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Role of Nrf2 in Protection against Intracerebral Hemorrhage Injury in Mice

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Abstract

Nrf2 is a key transcriptional factor for antioxidant response element (ARE)-regulated genes. While its beneficial role has been described for stroke, its contribution to intracerebral hemorrhage (ICH)-induced early brain injury remains to be determined. Using wildtype (WT) and Nrf2 knockout (Nrf2^{-/-}) mice, the role of Nrf2 in ICH induced by intracerebral injection of collagenase was investigated. The results showed that injury volume was significantly larger in Nrf2^{-/-} mice than in WT controls 24 h after induction of ICH ($p < 0.05$), an outcome that correlated with neurological deficits. This exacerbation of brain injury in Nrf2^{-/-} mice was also associated with an increase in leukocyte infiltration, production of reactive oxygen species, DNA damage, and cytochrome c release during the critical early phase of the post-ICH period. In combination, these results suggest that Nrf2 reduces ICH-induced early brain injury, possibly by providing protection against leukocyte-mediated free radical oxidative damage.

Keywords

DNA damage; Free radicals; Inflammation; NF-E2-related factor 2; Reactive oxygen species

Introduction

Clinical and animal studies have provided evidence that inflammation and oxidative stress from reactive oxygen species (ROS) are involved in the progression of intracerebral hemorrhage (ICH)-induced early brain injury [1–3]. In addition, recent research has demonstrated that oxidative stress can modulate inflammatory responses during tissue injury, possibly through activation of nuclear factor erythroid 2-related factor 2 (Nrf2), a key transcriptional factor for antioxidant response element (ARE)-regulated genes [4].

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Nrf2 is regarded as a protector for many organs, including brain (reviewed in [5]). It has been reported that Nrf2, a key regulator of cell survival [6,7], can induce and up-regulate cytoprotective and antioxidant genes that attenuate tissue injury [8,9]. Sulforaphane, a naturally occurring isothiocyanate that induces the expression of multiple Nrf2-responsive genes, has been shown to be neuroprotective against focal cerebral ischemia in rats [10]. In addition, activation of the Nrf2 pathway, either by sulforaphane itself or by Nrf2 overexpression, was able to protect neurons from oxidative stress damage [11]. Furthermore, primary cultured neurons derived from Nrf2 knockout (Nrf2^{-/-}) mice were shown to be more vulnerable to oxidative stress than neurons from control animals. However, when the neurons were transfected with a functional Nrf2 construct, they become more resistant to free radicals [12]. Consistent with the results of these studies, dominant-negative-Nrf2 and siRNA-Nrf2-stable neuroblastoma cell lines were more prone to apoptosis than cells transfected with vector only because of the down-regulation of ARE-mediated protective genes [13].

Previous studies have shown that increasing Nrf2 activity provides protection against cerebral ischemia in vivo [10,11,14], but the role of Nrf2 activity during hemorrhage has not yet been examined. In this study, we hypothesized that Nrf2 would be protective in intracerebral hemorrhage (ICH). To test this hypothesis, we subjected wildtype (WT) and Nrf2^{-/-} mice to an ICH model that caused disruption of blood vessels and entry of blood into the brain parenchyma [3]. Then we compared the outcomes in terms of brain injury volume, number of degenerating neurons, neurologic function, inflammatory response, and ROS production.

Materials and methods

Animals

This study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals. Experimental protocols were approved by the Johns Hopkins University Animal Care and Use Committee. Nrf2^{-/-} and WT mice on a CD1 background were generated as described previously [15,16] and were maintained in our facilities. All mice were subjected to genotyping for Nrf2 status by PCR amplification of genomic DNA extracted from tail tips [17]. Three primers were used to perform PCR amplification: 5'-TGGACGGGACTATTGAAGGCTG-3' (sense for both genotypes), 5'-CGCCTTTTCAGTAGATGGAGG-3' (antisense for WT mice), and 5'-GCGGATTGACCGTAATGGGATAGG-3' (antisense for LacZ). These CD1 mice were fed with an AIN-76A diet, given water ad libitum, and housed under controlled conditions (23 ± 2°C; 12 h light/dark cycle).

