Heme Oxygenase-2 Modulates Early Pathogenesis after Traumatic Injury to the Immature Brain

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Key Words

Oxidative damage • Cell death • Motor recovery • Neuroprotection • Murine • Cortical lesion • Dentate

Abstract

We determined if heme oxygenase-2 (HO-2), an enzyme that degrades the pro-oxidant heme, confers neuroprotection in the developing brain after traumatic brain injury (TBI). Male HO-2 wild-type (WT) and homozygous knockout (KO) mice at postnatal day 21 were subjected to TBI and euthanized 1, 7, and 14 days later. Relative cerebral blood flow, measured by laser Doppler, cortical and hippocampal pathogenesis, and motor recovery were evaluated at all time points. Cerebral blood flow was found to be similar between experimental groups. Blood flow significantly decreased immediately after injury, returned to baseline by 1 day, and was significantly elevated by 7 days, post-injury. Nonheme iron preferentially accumulated in the ipsilateral cortex, hippocampus, and external capsule in both WT and KO brain-injured genotypes. There were, however, a significantly greater number of TUNEL-positive cells in the hippocampal dentate gyrus and a significantly greater cortical lesion volume in KOs relative to WTs within the first week post-injury. By 14 days post-injury, however, cortical lesion volume and cell density in the

hippocampal CA3 region and dorsal thalamus were similar between the two groups. Assays of fine motor function (grip strength) over the first 2 weeks post-injury revealed a general pattern of decreased strength in the contralateral forelimbs of KOs as compared to WTs. Together, these findings demonstrate that deficiency in HO-2 alters both the kinetics of secondary damage and fine motor recovery after TBI.

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Introduction

It was originally believed that the immature brain, which has the capacity for substantial plasticity, exhibits remarkable recovery after brain injury. This belief has been challenged, however, by studies which have demonstrated that children less than 4 years of age exhibit poorer motor outcome and cognitive function as compared to older children after brain injury [1–4]. The determinants of this vulnerability in young children remain unclear.

We hypothesize that heme oxygenase-2 (HO-2) could be one of the determinants of secondary pathogenesis and motor recovery after traumatic injury to the developing murine brain. We have previously shown that HO-2 confers protection in the adult brain after traumatic brain

injury (TBI) [5]. HO-2 deficient mice exhibit greater neuronal cell loss, impaired motor recovery, and an increased susceptibility to lipid peroxidation as compared to brain-injured wild-type (WT) littermates. What remains unclear is the extent to which HO-2 may modulate injury and recovery mechanisms in the injured, developing brain.

HO, first characterized by Tenhunen et al. [6], catalyzes the degradation of heme to biliverdin, carbon monoxide and ferrous iron (Fe^{2+}) [7]. HO activity and the degradation products of heme have been implicated in a variety of biologic processes including those associated with both cytoprotection and toxicity, neurotransmission, and inflammation [8–12].

The two principal isoforms of HO are HO-1 and HO-2. HO-1 (HSP-32) is preferentially expressed in neurons in the hilus of the dentate gyrus and the ventromedial hypothalamus [13] and is markedly induced under conditions of cellular stress [14–26], including those associated with TBI [20, 27] where it is primarily localized to glia [5, 20]. In contrast, HO-2 is constitutively expressed in neurons throughout the forebrain, hippocampus, basal ganglia, thalamus, cerebellum, and brain stem [13, 28], and is typically not responsive to conditions associated with cellular stress. HO-2 is regulated by adrenal glucocorticoids via a glucocorticoid responsive element within the promoter region of the gene [17, 28–32]. Two pseudogenes, HO-3a and HO-3b, have recently been identified and are thought to be nonfunctional derivatives from HO-2 transcripts [33].

We have developed and characterized a model of TBI in the mouse at postnatal day (PD) 21, an age that approximates the toddler-aged child [34]. In this study, we show that HO-2 deficient mice, subjected to TBI at PD 21, exhibit significantly greater neuronal injury in the hippocampus and a larger cortical lesion volume as compared to brain-injured WT littermates within the first week post-injury. Moreover, this pronounced period of pathogenesis coincides with a deficit in forelimb grip strength. Together, these findings suggest that HO-2 modulates the early kinetics of secondary pathogenesis in the injured, immature brain.

