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Oxidative stress induced by loss of Cu,Zn-superoxide dismutase (SOD1) or superoxide-generating herbicides causes axonal degeneration in mouse DRG cultures

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Abstract

Axonal degeneration is a common pathologic feature in peripheral neuropathy, neurodegenerative disease, and normal aging. Oxidative stress may be an important mechanism of axonal degeneration, but is underrepresented among current experimental models. To test the effects of loss of the antioxidant enzyme Cu,Zn-superoxide dismutase (SOD1) on axon survival, we cultured dorsal root ganglion (DRG) neurons from SOD1 knockout mice. Beginning as early as 48–72 h, we observed striking degeneration of *Sod1*^{−/−} axons that was prevented by introduction of human SOD1 and was attenuated by antioxidant treatment. To test susceptibility to increased superoxide production, we exposed wild-type DRGs to the redox-cycling herbicides paraquat and diquat (DQ). Dose-dependent axon degeneration was observed, and toxicity of DQ was exacerbated by SOD1 deficiency. MTT staining suggested that DRG axons are more susceptible to injury than their parent cell bodies in both paradigms. Taken together, these data demonstrate susceptibility of DRG axons to oxidative stress-mediated injury due to loss of SOD1 or excess superoxide production. These in vitro models provide a novel means of investigating oxidative stress-mediated injury to axons, to improve our understanding of axonal redox control and dysfunction in peripheral neuropathy.

Keywords

Axon; Axonal degeneration; Oxidative stress; Neuropathy; Superoxide dismutase; Dorsal root ganglion

Introduction

Axonal degeneration underlies numerous inherited and sporadic forms of peripheral neuropathy, and is increasingly recognized as a key pathologic feature and therapeutic target in neurodegenerative disease [13, 52]. Growing evidence from models of motor neuron disease [22], Parkinson's disease [11], Alzheimer's disease [65], and Huntington's disease [40] demonstrates axonal defects that occur prior to neuronal death and correlate closely with functional decline. But despite progress in understanding common downstream pathways of axonal demise [12], the initial triggers for axon degeneration in disease remain largely unknown.

Aging is the most prominent risk factor for degenerative diseases of the nervous system, even in patients with disease-causing mutations. Normal aging may, in fact, represent the most common cause of peripheral neuropathy, affecting an estimated 7 percent of individuals 65–84 [4]. Spontaneous, age-related peripheral neuropathy is also a well-described phenomenon in laboratory mice [10, 67] and rats [62]. One obvious factor that may increase susceptibility to neuropathy with aging is oxidative stress.

There is experimental evidence to support the idea that oxidative stress causes axonal degeneration. Hyperglycemia-induced free radical production is a widely used model of diabetic neuropathy (reviewed in [20]). Several in vitro models of oxidative stress-mediated axonal degeneration have also been developed using exogenous agents, such as rotenone [49], H₂O₂ [41], and 4-hydroxynonenal [1]. Although localized knockdown of manganese-superoxide dismutase (SOD2) has been shown to cause optic neuropathy [51], models of peripheral neuropathy due to loss of antioxidant defenses are lacking. This may be related to the lethal consequences of loss of some antioxidant enzymes [15, 39], and the comparatively mild effects of others, presumably due to redundancy or compensation [9, 31].

SOD1 is an abundant and highly conserved enzyme found in the cytoplasm, nucleus, and mitochondrial inter-membrane space that converts superoxide (O₂^{•-}) to H₂O₂ and O₂ [43]. Loss of SOD1 in mice is not lethal, but causes a 30% reduction in lifespan [17] and accelerated age-related pathology, including cataract formation [54], cochlear hair cell loss [44], retinal degeneration [30], and skeletal muscle atrophy [45]. Anti-sense knockdown of SOD1 has been shown to cause death of PC12 cells [69] and motor neurons in spinal cord slice culture [57], although axons were not evaluated in these studies. Here, we tested whether SOD1 may be required for survival of axons in vitro, using the DRG culture model that we have used previously in studies of chemotherapy-induced neuropathy [72, 73].

