Materials and Methods

Monomerisation of HA and synthesis of Cy3B-labelled HA

HA was from Bachem AG. Monomerisation was achieved by heating a 10 M solution of HA in 0.1 N HCl for 5 minutes to 95°C. After neutralisation with NaOH to pH 7.0, samples were stored frozen at –20°C and used 2-3 times only (Bodenmuller et al., 1986). For labelling Cy3B, 150 nmoles of monomerised HA were lyophilised and dissolved in 100 l dimethylformamide containing 0.2% *N*-methylmorpholine. Cy3B-mono-*N*-hydroxysuccinimide (NHS) ester (Amersham Biosciences) was dissolved in the same buffer (0.5 mg in 50 l) and incubated with HA overnight in the dark. The Cy3B-labelled HA was purified by C18 reverse-phase HPLC, yielding approximately 30-40 nmoles of Cy3B-labelled HA.

Molecular biology

Human GPR37 cDNA was inserted into pcDNA 3.1 (+) and into pcDNA3-FLAG-His6C as described earlier (Imai et al., 2001). GPR37 and GPR37-FLAG were subcloned into the dual-function vector pXOON, a kind gift from T. Jespersen, optimised for expression both in frog oocytes and in mammalian cells (Jespersen et al., 2002). GPR37-FLAG was introduced into CHO-K1 cells stably expressing G 16 and apoaequorin (CHO-G 16-AEQ) (Stables et al., 1997) with the vector pIRES-P, a kind gift from S. Hobbs (Hobbs et al., 1998). Stable integration was monitored by immunostaining with antibodies against FLAG (Sigma-Aldrich). For inducible expression, GPR37 was transfected into HEK-293 cells using the Flp-In T-REx system of Invitrogen (Karlsruhe, Germany). The concatemeric construct between GIRK1 and GIRK2 (GIRK1/2) was kindly provided by A. Karschin (Wischmeyer et al., 1997). All constructs were confirmed by sequencing.

Expression of GPR37 in *Xenopus laevis* oocytes and electrophysiology

For functional expression in frog oocytes, the GPR37 cRNA was transcribed in vitro with T7 polymerase from the *Xba*I-linearised pXOON-GPR37-FLAG vector and co-injected at a ratio of 5:1 with cRNA of the concatemeric GIRK1/2 construct transcribed from the *Nhe*I-linearised plasmid. For recordings, oocytes were superfused with ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5). Two-electrode voltage-clamp recordings were performed with electrodes pulled to a tip resistance of 0.5-2.0 M . A Gene Clamp 500B amplifier (Axon Instruments), pClamp9 (Axon Instruments) and Origin (Microcal Software) served for data acquisition and analysis. Whole cells were clamped at –100 mV. For agonist measurements, the medium was changed to high K^+ (ND-96 with 96 mM KCl, 2 mM NaCl). After the initial inward current had reached a plateau, agonists were applied in high K^+ medium. Agonist treatment was terminated by wash-out with low K^+ to control intactness of the oocyte membrane. All recordings were performed at room temperature.

Cell culture, transfection and immunostaining

NH15-CA2, HT22 and COS-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), HEK-T-REx-GPR37 cells with 10% newborn calf serum (tetracycline-free) and CHO-K1 cells in DMEM-F12 with 5% FCS. For routine culture, 100 U ml^{-1} penicillin, 100 g ml^{-1} streptomycin and 10 mM Hepes, pH 7, were added to these media. CHO-G 16-AEQ cells stably expressing GPR37 required the addition of 750 g ml^{-1} geneticin, 200 g ml^{-1} hygromycin and 5 g ml^{-1} puromycin. HEK-T-REx-GPR37 cells were induced to express GPR37 by incubation in 1 g ml^{-1} doxycycline. Lipofectamine 2000 (Invitrogen), Fugene 6 (Roche Diagnostics), or electroporation were used for transfection. To assay ligands, cells were transferred overnight into serum-free defined medium consisting of the respective basal media to which 5 g ml^{-1} insulin, 30 g ml^{-1} transferrin, 20 M ethanolamine, 30 nM sodium selenite, 1 M sodium pyruvate, 1% non-essential amino acids and 2 mM glutamine were added.

