, the mutant SOD1 is thought to have a gain of toxic function (Cleveland and Rothstein, 2001). In another animal model of motoneuron degeneration, peripheral nerve avulsion exhibits extensive loss of motoneurons in adult rats (Søreide, 1981; Wu, 1993; Koliatsos et al., 1994; Watabe et al., 2000; Sakamoto et al., 2000, 2003a,b; Ikeda et al., 2003; Moran and Graeber, 2004). The mechanism of motoneuron degeneration after avulsion also remains unclear, but peroxinitrite-mediated oxidative damage and perikaryal accumulation of phosphorylated neurofilaments have been demonstrated in injured motoneurons after avulsion (Martin et al., 1999). Both of these pathological features have also been shown in

Contract grant sponsor: Ministry of Education, Culture, Sports, Science and Technology, Japan; Contract grant sponsor: Research on Specific Diseases, Health Sciences Research Grants, Ministry of Health, Labor and Welfare, Japan; Contract grant sponsor: Research on Psychiatric and Neurological Diseases and Mental Health, H16-kokoro-017, Ministry of Health, Labor and Welfare, Japan.

*Correspondence to: Kazuhiko Watabe, MD, PhD, Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan.
E-mail: kazwtb@tmin.ac.jp

Received 6 April 2005; Revised 31 May 2005; Accepted 30 June 2005

Published online 17 August 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20621

spinal motoneurons in mutant SOD1-tg animals as well as in patients with familial and sporadic ALS (Estévez et al., 1998; Cleveland, 1999; Cleveland and Rothstein, 2001). If motoneuron degeneration after peripheral nerve avulsion shares any underlying mechanisms of motoneuron death associated with SOD1 mutation, motoneurons in presymptomatic mutant SOD1-tg animals may be more susceptible to pathological insults following avulsion compared with their non-tg littermates. If this is so, we may be able to utilize facial nerve avulsion as an animal model for understanding the mechanisms of motoneuron degeneration in ALS. In the present study, we examined injured motoneurons after facial nerve avulsion in presymptomatic mutant human SOD1-tg rats and their littermates.

MATERIALS AND METHODS

Animals and Surgical Procedures

The experimental protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Metropolitan Institute for Neuroscience and Tohoku University Graduate School of Medicine. The tg rats expressing human mutant SOD1 (H46R, G93A) were generated as described previously (Nagai et al., 2001). Two types of rats with SOD1 mutations, H46R and G93A, were used for experiments. The H46R-tg rats develop motor deficits at about 140 days of age and die after 3 weeks, and G93A-tg rats show the clinical signs at around 120 days of age and die after 10 days (Nagai et al., 2001).

The presymptomatic female H46R (90 days old)- and G93A (80 days old)-tg rats were anesthetized with inhalation of halothane. Under a dissecting microscope, the right facial nerve was exposed at its exit from the stylomastoid foramen. With microhemostat forceps, the proximal facial nerve was avulsed by gentle traction and removed from the distal facial nerve as described elsewhere (Sakamoto et al., 2000, 2003a,b; Ikeda et al., 2003). As for axotomy, the right facial nerve was transected at its exit from the stylomastoid foramen, and a distal portion of the nerve, 5 mm in length, was cut and removed. The wound was covered with a small piece of gelatin sponge (Gelfoam; Pharmacia Upjohn, Bridgewater, NJ) and closed by fine suture.

Motoneuron Cell Counting

At 2 weeks postoperation, rats were anesthetized with a lethal dose of pentobarbital sodium and transcardially perfused with 0.1 M phosphate buffer, pH 7.4 (PB), followed by 4% paraformaldehyde in 0.1 M PB. The brainstem tissue was excised, postfixed in the same fixative for 2 hr, dehydrated, and embedded in paraffin, and serial transverse sections (6-μm thickness) were made. Every fifth section (24-μm interval) was collected, deparaffinized, and stained with cresyl violet (Nissl staining), and facial motoneurons having nuclei containing distinct nucleoli on both sides of the facial nuclei were counted in 25 sections as described elsewhere (Sakamoto et al., 2000, 2003a,b; Ikeda et al., 2003). The data were expressed as the mean ± SEM, and statistical significance was assessed by Mann-Whitney U-test.