We report spontaneous axonal degeneration in *Sod1*^{-/-} DRGs. We also found that exposure to paraquat (PQ) and diquat (DQ), redox-cycling herbicides that increase intracellular superoxide [59], caused robust, dose-dependent axon degeneration. Toxicity in this paradigm was exacerbated by SOD1 deficiency. Finally, we provide evidence using vital staining to suggest that DRG axons are more vulnerable to injury than neuronal cell bodies in these models. We conclude that DRG axons in vitro are highly susceptible to oxidative stress-mediated degeneration due to loss of SOD1 or increased superoxide production.

These data reinforce the idea that oxidative stress may contribute to the pathogenesis of axonal degeneration in peripheral neuropathy.

Materials and methods

Animals

Sod1^{−/−} mice, generated by Huang et al. [32], were obtained from Marie Csete (Emory University) [45]. Transgenic mice overexpressing wild-type human SOD1 (*WTSOD1*) were obtained from Jackson (stock number 002298). Both lines are maintained in our colony on a C57BL/6 background. For the cross breeding experiment (Fig. 2), *Sod1*^{−/−} males were mated with *WTSOD1* females to generate *WTSOD1*, *Sod1*^{+/-} mice. *WTSOD1*, *Sod1*^{+/-} mice were then crossed with *Sod1*^{+/-} mice to generate *WTSOD1*, *Sod1*^{−/−} mice and littermate controls. Animals were housed in microisolator cages on a 12-h light–dark cycle and given free access to food and water. Genotyping was by standard PCR analysis on tail snip DNA. All animal procedures were approved by the Emory University Institutional Animal Care and Use Committee.

SOD1 expression

Spinal cord was removed from animals used for DRG cultures and homogenized in lysis buffer containing 1.5% NP-40, 1 mM EGTA, 2 mM Na₃VO₄, 6 mM NaF, and complete protease inhibitor cocktail (Roche). Protein concentration was determined by the BCA Assay (Pierce). SOD1 protein was detected by standard immunoblotting using sheep anti-SOD1 (1:5,000, Calbiochem). Mouse anti-β-actin (1:5,000, Sigma) was used as a loading control. Membranes were scanned on an Odyssey infrared imaging system (LI-COR Biosciences).

DRG cultures

DRG explants were derived from postnatal day 4 (P4) mice and plated four per dish in 35 mm tissue culture-treated dishes. Dishes were coated with a thin layer of rat-tail collagen type 1 (BD Bioscience, No. 354236), dried overnight at room temperature and rehydrated with DMEM and 1% FBS for at least 1 h prior to use. Growth medium was changed weekly and consisted of DMEM (with 4.5 g/L glucose and L-glutamine) supplemented with N2 (Invitrogen) and 100 ng/mL 7S NGF (Alomone Labs, Jerusalem, Israel). Non-neuronal cells were not depleted due to toxicity of anti-mitotic agents in preliminary studies (not shown). For culture at 6% O₂ (5% CO₂), cells were placed in modular incubators (Billups-Rothenberg, DelMar CA, USA) after equilibration in a gas-controlled environment. Incubators were re-equilibrated to 6% O₂ every 24 h.

DRG morphology

Serial digital images were taken at regular intervals on an Olympus CK40 inverted light microscope equipped with a Cohu 2222-1040 digital camera. Due to the large size of DRG explants, overlapping frames were captured and reassembled in Photoshop CS (Adobe) using the automated photomerge feature. The length of the longest axon and DRG area were measured using ImageJ software (<http://rsb.info.nih.gov/ij/>). Axon length was determined by drawing a straight line from the center of the ganglion to the tip of the longest axon. DRG

area was measured by tracing around the outer limit of the axons surrounding each ganglion. To normalize for variation in starting size between different DRGs, measurements were expressed as percent of day 2 (the first day of measurement), or as percent of baseline (day 0 of treatment) [72, 73]. DRGs grown in 6% O₂ were not removed for serial imaging, so data are reported as absolute measurements acquired on day 6 (final day in culture).

MTT assay

Viability of axons versus cell bodies was evaluated using the MTT assay, as previously described [42, 64]. A 5 mg/ml stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma M5655) was prepared in DMEM, and added directly to culture dishes at a 1:10 dilution. Cells were returned to the incubator for 2 h, and then fixed with 4% PFA/PBS for 15 min. Viability was assessed qualitatively by light microscopy, to evaluate NADPH and NADH-dependent conversion of the yellow tetrazolium dye to dark purple MTT formazan crystals [5].