Materials and Methods

Male HO-2 knockout (KO) and WT mice at PD 21 were evaluated in this study. All procedures were approved by the Institutional Animal Care and Use Committee at the University of California at San Francisco.

Surgical Procedures

Animals were anesthetized with Avertin (1.25% 2, 2, 2-tribromoethanol diluted in isotonic saline at 0.02 ml/g of body weight, i.p.) and subjected to a controlled cortical impact injury or sham surgery to the left frontoparietal cortex as we have previously described [35]. Body temperature was monitored with a rectal probe and maintained at 35.9–37°C using a circulating water heating pad. All studies described below were conducted with the observer blinded to the experimental group.

Relative Cortical Blood Flow

Relative cortical blood flow was determined using a laser Doppler probe (LaserFlo BPM2; Vasamedics, St. Paul, Minn., USA) in WT, KO, and sham-operated animals for each genotype ($n = 5$ animals per group at each time point). Anesthetized animals were positioned on a warming blanket to maintain normothermia. A fiber optic needle probe, mounted on a stereotactic manipulator, was positioned 0.5 mm above the dura mater. Care was taken to avoid placement directly over visibly large superficial vessels. Measurements over a period of 3 min were made in all animals before injury and at the same location immediately post-injury. Animals were then euthanized either 1 or 7 days later. Immediately prior to euthanasia, the probe was repositioned over the site of the injury and measurements from the cerebral cortex were again recorded for 3 min. In sham controls, measurements were recorded after craniectomy and again at either 1 or 7 days post craniectomy. In all animals subjected to TBI, values are expressed as a percentage change from baseline obtained from the intact cortex.

Immunocytochemistry

At 1, 7, and 14 days post-injury, WT and KO mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in 0.05 M phosphate-buffered saline (PBS). Each brain was removed, fixed in PFA at 4°C for 4 h and then returned to PBS overnight. Coronal sections, 50 μm in thickness, were cut with a vibratome and every fourth (14 day group) or fifth (1 and 7 day groups) section was collected in wells containing 0.05 M PBS and prepared for histologic studies.

Neurons were identified using a modified protocol for a monoclonal antibody to neuronal nuclei (NeuN; Chemicon International, Temecula, Calif., USA). A mouse-on-mouse (MOM) immunodetection kit (Vector Laboratories) was used to avoid nonspecific staining associated with the mouse-derived primary antibody. Sections were first rinsed in 0.05 M PBS two times for 5 min each and incubated in 1% H_2O_2 for 10 min to quench any endogenous peroxidase activity, and incubated in the following solutions: MOM mouse blocking reagent, 1 h; PBS, three times for 5 min each; MOM diluent, 5 min; NeuN in MOM diluent, 1:1,000 dilution, 30 min; PBS, three times for 5 min each; MOM biotinylated anti-mouse IgG reagent, 1:250 dilution, 10 min; PBS, three times for 5 min each; and Vectastain Elite ABC reagent (Vector Laboratories), 5 min. All sections were rinsed with PBS three times for 5 min each and reacted with 0.05% DAB in 0.02% H_2O_2 for 5 min.

Analysis of Lesion Volumes

Cortical lesion volumes were determined at 7 (KO, $n = 6$; WT, $n = 10$) and 14 (KO, $n = 21$; WT, $n = 15$) days post-injury from three sections, stained with cresyl violet, in the region of maximal cortical damage. In each section, lesion area (LA) was determined as follows: LA = area of contralateral hemisphere – area of ipsilat-

eral hemisphere. The lesion volume ratio was then defined as follows: lesion volume ratio = (LA1 + LA2 + LA3) $\times$ 250 (d7) or 200 (d14) μ m/whole brain volume of the three sections, where LA1, LA2, and LA3 represent LAs of each individual section and 200 μ m reflects the sum of the thickness of the section (50 μ m) and the distance between the sections (150 μ m).