Immunohistochemistry

Immunohistochemistry on paraffin sections was performed with the following primary antibodies: sheep anti-human SOD1 (1:1,000; Calbiochem, San Diego, CA), rabbit anti-human SOD1 (1:10,000; kindly provided by Dr. K. Asayama; Asayama and Burr, 1984), mouse monoclonal anti-phosphorylated neurofilament SMI-31 (1:1,000; Sternberger Monoclonals, Lutherville, MD), rabbit anti-ubiquitin (1:1,000; Dako, Glostrup, Denmark), rabbit anti-glial fibrillary acidic protein (GFAP; 1:1,000; Dako), rabbit anti-activating transcription factor-3 (ATF3; sc-188, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-c-Jun (sc-1694, 1:200; Santa Cruz Biotechnology), mouse monoclonal anti-phosphorylated c-Jun (sc-822, 1:200; Santa Cruz Biotechnology), rabbit anti-heat shock protein (Hsp) 25 that reacts with rat Hsp27 (SPA-801, 1:200; Stressgen, Victoria, British Columbia, Canada), and rabbit anti-phosphospecific (Ser^{15})Hsp27 (1:200; Oncogene, San Diego, CA). For immunohistochemistry, deparaffinized sections were pretreated with 0.3% H_2O_2 in methanol and preincubated with 3% heat-inactivated goat or rabbit serum in 0.1% Triton X-100 in phosphate-buffered saline (T-PBS). In cases of immunostaining with mouse primary antibodies, MOM blocking kit (Vector, Burlingame, CA) was used according to the manufacturer's instructions to reduce nonspecific background staining. Sections were then incubated overnight at 4°C with the primary antibodies diluted in T-PBS, followed by the incubation with biotinylated rabbit anti-sheep, goat anti-rabbit, or goat anti-mouse IgG at a dilution of 1:200 and with ABC reagent (Vector), visualized by 3,3′-diaminobenzidine tetrahydrocholoride (DAB)-H_2O_2 solution and counterstained with hematoxylin. For negative controls, the primary antibodies were omitted or replaced by nonimmunized animal sera.

RESULTS

Two weeks after avulsion of the right facial nerves in non-tg littermates, the number of surviving facial motoneurons declined to ~70% of that on the contralateral side, similar to that in normal rats, as described previously (Sakamoto et al., 2000). In SOD1-tg rats, only ~30–50% of motoneurons survived 2 weeks after avulsion, indicating that the loss of motoneurons was exacerbated in SOD1-tg rats compared with their non-tg littermates (Fig. 1, Table I). The numbers of surviving motoneurons in G93A-tg rats after avulsion (~35% of contralateral side) were significantly less than those in H46R-tg rats (~50% of contralateral side; Table I). The numbers of intact motoneurons at contralateral sides did not differ between tg rats and non-tg littermates, indicating that cell loss does not happen at this moment in the course of the disease with SOD1 mutations (Table I). Facial nerve axotomy did not induce significant loss of injured motoneurons in tg rats and non-tg littermates at 2 weeks postoperation (Fig. 1, Table I).