Paraquat/diquat toxicity

Paraquat (PQ; 1,1'-Dimethyl-4,4'-bipyridinium dichloride) and diquat (DQ; 1,1'-Ethylene-2,2'-bipyridyldiylidinium dibromide) were obtained from Sigma. Stock solutions were prepared in ddH₂O and stored at 4°C. To evaluate toxicity in DRG cultures, a single dose of PQ (25, 50, or 100 μM) or DQ (50 μM) was added to wild-type DRGs on day 14 in culture ("day 0" for axon measurements). DRGs were imaged at regular intervals following PQ/DQ exposure and axon length and DRG area were measured. To determine the effect of endogenous SOD1 expression, *Sod1*^{+/+} DRGs were compared to DRGs from *Sod1*^{+/-} littermates.

Statistical analysis

Data are shown as mean ± SEM unless otherwise indicated, and represent pooled data from at least three replicates per genotype or treatment condition. Differences between groups were evaluated by one- and two-way ANOVA followed by Tukey post hoc analysis ($\alpha = 0.05$), using Prism software (GraphPad).

Results

SOD1 is required for DRG survival in vitro

To test the effect of loss of SOD1 on DRG survival, DRG explants were cultured from P4 *Sod1*^{-/-} mice and their *Sod1*^{+/-} and *Sod1*^{+/+} littermates (Fig. 1). During the first 48 h, *Sod1*^{-/-} DRGs extended axons that were morphologically identical to wild-type DRGs (Fig. 1a). No significant difference in axon length was seen between *Sod1*^{-/-} (0.94 ± 0.05 mm), *Sod1*^{+/-} (1.34 ± 0.06 mm), and *Sod1*^{+/+} (1.27 ± 0.05 mm) axons on day 2 ($P = 0.072$). Between days 2 and 6, *Sod1*^{-/-} axons fragmented and degenerated, in contrast to *Sod1*^{+/+} and *Sod1*^{+/-} axons that exhibited rapid growth (Fig. 1b). Morphology was quantified by serial measurement of the longest axon and the total DRG area, including the ganglion and its surrounding halo of axons (Fig. 1c, d). By day 6, *Sod1*^{+/+} and *Sod1*^{+/-} DRG area increased to 231 ± 11 and $243 \pm 13\%$ of day 2, respectively, while *Sod1*^{-/-} DRG area fell to $49 \pm 5\%$ ($P < 0.001$). Western blot of spinal cord homogenates was carried out to

confirm absence of SOD1 protein in *Sod1*^{-/-} mice and an intermediate level of SOD1 expression in *Sod1*^{+/-} mice (Fig. 1e).

To confirm that degeneration in *Sod1*^{-/-} DRG cultures is a specific effect due to genetic deletion of SOD1, we tested whether introduction of wild-type human SOD1 would rescue *Sod1*^{-/-} DRGs (Fig. 2). *Sod1*^{-/-} mice were crossed with transgenic mice overexpressing wild-type human SOD1 (*WTSOD1*) in a two-step breeding process to yield *WTSOD1*, *Sod1*^{-/-} mice and controls (see “Materials and methods”). SOD1 protein expression in spinal cord was determined by Western blot and found to correspond with genotype (Fig. 2a). DRGs cultured from *WTSOD1*, *Sod1*^{-/-} animals showed robust protection by human SOD1 when compared to DRGs from *Sod1*^{-/-} littermates (Fig. 2b). Morphological analysis showed that *WTSOD1*, *Sod1*^{-/-} axon length (Fig. 2c) and DRG area (Fig. 2d) were identical to *Sod1*^{+/+} and *WTSOD1*, *Sod1*^{+/+} controls. *WTSOD1*, *Sod1*^{+/+} DRGs did not show enhanced axon outgrowth compared to *Sod1*^{+/+} DRGs.

Axon degeneration precedes death of cell bodies

Viability of DRG axons and neuronal cell bodies was evaluated qualitatively using the MTT assay. On days 2, 4, and 6, MTT dye was added to the culture medium, and MTT reduction product indicating metabolic activity was visualized by bright field microscopy (Fig. 3). In *Sod1*^{+/+} and *Sod1*^{+/-} DRGs, MTT staining was readily visualized in the ganglion and throughout the axons. *Sod1*^{-/-} DRGs had a similar appearance on day 2. By day 4, numerous *Sod1*^{-/-} axons were devoid of MTT staining (Fig. 3, arrowheads), in contrast to the cell bodies and some axons that were still stained (Fig. 3, arrows). By day 6, the majority of *Sod1*^{-/-} axons were MTT-negative, while many ganglia were still MTT-positive (for an example of an MTT-negative ganglion, see Fig. 4f). Taken together, these data demonstrate that SOD1 is required for survival of DRG axons in vitro and suggest that axonal degeneration due to loss of SOD1 precedes degeneration of neuronal cell bodies.