ICH model

The procedure for inducing ICH by collagenase injection in mice, adapted from an established rat protocol [18], has been described previously [19,20]. Age- and weight-matched adult male mice (26–33 grams) were anesthetized by intraperitoneal injection with Avertin (2-2-2 tribromoethanol; Sigma, St. Louis, MO; 0.5 mg/g body weight). To induce hemorrhage, mice were injected unilaterally into the caudate putamen with collagenase VII-S (0.1 U in 500 nl saline, Sigma) at the following stereotactic coordinates: 0.8 mm anterior and 2.5 mm lateral of the bregma, 2.5 mm in depth. Collagenase was delivered over 5 min, and the needle was left in place for an additional 25 min to prevent any reflux. Rectal temperature was maintained at 37.0 ± 0.5°C throughout the experimental and recovery periods. Because the focus of our study was the early brain injury in ICH, mice were sacrificed for analysis 24 h later, after being tested for neurologic deficits.

Neurologic deficit

An experimenter blinded to the mouse genotype scored all mice (10 WT; 7 Nrf2^{-/-}) for neurologic deficits with a 24-point neurologic scoring system [21] 24 h after collagenase injection. The tests included body symmetry, gait, climbing, circling behavior, front limb symmetry, and compulsory circling. Each test was graded from 0 to 4, establishing a maximum deficit score of 24. Immediately after the testing, the mice were sacrificed for injury analysis.

Hemorrhagic injury analysis

All processing and analysis of tissue sections as described in this and the following sections were conducted by an observer blind to the genotype of the mice. Nrf2^{-/-} ($n = 7/\text{group}$) and WT ($n = 10/\text{group}$) mice were euthanized, and their brains were harvested, fixed in 4% paraformaldehyde for 24 h, and cryoprotected in serial phosphate-buffered sucrose solutions (20, 30, and 40%) at 4°C. Then the brains were cut into 50- μm sections with a cryostat. Sections were stained with Luxol fast blue and Cresyl Violet [20] before being quantified for injury area with SigmaScan Pro software (version 5.0.0 for Windows; Systat, Port Richmond, CA). Six to eight coronal slices from different levels of the injured hemorrhagic area were summed, and the volumes in cubic millimeters were calculated by multiplying the thickness by the measured areas [20].

Histology

Luxol fast blue/Cresyl Violet, and Fluoro-Jade B (FJB) staining were performed according to published protocols [22,23]. Cells permeable to FJB are marked as degenerating neurons. To perform the quantification analysis, three sections per mouse with similar areas of hematoma were chosen from three WT and three Nrf2^{-/-} mice with similar brain injury volumes, and positively stained cells were counted in four different comparable fields adjacent to the hematoma. Three sections per animal over a microscopic field of 40 x were averaged and expressed as cells/field, as previously reported [20]. Stained sections were examined with a fluorescence microscope; the images were captured and analyzed by SPOT image software (Diagnostic Instruments Inc., Sterling Heights, MI). Areas with large blood vessels were avoided.

Immunofluorescence

Immunofluorescence was carried out as described previously [24]. Briefly, free-floating sections were washed in PBS for 20 min, blocked in 5% normal goat serum, and incubated overnight at 4°C with primary antibodies: rabbit anti-myeloperoxidase (MPO, neutrophil marker; 1:100; DAKO, UK); rabbit anti-Iba 1 (microglia marker; 1:1000; Wako Chemicals, Richmond, VA); mouse anti-Nitrotyrosine (peroxynitrite marker; 1:1000; Upstate, Lake Placid, NY); mouse anti-8-hydroxyguanosine (8-OHG; 10 $\mu\text{g}/\text{ml}$, Oxis International Inc, Portland, OR); mouse anti-cytochrome c (1:1000; BD Pharmingen, San Diego, CA). To assess the cellular source of markers of oxidative stress (nitrotyrosine and 8-OHG) and cytochrome c after ICH, double immunofluorescence was performed with one of these markers and an antibody against microtubule-associated protein-2 (MAP2, neuronal marker; 1:1000; Chemicon, Temecula, CA). Sections then were incubated with Alexa488 (1:1000; Molecular Probes) and/or Cy3 (1:1000; Jackson ImmunoResearch, West Grove, PA)-conjugated secondary antibody. Three sections per mouse with similar areas of hematoma were chosen from WT and Nrf2^{-/-} mice (three mice per group) with similar brain injury volumes, and positively stained cells were counted in four different comparable fields adjacent to the hematoma. Three sections per animal over a microscopic field of 60 x (for neutrophils) or 40 x (for microglia/macrophages) were averaged and expressed as cells/field. Stained sections were examined with a fluorescence microscope as described above. Control sections were processed by the same method, except that primary antibodies were omitted.