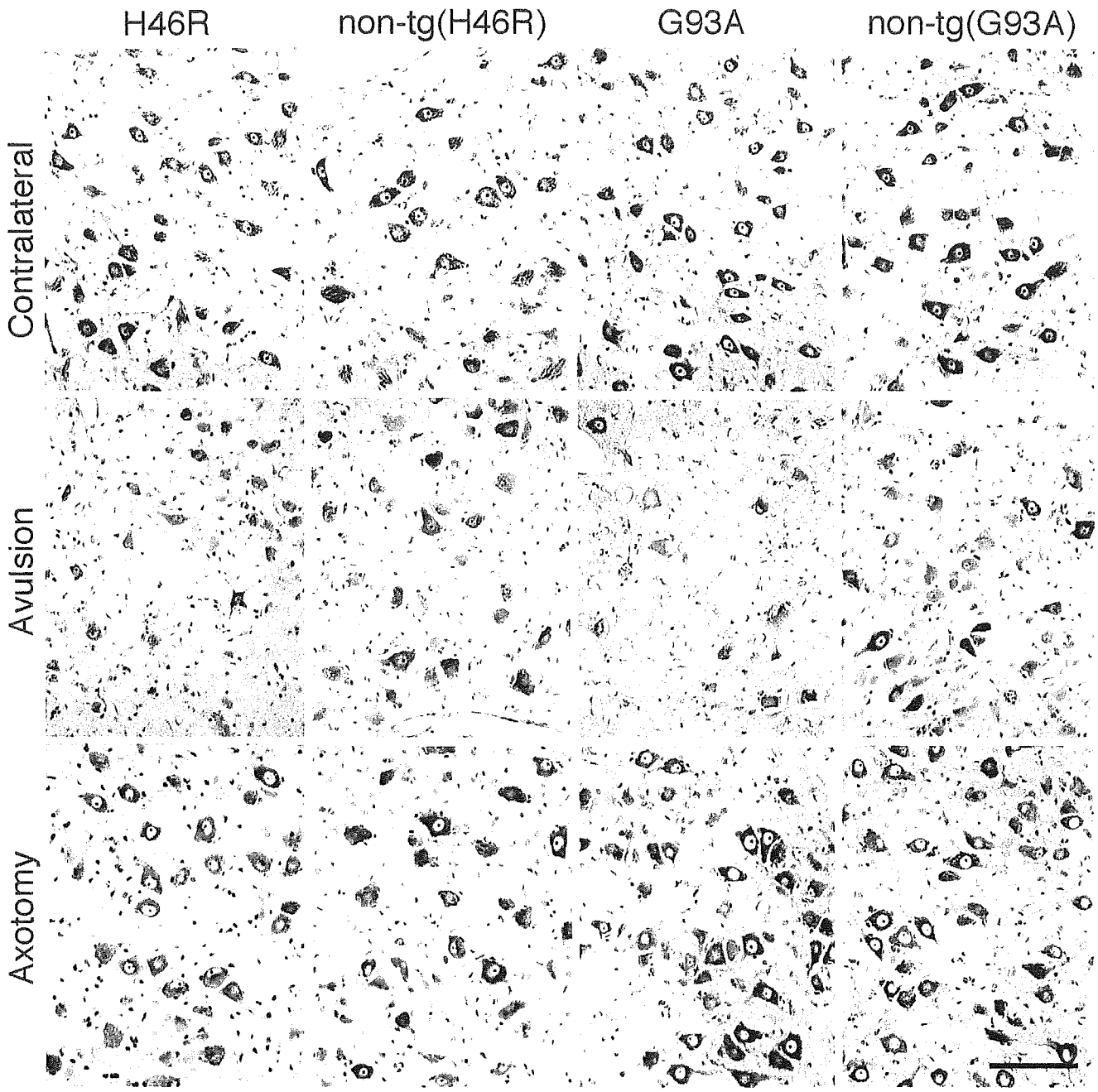

Fig. 1. Facial motoneurons of H46R- and G93A-transgenic (tg) rats and their non-tg littermates on the contralateral and ipsilateral (avulsion or axotomy) sides 2 weeks after facial nerve avulsion or axotomy. Nissl stain. Scale bar = 100 μm.

Examination of sections immunostained for SOD1 showed intense cytoplasmic immunolabeling for SOD1 in injured motoneurons after avulsion in H46R- and G93A-tg rats compared with uninjured motoneurons on the contralateral side that were not or were very faintly immunoreactive for SOD1 (Fig. 2). We used sheep and rabbit anti-SOD1 antibodies, both of which gave identical results. The cytoplasmic SOD1 immunolabeling patterns of injured motoneurons appeared diffuse in H46R-tg rats, whereas they were granular in G93A-tg rats. In G93A-tg rats, there were axons and vacuolar changes in the neuropil consistently immunoreactive for SOD1 at both uninjured and injured sides of facial nuclei (Fig. 2). There was no definite immunolabeling for SOD1 in either injured or uninjured motoneurons and their axons in non-tg littermates (Fig. 2). Facial nerve axotomy did not increase immunoreactivity for SOD1 in injured motoneurons of tg rats and non-tg littermates at 2 weeks

TABLE I. Survival of Motoneurons After Facial Nerve Avulsion and Axotomy†

Rat (n)	Ipsilateral motoneuron number	Contralateral motoneuron number	Survival %
Avulsion			
NL (H46R) (n = 10)	598 ± 18	813 ± 26	73.7 ± 1.2
H46R (n = 8)	402 ± 36*	839 ± 27	47.5 ± 2.8*
NL (G93A) (n = 6)	637 ± 56	822 ± 47	76.7 ± 3.0
G93A (n = 7)	306 ± 37*	884 ± 44	34.7 ± 3.6*,**
Axotomy			
H46R (n = 5)	751 ± 19	843 ± 23	89.2 ± 1.4
NL (G93A) (n = 6)	743 ± 15	835 ± 12	88.9 ± 0.6
G93A (n = 5)	741 ± 42	781 ± 45	94.9 ± 1.2

†Numbers of facial motoneurons and the percent survival at the ipsilateral (lesion) side relative to the contralateral (control) side 2 weeks after avulsion or axotomy. Results are presented as mean ± SEM. Statistical comparison was done by Mann-Whitney U-test. n = number of animals. NL. nontransgenic littermates.
*$P < 0.01$ vs. NL (H46R) and NL (G93A) rats after avulsion.
**$P < 0.05$ vs. H46R-transgenic rats after avulsion.

postoperation (Fig. 2). In contrast, immunohistochemical examination showed perikaryal accumulation of phosphorylated neurofilaments in injured motoneurons both after axotomy and after avulsion, as described previously (Koliatsos et al., 1989, 1994; Koliatsos and Price, 1996). There were no hyaline inclusions identifiable in HE-stained sections or ubiquitin-immunoreactive structures in both H46R- and G93A-tg rats and their non-tg littermates on either operated or contralateral sides (data not shown). Proliferation of astrocytes as evidenced by immunostaining for GFAP was observed at the injured sides in all the animals after avulsion and axotomy, and the degree of the astrocytic response appeared to correlate with the extent of motoneuron loss after avulsion; i.e., more intense GFAP immunostaining was demonstrated when less neuronal survival was observed (Fig. 3).

It has been shown that ATF3 is expressed, and c-Jun and Hsp27 are up-regulated and phosphorylated, in injured motoneurons after axotomy (Tsujino et al., 2000; Casanovas et al., 2001; Benn et al., 2002; Kalmár et al., 2002). Several reports have documented that ATF3, c-Jun, and Hsp27 cooperate to promote neuronal survival in vitro and in vivo, suggesting neuroprotective roles of these molecules (Pearson et al., 2003; Nakagomi et al., 2003). We then examined the expression of ATF3, c-Jun, and Hsp27 in injured motoneurons after facial nerve avulsion that causes extensive neuronal loss. In wild-type adult rats, intact facial motoneurons were constitutively immunoreactive for c-Jun and Hsp27 but not for ATF3, phosphorylated c-Jun, or phosphorylated Hsp27, whereas injured motoneurons become immunoreactive for ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 within 1 day after facial nerve avulsion and remain positive up to 4 weeks (Watabe et al., unpublished observations). In a similar manner, virtually all injured motoneurons were immunostained for ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 in H46R- and G93A-tg rats and their non-tg littermates 2 weeks after avulsion and axotomy as examined in this study (Fig. 3).