For immunocytochemistry, cells were fixed either with 4% formaldehyde in PBS for 30 minutes at room temperature or with ice-cold 1% acetic acid in ethanol for 5 minutes. After washing with 0.1% Triton X-100 and pre-absorption with 1% bovine serum albumin, first and second antibodies were applied. For cell-surface staining, living cells were incubated with ligand and/or antisera for 20-30 minutes on ice, washed, fixed and visualised as indicated. No Triton X-100 was added to prevent permeabilisation. For western blotting, cells were harvested by treatment with 2 mM EDTA in PBS for 10 minutes, collected by centrifugation, and ultrasonicated for 20 seconds in Tris-HCl buffer, pH 7.4, containing 2 mM EDTA and a protease-inhibitor cocktail (Roche Diagnostics). After centrifugation at 100,000 *g*, the membrane pellets were dissolved in sample buffer and separated by SDS-PAGE. The monoclonal mouse anti-GPR37 antibody, recognising an extracellular domain of recombinant human GPR37, was used at a dilution of 1:400, the polyclonal rabbit antisera against the intracellular C-terminal domain of GPR37, anti-GPR37(R1) and anti-GPR37(R2), were diluted 1:1000 and 1:2000, respectively. All GPR37-specific antibodies were produced in the laboratory of Takahashi and have been described previously (Imai et al., 2001). The antibody against FLAG (M2) was from Sigma-Aldrich and that against phospho-histone H3 from Biomol. Cy2 or Cy3 secondary antibodies were used for confocal analysis, and alkaline phosphatase- or peroxidase-conjugated secondary antibodies were used for light microscopy and western blotting. Western blots were visualised by ECL. Biotinylated proteins were detected with an avidin-peroxidase conjugate (Bio-Rad).

FRET analysis

For fluorescence resonance energy transfer (FRET) experiments, HA was reacted with the highly specific HA antiserum 102.8, which binds to HA in the picomolar range and was described earlier (Schaller et al., 1984). It was used at a dilution of 1:3000 and visualised with Alexa Fluor 488 goat anti-rabbit as donor (Invitrogen). GPR37 was detected with anti-GPR37 antibody and visualised with Cy3 anti-mouse antibody (Amersham) as acceptor. The energy transfer was detected as increase in donor fluorescence (Alexa Fluor 488) after complete photobleaching of the acceptor molecule (Cy3). Initial images were recorded after excitation at 488 and 568 nm. A discrete area of the sample was illuminated with intense 568 nm light (laser power 100%) for a few minutes to destroy completely the acceptor fluorescence. The cell was then rescanned using excitation at 488 nm. An increase within the photobleached area was used as a measure for the amount of FRET obtained. The efficiency of energy transfer (E) was expressed as E=1-(D1/D2), where D1 is the donor fluorescence before, and D2 after, photobleaching. Data were collected for 4-5 different fields from a single coverslip; 2-3 coverslips were used for each measurement; the experiment was repeated at least three times.

Biotinylation of surface proteins

COS-7 cells were transiently transfected with GPR37-FLAG using the Fugene 6 reagent (Roche Diagnostics). 48 hours after transfection, cells were washed 2 with PBS and biotinylated for 30 minutes at room temperature with 1 mM S-NHS-biotin (Perbio Science). The reaction was stopped by addition of 0.5 M Tris-HCl, pH 7.5, for 5 minutes at room temperature, and the cells were washed with PBS to remove free biotin. Cell lysates were prepared in a buffer consisting of 5 mM EDTA, 10 mM Tris-HCl, pH 7.4, and protease-inhibitor cocktail. Samples were ultrasonicated for 20 seconds and centrifuged at 100,000 *g* for 30 minutes. Pellets were solubilised in buffer containing 1% Triton X-100, 0.5% NP40, 150 mM NaCl, 7 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4, and protease-inhibitor cocktail for 30 minutes on ice, followed by centrifugation at 16,000 *g* for 15 minutes at 4°C. The supernatant was used for immunoprecipitation.