Axonal degeneration is mediated by oxidative stress

To verify a role for oxidative stress in degeneration of *Sod1*^{-/-} axons, we tested several antioxidant strategies (Fig. 4). *N*-acetylcysteine (NAC) is hydrolyzed to cysteine inside cells, where it acts as a precursor for the synthesis of GSH, a free radical-scavenger. NAC also has GSH-independent effects on the thiol redox state [34, 60]. When a single dose of 1 or 5 mM NAC was added to *Sod1*^{-/-} DRGs at the time of plating, increased axon outgrowth was observed (Fig. 4a–c). Protection persisted over time in the 5 mM group, while axonal degeneration still occurred in the 1 mM group from days 2–6. On day 6, area of *Sod1*^{-/-} DRGs treated with 5 mM NAC was $117 \pm 10\%$ of day 2 ($P < 0.01$), compared to $60 \pm 7\%$ in 1 mM NAC-treated DRGs and $56 \pm 6\%$ in untreated DRGs.

Next, we treated *Sod1*^{-/-} DRGs with β -mercaptoethanol (BME), which facilitates cysteine uptake for GSH synthesis [33]. When 50 or 100 μ M BME was added at the time of plating, a 58% ($P < 0.05$) and 108% ($P < 0.01$) increase in DRG area was observed, respectively (Fig. 4d).

Finally, *Sod1*^{-/-} DRGs were cultured at low (6%) oxygen, which more closely approximates physiologic oxygen levels [66], and reduces oxidative stress in cultured cells [28]. On day 6, morphology and viability were compared to DRGs cultured in standard conditions (20% O₂). *Sod1*^{-/-} DRGs cultured at 6% O₂ showed an approximately twofold increase in DRG area compared to those cultured at 20% O₂ (0.94 ± 0.13 vs. 0.52 ± 0.8 mm² ($P < 0.05$), Fig. 4e). Viability of axons and cell bodies was preserved in 6% O₂, as determined by the MTT assay (Fig. 4f). These data support the conclusion that axonal degeneration in the *Sod1*^{-/-} model is due to oxidative stress.

PQ and DQ cause axonal degeneration

PQ and DQ are structurally similar herbicides that cause toxicity by a well-described redox-cycling mechanism [8, 35, 59]. Following reduction to a cationic radical (PQ⁺), PQ/DQ are rapidly reoxidized in the presence of O₂, resulting in superoxide production. To determine whether increased superoxide, as opposed to loss of SOD1, is toxic to DRGs, we exposed wild-type DRGs to PQ on day 14 in culture. Dose-dependent axon degeneration was observed (Fig. 5a, b). By day 8, the area of PQ-treated DRGs fell to $88 \pm 3\%$ ($P < 0.05$), $51 \pm 8\%$ ($P < 0.001$), and $16 \pm 5\%$ of day 0 ($P < 0.001$) in 25, 50, and 100 μ M-treated groups, respectively. Toxicity of 50 μ M DQ was similar in severity to 50 μ M PQ in wild-type DRGs (Fig. 5a, c). When we compared susceptibility of *Sod1*^{+/+} and *Sod1*^{+/-} DRGs to DQ, we found that *Sod1*^{+/-} DRGs were significantly more susceptible (Fig. 5c). By day 6 of DQ exposure, *Sod1*^{+/+} DRG area was $60 \pm 8\%$ of day 0, while *Sod1*^{+/-} DRG area was only $9 \pm 3\%$ of day 0 ($P < 0.001$). These data suggest that SOD1 plays an important role in protection against DQ toxicity. Additional support for this conclusion comes from experiments (not shown) in which *WTSOD1* DRGs were exposed to PQ. After 8 days of exposure to 50 μ M PQ, the area of *WTSOD1* DRGs was reduced to $50 \pm 6\%$ of day 0, compared to $28 \pm 4\%$ in wild-type DRGs ($P < 0.001$).

