Published in final edited form as:

Neurobiol Dis. 2013 June ; 54: 329–338. doi:10.1016/j.nbd.2013.01.003.

Deletion of Nrf2 impairs functional recovery, reduces clearance of myelin debris and decreases axonal remyelination after peripheral nerve injury

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Abstract

Oxidative stress is generated in several peripheral nerve injury models. In response to oxidative stress, the transcription factor Nrf2 is activated to induce expression of antioxidant responsive element (ARE) genes. The role of Nrf2 in peripheral nerve injury has not been studied to date. In this study, we used a sciatic nerve crush model to examine how deletion of Nrf2 affects peripheral nerve degeneration and regeneration. Our study demonstrated that functional recovery in the Nrf2^{-/-} mice were impaired compared to the wild type mice after sciatic nerve crush. Larger myelin debris were present in the distal nerve stump of the Nrf2^{-/-} mice than in the wild type mice. The presence of larger myelin debris in the Nrf2^{-/-} mice coincides with less macrophages accumulation in the distal nerve stump. Less accumulation of macrophages may have contributed to slower clearance of myelin and thus resulted in the presence of larger myelin debris. Meanwhile, axonal regeneration is comparatively lower in the Nrf2^{-/-} mice than in the wild type mice. Even after 3 months post the injury, more thinly myelinated axon fibers were present in the Nrf2^{-/-} mice than in the wild type mice. Taken collectively, these data support the concept of therapeutic intervention with Nrf2 activators following nerve injury.

Keywords

Nrf2; sciatic nerve crush; myelin clearance; axonal regeneration; remyelination

Introduction

Lesion to the peripheral nerve elicits a cascade of events, including degeneration of the distal axon stump, loss of synaptic terminals, activation of Schwann cells and infiltration of macrophages (Chen et al., 2007; Coleman, 2005; Coleman and Freeman, 2010; Dubovy, 2011; Hoke, 2006). Several reports suggested that generation of oxidative stress is involved after peripheral nerve injury. For example, crush or chronic constriction injury to the sciatic

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nerve increases lipid peroxidation in the sciatic nerve (Naik et al., 2006; Senoglu et al., 2009). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in muscle after sciatic nerve transection or chronic constriction injury (Abruzzo et al., 2010; Bhattacharya et al., 2009; Tan et al., 2009). Sciatic nerve transection in rats led to an increase in ROS and RNS levels in the spinal cord (Guedes et al., 2008; Guedes et al., 2009). Generation of mitochondrial ROS is also observed in the dorsal horn neurons and microglia after L5 spinal nerve transection (Kim et al., 2010; Park et al., 2006).

In response to oxidative stress, mammalian cells activate expression of phase-II detoxification and antioxidant enzymes to combat toxic damage. Transcriptional activation of these antioxidant responsive element (ARE) genes generally relies on the transcription factor nuclear factor erythroid-2-related factor 2 (Nrf2) (Johnson et al., 2008; Vargas and Johnson, 2009). Nrf2 belongs to the Cap'n'Collar (CNC) family of basic-leucine-zipper (b-Zip) transcription factors and heterodimerizes with other b-Zip family proteins, such as small Maf proteins, for regulation of ARE gene expression (Jaiswal, 2004; Nguyen et al., 2003). In unstressed conditions, Nrf2 is retained in the cytoplasm by its inhibitor kelch-like ECH-associated protein 1 (Keap1) (Kensler et al., 2007; Motohashi and Yamamoto, 2004; Nguyen et al., 2009). Oxidative/electrophilic stress releases Nrf2 from Keap1, enables its nuclear translocation, and thereby induces the transcriptional activation of protective genes such as NAD(P)H:quinone oxidoreductase-1 (NQO1), hemeoxygenase 1 (HO1), glutamate-cysteine ligase catalytic (GCLC) and glutamate-cysteine ligase modifier (GCLM) (Johnson et al., 2008; Lee et al., 2005).

Despite the fact that oxidative stress is generated in several peripheral nerve injury models, little has been explored regarding the role of Nrf2 in this process. It is not clear whether the Nrf2-ARE pathway would exert a beneficial or deleterious effect on recovery after peripheral nerve injury. Therefore, we sought to determine how deletion of Nrf2 expression would impact regeneration after peripheral nerve injury. In this study, we performed sciatic nerve crush on ARE-reporter mice, and wild type mice as well as mice deficient for Nrf2 expression. Our results suggest that the Nrf2-ARE pathway is activated in muscle upon sciatic nerve crush. Ablation of Nrf2 led to slightly higher loss in neuromuscular junction (NMJ) innervation, more deposits of larger myelin debris, less accumulation of macrophages, relatively less axonal sprouting and remyelination. These may have collectively contributed to the slower functional recovery of the Nrf2 KO mice.

Materials and Methods

Animals

Nrf2 (-/-) mice were generated by replacing the b-Zip domain with a LacZ reporter (Chan et al., 1996) and were generously provided by Dr. Yuet Wai Kan (University of California, San Francisco). Both wild type [WT] mice and Nrf2 (-/-) [KO] mice were maintained on the B6/SJL background. Mice for experiments were bred as follows: 1) WT mice were crossed with Nrf2 (-/-) [KO] mice to obtain Nrf2 (+/-) mice. 2) Nrf2 (+/-) males were bred with B6/SJL females to obtain WT mice. Meanwhile, Nrf2 (+/-) males were bred with Nrf2 (-/-) females to generate KO mice. 3) WT mice from step 2 were bred together to generate experimental WT mice. Meanwhile, KO mice from step 2 were bred together to generate experimental KO mice. All animal procedures are approved by the Animal Care and Use Committee (IACUC) at University of Wisconsin-Madison.