DISCUSSION

We demonstrated that only 50% (H46R-tg rats) or 35% (G93A-tg rats) of motoneurons in mutant SOD1-tg rats survived 2 weeks after avulsion at their presymptomatic stage compared with 70% survival of motoneurons in their non-tg littermates, indicating that motoneuron degeneration after avulsion is significantly more severe in these presymptomatic mutant SOD1-tg rats. It is interesting to note that the loss of motoneurons in G93A-tg rats was significantly greater than that in H46R-tg rats after avulsion, insofar as the onset of paralysis is earlier and the disease progression is more rapid in G93A-tg rats compared with the H46R rats used in the present study (Nagai et al., 2001). The clinical courses of these tg rats are also likely to be relevant to those of human mutant SOD1-mediated familial ALS, in that the human H46R cases progress very slowly compared with the G93A cases (Nagai et al., 2001; Aoki et al., 1993, 1994). In contrast, we did not see significant motoneuron loss in the presymptomatic SOD1-tg rats and their non-tg littermates 2 weeks after facial nerve axotomy. Unlike avulsion, axotomy does not generally induce significant motoneuron death in adult rodents (Lowrie and Vrbová, 1992; Moran and Graeber, 2004), except that, in the case of adult Balb/C mice, the facial nerve axotomy leads to loss of >50% of the motoneurons at 30 days postoperation (Hottinger et al., 2000), and C57BL mice show late motoneuron loss (~60%) 8 weeks after facial nerve axotomy (Angelov et al., 2003). Mariotti et al. (2002) axotomized facial nerves of G93A-tg mice and their non-tg littermates at their presymptomatic stage and observed loss of facial motoneurons that was higher in G93A-tg mice than in non-tg littermates at 30 days postaxotomy; these data are relevant to our present data acquired from avulsion, but not axotomy, in rats, which probably is due to the use of different animal species. In contrast, Kong and Xu (1999) described axotomy of lumbar spinal or sciatic nerve in G93A-tg mice at the presymptomatic stage reducing the extent of axon degeneration at the end stage of the disease. They did

