Histological assessment and immunohistochemistry

Mice were anesthetized with sodium pentobarbital and perfused through the aortic cone with phosphate-buffered saline (PBS), followed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) at pH 7.4. Brains and spinal cords were removed and post-fixed in the same fixative for 2 h, and then immersed in 20% sucrose in PB overnight at 4℃. The tissue was sectioned at 20 μm on a freezing microtome. For paraffin-embedded section, tissues were transferred to 70% ethanol and embedded in paraffin as described (Ichikawa et al., 1997). Serial brain or spinal cord sections were cut at 5 μm. Sections were stained for Nissl substance with cresyl violet, or Fluoro-Jade B (Chemicon) according to the manufacturer's protocol. For staining of SOD2, SOD1, ChAT, and CHT, paraffin-embedded sections were immunohistochemically processed as described elsewhere (Ichikawa et al., 1997) with diaminobenzidine (DAB) as a chromogen followed by post-staining with hematoxylin. Antibodies used are rabbit polyclonal anti-SOD2 antibody (1:2,000; Stressgen Biotechnologies), rabbit polyclonal anti-SOD1 antibody (1:2,000; Stressgen Biotechnologies), rabbit polyclonal anti-ChAT (1:10,000; Ichikawa et al., 1997), and rabbit polyclonal anti-CHT antibody (50 ng/ml; Misawa et al., 2001). To estimate frequencies of Cre-mediated recombination of the floxed SOD2 alleles, serial paraffin sections were stained with SOD2 and ChAT as above and the number of positive cells were counted. More than 500 cells were analyzed in the spinal cord ventral horn and 200 cells in each of the brainstem motor nucleus (from at least 3 mice at respective age). Double labeling for SOD2 and SMI-32 was performed by immunofluorescence. Sections were

incubated simultaneously with rabbit polyclonal anti-SOD2 antibody (1:500; Stressgen Biotechnologies) and mouse SMI-32 monoclonal antibody to neurofilaments (1:1,000; Sternberger Monoclonals). Texas red-conjugated goat anti-mouse IgG was used to detect SOD2-positive cells and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was used to detect SMI-32-expressing cells (1:200; Jackson Immunoresearch Labs).

In situ detection of $O_2^{\cdot -}$ production

The spatial production of $O_2^{\cdot -}$ was investigated by in situ detection of oxidized hydroethidine (HEt; Molecular Probes) as previously described (Murakami et al. 1998). HEt is oxidized to a red fluorescent dye (ethidium) in living cells selectively by $O_2^{\cdot -}$, but not by other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or peroxynitrite (Bindokas et al., 1996). Briefly, HEt solution (0.2 ml; stock solution of HEt, 100 mg/ml in DMSO, diluted to 1 mg/ml in PBS) was intravenously injected 30 min before the animals were sacrificed. The animals were perfused with 4% paraformaldehyde as described above. Brain and spinal cord sections (20 μm) were cut on a cryostat and processed for fluorescent microscopy.

Hypoglossal nerve axotomy

Nine-month-old mice ($SOD2^{lox/lox}$;$Cre^{slow/-}$ or $SOD2^{lox/lox}$;$Cre^{-/-}$) were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). The right

hypoglossal nerve was exposed under the digastric muscle and transected with scissors. After 5 weeks, the animals were re-anesthetized and perfused with 4% paraformaldehyde. The brainstem was removed, 5 μm serial paraffin-embedded sections were prepared and stained with cresyl violet as described above. Hypoglossal motor neurons with distinct clear nuclei in every eighth section (total 8 sections per animal) were counted.

Analysis of Wallerian degeneration

Two days after unilateral transection of hypoglossal nerves as described above, mice were sacrificed by over-dose of sodium pentobarbital, the swollen first 2 mm of the distal nerve was discarded, the next 2 mm was used for morphological analysis, and a segment 4 - 7 mm distal to the lesion site was used for Western blotting. For morphological analysis, the nerve segments were fixed for 1 day in 2% paraformaldehyde, 2% glutaraldehyde in 50 mM phosphate buffer, pH 7.4 (PB). Samples were treated in 1% OsO_4 in PB, washed, dehydrated with ethanol and then propylene oxide, and finally embedded in Quetol 812 epoxy resin (Nisshin EM, Tokyo, Japan). Semithin cross-sections (0.5 μm) for light microscopy were stained with toluidine blue.