Surgical procedure

Mice were anaesthetized with isoflurane gas anesthesia system and kept in a constant flow of isoflurane inhalation. Buprenorphine (0.05 mg/kg) was administrated intra-peritoneally

for pain. The skin in the up left leg was shaved and prepared with 10% Betadine solution. An incision was made on the mid-thigh muscle of the hind left leg to expose the sciatic nerve. The sciatic nerve was carefully lifted and crushed using a No. 5 Jeweler's forceps for a period of 30 s, stopped for a 10 s interval and then crushed at the same site again for a 30 s period. For sham, the nerve was exposed but not crushed. The incision in the muscle was closed with 5-0 vicryl suture followed by closure of the skin with 5-0 silk. The right contralateral nerve served as controls.

Immunohistochemistry

Frozen nerve cross sections ~3 mm from the injury site (20 μ m) were permeabilized and blocked with 1% BSA, 0.3% Triton X-100 and 10% goat serum for 1 h at room temperature. Sections were incubated with the following primary antibodies overnight at 4°C: anti-Mac2 (1:1000, Cedarlane Labs), anti-CD68 (1:500, Santa Cruz), anti-MAG (1:500, cell signaling). Slides were washed with PBS and incubated in Alexa-fluor 488 or Alexa-Fluor 594 or Alexa Fluor 633 conjugated secondary antibodies for 1 h at room temperature. Sections were then counterstained with Hoechst and mounted with FLUORO-GEL (EMS) and images were captured by an Olympus Fluoview 1000 confocal microscope.

For neuromuscular junction (NMJ) staining, tibialis anterior (TA) muscle from contralateral and ipsi-lateral legs were harvested. Muscles were flash frozen in isopentane and 25 μ m longitudinal sections were cut by a microtome cryostat. Sections were rinsed with PBS and blocked in 1% BSA, 10% goat serum and 0.1% triton for 1 h at room temperature. After blocking, sections were stained with 1 μ g/ml Alexa-fluor 594 conjugated α -bungarotoxin (Invitrogen), mouse neurofilament M (NF-M) antibody (1:1000, Abcam) and rabbit synaptophysin antibody (1:1000, Abcam) overnight at 4°C. Sections were washed with PBS three times and then incubated with Alexa-Fluor 488 anti-rabbit and anti-mouse secondary antibodies for 2 h at room temperature. After washing, sections were mounted using FLUORO-GEL (EMS) and images were captured by Olympus Fluoview 1000 confocal microscope.

Western blot

Tissue were homogenized and lysed by TMNC buffer (50 mM Tris, 5 mM MgCl₂, 100 mM NaCl and 4% CHAPS). Protein concentration of lysates were quantified by BCA assay (Pierce) and ~20 μ g protein was loaded onto SDS-PAGE gel for electrophoresis. After running, proteins were transferred onto PVDF membranes (GE Healthcare) and the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline-Tween 20 (TBST) for 1 h at room temperature. Membranes were incubated with the following primary antibodies overnight at 4°C: anti-Tubulin (1:2000, DSHB), anti-CD11b (1:1000, Millipore), anti-Mac2 (1:2000, Cedarlane), anti-NF-M (1:1000, Abcam) and anti-GAP43 (1:1000, Cell Signaling). After washing with TBST, the membranes were incubated with peroxidase conjugated anti-mouse, anti-rabbit or anti-rat secondary antibodies for 1 h at room temperature. Membranes were washed with TBST and visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Walking track analysis

Mice were tested in a confined 18 $\times$ 2.5 inch walkway with a dark box at the end. The hind paws of the mice are pressed to a black-ink soaked sponge and allowed to walk down the walkway lined with white paper. Print length (distance from the heel to the third toe, abbreviated as PL) and toe spread (the distance between the first and fifth toes, abbreviated as TS) were taken from the footprints of the normal side (N) and experimental side (E). Sciatic functional index (SFI) is calculated using the following formula: $SFI = 118.9 [(ETS - NTS)/NTS] - 51.2 [(EPL - NPL)/NPL] - 7.5$ (Insera et al., 1998; Magill et al., 2007).

TreadScan

Gait analysis was performed by the TreadScan system (Clever Systems) as described previously (Wooley et al., 2009). In brief, animals were trained in the testing chamber for a week. 8 mice in the WT group and 8 mice in the KO group were included for the training. For data collection, a mouse is placed into the testing chamber and allowed to run on the treadmill at 11 cm/s for 20 seconds. Data was collected before surgery, 1w, 2w and 3w post-surgery. The TreadScan software was then used to collect gait parameters, such as stance and swing time.

Withdrawal reflex latency

Pain nociception was measured using a Columbus instruments Analgesia Hotplate. The hotplate was set to 55 °C. An individual mouse was placed on the hot plate and the experimenter observed the mouse very closely, carefully watching for licking directed responses to the hind paws. After the mouse made characteristic lifting and licking responses to each hind paw, it was removed from the hotplate. The entire trial was videotaped and scored later for withdrawal reflex latency.

Electron microscopy

The contralateral and ipsilateral sciatic nerves were harvested and fixed in ice-cold 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (PB, pH=7.4) overnight at 4 °C, postfixed in 1% osmium tetroxide plus 1% potassium ferrocyanide in PB, dehydrated in a graded ethanol series, and infiltrated/embedded in PolyBed 812 resin (Polysciences Inc.). One μ m semi-thin sections were cut (~3 mm from the injury site) and stained with Methylene blue/Azure II with 1% borax. Light microscopy images were captured by Axiovision software using a Zeiss microscope. For ultra-structural examinations using electron microscopy, 90 nm thin-sections were collected on Pioloform-coated 1×2 mm Cu slot grids, post-stained with uranyl acetate and lead citrate. Images were documented at 80kV using a Philips CM120 transmission electron microscope with a MegaView III digital camera (Olympus Soft Imaging Systems). Number of remaining fibers (n=4 each condition), axonal sprouts (n=3 each condition), myelin debris (n=3 each condition) and size of myelin debris (n=3 each condition) were quantified by the ImageJ software from NIH. For morphological analysis of axon fiber, g-ratio (total axonal area divided by total fiber area) was calculated using the g-ratio plug-in for ImageJ. G-ratio was calculated for 100 fibers per mouse for three mice in each experimental condition.