Immunoprecipitation with anti-FLAG M2-agarose

Since high concentrations of NP40 inhibited binding to FLAG-agarose, the supernatant from the NP40-solubilised and biotinylated COS-7 cells was diluted fivefold with TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) to reduce the NP40 concentration to 0.1%. Samples were incubated with 100 l anti-FLAG M2-agarose (Sigma-Aldrich) overnight at 4°C and then centrifuged at 1500 *g* for 5 minutes at 4°C. Pellets were resuspended in 1 ml TBS and centrifuged again at 16,000 *g* for 2 minutes at 4°C. After washing with TBS, pellets were dissolved in 50 l sample buffer and subjected to western blotting.

Electrophysiology with mammalian cells

For electrical recordings, COS-7 cells were microinjected with 50 ng l^{-1} GPR37-pcDNA3 and 5 ng l^{-1} EGFP-N1-pcDNA3, the latter being used to facilitate detection of successfully transfected cells. Membrane currents were recorded in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) or the perforated-patch configuration with nystatin (Horn and Marty, 1988). An EPC9 patch-clamp amplifier was used in conjunction with the PULSE-stimulation and data-acquisition software (HEKA Elektronik). The patch electrodes were made from 1.5 mm diameter borosilicate glass capillaries with resistances of 2.5-4 M . Data were low-pass filtered at 3 kHz and compensated for both fast and slow capacity transients. Series resistance was compensated by 75-90%. All experiments were performed at room temperature (22-25°C). The pipette solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM EGTA, 10 mM HEPES and had a calculated free Ca^{2+} concentration of 66 nM. The pH was adjusted to 7.3 with KOH. The standard external solutions contained 140 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES and 10 mM glucose, buffered to pH 7.3 with NaOH. Nystatin was dissolved in dimethyl sulfoxide (DMSO). Its final concentration in the standard pipette solution was 0.2 mg ml^{-1}. All chemicals for electrophysiology were purchased from Sigma-Aldrich.

Statistical analysis

The results are expressed as means of 3-6 determinations ± s.d. Curve fittings were performed with the Prism program (GraphPad). Each experiment was repeated at least three times.

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Regular Article (NBD-05-378-Revised)

Conditional knockout of Mn superoxide dismutase in postnatal motor neurons reveals resistance to mitochondrial generated superoxide radicals

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Abstract

Mitochondrial dysfunction and oxidative damage are implicated in the pathogenesis of neurodegenerative disease. Mice deficient in the mitochondrial form of superoxide dismutase (SOD2) die during embryonic or early postnatal development, precluding analysis of a pathological role for superoxide in adult tissue. Here we generated postnatal motor neuron-specific SOD2 knockouts by crossing mice with floxed SOD2 alleles to VAChT-Cre transgenic mice in which Cre expression is restricted to postnatal somatomotor neurons. SOD2 immunoreactivity was specifically lost in a subset of somatomotor neurons resulting in enhanced superoxide production. Yet extensive histological examination revealed no signs of oxidative damage in animals up to 1 year after birth. However, disorganization of distal nerve axons following injury was accelerated in SOD2-deficient motor neurons. These data demonstrate that postnatal motor neurons are surprisingly resistant to oxidative damage from mitochondrial-derived superoxide radicals, but that such damage may sensitize axons to disorganization following nerve injury.

Key Words: Motor neurons; Oxidative stress; Mitochondria; Nerve injury; Conditional knockout; SOD2; Amyotrophic lateral sclerosis

Introduction

Oxygen radicals, of which superoxide ($O_2^{\cdot -}$) is the most abundant, are a natural byproduct of oxygen consumption by the respiratory chain in aerobic ATP production. The superoxide dismutases (SODs) are enzymes that catalyze the conversion of $O_2^{\cdot -}$ to hydrogen peroxide and thus help prevent the build up of toxic $O_2^{\cdot -}$ levels. Three SOD isoforms are expressed in mammalian cells: copper/zinc SOD (SOD1) located in the cytoplasm (McCord and Fridovich, 1969), manganese SOD (SOD2) located in the mitochondrial matrix (Weisiger and Fridovich, 1973) and extracellular SOD (SOD3) (Marklund, 1982; Hjalmarsson et al., 1987). A small fraction of SOD1 is also reported to reside in the intermembrane space of mitochondria (Okado-Matsumoto and Fridovich, 2001; Mattiazzi et al., 2002; Okado-Matsumoto and Fridovich, 2002).