Histochemistry for Nonheme Iron

Nonheme iron was identified using a modified Perl's solution (2% potassium ferrocyanide and 6% hydrochloride in H₂O) in the brains of WT and KO mice at 7 and 14 days post-injury. Sections, prepared at the level of the impact injury, were incubated in modified Perl's solution for 30 min, washed with 0.05 M PBS three times for 5 min each, and reacted with 0.05% DAB in 0.02% H₂O₂ for 15 min. The sections were then mounted on slides and prepared for light microscopic evaluation. Substrate controls consisted of incubation in DAB and H₂O₂ only.

TUNEL Staining

TUNEL staining was used to localize irreversibly injured cells. Sections were first mounted on slides and dried at room temperature for 3 days. A commercial kit (TdT-FragEL DNA Fragmentation Detection Kit; Oncogene, La Jolla, Calif., USA) was used to label cells with methyl green as a counter stain. Positive controls were pretreated with DNase I (1 μ g/ml) to introduce nonspecific strand breaks. Negative controls consisted of the same procedure for TUNEL in the absence of terminal deoxynucleotidyl transferase.

TUNEL-positive cell counting (1d KO, n = 7; 1d WT, n = 10; 7d KO, n = 6; 7d WT, n = 6) and analysis were performed on images captured with a Nikon microscope at 40 $\times$ magnification with a mounted CCD camera (SPOT-1; Diagnostic Instruments, Sterling Heights, Mich., USA) using MicroBright Fields Neurolucida Software. Three consecutive coronal sections, centered at the level of hippocampal structures of interest, were chosen for analysis. The first section was taken from the same anatomical plane which was 1.28 mm anterior from the bregma in each animal. The hippocampal dentate gyrus (upper and lower blade) was contoured in each section and the number of TUNEL-positive cells was determined within the contoured area using Fractionator. The counting frame was 20 $\times$ 20 μ m and the Scan Grid was 50 $\times$ 50 μ m. Values are expressed as the number of cells per unit area.

NeuN Staining

The number of NeuN-positive neurons in the dorsal thalamus of KO and WT mice was determined at 14 days post-injury (KO, n = 21; WT, n = 15). Three consecutive coronal sections, which were centered at the level of the dorsal thalamus, were chosen for analysis. Images were captured with a Nikon microscope at 20 $\times$ magnification with a mounted CCD camera (SPOT-1; Diagnostic Instruments). The number of neurons, exhibiting distinctly stained nuclei, was determined within the lateral dorsal thalamic nucleus. Neuronal density was expressed as the ratio of the number of cells in the ipsilateral relative to the contralateral hemisphere, and these values were then averaged for each animal.

Assessment of Motor Recovery

Performance on a rotarod and grip strength were evaluated in brain-injured WT and KO mice. All mice were pretrained on the rotarod (Rota-Rod Treadmill; Stoelting Co., Wood Dale, Ill., USA)

for 3 days prior to the day of trauma. Animals were then evaluated at 1, 2, 4, 7, 10, and 14 days post-injury. On each day, the tests were performed three times with 10-min intervals between each test. The rotarod was set to start at 4 m/min and accelerated to 40 m/min over 300 s. The performance score was measured 0–600 s as the time successfully spent on the rotating rod without falling off. Values are expressed relative to pre-injury performance.

Grip strength was evaluated 1, 2, 4, 7, 10, and 14 days post-injury. Each animal was suspended by its tail within reach of a calibrated bar (Digital Grip Strength Meter; Columbus Instruments International Co., Columbus, Ohio, USA). The strength of each forelimb was measured as the animal grasped the bar. Values are expressed relative to pre-injury performance.

Statistical Analyses

Two-way analysis of variance (ANOVA) was used to compare blood flow and motor performance between genotypes over time. Morphologic assessments were analyzed by unpaired two-tailed t tests. Data are expressed as the mean $\pm$ SEM. Significance was defined at $p < 0.05$.

Results

Relative Cortical Blood Flow Is Similar between Genotypes after TBI

Relative cortical blood flow, as measured by laser Doppler, was evaluated in the ipsilateral cortex of WT and KO groups prior to and immediately after injury or sham surgery as well as 1 and 7 days later (fig. 1). In sham-operated animals, there were no changes in blood flow immediately after surgery nor were there changes associated with brain maturation within the first week after surgery. Relative blood flow was approximately 30 ml/min at 1, 3 and 7 days post-surgery (data not shown).