MTT staining in PQ and DQ-treated DRGs again showed a distinction between vulnerability of axons versus cell bodies (Fig. 6). After 1 week of 50 μ M PQ or DQ exposure, marked loss of axonal staining was seen, but DRG cell bodies were still MTT-positive. Thus, increased superoxide production also causes toxicity to DRGs in vitro, and qualitative evidence from MTT staining suggests that axons are more susceptible than cell bodies.

Discussion

Growing evidence supports a role for oxidative stress in the pathogenesis of neurodegeneration in aging and disease. In this study, we tested the effect of genetic deletion of the antioxidant enzyme SOD1 on the survival of mouse DRGs in vitro. We show that SOD1 is required for DRG axon survival, and that axon outgrowth in *Sod1*^{-/-} DRGs is improved by antioxidant treatment. We also found that exposure to the superoxide-generating herbicides PQ and DQ caused degeneration of wild-type DRGs, and that the degree of toxicity was directly related to the level of SOD1 expression. These novel in vitro models reinforce the idea that oxidative stress participates in the pathogenesis of peripheral neuropathy, and will be useful for future mechanistic and therapeutic studies of oxidative stress-mediated axon degeneration.

Superoxide-mediated neurotoxicity

Superoxide radical is continuously produced in cells as a byproduct of mitochondrial electron transport. If not efficiently neutralized by SOD enzymes, superoxide radical can undergo reactions that produce highly toxic free radicals such as peroxynitrite and hydroxyl radical. Neurodegeneration in *Sod1*^{-/-} mice has been reported in retinal neurons [30], and cochlear hair cells [44], and evidence from muscle pathology and EMG recordings in these mice is consistent with a possible motor neuropathy [24, 63]. *Sod1*^{-/-} neurons also show increased cell death in ischemic [37], excitotoxic [61], and traumatic injury models [53]. These data suggest an important role for SOD1 in maintaining neuronal viability under stress and during the aging process.

Superoxide-mediated toxicity may be particularly relevant to the pathogenesis of diabetic neuropathy, which most commonly presents as a symmetrical, distal sensory and autonomic neuropathy [68]. Hyperglycemia-induced mitochondrial superoxide production is thought to initiate multiple downstream pathways of tissue damage in diabetes [46]. Consistent with this hypothesis, *Sod2*^{+/-} DRG neurons exhibit increased superoxide production and decreased neurite outgrowth in hyperglycemic conditions, and *Sod2*^{+/-} mice are more susceptible to development of diabetic neuropathy [71]. The effect of SOD1 expression on susceptibility to diabetic neuropathy has not been reported, but SOD1 deficiency does exacerbate diabetic nephropathy [16] and cataract formation [47]. Genetic variations in SOD1 and SOD2 are linked to the risk of diabetic complications in humans [2, 23], and a reduction in plasma SOD activity was reported in diabetic patients [23].

Oxidative stress, and SOD1 specifically, are also implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS). Over 100 mutations in SOD1 have now been linked to the inherited form of this fatal motor neuron disease (ALS online database, <http://alsod.iop.kcl.ac.uk/Als/Index.aspx>) [76]. We and others have shown distal axon degeneration to be an early pathologic event in mutant SOD1 models of ALS [21, 26, 50]. The current consensus is that mutant SOD1 causes disease by a toxic gain of function, not a loss of antioxidant function (reviewed in [56]). Still, oxidative damage to proteins [19], lipids [48], and DNA [6] is increased in human ALS and in SOD1 mutant mice [3, 27], and antioxidants have provided robust extensions of life span in SOD1 mutant mice [14, 29]. Some proposed gains of toxic function by mutant SOD1 enzymes are likely to increase oxidative stress, including aberrant redox chemistry [75], alterations in metal binding [18], and formation of insoluble SOD1 aggregates in mitochondria [70]. Mechanistic comparisons between oxidative injury in the SOD1 mutant and SOD1 KO model with regard to axonal degeneration may be fruitful to pursue.