Statistical analysis

Each experiments was performed use at least three animals. All data are presented as mean $\pm$ standard error of mean (SEM). Statistical analysis was performed mainly using two-way ANOVA with Bonferroni's post-test (GraphPad Prism 4). In a few cases where one-way ANOVA or t-test is used, it is noted in the figure legend below the corresponding figure. *: p<0.05, **: p<0.01, ***: p<0.001.

Results

Ablation of Nrf2 impairs functional recovery after sciatic nerve crush

The role of Nrf2 in peripheral nerve injury has not been explored before, we therefore evaluated whether deletion of Nrf2 would affect functional recovery after sciatic nerve crush. Sciatic functional index (SFI) is frequently used to assess functional recovery after sciatic nerve crush (Insera et al., 1998). SFI was not affected in the sham operated control groups. The SFI score reached the lowest around 2 days post injury for mice experienced sciatic nerve crush, and then gradually increases as repair occurs (Fig. 1A). The KO mice

tend to have a slower recovery rate compared with the WT mice. At 1-week and 2-week post injury, the SFI score was significantly lower in the KO mice than in the WT mice. However, the difference reduces along with recovery and the SFI score was the same by 3-week post injury (Fig. 1A). While the biggest motor function difference between the WT and KO mice was observed at 1-week post injury (Fig. 1A), no difference in nociceptive response was observed at this time point. The crush itself significantly increased the latency to analgesia hot plate stimulation in both WT and KO mice, however, the KO mice behaved similarly to the WT mice (Fig. 1B).

In addition, we used TreadScan to obtain gait analysis parameters for evaluation of gait performance. Numerous gait parameters can be collected from TreadScan analysis and we only listed the ones with significant changes for the hind limbs in Table 1. At 1-week post injury, stance (time elapsed while the foot is in contact with the tread) is significantly longer while swing (time elapsed while the foot is in the air) is significantly shorter for the KO mice when comparing the ipsilateral limb to the contralateral limb, whereas the WT mice exhibited similar stance and swing time when comparing the two limbs. Print area (the average of the size of the foot print over the entire stance) is significantly increased in the injury side in both WT and KO mice at 1-week post injury. Increased print area for the injured foot was also observed from the walking track analysis and the print area is close to normal when fully functional recovery was achieved. Increase in the print area of the injured foot remained significant till 3 weeks after injury in KO mice, but was only significant at 1-week in WT mice (Table 1). Gait abnormality was also observed for homologous coupling (Homolog Cpl, the fraction of the stride of a given foot when the other foot on the same half starts its stride), lateral deviation minimum and maximum (LatD min and LatD max, minimal and maximal distance from the middle of paw print to the long axis). Significant difference in homologous coupling was observed in KO mice at 1-week and 2-week post injury but no difference was observed for the WT mice. LatD min and LatD max revealed that the distance of the injury limb to the long axis is decreased whereas that of the control limb is increased at 2-week post injury. The difference between the control and injury limb for LatD min occurred at both 1-week and 2-week post injury in KO mice, but only occurred at 1-week in the WT mice (Table 1). These results suggest that sciatic nerve crush led to more changes in gait parameters in the KO mice than in the WT mice, which indicates *Nrf2* may play an important role in coordinating gait performance after sciatic nerve crush.

Deletion of *Nrf2* slightly affects neuromuscular junction innervation

Loss of motor function is directly linked to denervation in the neuromuscular junction (NMJ). We therefore set out to determine if the lower SFI score is due to more denervation in KO mice. Three types of innervation patterns were shown in Figure 2A: innervated, partially innervated and denervated. Percentage of innervated NMJ in the contralateral muscle remained above 90% throughout 3 weeks post surgery (Supplementary Fig. S1). Since all the contralateral muscle have similar innervation patterns, we used the contralateral muscle from 1-d injured animals as control and plotted with data from ipsilateral muscle at different injury times in Fig. 2B. For the ipsilateral side, innervated NMJ dropped to ~20% at 1-day post crush, reached the lowest at 1-week post crush, then gradually increased as recovery takes place. At 3-week post injury, the percentage of innervated NMJ is close to the control limb (Fig. 2B). Since the biggest motor function difference between WT and KO mice was observed at 1-week post injury (Fig. 1A), we checked whether there is a difference in terms of innervation between the WT and KO mice at this time. At 1-week post injury, crush caused significant loss of NMJ innervation (Fig. 2C). Percentage of partial innervation in the crush side is slightly increased in KO mice but significantly increased in WT mice when compared to the control side. Denervated NMJ increased significantly due to crush in both WT and KO mice (Fig. 2C). Knockout *Nrf2* itself does not lead to significant

denervation ($p=0.2092$), however, an interaction between injury and Nrf2 deletion was observed ($p=0.0437$) from two-way ANOVA analysis. A Bonferroni post-hoc test indicates that the crush side of KO mice had significantly higher denervated NMJ than the crush side of WT mice (Fig. 2C and Supplementary Table S4). Our results indicate that ablation of Nrf2 enhanced crush resulted denervation. In addition, we performed an ANCOVA (analysis of covariance) analysis using number of axons (Fig. 6C) as a covariate. The ANCOVA analysis returns a p-value of 0.012 (with F value equals 15.365) (Supplementary Table S9). The p-value for the influence of NMJ to SFI is 0.015 and for gene to SFI is 0.011, indicating that both significantly contribute to the observed significant difference in SFI between WT and KO mice.

Deletion of Nrf2 led to deposition of larger myelin debris

After sciatic nerve crush, the majority of myelinated fibers in the distal nerve stump were gone, whereas fibers proximal to the crush site were still well preserved (Fig. 3A). When comparing the distal nerve at 1-week post injury, larger blue deposits were observed in the KO mice as compared to the WT mice (Fig. 3A, ellipse). Ultra-structural analysis by electron microscopy (EM) indicates that macrophages (Fig. 3B, arrow) infiltrate to the injured nerve and help to clear the myelin debris (Fig. 3B, star). Quantification of the number of myelin debris in the distal nerve stump indicates that the total number of myelin debris was similar in the WT and KO mice (Fig. 3C). While deletion of Nrf2 does not seem to impact the number of myelin debris present, the average size of myelin debris was much greater in the KO mice than in the WT mice (Fig. 3D).