Both genotypes showed similar changes in blood flow after injury. Blood flow was reduced immediately post-injury, returned to baseline by 1 day, and significantly increased thereafter by 7 days post-injury (fig. 1).

Nonheme Iron Accumulates at Impact Site and along Adjacent External Capsule

Excessive iron accumulation may be a determinant of vulnerability after TBI. We therefore examined the temporal and regional pattern of nonheme iron accumulation after injury to WT and KO animals (fig. 2). There were no qualitative differences noted in the temporal and regional patterns of staining for iron between genotypes. In both genotypes, no overt staining for iron was noted at 1 day post-injury. By 7–14 days, iron staining was preferentially localized to the injured cortex and the adjacent external capsule where it exhibited a diffuse pattern, presumably reflecting an extracellular distribution, and a

Fig. 1. Relative cerebral blood flow, as measured by laser Doppler, was evaluated in brain-injured HO-2 WT (+/+) and (-/-) mice. There was a marked reduction in blood flow to 65% of the baseline immediately post-injury in both groups (Day 0). By 1 day post-injury, cortical blood flow recovered to that of pre-injury levels (dashed horizontal line). The effects of genotype and time post-injury on relative blood flow was evaluated by two-way ANOVA. There was no significant overall interaction between these factors ($p = 0.81$). Main effects analysis showed a statistically significant time post-injury effect ($p = 0.0001$), but not a significant difference for genotypes ($p = 0.48$). Based on multiple comparison tests among the days post-injury, blood flow immediately after injury (Day 0) was significantly lower relative to all other time points in both the WT ($p < 0.01$) and KO ($p < 0.05$) groups. There was also a significant increase in blood flow at 7 days relative to 1 day in both the WT ($p < 0.01$) and KO ($p < 0.05$) groups. Values are means $\pm$ SEM.

cellular localization, including structural elements related to the vascular compartment. Iron-positive cells with either a glial or macrophage-like phenotype were most apparent in the external capsule and were typically oriented parallel to the fiber tracts.

Early Cell Injury Is Enhanced in the Brain-Injured HO-2 KO

Sections prepared from brain-injured WT and KO mice were stained with TUNEL to define regional patterns of cell injury at 1 and 7 days post-injury in the thalamus and hippocampus. TUNEL-positive cells were not evident in the thalamus of either group at each of the time points (data not shown). In contrast, prominent labeling of TUNEL-positive cells was evident in the hippocampus in both genotypes particularly at the 1 day time point (fig. 3).

The hippocampus exhibited a very distinctive qualitative pattern of TUNEL-positive cells. In both genotypes, TUNEL-positive cells dominated in the upper and lower

blades of the dentate gyrus at 1 day post-injury (fig. 3). On occasion, TUNEL-positive cells were also evident in the CA1, CA2, and CA3 regions. By 7 days post-injury, there appeared to be fewer labeled cells in the hippocampus in each of the genotypes (fig. 3).

We next determined if the number of TUNEL-positive cells in the dentate gyrus differed between groups (fig. 4). The number of TUNEL-positive cells in the dentate gyrus (upper + lower blades) was significantly greater in HO-2 KO compared to WT mice at 1 day post-injury (fig. 4, $p = 0.03$). However, no differences were noted between these groups by 7 days post-injury (fig. 4, $p = 0.89$).

Evolution of Cortical Lesion Volume Shows Genotype-Related Differences

At 7 and 14 days post-injury, a large left frontoparietal cortical defect extending to the external capsule was evident at the site of impact in both genotypes (fig. 5). Lesion volume was unchanged at each of the time points in the KOs (6.37 ± 0.63 at 7 days, 5.83 ± 0.49 at 14 days, $p = 0.59$). In contrast, lesion volume was significantly increased in the WTs at 14, as compared to 7, days post-injury (3.92 ± 0.48 at 7 days, 5.92 ± 0.36 at 14 days, $p = 0.003$).

Lesion volume was compared in the KOs versus the WTs at each time point (fig. 5). Lesion volume was significantly greater in the brain-injured KO as compared to WT animals at 7 days post-injury (fig. 5, $p = 0.008$). However, no differences were noted in these groups by 14 days post-injury.