Axonal degeneration due to PQ/DQ

The neurotoxicity of PQ is well established in neuron-like cell lines [25] and in primary dopaminergic [7], cortical [36], and motor neuron cultures [38]. The doses of PQ and DQ used here were selected based on previous primary neuron studies, whereas much higher doses are typically required to cause toxicity in cell lines. We observed dose-dependent toxicity of PQ in mouse DRGs. Moreover, we saw a close correspondence between the effect of DQ and PQ, which have similar chemical structures and mechanisms of toxicity

[7]. As opposed to previous studies, which focused primarily on cell death as an endpoint, we found that DRGs exposed to PQ or DQ showed severe axon fragmentation and loss of axonal MTT staining, at a time when the cell bodies were still MTT-positive. Axonal toxicity of DQ was exacerbated by SOD1 deficiency, consistent with previous reports that suggest SOD1 plays a key role in protection against PQ toxicity [32]. PQ toxicity has also been shown to be attenuated by GSH supplementation [38], similar to *Sod1*^{-/-} DRGs. PQ/DQ exposure may therefore be a useful adjunct to SOD1 deficiency, for experiments that require the ability to manipulate spatial and temporal aspects of oxidative injury to axons.

Axonal degeneration versus cell death

In the current study, we used axon length as a primary measure of toxicity, and provide preliminary data that suggest a relatively higher susceptibility of axons versus neuronal cell bodies to loss of SOD1 or increased superoxide production. However, the axonal degeneration we observed could also have been due to cell death not detected by our methods, or due to sublethal injury to the cell bodies, causing failure to maintain distal axons. As in previous studies of axonal degeneration [42, 64], we used the MTT assay as a way to measure viability without disrupting the spatial organization of the cultures. This assay depends on metabolic activity present throughout the cell, as opposed to cell death assays that rely on nuclear/nucleic acid staining and are unsuitable for comparison of cell bodies and axons. The current data are by no means conclusive, but our observation of apparent axonal > neuronal toxicity in both the *Sod1*^{-/-} and PQ/DQ models suggests that further evaluation of relative susceptibility to oxidative stress is warranted.

The issue of axonal versus neuronal susceptibility may be important for understanding why, in diabetic (and other) neuropathies, length-dependent, distal to proximal axonal degeneration occurs in the absence of significant cell death [58, 74]. Recent studies support the idea that axons may be preferentially susceptible to oxidative stress. DRG neurons isolated from diabetic rats and subjected to hyperglycemia showed increased oxidative stress in axons, but not cell bodies, and an associated reduction in axon length [77]. Similarly, the concentration of 4-hydroxynonenal, a product of lipid peroxidation, required for 50% reduction of axon outgrowth in cultured DRG neurons had no effect on cell viability [1]. In an in vivo model of diabetic neuropathy, markers of oxidative damage in DRG neurons were significantly increased, yet few apoptotic neurons were detected [78]. It is unclear why axons may be more susceptible to oxidative stress-mediated injury than their parent cell bodies, although sciatic nerves of normal rats were reported to have tenfold less total GSH content than brain [55]. This suggests that peripheral nerve may be highly susceptible to oxidative stress at baseline due to a low GSH reserve.

In summary, we demonstrated that the antioxidant enzyme SOD1 is required for survival of DRG axons in vitro, and also showed susceptibility of DRG axons to increased superoxide production by the redox-cycling herbicides PQ and DQ. These in vitro models provide a novel means of investigating oxidative stress-mediated injury in mammalian axons, to improve our understanding of axonal redox control and dysfunction as it relates to human aging and disease.

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Fig. 1.

SOD1 is required for DRG axon outgrowth and survival in vitro. DRGs were cultured from P4 *Sod1*^{-/-} mice and their *Sod1*^{+/-} and *Sod1*^{+/+} littermates, and serial images taken every 2 days for morphologic analysis. **a** DRGs from *Sod1*^{+/+} and *Sod1*^{-/-} mice on day 2 in culture show similar axon length and morphology. **b** By day 6, *Sod1*^{-/-} axons have degenerated while *Sod1*^{+/+} axons have markedly increased in length (please note the difference in scale for days 2 and 6). **c** Length of the longest axon, expressed as percent of day two ($***P < 0.001$ vs. *Sod1*^{+/+} and *+/-*, two-way ANOVA). **d** DRG area (including the ganglion and surrounding axons), expressed as percent of day two ($***P < 0.001$ vs. *Sod1*^{+/+} and *+/-*, two-way ANOVA). In **c** and **d**, $n = 4-9$ animals per group (4-8 DRGs per animal). **e** Western blot of spinal cord from the same animals used for DRG cultures demonstrates absence of SOD1 protein in *Sod1*^{-/-} mice and intermediate expression in *Sod1*^{+/-} mice

Fig. 2.