Electron microscopy

Animals were perfused with 2% glutaraldehyde, 2% paraformaldehyde, 5% sucrose in 50 mM phosphate buffer (pH 7.4). The brain and spinal cord were removed and postfixed in

the same fixative for 1 d at 4℃. The facial nucleus and spinal cord ventral horn were cut into 1-2 mm square pieces, fixed in 1% osmium tetroxide for 1 h at 4℃, dehydrated through a graded series of ethanol solutions and into propylene oxide, and embedded in Quetol 812 (Nissin EM). Ultra-thin sections were stained with lead citrate and uranyl acetate and examined with an electron microscope (H7500; Hitachi, Tokyo, Japan) at 10,000x magnification.

Analysis of muscle atrophy and denervation

Fresh skeletal muscle biopsies were obtained and frozen by immersion in isopentane cooled in liquid nitrogen. Sections were cut at 10 μm and processed for hematoxylin-eosin (H & E) or Gomori trichrome staining.

Western blotting

Cytoskeletal protein preservation was determined as described by Mark et al. (2001). Briefly, axotomyzed or control (uncut) hypoglossal nerves (3-mm length) were homogenized in 50 μl 25 mM Tris-HCl (pH 7.5), 2% SDS, 1 mM EDTA, 1x Complete protease inhibitor cocktail (Roche). Proteins (20 μl each) were separated using a 5-20% gradient polyacrylamide-SDS gel and semi-dry transferred onto a nylon membrane (Immobilon-P; Millipore). Loading and transfer was checked by staining with Ponceau S (Sigma). The membranes were incubated with monoclonal N52 antibody (Sigma) against neurofilament

heavy chain diluted at 1:3,000 in 5% nonfat skim milk/0.1% Tween 20 in PBS, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (Bio-Rad), and visualized with ECL Western Blotting Detection Reagent (Amersham Pharmacia Biosciences). The same blots were re-probed with monoclonal antibody β-tub 2.1 (1:10,000; Sigma) against β-tubulin. In order to compare SOD2 expression between $SOD2^{lox/lox}$;$Cre^{slow/-}$ and $SOD2^{lox/lox}$;$Cre^{-/-}$ mice, ventral halves of the cervical spinal cord (5-mm length) were micro-dissected, homogenized and processed for immunoblot analysis as described above with rabbit polyclonal anti-SOD2 antibody (1:10,000; Stressgen Biotechnologies), rabbit polyclonal anti-SOD1 antibody (1:10,000; Stressgen Biotechnologies) and mouse monoclonal anti-actin antibody (1 μg/ml; Chemicon).

Results

Generation of motor neuron-specific SOD2 knockout mice

To generate postnatal motor neuron-specific SOD2 knockout mice, we crossed mice homozygous for floxed SOD2 alleles (Ikegami et al., 2002) with VAChT-Cre.Slow mice in which Cre expression is restricted in postnatal somatomotor neurons (Misawa et al., 2003). The VAChT-Cre.Fast line was not used in this study because the transgene integrated on the same chromosome as SOD2 (chromosome 17). Double heterozygote animals ($SOD2^{lox/+}$;$Cre^{slow/-}$) were again mated with homozygous floxed SOD2 mice. $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice were born at a Mendelian ratio and survived to adulthood with no

gross defects (data not shown). Furthermore, no signs of motor deficits, including tremor and paralysis, or muscle weakness were observed for up to 12 months as revealed by a rota-rod test and grip strength measurements, respectively. Motor neurons in $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice showed normal cell morphology, including soma size, and normal immunoreactivity for cholinergic markers such as choline acetyltransferase (ChAT; Fig. 1A and Fig. 2A-F), vesicular acetylcholine transporter (VAChT) and high-affinity choline transporter (CHT). In the brain stem and spinal cord, approximately 50% of ChAT-positive motor neurons lost mitochondrial SOD2-immunoreactivity, suggesting successful targeting of the SOD2 gene (Fig. 1A-J). The SOD2-negative motor neurons were observed in various somatomotor nuclei of the brainstem and spinal cord, but not in visceromotor nuclei such as the dorsal motor nucleus of the vagus. Also double immunofluorescence for SOD2 and SMI-32 (a marker for motor neurons) reveals that SOD2-immunoreactivity was lost specifically in SMI-32 positive motor neurons and not in SMI-32 negative spinal interneurons (Fig. 1H-J).