As the thalamus may show a reduction in neurons due to deprivation of its cortical target, we next determined the number of neurons in the ipsilateral dorsal thalamus, relative to the contralateral side, at 14 days post-injury (fig. 5). There were no differences between groups. There was an 18–24% reduction in the number of neurons in the KO and WT groups, respectively, at 14 days post-injury.

Grip Strength Is More Impaired in the Brain-Injured HO-2 KO

Grip strength and performance on a rotarod were examined over a period of 2 weeks in each genotype (fig. 6). There was a significant effect of both genotype and time post-injury on right (contralateral to the injury) grip strength. All animals improved with time and scores for WT animals were on average higher than KO animals. There was no significant interaction nor was there a significant genotype effect of time post-injury on left (ipsi-

Fig. 2. Nonheme iron was localized in HO-2 KO and WT mice at 7 (**a, b**) and 14 days (**c, d**) after brain injury. There were no overt differences in the pattern of iron localization in WT as compared to KO animals nor in the patterns of staining at 7 and 14 days post-injury. As might be expected, robust staining occurred preferen-

tially within the injured cortex where it appeared in both a punctate pattern, presumably reflecting cellular localization, and a diffuse pattern (**c**). In addition, iron was also localized to penetrating arterioles (**insets b** and **c**) as well as in cells that were densely distributed along the external capsule (**insets a** and **b**). Scale bar: 500 μ m.

lateral to injury) grip strength. Assessment of performance on the rotarod revealed no significant effect of genotype ($p = 0.52$), but a significant effect of time post-injury ($p < 0.0001$).

Discussion

TBI at PD 21 results in an extended period of pathogenesis that begins early after injury and continues thereafter during brain maturation [35, 36]. We report that

HO-2 deficiency alters early secondary pathogenesis and fine motor recovery in the traumatized, immature brain. Cortical lesion volume and hippocampal cell injury were significantly greater in the HO-2 KO relative to the WT during the first week post-injury. Thereafter, lesion volume remained unchanged in the KO but continued to expand in the WT. As a result of this difference in kinetics, lesion volume was indistinguishable between the genotypes by 2 weeks post-injury. We further show that KO mice tended to show reduced contralateral forelimb grip strength relative to WT over the first 2 weeks post-inju-

Fig. 3. TUNEL-positive cells in the hippocampal dentate gyrus of brain-injured HO-1 WT (a–c) and KO (d–f) mice were examined at 1 and 7 days post-injury. Enclosed areas in a and d are shown at higher magnification in b, c, e and f, respectively. Note that

there appear to be a greater number of TUNEL-positive cells in the dentate gyrus of the KO (e, f) as compared to the WT (b, c) animal at 1 day, whereas very low numbers of TUNEL-positive cells are apparent in each of the genotypes. Scale bars: 50 μ m.

ry, thus supporting a role for HO-2 in early functional recovery after TBI. Together, these findings suggest that HO-2 provides early neuroprotection to the injured, immature brain, but is not involved in delayed neurodegeneration.

The Developing Brain and Susceptibility to Oxidative Stress

HO catalyzes the conversion of the pro-oxidant heme to biliverdin, carbon monoxide (CO), and Fe^{2+} . Biliverdin is rapidly transformed to bilirubin, which at low concentrations functions as a neuroprotectant [37, 38]. CO is thought to act as a neurotransmitter, vasodilator, and anti-apoptotic factor [39–41]. Iron is known to promote oxidative damage via the Fenton reaction. However, excess Fe^{2+} may be sequestered by iron storage proteins such as ferritin, transferrin, and ceruloplasmin [42]. In the setting of TBI, it is unclear as to whether or not the iron regulatory capacity is adequate or if in fact is overwhelmed. The latter is implicated in studies showing that the iron chela-

tor, deferoxamine, is neuroprotective after traumatic injury to the adult [43, 44] and immature brain [45], as well as in models of intracerebral hemorrhage [46].