Oxidative stress has been implicated in various neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS). Though it remains unclear whether oxidative stress is a major cause or merely a consequence of cellular dysfunction associated with neurodegenerative diseases (Andersen, 2004), an accumulating body of evidence implicates impaired mitochondrial energy production and increased mitochondrial oxidative damage in early pathological events leading to neurodegeneration (Beal, 1996). Mitochondria are both a major source of reactive oxygen species (ROS) production as well as a major target of ROS-induced cellular injury. Thus mitochondrial localized superoxide dismutase (SOD2) is thought to play an important role in cellular defense against oxidative damage by ROS.

Loss of SOD2 results in embryonic or early postnatal lethality that varies with genetic

background. SOD2 knockout mice on a CD-1 background die either in utero or within 24 hours after birth from severe dilated cardiomyopathy (Li et al., 1995). Similarly, C57BL/6 SOD2 knockout mice die at late embryonic or early neonatal stages from dilated cardiomyopathy (Huang et al., 2001; Ikegami et al., 2002). On a mixed C57BL/6 and 129/Sv background, SOD2 mutant mice survive for up to 18 days, develop a milder-form of dilated cardiomyopathy and display a neurological phenotype (Lebovitz et al., 1996). In contrast, DBA/2J (D2) SOD2 mutant mice do not develop cardiomyopathy but instead develop severe metabolic acidosis and survive an average of 8 days (Huang et al., 2001). This phenotypic variation suggests that sensitivities to SOD2 deficiency are highly dependent on genetic modifiers that differ across strain and cell type.

Motor neurons are believed to be particularly susceptible to oxidative damage given the high metabolic requirement to sustain a large cell size and long axonal processes. Although motor neurons in cell culture are vulnerable to cell death mediated via calcium influx after exposure to glutamate, it is unclear how motor neurons respond to the overproduction of mitochondrial-derived ROS in vivo. To circumvent the early lethality of SOD2 knockout mice, we used a conditional gene deletion approach in which mice with floxed SOD2 genes (Ikegami et al., 2002) were mated with VAChT-Cre mice (Misawa et al., 2003) that express Cre recombinase in approximately 50 % of postnatal somatic motor neurons. Here we report that conditional loss of SOD2 in postnatal motor neurons results in elevated mitochondrial oxidative stress that fails to triggers signs of neurodegeneration under non-pathological conditions. In contrast, nerve axotomy revealed accelerated nerve disorganization, suggesting that adult motor neurons have relative resistance to mitochondrial-generated

superoxide radicals unless stressed.

Materials and Methods

Mice

C57BL/6 mice carrying the VAChT-Cre transgene (VAChT-Cre.Fast and VAChT-Cre.Slow) have been described previously (Misawa et al., 2003). C57BL/6 mice with floxed SOD2 alleles have been described elsewhere (Ikegami et al., 2002). Localization of the VAChT-Cre transgene in the VAChT-Cre.Slow mouse line to chromosome 4 was determined by FISH analysis (data not shown), and VAChT-Cre.Slow mice were used to direct motor neuron-specific Cre expression in this study. Homozygous floxed SOD2 mice (lox/lox) were crossed with VAChT-Cre.Slow heterozygote animals. The resulting double heterozygote animals ($SOD2^{lox/+}$;$Cre^{slow/-}$) were selected and mated with homozygote floxed SOD2 mice. All animals were genotyped for SOD2 allele (Ikegami et al., 2002) and the Cre trensgene (Misawa et al., 2003) using tail DNA as described previously. Motor performance was analyzed using a rota-rod treadmill (MK-600; Muromachi Kikai, Tokyo, Japan) at 28 rpm. Grip strength was measured using a Grip Strength Meter for Mouse (Model 57106; Stoelting, Wood Dale, IL). All animal protocols were approved by the Tokyo Metropolitan Institute for Neuroscience Institutional Animal Care and Use Committee.

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