Human SOD1 rescues *Sod1*^{-/-} DRGs. *Sod1*^{-/-} mice were crossed with transgenic mice overexpressing wild-type human SOD1 protein (*WTSOD1*), and DRGs were cultured from P4 offspring. **a** Western blot showing SOD1 expression in spinal cord from animals used for cultures. Mouse SOD1 (m) migrates slightly further than human SOD1 (h) on SDS-PAGE. **b** Representative DRG explants on day 6. *WTSOD1, Sod1*^{-/-} DRGs extended long axons that did not degenerate and were morphologically identical to controls. **c** Length of the longest axon, expressed as percent of day 2 (****P* < 0.001 vs. all other genotypes, two-way ANOVA). **d** DRG area, expressed as percent of day 2 (****P* < 0.001 vs. all other genotypes, two-way ANOVA).

Fig. 3.

Axonal degeneration due to loss of SOD1 precedes cell body degeneration. Viability of *Sod1*^{+/+} and *Sod1*^{-/-} DRGs was evaluated every 2 days via the MTT assay. On day 2, ganglia and axons in both groups were metabolically active (marked by the dark MTT reduction product). By day 4, some distal axons in *Sod1*^{-/-} DRGs were devoid of MTT staining (*arrowheads*), in contrast to axons that were still viable (*arrows*). By day 6, all *Sod1*^{-/-} axons were MTT-negative (*arrowheads*), but many ganglia were still MTT-positive. Representative bright field images are shown

Fig. 4.

Sod1^{-/-} DRGs are amenable to antioxidant protection. **a** Representative *Sod1*^{-/-} DRGs after 6 days of treatment with 0, 1, or 5 mM NAC. Both 1 and 5 mM NAC improved axon outgrowth, but axonal protection was only maintained over time in the 5 mM group. Scale bar 250 μm. **b** Length of the longest axon in NAC-treated DRGs, expressed as percent of day 2 (****P* < 0.001 vs. untreated, two-way ANOVA). **c** Area of NAC-treated DRGs, expressed as percent of day 2 (***P* < 0.01 vs. untreated, two-way ANOVA). In **b** and **c**, *n* = 8–11 animals per group (4 DRGs per animal). **d** *Sod1*^{-/-} DRG area after 6 days of treatment with 0, 50, or 100 μM BME (**P* < 0.05, ***P* < 0.001, one-way ANOVA). **e** *Sod1*^{-/-} DRG area after 6 days in 20 or 6% O₂ (**P* < 0.05, Student's *t* test). **f** MTT-stained DRGs from the same *Sod1*^{-/-} mouse, cultured for 6 days in either 20 or 6% oxygen. Note the complete loss of viability of axons and ganglia at 20% O₂, which was prevented by culturing at 6% O₂. Scale bar 50 μm

Fig. 5.

Superoxide-generating herbicides paraquat (PQ) and diquat (DQ) are toxic to DRGs in vitro. DRGs were cultured for 2 weeks, at which time PQ or DQ was added to the culture media. **a** Representative images of degenerating axons after 8 days of PQ exposure or 6 days of DQ exposure. **b** DRG area in PQ-exposed wild-type DRGs. Dose dependent toxicity was seen. $n = 4$ mice per group (4 DRGs per animal). **c** DRG area in DQ-exposed *Sod1*^{+/+} and *Sod1*^{+/-} DRGs. DQ toxicity was significantly more severe in *Sod1*^{+/-} DRGs. $n = 3$ mice per group (4 DRGs per animal). * $P < 0.05$, *** $P < 0.001$ vs. untreated, # $P < 0.001$ between the indicated groups (two-way ANOVA). Scale bar 100 μ M

Fig. 6.

DRG axons are more sensitive to PQ and DQ toxicity than their parent cell bodies. Viability of wild-type DRGs exposed to 50 μM PQ or 50 μM DQ for 1 week was evaluated by the MTT assay. MTT staining was retained in the ganglia, which appeared similar in intensity to control (untreated) DRGs. In contrast, PQ- and DQ-exposed axons from these same DRGs were devoid of MTT staining (*arrowheads*)