Here we consider HO-2 as a determinant of vulnerability of the immature brain after a traumatic injury. A recent review [42] has addressed the developmental expression/regulation of HO-2 in the developing brain. HO-2 mRNA is at relatively low values within the first postnatal week in the rodent brain. Thereafter, there is a sharp rise in mRNA reaching maximal values at adulthood. It has been speculated that adrenal glucocorticoids, acting through the glucocorticoid response element of the HO-2 gene, regulate the expression of HO-2. Consistent with this hypothesis, adrenal glucocorticoids show a similar developmental expression to that of HO-2, and administration of corticosterone increases both HO-2 mRNA and protein during brain development [5].

As HO-2 degrades the pro-oxidant heme, we hypothesized that mice deficient in this enzyme would show greater tissue damage. There are several likely intracel-

Fig. 4. Cell density of TUNEL-positive cells in the hippocampal dentate gyrus after TBI in HO-2 WT (+/+) and KO (-/-) mice. **a** The number of TUNEL-positive cells in the dentate gyrus is significantly greater in KO compared to WT mice at 1 day post-injury ($p = 0.031$). **b** There is a significant reduction in the number of TUNEL-positive cells in both KO and WT mice at 7 days post-injury as compared to 1 day post-injury (WT, $p = 0.0001$; KO, $p = 0.002$). There are, however, no differences between genotypes at this time point ($p = 0.891$). Values are means $\pm$ SEM.

Fig. 5. Typical appearance of the cortical lesion in brain-injured HO-2 WT and KO mice at 7 (**a**, **b**) and 14 days (**c**, **d**) post-injury that have been stained with cresyl violet. Whereas the cortical lesion appears to be qualitatively larger in KO relative to the WT at 7 days post-injury, no obvious differences are evident by 14 days post-injury. Scale bar: 500 μ m. Cortical lesion volume was quantified in brain-injured HO-2 WT (+/+) and KO (-/-) mice. Lesion volume was significantly greater in KO as compared to WT animals at 7 days post-injury (**e**, * $p = 0.008$). However, by 14 days

post-injury, no differences were noted between genotypes (**e**, $p = 0.59$). Note that lesion volume increased in the WT group from 7 to 14 days post-injury (**e**, **f**, $p = 0.03$), whereas there was no similar expansion of lesion volume in the KO group (**e**, **f**, $p = 0.59$). NeuN-positive neurons were quantified in the lateral dorsal thalamus (**g**) at 14 days post-injury in HO-2 WT (+/+) and KO (-/-) mice. There were no differences in relative neuronal density between genotypes ($p = 0.49$). Values are expressed as means $\pm$ SEM.

lular and extracellular sources of heme in the injured brain [42]. These include cytoplasmic hemoproteins and mitochondrial cytochromes, hemoproteins that are released into the extracellular space by dying cells, and heme that is generated from the breakdown of hemoglo-

bin in areas of intraparenchymal and subarachnoid hemorrhage [42].

The brain is especially susceptible to oxidative damage due to its high fatty acid content and proportionately large share of total body oxygen consumption [47]. Vul-

Fig. 6. Motor performance was assessed in brain-injured HO-2 WT (+/+) and KO (-/-) mice at 1–14 days post-injury. The effects of genotype and time post-injury on motor performance (rotarod and grip strength) were evaluated with two-way repeated measures ANOVA. There was no significant interaction between these factors ($p = 0.62$) when comparing performance on a rotarod. Main effects analysis showed a statistically significant time post-injury effect ($p < 0.001$), but not a significant difference for genotypes ($p = 0.52$). There was no interaction between genotype

and time post-injury in left forelimb grip strength (ipsilateral to injury, $p = 0.52$) or right forelimb grip strength (contralateral to injury, $p = 0.51$). Main effects analysis for right forelimb grip showed a significant time post-injury effect ($p < 0.0001$), but not a significant difference for genotypes ($p = 0.85$). In contrast, right forelimb grip showed both a statistically significant time post-injury effect ($p < 0.001$) and genotype effect ($p = 0.02$). Values are means $\pm$ SEM.

nerability is further compounded in the immature brain due to a lower antioxidant reserve [48, 49]. In this context, HO activity in the murine brain, which reaches adult values by 1 week after birth [50], may be in a key position to mitigate secondary damage. Although there are several members of the HO family, HO-2 is the principal source of HO activity in the brain [42].