Ablation of Nrf2 led to less macrophages accumulation in the injured nerve

Clearance of myelin debris is believed to involve both Schwann cells and macrophages, with macrophages being the principle cells for the final removal of degenerated myelin (Dubovy, 2011). Axonal growth inhibitors such as myelin associated glycoprotein (MAG) are normally present in the myelin debris (Schafer et al., 1996; Vargas and Barres, 2007). At 1-week post injury, the ipsilateral nerve in the KO mice had obviously higher MAG protein than in the WT mice (Fig. 4A). In accordance, less MAC2 and CD68 (both are markers for macrophages) positive cells were present in the injured nerve of KO mice at 1-week post injury (Fig. 4B). Quantification of CD68 positive cells suggests that more macrophages were present in the injured nerve in WT mice than in KO mice (Fig. 5A). In WT mice, CD11b (also known as MAC1, a macrophage marker) levels continuously accumulate, reached a peak at 1-week and remained significantly high at 2-week post injury in the WT mice (Fig. 5B and 5C), its level in the KO mice remained similar through this time course (Fig. 5B and 5C). In KO mice, though CD11b level showed significant increased as early as 3 days after crush, its level remained similar at 1-week and 2-week post injury (Fig. 5B and 5C). MAC2 expression pattern along injury time was quite different from CD11b. MAC2 level in the ipsilateral nerve was comparable at 3 days after injury in WT and KO mice, but its level was lower in the KO mice at 1-week post injury (Fig. 5B and 5D). MAC2 expression in the ipsilateral nerve of KO mice started to catch up after 1-week and was even slightly higher at 2-week post injury (Fig. 5B and 5D). Taken together, the presence of Nrf2 seems to be important for clearance of myelin debris in a timely fashion.

Ablation of Nrf2 resulted in slightly less preserved fibers and new axonal sprouts

While most myelinated fibers were degenerated, a few fibers escaped degeneration and were preserved in the distal nerve (Fig. 6A, arrow). The number of preserved fibers in the injured nerve in KO mice was slightly but not significantly lower than that in WT mice (Fig. 6C). In addition, new axonal sprouts have appeared in the distal end of the injured nerve at 1-week post injury in both WT and KO mice (Fig. 6B, star). Quantification for the number of newly sprouting axons suggests that the total axonal sprouts in KO mice was also slightly less

though not statistically significant when compared to WT mice (Fig. 6D). We also assessed the expression of neurofilament-M (NF-M, neuronal marker) and GAP43 (marker for axonal sprouting or elongation) for axonal regeneration. Nerve crush caused axonal degeneration and therefore loss of NF-M. At the same time, GAP43 positive new axons start to sprout to the distal end and the expression of GAP43 increased dramatically after nerve crush (Fig. 6E). The increase in GAP43 protein level is significantly higher in both WT and KO mice when comparing the ipsilateral nerve to the contralateral nerve (Fig. 6F). When the ratio of the ipsilateral nerve to the corresponding contralateral nerve (ip/con) for each mouse is taken, the fold increase is 48.2 ± 7.65 (mean $\pm$ SEM, $n=3$) in the WT group mice and 22.0 ± 4.54 (mean $\pm$ SEM, $n=3$) in the KO mice group, with WT significantly higher than KO mice (Fig. 6F), suggesting that more axonal growth is promoted in the injury side of WT mice. The reduction of NF-M protein level in KO mice is significantly higher than that in the WT mice (Fig. 6E and 6F), indicating that the number of axons is less in the KO mice. Taken together, these results indicate that deletion of Nrf2 preserves slightly few fibers and renders relatively less axonal regeneration.

Deletion of Nrf2 decreases axonal remyelination

While motor function is mostly recovered at 3-week post sciatic nerve crush (Fig. 1A) and neuromuscular junction innervation pattern is comparable to controls at this time point (Fig. 2B), the injured nerve still look quite different than control nerves (Fig. 7A). At 3-week post injury, the regenerated axon fibers were still thinly myelinated and a number of myelin debris still exist (Fig. 7A). Even at 6-week post injury, the size of regenerated fibers still look much smaller than the ones in control (Fig. 7A). Therefore, a 3-month long time was given to the animals to recover from the nerve crush and axonal morphology was examined at that time point. Axonal morphology of the injured nerve appears close but still smaller and thinner to the control nerve at 3-month post injury for both WT and KO mice (Fig. 7B). G-ratio (total axonal area divided by total fiber area, or inner diameter of fiber divided by the outer diameter of the fiber) gives a good estimate for myelination of axon fibers. The lower the g-ratio value, the thicker the myelination sheath is. The majority of axon fibers have a g-ratio in the range of 0.6-0.8 (Fig. 7C). Even at 3-month after injury, a significantly higher number of thinly myelinated axon fibers (in the range of 0.8-1.0) is present in the ipsilateral nerve in both WT and KO mice, and the percentage is even higher in the KO mice (Fig. 7C). When comparing the ipsilateral side to the contralateral side, a significantly lower number of axon fibers in the G-ratio range of 0.5-0.6 and 0.6-0.7 were observed in the KO mice, but not WT mice (Fig. 7C). Collectively, these results demonstrated that knock out Nrf2 has slowed the myelination of regenerated axon fibers