Increased production of $O_2^{\cdot -}$ by mitochondria in $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice

To examine the effect of loss of SOD2 on superoxide production, we compared the spatial production of $O_2^{\cdot -}$ between $SOD2^{lox/lox}$;$Cre^{slow/-}$ and $SOD2^{lox/lox}$;$Cre^{-/-}$ mice by using HEt, a $O_2^{\cdot -}$-specific fluorescent dye (Fig. 1K-N). In the brainstem and spinal cord, punctate ethidium signals in the cytosol reflecting mitochondrial production of $O_2^{\cdot -}$ were detected in motor neurons with large somas, suggesting a relatively high mitochondrial respiratory rate in

these cells under normal physiological conditions. The cytosolic punctate fluorescence of oxidized HEt was more intense in $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice compared to $SOD2^{lox/lox}$;$Cre^{-/-}$ mice in a subset of hypoglossal and spinal cord motor neurons, confirming enhanced production of $O_2^{\cdot -}$ by mitochondria in $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice. On the other hand, this increase in HEt fluorescence was not observed in visceral motor neurons such as the dorsal motor nucleus of the vagus in $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice (data not shown).

Normal neurological and neurochemical profile of $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice

Despite the loss of SOD2 expression and increased superoxide production in motor neurons by 3-months of age (Fig. 2A-F), the number of ChAT-positive motor neurons in the cranial and lumber spinal cord was not significantly different between $SOD2^{lox/lox}$;$Cre^{slow/-}$ and $SOD2^{lox/lox}$;$Cre^{-/-}$ mice at either 5 or 9 months of age (Fig. 2G). Neither overt cell loss nor vacuolar changes in neurons or neuropil were observed as revealed by Nissl-staining of SOD2-negative motor neurons (Fig. 3A-D). Also analyzed was neuronal degeneration by Fluoro-Jade B (Schmued et al., 1997). No Fluoro-Jade B-positive neurons were detected in brain and spinal cord sections from either $SOD2^{lox/lox}$;$Cre^{slow/-}$ or $SOD2^{lox/lox}$;$Cre^{-/-}$ mice (not shown).

As free radicals are a potential source of damage to cellular constituents such as DNA, lipids and proteins, we evaluated $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice for histochemical signs of oxidative injury and stress. Yet SOD2-deficient motor neurons failed to react with any of the following antibodies: anti-SMI-31 monoclonal antibodies (Sternberger Monoclonals) to

phosphorylated neurofilaments which are shown to be accumulated in nerve cell bodies under pathological conditions; a monoclonal antibody against 8-hydroxy-2-deoxyguanosine (8-OHdG; JAICA, Shizuoka, Japan) to oxidative DNA damage; a polyclonal anti-malondialdehyde antibody (MDA; Alpha Diagnostic International, San Antonio, TX) to lipid peroxidation-related MDA-protein adduct; or a monoclonal anti-nitrotyrosine antibody (Upstate Cell Signaling, Lake Placid, NY) to peroxynitrite-mediated protein modification.

A small fraction of SOD1 is reported to reside in the intermembranous space of mitochondria (Okado-Matsumoto and Fridovich, 2001; Mattiazzi et al., 2002; Okado-Matsumoto and Fridovich, 2002) where it may work as an additional line of defense against $O_2^{\cdot -}$. However, immunohistochemical staining showed no obvious compensatory overexpression of SOD1 in the SOD2-deficient motor neurons (Fig. 3E and F). It is still tempting to speculate that an increased amount of SOD1 is accumulated in mitochondria under the SOD2-deficient conditions. A detailed analysis of SOD2 subcellular localization using confocal microscopy or immunoelectron microscopy will be needed to address the possibility.

Next we analyzed SOD2 expression in ventral halves of the cervical spinal cord micro-dissected from 9-month old $SOD2^{lox/lox}$;$Cre^{slow/-}$ and $SOD2^{lox/lox}$;$Cre^{-/-}$ mice by immunoblot analysis (Fig. 3G). A significant decrease (30% by densitometry) in SOD2 content was evident in $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice, although again SOD1 expression was unchanged. Even though SOD2 immunoreactivity is most strong in motor neurons in spinal cord sections, the homogenates include mitochondria from various other cell types such as glia and interneurons. Thus the result shows that SOD2 expression was lost in 30% of the

spinal motor neurons at the very least.