Statistics

All data are expressed as means $\pm$ SD. Differences between groups were determined by Student's *t*-test. Statistical significance was set at $p < 0.05$.

Results

Nrf2^{-/-} mice have larger brain injury volumes and greater neurologic deficit than WT mice after ICH

From previous in vitro and in vivo studies that demonstrated a neuroprotective role for Nrf2, we hypothesized that Nrf2 gene deletion would lead to increased brain injury after ICH. Quantification of brain injury with Luxol fast blue/Cresyl Violet staining confirmed that injury volume of Nrf2^{-/-} mice ($24.1 \pm 7.4 \text{ mm}^3$) was larger than that of WT mice ($14.7 \pm 4.4 \text{ mm}^3$, $p = 0.015$) 24 h after ICH (Fig. 1A and 1B). These results are consistent with our previous studies [21,24]. No detectable bleeding was observed in sham-operated mice (data not shown).

To further determine whether the greater ICH-induced brain injury in Nrf2^{-/-} mice correlated with greater neurobehavioral deficits, assessment of neurologic function of the animals was performed at 24 h after collagenase injection. Nrf2^{-/-} mice showed more severe neurologic deficits than WT mice after ICH (15.5 ± 4.0 vs. 10.9 ± 1.9 , $p = 0.006$) (Fig. 1C). The largest differences in scores between Nrf2^{-/-} and WT mice were in the attributes of body symmetry, circling behavior, and compulsory circling. We have previously observed that anesthesia alone has no effect on the neurologic function of mice [21].

To examine whether neuronal death was more evident at the site of hemorrhage in Nrf2^{-/-} mice, we used FJB histological staining, a specific marker for degenerating neurons [20,23]. The results suggest a trend toward more degenerating neurons in Nrf2^{-/-} than in WT mice (Fig. 2A), though they did not reach statistical significance (32.7 ± 5.4 vs. 23.2 ± 5.0 cells/field, $n = 3/\text{group}$, $p = 0.08$) (Fig. 2B). FJB-positive neurons were not observed in the contralateral side or normal brain, but were occasionally observed along the needle track in sham-operated WT and Nrf2^{-/-} mice (data not shown).

Nrf2 deletion increases leukocyte infiltration

Acute inflammation is a normal response to brain injury. As indicated by immunoreactive MPO, ICH produces a robust infiltration of neutrophils into the affected striatum that can be observed as early as 4 h after ICH [24]. Although infiltrating neutrophils were evident in and around the injury site in WT and Nrf2^{-/-} mice 24 h post-ICH (Fig. 3A and 3B), Nrf2^{-/-} mice had significantly more neutrophils (Fig. 3E, 39.2 ± 4.0 vs. 30.4 ± 3.4 cells/field, $n = 3/\text{group}$, $p = 0.04$).

Microglial/macrophage activation contributes to ICH-induced early brain injury [1–3]. To clarify the effect of Nrf2 on the state of microglial/macrophage activation after ICH, Iba1, a marker for microglia/macrophages, was used [25]. The results showed that resting microglial cells were sparse, but distributed similarly in WT and Nrf2^{-/-} mice on the uninjected side 24 h after ICH (data not shown). Similarly, no differences were apparent in the distribution of activated microglia/macrophages around the injury site in WT and Nrf2^{-/-} mice (Fig. 3C–3E).