Discussion

This study demonstrated that activation of the Nrf2-ARE pathway plays an important role in peripheral nerve degeneration and regeneration. Nrf2 is essential for activation of ARE genes and lack of Nrf2 may cause accumulation of oxidative stress and subsequently damage to tissues. Our data suggest that deletion of Nrf2 had several adverse effects on recovery after sciatic nerve crush. Lack of Nrf2 delays functional recovery, leads to less macrophage accumulation, causes deposition of larger myelin debris, partially reduces axonal regeneration and also decreased axonal remyelination. Recovery after peripheral nerve injury requires efficient removal of damaged tissue, such as myelin debris which contains inhibitory factors for axonal growth, to create a regeneration friendly environment. While the clearance of myelin debris in the WT mice seems to be efficient, removal of myelin debris in the KO mice is much slower and larger myelin debris were observed in the KO mice as compared to the WT mice (Fig. 3B). Myelin phagocytosis is achieved by the coordinated action of both Schwann cells and macrophages. The first step of myelin removal

is carried out by Schwann cells and resident macrophages within 2~3 days after nerve injury (Dubovy, 2011; Gaudet et al., 2011). Initial breakdown of the myelin sheath is carried out by Schwann cells (Hirata and Kawabuchi, 2002). MAC-2, a marker initially identified for macrophages, was highly induced in Schwann cells after nerve degeneration (Reichert et al., 1994; Saada et al., 1996). Along with myelin breakdown by Schwann cells, resident macrophages start to proliferate and actively phagocytose degenerating myelin before hematogenous macrophages are recruited to the injured nerve (Mueller et al., 2003; Mueller et al., 2001). Within a few days post injury, hematogenous macrophages infiltrate to the injured nerve and make significant contribution towards clearance of myelin debris (Bruck, 1997). MAC-2 expression was also induced in macrophages in addition to Schwann cells during nerve degeneration (Reichert et al., 1994; Rotshenker, 2003; Saada et al., 1996). Another macrophage marker, MAC-1 (or CD11b), has been shown to be critical for myelin phagocytosis. Delivery of MAC-1 antibody led to significant reduction in myelin phagocytosis after nerve transection (Bruck and Friede, 1991) and selective deletion of CD11b positive macrophages was shown to decrease clearance of myelin debris after sciatic nerve crush (Barrette et al., 2008).

Activation of macrophages is a self-defense response that helps the injured nerve to clear myelin debris and promote axonal regeneration. In this study, we observed that the levels of CD11b and MAC-2 were comparable at 3 days post injury in WT mice and KO mice (Fig. 5B, 5C and 5D), indicating that the early breakdown of myelin by Schwann cells and resident macrophages were not affected by deletion of Nrf2. However, CD11b positive cells continuously accumulate in the injured nerve within the first week post injury in WT but not in KO mice (Fig. 5B and 5C). Less accumulation of CD11b positive cells in the KO mice may be due to decreased infiltration of macrophages at later time points or loss of viability of the already infiltrated cells. Ablation of Nrf2 resulted in more oxidative stress, as indicated by the higher 4-Hydroxynonenal (4-HNE) content in the injured nerve of the KO mice (Supplementary Fig. S2). Such oxidative stress may be harmful to the infiltrated macrophage, therefore possibly resulted in a lower number of CD11b positive macrophages in the injured nerve of the KO mice. MAC-2 positive cells were much less (Fig. 4B) and its protein level is lower (Fig. 5B and 5D) in the KO mice at 1-week post injury. MAC-2 protein is even lower at 1-week than at 3-d post injury in the KO mice, which suggest that some MAC-2 positive cells is probably eliminated via local apoptosis and/or circulation to local lymph nodes and the spleen (Kuhlmann et al., 2001). Unlike CD11b, MAC-2 protein showed a trend of increase at later time. MAC-2 level in the KO mice seems to catch up with the WT mice at 2-week post injury (Fig. 5B and 5D), which may have helped to speed up the clearance of myelin debris, facilitate axonal regeneration and catch up on functional recovery at 3-weeks (Fig. 1A).

In addition to reduced clearance of myelin debris, axonal remyelination was also decreased in KO mice (Fig. 7B and 7C), suggesting Nrf2 plays an important role in regulating myelin turnover and regeneration. Indeed, another report by Hubbs et.al suggested that markers of oxidative stress accumulate within the myelin sheath in the absence of Nrf2 and therefore lead to myelin degeneration in the brain (Hubbs et al., 2007). The myelin sheath is formed by myelinating Schwann cells in the peripheral nerves. Thickness of the myelin sheath is known to be regulated by the neuregulin-ErbB signaling pathway in Schwann cells (Sherman and Brophy, 2005). Mice lacking ErbB2 receptor expression have thinner myelin sheath and fewer myelin wraps (Garratt et al., 2000). One study showed that silencing of Nrf2 represses ErbB2 expression in ovarian carcinoma cells (Manandhar et al., 2012). In our study, we observed that myelin sheath is thinner in Nrf2 knock-out mice. It is likely that the deletion of Nrf2 negatively impacts the neuregulin-ErbB2 signaling in Schwann cells and resulted in thinner myelination in the KO mice.

In summary, we demonstrated that proper activation of the Nrf2-ARE pathway facilitates functional recovery after sciatic nerve crush. Our data suggested that deletion of Nrf2 reduced the efficiency of macrophage accumulation in the injured nerve, impaired clearance of myelin debris and decreased axonal remyelination which may contribute to the delayed regeneration in the KO mice. Though the difference in terms of motor functional recovery is significant but not dramatically different between the WT and KO mice, more gait abnormalities were observed in the KO mice compared to the WT mice. Moreover, regeneration after crush injury is usually fast and far better than more severe injuries such as transection injury. Exaggerated difference between the WT and KO mice is likely when a transection injury is applied. Taken collectively, these data support the concept of therapeutic intervention with Nrf2 activators following nerve injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by R01ES08089 from the National Institute of Environmental Health Sciences (JAJ). Animal behavioral studies were supported in part by a core grant to the Waisman Center from the National Institute of Child Health and Human Development (P30 HD03352). We would like to thank Dr. Matthew E. Andrzejewski from the Rodent Models Core at the Waisman Center at University of Wisconsin-Madison for performing the TreadScan and analgesia hotplate analysis. We also thank Dr. Randall Massey from the EM facility at University of Wisconsin-Madison for the EM imaging.