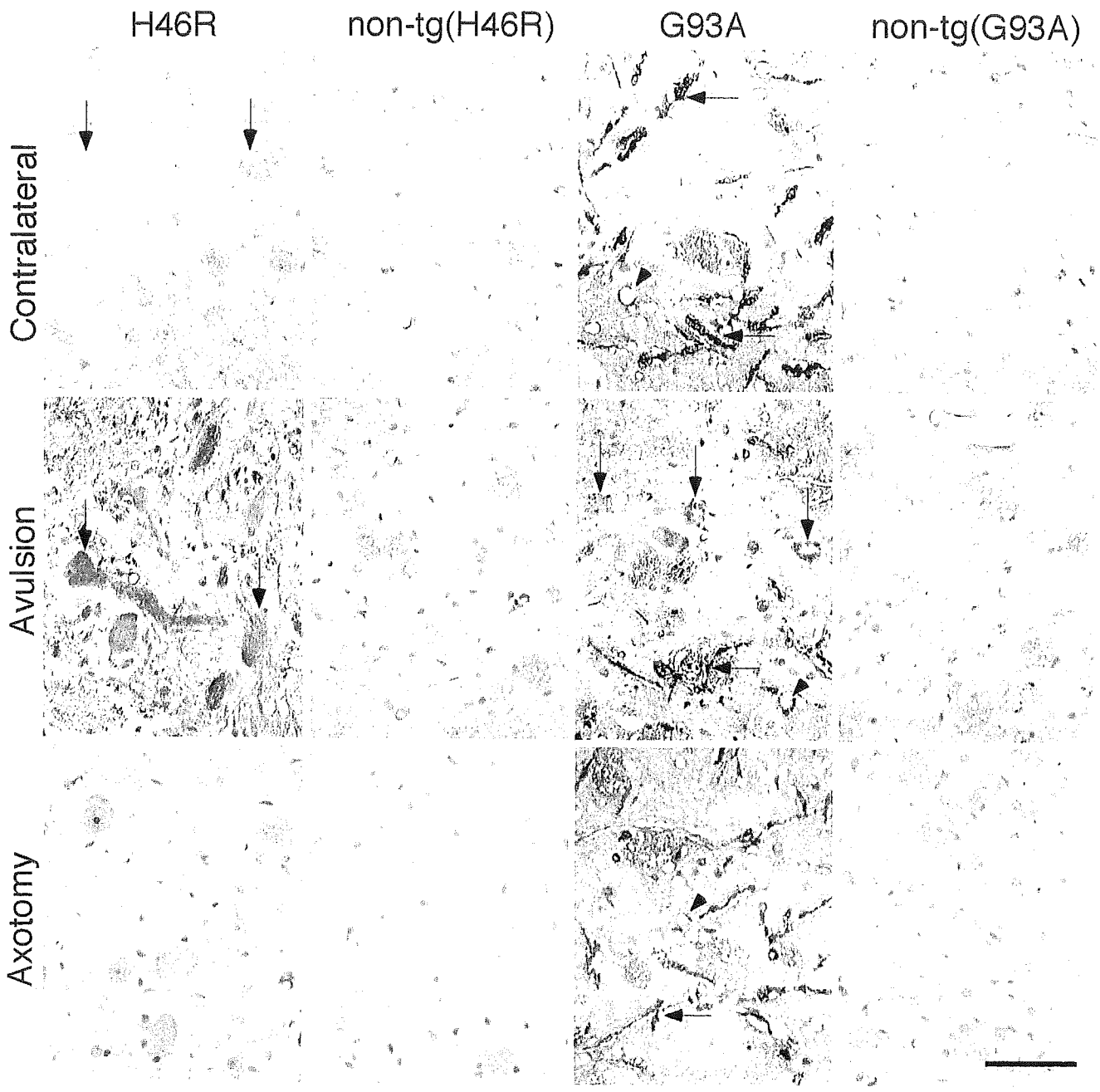

Fig. 2. SOD1 immunohistochemistry of facial motoneurons of H46R- and G93A-tg rats and their non-tg littermates on the contralateral and ipsilateral (avulsion or axotomy) sides 2 weeks after facial nerve avulsion or axotomy. Counterstained with hematoxylin. Note immunostained motoneurons (vertical arrows), axons (horizontal arrows), and vacuoles in neuropil (arrowheads) in H46R- and G93A-tg rats. Scale bar = 50 μm.

not evaluate the response of the cell bodies of spinal motoneurons, so it remains unknown whether SOD1 mutation affects the viability of spinal motoneurons after axotomy. In the present study, we demonstrated that motoneuron degeneration after facial nerve avulsion, but not after axotomy, is exacerbated in presymptomatic mutant SOD1-tg rats at 2 weeks postoperation. These data clearly indicate the increased vulnerability of facial motoneurons to proximal nerve injury in the presymptomatic SOD1-tg rats.

It has been shown that SOD1 is abundantly expressed in cell bodies, dendrites, and axons of wild-type mouse and rat motoneurons in vivo (Pardo et al., 1995; Moreno et al., 1997; Yu, 2002). In the present