Nrf2 deletion increases ROS production, DNA damage, and cytochrome c release

ROS are thought to play a major role in the various mechanisms of ICH-induced brain injury [1,24]. Peroxynitrite (ONOO⁻) is one of the ROS produced by the interaction of nitric oxide (NO) and superoxide. ONOO⁻, acting as an oxidant, is more stable than NO or superoxide and can readily diffuse across phospholipid membranes [26]. We detected ONOO⁻-positive cells around the injury site 24 h post-ICH in WT and Nrf2^{-/-} mice as indicated by the ONOO⁻

staining in Figure 4A. We did not detect ONOO⁻-positive cells on the contralateral side or in normal brain. Double labeling of nitrotyrosine and MAP2 demonstrated that nearly all the ONOO⁻-positive cells in WT mice were neurons (Fig. 4B). Quantification analysis showed that Nrf2^{-/-} mice had significantly more ONOO⁻-positive cells than WT mice around the border region of injury (Fig. 4C, 17.0 ± 1.7 vs. 12.6 ± 1.5 cells/field, $n = 3/\text{group}$, $p = 0.03$). ONOO⁻-positive cells were observed very rarely in sham-operated WT and Nrf2^{-/-} mice (data not shown).

8-hydroxyguanosine (8-OHG) is a reliable and commonly used biomarker for oxidative DNA damage caused by superoxide anion, as shown previously after various forms of brain injury [27–29], including ICH [28,30]. Here, 8-OHG-positive cells were detected around the injury site 24 h post-ICH in WT and Nrf2^{-/-} mice (Fig. 5A). Double labeling of 8-OHG and MAP2 demonstrated that nearly all the 8-OHG-positive cells in WT mice were neurons (Fig. 5B). Analysis showed that Nrf2^{-/-} mice had more 8-OHG-positive cells than did WT mice around the border region of injury at 24 h post-ICH (Fig. 5C, 19.3 ± 2.6 vs. 9.2 ± 2.0 cells/field, $n = 3/\text{group}$, $p = 0.006$). 8-OHG-positive cells were not observed in the contralateral side or in normal brain, but were observed very rarely in sham-operated WT and Nrf2^{-/-} mice (data not shown).

To understand the mechanisms implicated in cell death after ICH, we examined the immunoreactive cytosolic cytochrome c. Release of mitochondrial cytochrome c to the cytosol has been linked to apoptotic cell death [31], a significant contributor to ICH-induced brain damage [20,32,33]. We did not detect cytochrome c in the control hemisphere. In contrast, we did detect cytochrome c immunoreactivity in the cytosol of the cells around the border region of injury 24 h post-ICH (Fig. 6A). Double labeling of cytochrome c and MAP2 in WT mice demonstrated that most cytochrome c-positive cells were neurons (Fig. 6B). Analysis suggested that around the border region of injury, Nrf2^{-/-} mice had more cytochrome c-positive cells than did WT mice (Fig. 6C, 23.1 ± 4.2 vs. 11.2 ± 0.8 cells/field, $n = 3/\text{group}$, $p = 0.03$). Cytochrome c-positive cells were observed very rarely in sham-operated WT and Nrf2^{-/-} mice (data not shown).

Discussion

This study revealed that Nrf2^{-/-} mice are significantly more prone to hemorrhagic brain injury and neurologic deficits than their WT counterparts. Furthermore, we found that Nrf2^{-/-} mice have more neuronal cell death, neutrophil infiltration, ROS production, DNA damage, and cytochrome c release. Although previous work has shown that after a permanent stroke model (permanent middle cerebral artery occlusion without reperfusion) Nrf2^{-/-} mice suffered more stroke damage than WT controls [34], a finding that we have confirmed and extended in a transient stroke model (middle cerebral artery occlusion with reperfusion) [35]; to our knowledge, these findings reported here provide the first clear evidence that Nrf2 plays a critical role in limiting the cascade of events leading to ICH-induced early brain injury.

Oxidative stress from ROS contributes to ICH-induced early brain injury [1–3]. In this study, ONOO⁻ (a marker for ROS) and 8-OHG (a marker for DNA oxidation) were found mostly in neurons bordering the injury site at 24 h post-ICH; however, more ONOO⁻ and 8-OHG-positive neurons were observed in Nrf2^{-/-} mice than in WT mice. It is therefore likely that the exacerbated injury from hemorrhage observed in Nrf2^{-/-} mice is, at least in part, attributable to the increase in post-ICH ROS production.