References

- Abruzzo PM, et al. Oxidative stress in the denervated muscle. *Free Radic Res.* 2010; 44:563–76. [PubMed: 20298122]
- Barrette B, et al. Requirement of myeloid cells for axon regeneration. *J Neurosci.* 2008; 28:9363–76. [PubMed: 18799670]
- Bhattacharya A, et al. Denervation induces cytosolic phospholipase A2-mediated fatty acid hydroperoxide generation by muscle mitochondria. *J Biol Chem.* 2009; 284:46–55. [PubMed: 19001413]
- Bruck W. The role of macrophages in Wallerian degeneration. *Brain Pathol.* 1997; 7:741–52. [PubMed: 9161725]
- Bruck W, Friede RL. The role of complement in myelin phagocytosis during PNS wallerian degeneration. *J Neurol Sci.* 1991; 103:182–7. [PubMed: 1880536]
- Chan K, et al. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc Natl Acad Sci U S A.* 1996; 93:13943–8. [PubMed: 8943040]
- Chen ZL, et al. Peripheral regeneration. *Annu Rev Neurosci.* 2007; 30:209–33. [PubMed: 17341159]
- Coleman M. Axon degeneration mechanisms: commonality amid diversity. *Nat Rev Neurosci.* 2005; 6:889–98. [PubMed: 16224497]
- Coleman MP, Freeman MR. Wallerian degeneration, wld(s), and nmnat. *Annu Rev Neurosci.* 2010; 33:245–67. [PubMed: 20345246]
- Dubovy P. Wallerian degeneration and peripheral nerve conditions for both axonal regeneration and neuropathic pain induction. *Ann Anat.* 2011; 193:267–75. [PubMed: 21458249]
- Garratt AN, et al. A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. *J Cell Biol.* 2000; 148:1035–46. [PubMed: 10704452]
- Gaudet AD, et al. Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. *J Neuroinflammation.* 2011; 8:110. [PubMed: 21878126]
- Guedes RP, et al. Increase in reactive oxygen species and activation of Akt signaling pathway in neuropathic pain. *Cell Mol Neurobiol.* 2008; 28:1049–56. [PubMed: 18379870]

- Guedes RP, et al. Sciatic nerve transection increases glutathione antioxidant system activity and neuronal nitric oxide synthase expression in the spinal cord. *Brain Res Bull.* 2009; 80:422–7. [PubMed: 19683561]
- Hirata K, Kawabuchi M. Myelin phagocytosis by macrophages and nonmacrophages during Wallerian degeneration. *Microsc Res Tech.* 2002; 57:541–7. [PubMed: 12112437]
- Hoke A. Neuroprotection in the peripheral nervous system: rationale for more effective therapies. *Arch Neurol.* 2006; 63:1681–5. [PubMed: 17172606]
- Hubbs AF, et al. Vacuolar leukoencephalopathy with widespread astrogliosis in mice lacking transcription factor Nrf2. *Am J Pathol.* 2007; 170:2068–76. [PubMed: 17525273]
- Insera MM, et al. Functional indices for sciatic, peroneal, and posterior tibial nerve lesions in the mouse. *Microsurgery.* 1998; 18:119–24. [PubMed: 9674927]
- Jaiswal AK. Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radic Biol Med.* 2004; 36:1199–207. [PubMed: 15110384]
- Johnson JA, et al. The Nrf2-ARE pathway: an indicator and modulator of oxidative stress in neurodegeneration. *Ann N Y Acad Sci.* 2008; 1147:61–9. [PubMed: 19076431]
- Kensler TW, et al. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol.* 2007; 47:89–116. [PubMed: 16968214]
- Kim D, et al. NADPH oxidase 2-derived reactive oxygen species in spinal cord microglia contribute to peripheral nerve injury-induced neuropathic pain. *Proc Natl Acad Sci U S A.* 2010; 107:14851–6. [PubMed: 20679217]
- Kuhlmann T, et al. Macrophages are eliminated from the injured peripheral nerve via local apoptosis and circulation to regional lymph nodes and the spleen. *J Neurosci.* 2001; 21:3401–8. [PubMed: 11331370]
- Lee JM, et al. Nrf2, a multi-organ protector? *FASEB J.* 2005; 19:1061–6. [PubMed: 15985529]
- Magill CK, et al. Reinnervation of the tibialis anterior following sciatic nerve crush injury: a confocal microscopic study in transgenic mice. *Exp Neurol.* 2007; 207:64–74. [PubMed: 17628540]
- Manandhar S, et al. NRF2 inhibition represses ErbB2 signaling in ovarian carcinoma cells: implications for tumor growth retardation and docetaxel sensitivity. *Free Radic Biol Med.* 2012; 52:1773–85. [PubMed: 22387177]
- Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med.* 2004; 10:549–57. [PubMed: 15519281]
- Mueller M, et al. Macrophage response to peripheral nerve injury: the quantitative contribution of resident and hematogenous macrophages. *Lab Invest.* 2003; 83:175–85. [PubMed: 12594233]
- Mueller M, et al. Rapid response of identified resident endoneurial macrophages to nerve injury. *Am J Pathol.* 2001; 159:2187–97. [PubMed: 11733369]
- Naik AK, et al. Role of oxidative stress in pathophysiology of peripheral neuropathy and modulation by N-acetyl-L-cysteine in rats. *Eur J Pain.* 2006; 10:573–9. [PubMed: 16214382]
- Nguyen T, et al. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem.* 2009; 284:13291–5. [PubMed: 19182219]
- Nguyen T, et al. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu Rev Pharmacol Toxicol.* 2003; 43:233–60. [PubMed: 12359864]
- Park ES, et al. Levels of mitochondrial reactive oxygen species increase in rat neuropathic spinal dorsal horn neurons. *Neurosci Lett.* 2006; 391:108–11. [PubMed: 16183198]
- Reichert F, et al. Peripheral nerve injury induces Schwann cells to express two macrophage phenotypes: phagocytosis and the galactose-specific lectin MAC-2. *J Neurosci.* 1994; 14:3231–45. [PubMed: 8182468]
- Rotshenker S. Microglia and macrophage activation and the regulation of complement-receptor-3 (CR3/MAC-1)-mediated myelin phagocytosis in injury and disease. *J Mol Neurosci.* 2003; 21:65–72. [PubMed: 14500997]
- Saada A, et al. Granulocyte macrophage colony stimulating factor produced in lesioned peripheral nerves induces the up-regulation of cell surface expression of MAC-2 by macrophages and Schwann cells. *J Cell Biol.* 1996; 133:159–67. [PubMed: 8601605]