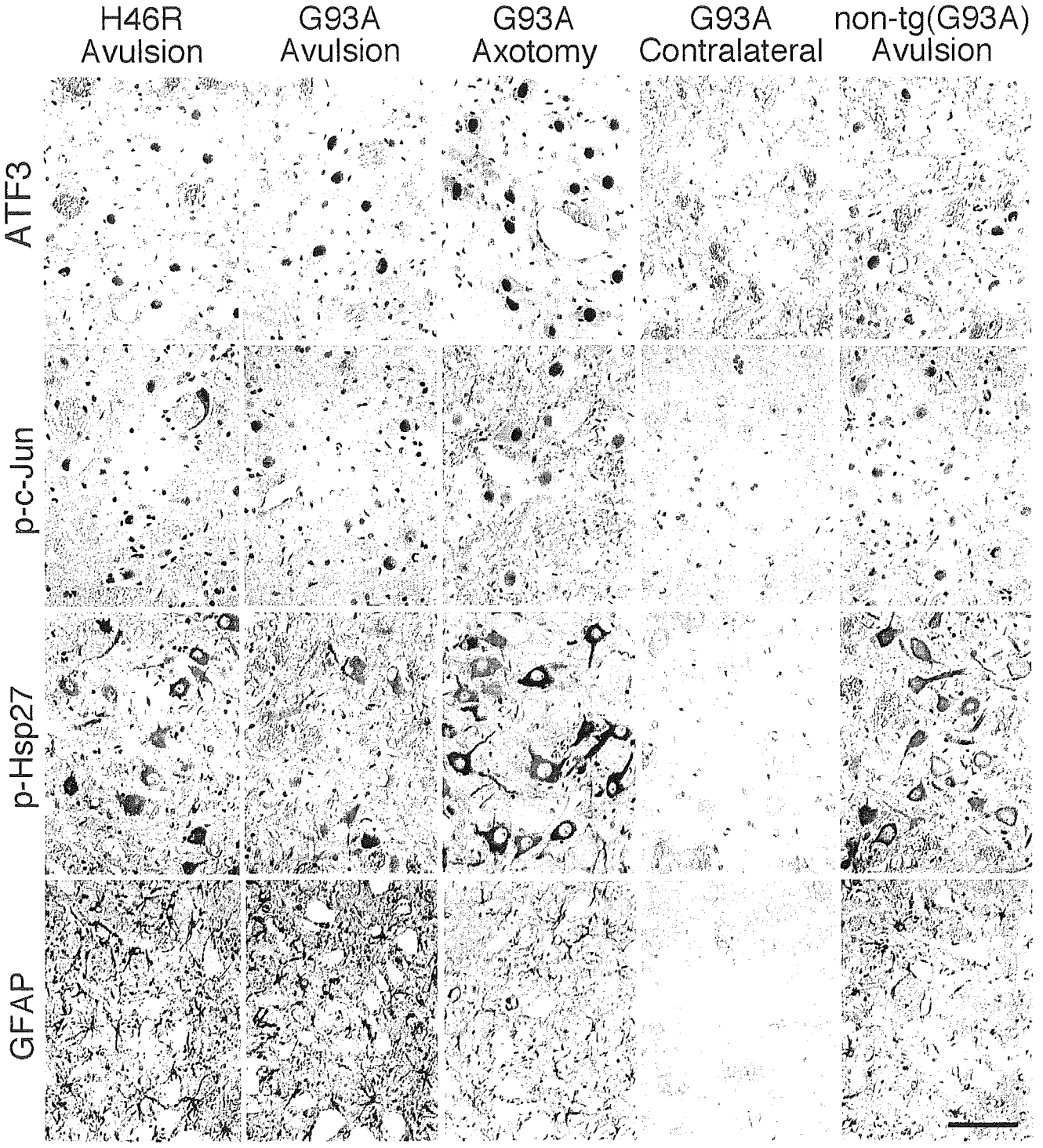

Fig. 3. Immunohistochemistry for ATF3, phosphorylated c-Jun (p-c-Jun), phosphorylated Hsp27 (p-Hsp27), and GFAP of facial nuclei in H46R-tg rat, G93A-tg rat, and non-tg littermate on the ipsilateral (avulsion or axotomy) and contralateral sides 2 weeks after facial nerve avulsion or axotomy. All injured motoneurons are immunostained for ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 in these rats after avulsion or axotomy. The intensity of GFAP immunoreactivity appears parallel to the extent of motoneuron loss (see also Fig. 1). Counterstained with hematoxylin. Scale bar = 50 μm.

study, we did not observe immunoreactivity for SOD1 in facial motoneurons of nontransgenic littermates with sheep and rabbit anti-human SOD1 antibodies; it is postulated that the antibody concentrations (i.e., 1:1,000–10,000) used in this study are below the detection levels for immunostaining rat SOD1 antigen on paraffin sections. Instead, we demonstrated some facial motoneurons showing very faint immunoreactivity for SOD1 in H46R-tg rats on paraffin sections. In G93A-tg rats, axons and vacuoles in neuropil were intensely immunoreactive for SOD1 at both uninjured and injured sides. The increased immunostaining for SOD1 in injured motoneurons of SOD1 (H46R and G93A)-tg rats may therefore indicate that human mutant SOD1 protein is accumulated in the cytoplasm of facial motoneurons after avulsion. When several mutant SOD1 genes that include G93A were transfected to COS7 cells, the mutant SOD1s, but not wild-type SOD1, aggregated in association with the endoplasmic reticulum (ER) and induced ER stress (Tobisawa et al., 2003). Accumulation of mutant SOD1 in injured motoneurons after avulsion may therefore potentiate ER stress and exacerbate motoneuron death in the presymptomatic mutant SOD1-tg rats, although the mechanism of accumulation of SOD1 remains unknown. Whether up-regulation of cytoplasmic mutant SOD1 expression or retrograde accumulation of mutant SOD1 from injured axons was induced in these neurons awaits further investigations. In addition, facial nerve axotomy, as opposed to avulsion, did not increase immunoreactivity for SOD1 in injured motoneurons of SOD1-tg rats and their non-tg littermates, which seems consistent with the absence of significant motoneuron loss in these rats as described above. As for wild-type SOD1, previous reports documented no change in SOD1 mRNA levels or SOD1 immunoreactivity in injured motoneurons after facial or sciatic nerve axotomy in wild-type rats (Yoneda et al., 1992; Rosefeld et al., 1997).

It has been demonstrated that ATF3 is expressed, and c-Jun and Hsp27 are up-regulated and phosphorylated, in injured adult motoneurons after axotomy (Tsujino et al., 2000; Casanovas et al., 2001; Benn et al., 2002; Kalmár et al., 2002). As for the neuroprotective nature of these molecules, it has been reported that ATF3 enhances c-Jun-mediated neurite sprouting in PC12 and Neuro-2a cells (Pearson et al., 2003), and ATF3 and Hsp27 cooperate with c-Jun to prevent death of PC12 cells and superior cervical ganglion neurons (Nakagomi et al., 2003). Hsp27 is induced and phosphorylated in adult, but not in neonatal, motoneurons after axotomy, and axotomized neonatal motoneurons that lack Hsp27 die by apoptosis, suggesting that phosphorylated Hsp27 is necessary for motoneuron survival after peripheral nerve injury (Benn et al., 2002). However, there have been no reports concerning the expression of ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 in injured motoneurons after avulsion. In the present study, we have demonstrated that, even after avulsion that causes extensive motoneuron death, ATF3, phosphorylated c-Jun, and phosphorylated Hsp27 were fully up-regulated in both SOD1-tg and non-tg rats. These results suggest that neuroprotective effects of Hsp27 cannot overcome yet unidentified stress(es) induced by facial nerve avulsion. On the other hand, a recent report demonstrated that facial motoneurons of c-Jun-deficient mice are resistant to axotomy-induced cell death, suggesting that c-Jun promotes posttraumatic motoneuron death (Raivich et al., 2004). In addition, it has been shown that mutant SOD1 binds to Hsp27 and forms aggregates, suggesting that this binding of Hsp27 to mutant SOD1 blocks antiapoptotic function of Hsp27 and leads to motoneuron death (Okado-Matsumoto and Fridovich, 2002). The effects of phosphorylated c-Jun and Hsp27 and their association with mutant SOD1 accumulation should be further investigated to elucidate the mechanism of exacerbated motoneuron death in SOD1-tg rats after avulsion.