Finally an ultrastructural analysis of mitochondria in motor neurons was undertaken using electron microscopy. Sections of the facial nucleus and spinal cord from 9-month old $SOD2^{lox/lox}$;$Cre^{slow/-}$ and $SOD2^{lox/lox}$;$Cre^{-/-}$ mice were analyzed. Over 1,000 mitochondria in each brain regions were examined but no degenerative changes such as swelling, disorganization of the cristae or vacuolar formation were observed (n=3 for each genotype; data not shown).

As no obvious phenotype was detected in the cell bodies or organelles of SOD2-deficient motor neurons, we next analyzed the function of axonal processes by looking for muscle denervation and atrophy. Muscle biopsies from $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice revealed neither signs of muscle degeneration nor denervation/remodeling of motor axon terminals (Fig. 4). Furthermore, no evidence of reactive gliosis was revealed in $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice by GFAP staining (data not shown), despite its presence in the brainstem motor nuclei of SOD2-null mice (Melov et al., 1998; Lynn et al., 2005).

Motor neuron survival after hypoglossal nerve axotomy is unchanged

Motor neurons in SOD1-deficient mice show an increased vulnerability to facial nerve axotomy despite an otherwise normal phenotype (Reaume et al., 1996). To test whether SOD2-deficient motor neurons are also more vulnerable to nerve injury, we employed unilateral transection of the hypoglossal nerve. Hypoglossal motor neuron survival was then assessed 5 weeks following transection (Fig. 5). Cell number in the axotomized hypoglossal

nucleus decreased by approximately 10% compared to the contralateral control side, yet no statistical difference between $SOD2^{lox/lox}$;$Cre^{slow/-}$ and $SOD2^{lox/lox}$;$Cre^{-/-}$ mice was observed (Fig. 5). Furthermore, no difference in the number of SOD2-immuno-negative cells was detected in $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice after unilateral axotomy, and similar numbers of atrophic cells were seen in the transected nuclei of $SOD2^{lox/lox}$;$Cre^{slow/-}$ and $SOD2^{lox/lox}$;$Cre^{-/-}$ mice (data not shown).

Motor nerve axon disorganization is accelerated after hypoglossal nerve axotomy

Next, we analyzed the structural stability of the transected hypoglossal axon 2-4 mm distal to the lesion site 2 days post-operation. The distal segment of an injured nerve is known to undergo Wallerian degeneration within a few days. In the uncut contralateral nerve, we observed no difference in axon number and diameter between $SOD2^{lox/lox}$;$Cre^{slow/-}$ and $SOD2^{lox/lox}$;$Cre^{-/-}$ mice. However, $SOD2^{lox/lox}$;$Cre^{slow/-}$ axons did show an accelerated degeneration after nerve injury. When compared with $SOD2^{lox/lox}$;$Cre^{-/-}$ mice both cytoskeletal protein stability as revealed by Western blot and axon structure as revealed by histological analysis were significantly altered (Fig. 6).

Discussion

The present study demonstrates for the first time that SOD2 is not required for postnatal motor neurons survival and further reveals that motor neurons are, in fact, quite resistant to

mitochondrial generated $O_2^{\cdot -}$ in vivo. In the absence of SOD enzymatic activity, $O_2^{\cdot -}$ is relatively stable. Other antioxidants such as glutathione, ascorbate and tocopherols are relatively inefficient in removing superoxide radicals, and spontaneous dismutation occurs only very slowly. Because a portion of enzymatically active SOD1 is detected in the mitochondrial intermembranous space (Okado-Matsumoto and Fridovich, 2001; Mattiazzi et al., 2002; Okado-Matsumoto and Fridovich, 2002), we speculate that, although the physiological functions of SOD1 in the mitochondria are not fully understood, some SOD2 function is compensated by SOD1 potentially by removing $O_2^{\cdot -}$ generated from complex III (Han et al., 2001). And although we did not detect SOD1 up-regulation in SOD2-deficient motor neurons (Fig. 3E and F), endogenous levels of mitochondrial SOD1 may be sufficient to prevent both $O_2^{\cdot -}$-induced mitochondrial injury and $O_2^{\cdot -}$ release from mitochondria to cytosol. The physiological role and possible compensation of SOD2 loss by SOD1 can be addressed in future studies by crossing $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice to a SOD1-null background.