- Schafer M, et al. Disruption of the gene for the myelin-associated glycoprotein improves axonal regrowth along myelin in C57BL/Wlds mice. *Neuron*. 1996; 16:1107–13. [PubMed: 8663987]
- Senoglu M, et al. Intraperitoneal Alpha-Lipoic Acid to prevent neural damage after crush injury to the rat sciatic nerve. *J Brachial Plex Peripher Nerve Inj*. 2009; 4:22. [PubMed: 19939272]
- Sherman DL, Brophy PJ. Mechanisms of axon ensheathment and myelin growth. *Nat Rev Neurosci*. 2005; 6:683–90. [PubMed: 16136172]
- Tan EC, et al. The oxidative response in the chronic constriction injury model of neuropathic pain. *J Surg Res*. 2009; 152:84–8. [PubMed: 18708193]
- Vargas ME, Barres BA. Why is Wallerian degeneration in the CNS so slow? *Annu Rev Neurosci*. 2007; 30:153–79. [PubMed: 17506644]
- Vargas MR, Johnson JA. The Nrf2-ARE cytoprotective pathway in astrocytes. *Expert Rev Mol Med*. 2009; 11:e17. [PubMed: 19490732]
- Wooley CM, et al. Age, experience and genetic background influence treadmill walking in mice. *Physiol Behav*. 2009; 96:350–61. [PubMed: 19027767]

Highlights for review

- Deletion of Nrf2 impairs functional recovery after sciatic nerve crush
- Deletion of Nrf2 led to reduced clearance of myelin debris
- Deletion of Nrf2 led to less macrophages accumulation in the injured nerve
- Deletion of Nrf2 resulted in slightly less preserved fibers and new axonal sprouts
- Deletion of Nrf2 decreases axonal remyelination

Fig. 1. Ablation of Nrf2 delays motor function recovery but does not affect nociceptive reception. (A) Sciatic functional index (SFI) from walking track analysis before surgery, 2, 7, 14, and 21 days post surgery (n=7 for the sham groups and n=8 for the crush groups). Statistics performed by two-way ANOVA is shown in supplementary table S1. (B) Withdrawal reflex latency from analgesia hot plate analysis at 1-week post surgery (n=8). Sh: sham; cr: crush; con: contralateral hind foot; ip: ipsilateral hind foot. Statistics performed by two-way ANOVA is shown in supplementary table S2. **: p<0.01; ***: p<0.001.

Fig. 2.

Neuromuscular junction (NMJ) innervation pattern after nerve crush. (A) NMJ was stained with α -bungarotoxin (red), synaptophysin (green) and neurofilament M (green). Three innervation patterns were observed: innervated (A-1 to A-3), partially innervated (A-4 to A-6) and denervated (A-7 to A-9). (B) Percentage of innervated, partially innervated and denervated NMJ endplate at 1 day, 1 week, 2 weeks and 3 weeks after nerve crush in the contralateral and ipsilateral tibialis anterior muscle from WT mice ($n=4$). Statistics performed by one-way ANOVA is shown in supplementary table S3. (C) Percentages of innervated, partially innervated and denervated NMJ endplate at 1 week after crush from WT and KO mice ($n=6$). Statistics performed by two-way ANOVA is shown in supplementary table S4. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Fig. 3.

Axonal morphology 1-week post sciatic nerve injury. (A) Light microscopy images of semi-thin sciatic nerve sections stained with Methylene Blue/Azure II. The proximal end of the sciatic nerve mostly remains intact after crush, whereas the distal end is degenerated and most fibers are lost 1-week after injury. Bigger myelin debris seems to be present in the ipsilateral distal end nerve in the KO mice than in the WT mice (examples are given by ellipse). Scale bar: 20 μm . (B) Ultra-structural morphology of sciatic nerve by EM. The proximal end from both WT and KO remain well myelinated. Macrophages (denoted by arrow) infiltrate to the injured nerve and help to clear up myelin debris (denoted by stars). Bigger myelin debris exists in the distal end in the KO mice than in the WT mice. Scale bar: 5 μm . (C) Quantification for the number of myelin debris in the distal end of WT and KO mice ($n=3$). Statistics performed by t-test. P value is 0.209. (D) Quantification for size of myelin debris in the distal end of WT and KO mice ($n=3$). Statistics performed by t-test. P value is 0.004. **: $p<0.01$.

Fig. 4.

Clearance of myelin debris by macrophages in the nerve. (A) MAG (myelin associated glycoprotein) immunohistochemistry in contralateral and ipsilateral nerves (conN and ipN) 1 week after injury for WT and KO mice. Green: MAG. Scale bar: 10 μ m. (B) MAC2 (red) and CD68 (green) immunohistochemistry in conN and ipN one week after injury for WT and KO mice. Nerves were counterstained with Hoechst. Scale bar: 10 μ m.

Fig. 5.

Expression of macrophage markers after sciatic nerve crush. (A) Quantification of CD68 positive cells in the distal nerve stump of WT and KO mice at 1 week post injury. Statistics performed by t-test. P value is 0.048. *: $p < 0.05$. (B) Protein levels of CD11b and MAC2 in contralateral nerve (conN) and ipsilateral nerve (ipN) at 3 days, 1 week and 2 weeks post injury for WT and KO mice. (C-D) Quantification of CD11b (C) and MAC2 (D) protein levels in conN and ipN at 3 days, 1 week and 2 weeks post injury ($n=3$). Statistics performed by two-way ANOVA is shown in supplementary table S5 and S6. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Fig. 6.