In this study, we have demonstrated that motoneuron degeneration after facial nerve avulsion is exacerbated in presymptomatic mutant SOD1-tg rats compared with their non-tg littermates. Mutant SOD1 accumulation and its association with c-Jun and Hsp27 may have a key role leading to enhanced motoneuron death. In this context, motoneuron death after avulsion may share, at least in part, a common mechanism with the motoneuron degeneration associated with SOD1 mutation.

ACKNOWLEDGMENT

We are grateful to Dr. Kohtaro Asayama (University of Occupational and Environmental Health, Japan) for kindly providing rabbit anti-SOD1 antibody.

REFERENCES

Angelov DN, Waibel S, Guntinas-Lichius O, Lenzen M, Neiss WF, Tomov TL, Yoles E, Kipnis J, Schori H, Reuter A, Ludolph A, Schwartz M. 2003. Therapeutic vaccine for acute and chronic motor neuron diseases: implications for amyotrophic lateral sclerosis. Proc Natl Acad Sci U S A 100:4790–4795.

Aoki M, Ogasawara M, Matsubara Y, Narisawa K, Nakamura S, Itoyama Y, Abe K. 1993. Mild ALS in Japan associated with novel SOD mutation. Nat Genet 5:323–324.

Aoki M, Ogasawara M, Matsubara Y, Narisawa K, Nakamura S, Itoyama Y, Abe K. 1994. Familial amyotrophic lateral sclerosis (ALS) in Japan associated with H46R mutation in Cu/Zn superoxide dismutase gene: a possible new subtype of familial ALS. J Neurol Sci 126:77–83.

Asayama K, Burr IM. 1984. Joint purification of mangano and copper/zinc superoxide dismutase from a single source: a simplified method. Anal Biochem 136:336–339.

Benn SC, Perrelet D, Kato AC, Scholz J, Decosterd I, Mannion RJ, Bakowska JC, Woolf CJ. 2002. Hsp27 upregulation and phosphorylation is required for injured sensory and motor neuron survival. Neuron 36:45–56.

Casanovas A, Ribera J, Hager G, Kreutzberg GW, Esquerda JE. 2001. c-Jun regulation in rat neonatal motoneurons postaxotomy. J Neurosci 63:469–479.

Cleveland DW. 1999. From Charcot to SOD1: mechanisms of selective motor neuron death in ALS. Neuron 24:515–520.

Cleveland DW, Rothstein JD. 2001. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. Nat Rev Neurosci 2:806–819.

Estévez AG, Spear N, Manuel SM, Barbeito L, Radi R, Beckman JS. 1998. Role of endogenous nitric oxide and peroxynitrite formation in the survival and death of motor neurons in culture. Prog Brain Res 18: 269–280.

Hottinger AF, Azzouz M, Déglon N, Aebischer P, Zurn AD. 2000. Complete and long-term rescue of lesioned adult motoneurons by lentiviral-mediated expression of glial cell line-derived neurotrophic factor in the facial nucleus. J Neurosci 20:5587–5593.

Ikeda K, Sakamoto T, Kawazoe Y, Marubuchi S, Nakagawa M, Ono S, Terashima N, Kinoshita M, Iwasaki Y, Watabe K. 2003. Oral administration of a neuroprotective compound T-588 prevents motoneuron degeneration after facial nerve avulsion in adult rats. Amyotroph Lateral Scler Other Motor Neuron Disord 4:74–80.

Kalmár B, Burnstock G, Vrborá G, Greensmith L. 2002. The effect of neonatal injury on the expression of heat shock proteins in developing rat motoneurons. J Neurotrauma 19:667–679.

Koliatsos VE, Price DL. 1996. Axotomy as an experimental model of neuronal injury and cell death. Brain Pathol 6:447–465.

Koliatsos VE, Applegate MD, Kitt CA, Walker LC, DeLong MR, Price DL. 1989. Aberrant phosphorylation of neurofilaments accompanies transmitter-related changes in rat septal neurons following transection of the fimbria-fornix. Brain Res 482:205–218.

Koliatsos VE, Price WL, Pardo CA, Price DL. 1994. Ventral root avulsion: an experimental model of death of adult motor neurons. J Comp Neurol 342:35–44.