After brain injury, activated leukocytes and microglia/macrophages are major sources of ROS production [2,36–38], and available data from clinical and preclinical animal models support a role for activation of leukocytes and microglia/macrophages in ICH-induced early brain

injury [24,39–41]. In our study, more infiltrating neutrophils were observed in Nrf2^{-/-} mice at 24 h post-ICH, but no difference in microglia/macrophage activation was found between WT and Nrf2^{-/-} mice at the same time-point. These findings indicate that neutrophil infiltration, rather than microglial/macrophage activation, correlates with increased early brain injury in Nrf2^{-/-} mice. Infiltrating leukocytes damage brain tissue by increasing vascular permeability, releasing pro-inflammatory proteases, and generating ROS [24,42], which increase free radical oxidative damage in neurons. Therefore, increasing Nrf2 activity in the early stage of ICH may diminish additional recruitment of leukocytes and decrease leukocyte-mediated early brain injury.

Human and animal studies have provided evidence that apoptosis is a prominent form of cell death associated with ICH in the perihematoma region [20,33,43]. Using FJB staining as a marker for neuronal death, we observed a trend towards more degenerating neurons in Nrf2^{-/-} mice than in WT mice at 24 h post-ICH, although the difference was not statistically different. Oxidative stress from ROS has been shown to trigger cytochrome c release, which is often followed by DNA damage and cell death [44]. To explore further whether Nrf2 deficiency contributes to ROS-induced apoptosis in our ICH model, we investigated cytochrome c release as a means of predicting apoptosis [31]. When cytochrome c is released from the mitochondria into the cytosol as a result of increased mitochondrial permeability, it activates the initiator caspase-9, which then cleaves and activates caspase-3, finally leading to apoptotic cell death [31]. We found that 24 h after ICH, cytochrome c release was evident in neurons around the border of the injury site and was greater in Nrf2^{-/-} mice than in WT mice. The results support previous reports that neurons are more susceptible to ROS-induced DNA damage than other cell types in the brain [24,45], and also suggest that Nrf2 deficiency could enhance ICH-induced neuronal cell death possibly by apoptotic mechanisms.

One potential defense against the toxicity of oxidative stress is the induction of a family of phase II detoxification enzymes. ARE, a unique *cis*-acting regulatory sequence, is essential for the constitutive and induced expression of many antioxidant genes involved in the phase II pathway [46]. Available evidence suggests that Nrf2 is a major transcription factor responsible for upregulating ARE-mediated antioxidant gene expression such as NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione s-transferase (GST), heme oxygenase 1 (HO-1), glutamylcysteine ligase (the rate-limiting enzyme in glutathione synthesis), thioredoxin, and thioredoxin reductase 1 [4,12,47]. Basal NQO1 and GST activities were found to be lower in multiple brain regions of Nrf2^{-/-} mice, compared with WT mice [34]. Therefore, it is likely that deletion of the Nrf2 gene renders mice more susceptible to ICH-induced early brain injury because of decreased ability to induce phase II detoxification enzymes. Additional work is necessary to determine which ones or which combinations are responsible for the beneficial effect of Nrf2-mediated gene expression.

In conclusion, we have shown that Nrf2-deficient mice are significantly more susceptible to ICH-induced early brain injury than are control mice. The exacerbation of injury appears to be associated with an increase in leukocyte infiltration, ROS production, DNA damage, and cytochrome c release during the critical phase of the early post-ICH period. Taken together, these results suggest that Nrf2 deficiency contributes to ROS-induced DNA damage and apoptosis mostly in neurons in the early stage of ICH, and that activation of Nrf2 will serve to control the infiltration of leukocytes into the focus of the injury, preventing excessive free radical oxidative damage in the brain tissue. Although additional work with selective Nrf2 inducers and inhibitors is needed, the findings raise the possibility that Nrf2 will be a potential therapeutic target for the treatment of ICH.

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Abbreviations

8-OHG	8-hydroxyguanosine
ARE	antioxidant response element
FJB	Fluoro-Jade B
GST	glutathione s-transferase
ICH	intracerebral hemorrhage
IR	immunoreactive
NQO1, NAD(P)H	quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor
ONOO⁻	peroxynitrite
ROS	reactive oxygen species
WT	wildtype

Fig 1.