Remaining fibers, sprouting axons and deposits of myelin debris at 1-week after nerve crush. (A) Remaining fibers in the distal end of WT and KO mice at 1-week post injury (denoted by arrows). Scale bar: 5 μm . (B) New axonal sprouts appear in the distal end in both WT and KO mice (denoted by stars). Myelin debris present in the distal end of both WT and KO mice (denoted by pound key #). Scale bar: 5 μm . (C) Quantification of remaining fibers in the injured nerve of WT and KO mice at 1-week post injury ($n=4$). Statistics performed by t-test. P value is 0.147. (D) Quantification of axonal sprouts in the injured nerve of WT and KO mice at 1-week post injury ($n=3$). Statistics performed by t-test. P value is 0.144. (E) Protein levels for the neuron marker NF-M and axonal regeneration marker GAP43 in the contralateral and ipsilateral nerve (conN and ipN) 1-week after crush for WT and KO mice. (F) Quantification of NF-M and GAP43 protein levels at 1-week after crush for WT and KO mice. Statistics performed by two-way ANOVA is shown in supplementary table S7. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Fig. 7.

Axonal morphology and myelination after injury. (A) Light microscopy images of semi-thin sciatic nerve sections for contralateral nerve (conN) and ipsilateral nerves (ipN) at 1-week, 3-week and 6-week after injury in WT mice. Scale bar: 20 μ m. (B) Light microscopy images of semi-thin sciatic nerve sections for contralateral and ipsilateral nerves (conN and ipN) at 3-month after injury from WT and KO mice. Scale bar: 10 μ m. (C) G-ratio for regenerated axon fibers in the distal end of the sciatic nerve from WT and KO mice at 3-month after injury (n=3). Statistics performed by two-way ANOVA is shown in supplementary table S8. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Table 1

TreadScan parameters for WT and KO mice before surgery, 1-week, 2-week and 3-week post surgery (n=8).

Parameter	Time	WT-con		WT-ip		KO-con		KO-ip	
		Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM		Mean $\pm$ SEM	
Stance (ms)	pre	226 $\pm$ 23.1		229 $\pm$ 16.0		163 $\pm$ 7.95		202 $\pm$ 11.1	
	1w-post	216 $\pm$ 28.6		209 $\pm$ 4.06		145 $\pm$ 15.3		225 $\pm$ 2.60 *	
	2w-post	287 $\pm$ 24.6		255 $\pm$ 18.6		205 $\pm$ 17.9		227 $\pm$ 11.7	
	3w-post	234 $\pm$ 13.0		248 $\pm$ 14.2		238 $\pm$ 8.04		270 $\pm$ 15.7	
Swing(ms)	pre	174 $\pm$ 28.7		127 $\pm$ 12.3		213 $\pm$ 19.3		181 $\pm$ 15.1	
	1w-post	181 $\pm$ 14.5		159 $\pm$ 22.0		334 $\pm$ 32.6 #		165 $\pm$ 20.8 **	
	2w-post	112 $\pm$ 13.7		109 $\pm$ 13.9		185 $\pm$ 20.9 #		142 $\pm$ 8.41	
	3w-post	138 $\pm$ 30.6		99 $\pm$ 12.2		188 $\pm$ 24.9		132 $\pm$ 20.8	
Print area (mm ^2)	pre	553 $\pm$ 70.5		488 $\pm$ 36.6		379 $\pm$ 33.5		339 $\pm$ 44.0	
	1w-post	449 $\pm$ 93.1		796 $\pm$ 112 *		256 $\pm$ 19.1		609 $\pm$ 43.9 *	
	2w-post	684 $\pm$ 55.0		707 $\pm$ 69.3		434 $\pm$ 41.4 #		897 $\pm$ 70.4 ***	
	3w-post	704 $\pm$ 49.7		820 $\pm$ 49.6		555 $\pm$ 50.7		789 $\pm$ 55.0 *	
Homolog Cpl	pre	0.43 $\pm$ 0.02		0.46 $\pm$ 0.02		0.38 $\pm$ 0.03		0.50 $\pm$ 0.02	
	1w-post	0.43 $\pm$ 0.04		0.45 $\pm$ 0.03		0.28 $\pm$ 0.04 #		0.56 $\pm$ 0.03 ***	
	2w-post	0.45 $\pm$ 0.02		0.42 $\pm$ 0.02		0.39 $\pm$ 0.01		0.51 $\pm$ 0.01 *** ##	
	3w-post	0.52 $\pm$ 0.02		0.40 $\pm$ 0.01		0.46 $\pm$ 0.03		0.43 $\pm$ 0.02	
LatD min (mm)	pre	5.67 $\pm$ 1.04		6.14 $\pm$ 0.65		7.27 $\pm$ 1.48		6.15 $\pm$ 0.53	
	1w-post	3.30 $\pm$ 1.80		4.83 $\pm$ 0.75		6.89 $\pm$ 0.94		8.32 $\pm$ 0.80	
	2w-post	8.37 $\pm$ 1.06		1.72 $\pm$ 1.27 ***		11.0 $\pm$ 0.42		-0.41 $\pm$ 0.79 ***	
	3w-post	9.06 $\pm$ 0.69		4.97 $\pm$ 0.56		11.9 $\pm$ 1.66		4.50 $\pm$ 0.48 ***	
LatD max (mm)	pre	14.4 $\pm$ 0.47		14.0 $\pm$ 0.92		15.2 $\pm$ 0.58		14.1 $\pm$ 0.49	
	1w-post	13.0 $\pm$ 1.21		12.0 $\pm$ 0.77		13.1 $\pm$ 0.99		15.5 $\pm$ 0.46	
	2w-post	18.5 $\pm$ 0.93		10.2 $\pm$ 0.60 ***		20.3 $\pm$ 0.53		8.62 $\pm$ 0.72 ***	
	3w-post	16.7 $\pm$ 0.43		13.0 $\pm$ 1.05		19.7 $\pm$ 0.39		12.0 $\pm$ 0.60	

* : comparison between WT-con and WT-ip, or KO-con and KO-ip.
 * : p<0.5,
 ** : p<0.01,
 *** : p<0.001.
 # : comparison between WT-con and KO-con, or WT-ip and KO-ip.
 # : p<0.5,
 ## : p<0.01.