Kong J, Xu Z. 1999. Peripheral axotomy slows motoneuron degeneration in a transgenic mouse line expressing mutant SOD1 G93A. J Comp Neurol 412:373–380.

Lowrie MB, Vrbová G. 1992. Dependence of postnatal motoneurones on their targets: review and hypothesis. Trend Neurosci 15:80–84.

Mariotti R, Cristino L, Bressan C, Boscolo B, Bentivoglio M. 2002. Altered reaction of facial motoneurons to axonal damage in the presymptomatic phase of a murine model of familial amyotrophic lateral sclerosis. Neuroscience 115:331–335.

Martin LJ, Kaiser A, Price AC. 1999. Motor neuron degeneration after nerve avulsion in adult evolves with oxidative stress and is apoptosis. J Neurobiol 40:185–201.

Moran LB, Graeber MB. 2004. The facial nerve axotomy model. Brain Res Rev 44:154–178.

Moreno S, Nardacci R, Ceru MP. 1997. Regional and ultrastructural immunolocalization of copper-zinc superoxide dismutase in rat central nervous system. J Histochem Cytochem 45:1611–1633.

Nagai M, Aoki M, Miyoshi I, Kato M, Pasinelli P, Kasai N, Brown RH Jr, Itoyama Y. 2001. Rats expressing human cytosolic copper-zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. J Neurosci 21:9246–9254.

Nakagomi S, Suzuki Y, Namikawa K, Kiryu-Seo S, Kiyama H. 2003. Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. J Neurosci 23:5187–5196.

Okado-Matsumoto A, Fridovich I. 2002. Amyotrophic lateral sclerosis: a proposed mechanism. Proc Natl Acad Sci U S A 99:9010–9014.

Pardo CA, Xu Z, Borchelt DR, Price DL, Sisodia SS, Cleveland DW. 1995. Superoxide dismutase is an abundant component in cell bodies, dendrites, and axons of motor neurons and in a subset of other neurons. Proc Natl Acad Sci U S A 92:954–958.

Pearson AG, Gray CW, Pearson JF, Greenwood JM, During MJ, Dragunow M. 2003. ATF3 enhances c-Jun-mediated neurite sprouting. Brain Res Mol Brain Res 120:38–45.

Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, Hristova M, Nateri AS, Makwana M, Riera-Sans L, Wolfer DP, Lipp HP, Aguzzi A, Wagner EF, Behrens A. 2004. The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron 43:57–67.

Rosenfeld J, Cook S, James R. 1997. Expression of superoxide dismutase following axotomy. Exp Neurol 147:37–47.

Sakamoto T, Watabe K, Ohashi T, Kawazoe Y, Oyanagi K, Inoue K, Eto Y. 2000. Adenoviral vector-mediated GDNF gene transfer prevents death of adult facial motoneurons. Neuroreport 11:1857–1860.

Sakamoto T, Kawazoe Y, Shen J-S, Takeda Y, Arakawa Y, Ogawa J, Oyanagi K, Ohashi T, Watanabe K, Inoue K, Eto Y, Watabe K. 2003a. Adenoviral gene transfer of GDNF, BDNF and TGFβ2, but not CNTF, cardiotrophin-1 or IGF1, protects injured adult motoneurons after facial nerve avulsion. J Neurosci Res 72:54–64.

Sakamoto T, Kawazoe Y, Uchida Y, Hozumi I, Inuzuka T, Watabe K. 2003b. Growth inhibitory factor prevents degeneration of injured adult rat motoneurons. Neuroreport 14:2147–2151.

Søreide AJ. 1981. Variations in the axon reaction after different types of nerve lesion. Acta Anat 110:173–188.

Tobisawa S, Hozumi Y, Arawaka S, Koyama S, Wada M, Nagai M, Aoki M, Itoyama Y, Goto K, Kato T. 2003. Mutant SOD1 linked to familial amyotrophic lateral sclerosis, but not wild-type SOD1, induces ER stress in COS7 cells and transgenic mice. Biochem Biophys Res Commun 303:496–503.

Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K, Ochi T, Noguchi K. 2000. Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: a novel neuronal marker of nerve injury. Mol Cell Neurosci 15:170–182.

Watabe K, Ohashi T, Sakamoto T, Kawazoe Y, Takeshima T, Oyanagi K, Inoue K, Eto Y, Kim SU. 2000. Rescue of lesioned adult rat spinal motoneurons by adenoviral gene transfer of glial cell line-derived neurotrophic factor. J Neurosci Res 60:511–519.

Wu W. 1993. Expression of nitric-oxide synthase (NOS) in injured CNS neurons as shown by NADPH diaphorase histochemistry. Exp Neurol 120:153–159.

Yoneda T, Inagaki S, Hayashi Y, Nomura T, Takagi H. 1992. Differential regulation of manganese and copper/zinc superoxide dismutases by the facial nerve transection. Brain Res 582:342–345.

Yu WHA. 2002. Spatial and temporal correlation of nitric oxide synthase expression with CuZn-superoxide dismutase reduction in motor neurons following axotomy. Ann N Y Acad Sci 962:111–121.