Deletion of Nrf2 increases brain injury volume and neurologic deficits in mice subjected to intracerebral hemorrhage (ICH). Age- and weight-matched Nrf2 knockout (Nrf2^{-/-}) and wildtype (WT) mice were subjected to ICH, and brains were sectioned and stained with Luxol fast blue/Cresyl Violet. (A) Representative sections from Nrf2^{-/-} and WT mice 24 h after collagenase injection showing different areas of injury as represented by lack of staining. Scale bar = 100 μ m. (B) Quantification shows significantly larger brain injury volume in Nrf2^{-/-} mice ($n = 7$) compared with WT mice ($n = 10$) 24 h after collagenase injection. (C) An investigator blinded to genotype assessed the neurologic deficits of Nrf2^{-/-} and WT mice with a 24-point neurologic scoring system 24 h after collagenase injection. Neurologic deficits were

significantly more severe in Nrf2^{-/-} mice ($n = 7$) than in WT mice ($n = 10$). Values are means $\pm$ SD; * $p < 0.05$.

Fig 2. Deletion of Nrf2 increases the number of degenerating neurons in mice subjected to ICH. (A) Fluoro-Jade B histological staining of degenerating neurons in sections collected 24 h after collagenase injection shows intensely labeled neurons and processes in the peri-ICH region in WT and $Nrf2^{-/-}$ mice. Scale bar = 20 μ m. (B) Quantification analysis suggested that $Nrf2^{-/-}$ mice had more degenerating neurons than WT control mice, but the difference did not reach statistical significance ($n = 3/\text{group}$, $p = 0.08$). Values are means $\pm$ SD.

Fig 3.

Deletion of Nrf2 increases leukocyte infiltration, but does not affect microglial activation in mice subjected to ICH. (A–D) Infiltrating neutrophils (MPO-positive cells; scale bar: 40 μ m) and activated microglia (Iba1-positive cells; scale bar: 20 μ m) were apparent in or around the injury site in Nrf2^{-/-} and WT mice 24 h post-ICH. (E) Quantification analysis indicated that Nrf2^{-/-} mice had significantly more infiltrating neutrophils than did WT mice at 24 h post-ICH; the number of activated microglial cells around the injury site was similar in Nrf2^{-/-} and WT mice (both $n = 3/\text{group}$, * $p < 0.05$).

Fig 4.

Deletion of Nrf2 increases ROS production in mice subjected to ICH. Peroxynitrite (ONOO⁻) was used as a marker for ROS production. (A) Increased ONOO⁻ immunoreactivity (IR) was detected in the cytosol of cells around the injury site 24 h post-ICH in tissue sections from WT and Nrf2^{-/-} mice. Scale bar = 20 μ m. (B) Double labeling of nitrotyrosine and MAP2 in WT mice indicated that nearly all the ONOO⁻-positive cells were neurons. Scale bar = 30 μ m. (C) Quantification of ONOO⁻-immunopositive cells around the injury border region showed that Nrf2^{-/-} mice had significantly more positive cells than did WT mice ($n = 3$ /group, * $p < 0.05$). Values are the means $\pm$ SD.

Fig 5.

Deletion of Nrf2 increases DNA damage in mice subjected to ICH. 8-hydroxyguanosine (8-OHG) was used as a marker for DNA oxidation. (A) Increased 8-OHG immunoreactivity (IR) was detected in the cytosol of cells around the injury site 24 h post-ICH in tissue sections from WT and Nrf2^{-/-} mice. Scale bar = 20 μ m. (B) Double labeling of 8-OHG and MAP2 in WT mice indicated that nearly all the 8-OHG-immunopositive cells were neurons. Scale bar = 30 μ m. (C) Quantification of 8-OHG-positive cells around the injury border region showed that Nrf2^{-/-} mice had significantly more positive cells than did WT mice ($n = 3/\text{group}$, $*p < 0.01$). Values are means $\pm$ SD.

Fig 6. Deletion of Nrf2 increases cytochrome c release in mice subjected to ICH. (A) Increased cytochrome c immunoreactivity (IR) was detected in the cytosol of cells around the injury site 24 h post-ICH in WT and Nrf2^{-/-} mice. Scale bar = 40 μ m. (B) Double labeling of cytochrome c and MAP2 in WT mice indicated that most cytochrome c-immunopositive cells were neurons. Scale bar = 30 μ m. (C) Quantification of cytochrome c-positive cells around the injury border region showed that Nrf2^{-/-} mice had significantly more positive cells than did WT mice ($n = 3$ /group, $*p < 0.05$). Values are means $\pm$ SD.