Motor neurons in the SOD1-deficient mice are vulnerable to axotomy-induced oxidative burden (Reaume et al., 1996). In the present study, we have analyzed the effect of nerve transection on motor neurons lacking SOD2 expression. We demonstrate here that neuronal survival after axotomy is not affected, but that disorganization of distal nerve axons is accelerated in the SOD2-deficient motor neurons. Thus loss of SOD2 function is insufficient to kill transected motor neurons, but does trigger more rapid motor axon degeneration after nerve injury. Although we don't yet understand the mechanism underlying the observed accelerated disorganization, we speculate that abnormal

Ca^{2+}-handling in SOD2-deficient mitochondria results in lowered Ca^{2+}-buffering activity specifically in lesioned distal axons while leaving the cell bodies unaffected. Interestingly, neurofilament proteins are known to be particularly susceptible tyrosine nitration and lysine oxidation (Beckman et al., 1993). Our present study thus implicates a previously unrecognized link between mitochondrial oxidative stress and axonal vulnerability to injury.

ALS is a fatal adult-onset neurodegenerative disease characterized by the selective loss of upper and lower motor neurons. Although its cause is not fully understood, mutations in the SOD1 gene cause a familial form of ALS and recent studies show involvement of mitochondrial dysfunction and oxidative damage in ALS pathogenesis (Andersen, 2004; Bendotti and Carri, 2004; Bruijn et al., 2004; Xu et al., 2004). In animal models, mitochondrial abnormalities were seen in motor neurons of mice or rats expressing the SOD1 mutations $SOD1^{G93A}$ (Dal Canto and Gurney, 1994; Jaarsma et al., 2001; Howland et al., 2002) and $SOD1^{G37R}$ (Wong et al., 1995), but similar pathology was not detected in motor neurons expressing other types of mutant SOD1 (Bruijn et al., 1997; Nagai et al., 2001). Furthermore, Andreassen et al. (2000) reported that heterozygous loss of SOD2 exacerbates disease in mutant SOD1 transgenic mice. Recent studies show that mutant SOD1, rather than abrogating function, acquires a toxic function and that mutant SOD1 expression is required in both neurons and glia cells to induce motor neuron degeneration (Gong et al., 2000; Pramatarova et al., 2001; Lino et al., 2002; Clement et al., 2003). The present study also is consistent with a possible importance of interplay between neurons and glia cells in motor neuron survival.

In an ALS mouse model, expression of the neuronal isoform of nitric oxide synthase

(nNOS) is increased in astrocytes surrounding motor neurons in the spinal cord and brainstem (Cha et al., 1998). Also increased nitrotyrosine labeling in motor neurons and in the ventral horn has been reported in ALS and mutant SOD1-expressing mouse models (Abe et al., 1995; Beal et al., 1997; Ferrante et al., 1997; Cha et al., 2000). In the present study we did not find any nitrotyrosine immuno-positive cells in the brain and spinal cord of 9-month old $SOD2^{lox/lox}$;$Cre^{slow/-}$ mice. Furthermore, neither signs of reactive gliosis (GFAP-IR) nor peroxinitrite-mediated oxidative damage (nitrotyrosine-IR) in astrocytes surrounding SOD2-deficient motor neurons were evident. We speculate that elevated levels of $O_2^{\cdot -}$ in motor neurons is not by itself enough to trigger chronic cell injury, but NO produced from neighboring astrocytes resulting in peroxinitrite production may be a further requirement to trigger ROS-induced toxicity.

Axonal disorganization and reduced slow axonal transport are well-known hallmarks of ALS. Although our present results indicate that loss of SOD2 function is not by itself sufficient to kill motor neurons in vivo, it does modify axonal susceptibility to nerve injury. Recently Vande Velde et al. (2004) reported that Wld^s protein, the dominant neuroprotective factor that markedly delays Wallerian axonal degeneration after nerve injury, does not prevent SOD1-mediated motor neuron loss when introduced the Wld^s mutation into the $SOD1^{G37R}$ or $SOD1^{G85R}$ ALS mouse models. These results show that inhibiting axonal degeneration is not effective to ameliorate ALS pathogenesis induced by the mutant SOD1 protein.

Although the precise pathologic role of $O_2^{\cdot -}$ in motor neuron degeneration remains to be fully clarified, the present study is consistent with the possible involvement of nonneuronal cells in mitochondrial-derived, superoxide-induced injury in motor neurons. Thus a rational

therapeutic strategy that delivers antioxidants to surrounding astrocytes or microglia may significantly help motor neurons survive oxidative stress.

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