We found that HO-2 deficiency results in an early expansion of the cortical lesion, increased TUNEL-positive cells in the hippocampus, and a reduction in fine motor function as measured by a grip test. These findings are consistent with other *in vitro* and *in vivo* studies of HO-2. HO-2 protects against apoptotic cell death in cortical, hippocampal, and cerebellar granule cultures, as well as in *in vivo* models of ischemic brain injury [51, 52]. Moreover, adult brain-injured mice, genetically deficient in HO-2, show enhanced early lipid peroxidation, increased hippocampal cell loss, and impaired behavioral recovery [5]. Together, these findings in concert with our current observations support an early neuroprotective role for HO-2 in response to injury to both the immature and adult brains.

In this study we evaluated both grip strength and performance on a rotarod. While there was an effect of genotype on grip strength (contralateral to the injury), there were no genotypic differences on rotarod performance. The differences in these findings may reflect the nature of the parameters. Performance on a rotarod is a complex

motor and sensory task that involves coordinated movement of both fore- and hindlimbs and motor learning. In contrast, grip strength is primarily a metric for the fine intrinsic muscles of the forepaw. Our data suggest that HO-2 deficiency does not have a significant effect on the broader motor and sensory skills that are required to maintain position on a rotarod, but rather influences the more subtle changes in fine motor control.

Surprisingly, we also found that by 2 weeks post-injury, cortical lesion volumes and measures of fine grip strength were similar in the KO relative to age-matched WT controls. These findings suggest that HO-2 may not be involved in delayed neurodegeneration and that the latter is a key determinant of long-term neurologic recovery. In previous work, we have shown that the injured immature brain shows an extended period of pathogenesis during brain maturation [35, 36]. This is realized not only in the expansion of the cortical lesion, but also in progressive loss of neurons in the hippocampus. Given the complex clinical presentation of the brain-injured child [53], it is likely that TBI has broad effects on the structural integrity of the brain. In an experimental model of neonatal hypoxia-ischemia, *ex vivo* diffusion tensor imaging revealed connectivity-directed degeneration [54]. Focusing on the limbic system, the authors showed that neural injury is initially evident in the ipsilateral hippocampus, followed by degeneration of the

fimbria fornix, and finally the more delayed degeneration of the ipsilateral dorsolateral septal nuclei [54]. It is likely that degeneration of neural networks likewise occurs after trauma to the immature brain. In this study, loss of thalamic neurons likely reflects a response to target deprivation arising from the expanding cortical lesion. Of interest, HO-2 deficiency had no effect on the long-term loss of neurons in the thalamus. Such a negative finding is consistent with similar-sized cortical lesions in each of the genotypes.

It is also possible that these longer-term pathogenic events are a result of a hostile environment in which heme may only be a minor contributing factor. There is indirect evidence to support this hypothesis. Overexpression of the antioxidant glutathione peroxidase in the injured immature brain results in protection against delayed hippocampal neuronal loss and cognitive decline. Such findings suggest that adequacy of antioxidant reserves, resulting from increased glutathione peroxidase activity, is a determinant of long-term recovery. Candidates for promoting extended pathogenicity include inflammatory mediators, iron accumulation in the absence of adequate iron-binding proteins, and oxidative insults arising from reactive oxygen and nitrogen species [55].

There is a critical need to develop age-appropriate therapies for the brain-injured child. Here we describe a model of relatively severe traumatic injury to the immature brain. An early report described the 'double-hazard' effect in brain-injured children where severe injury at a young age yields the poorest clinical outcomes [56]. This position continues to be supported in more recent work where children who sustain severe TBI before 8 years of age achieve poorer outcomes on measures of verbal and nonverbal skills, attention, and intellectual ability [57]. Understanding pathogenesis in a framework of a maturing brain is key to achieving improved neurologic outcomes. While it is unlikely that a single therapy will provide optimal neurologic recovery, identifying synergism between candidate therapeutics while supporting endogenous neuroprotective mechanisms, including HO-2-directed early neuroprotection, will increase the likelihood of improved functional outcomes.

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