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Astrocyte Overexpression of Heme Oxygenase-1 Improves Outcome after Intracerebral Hemorrhage

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Abstract

Background and Purpose—Heme oxygenase-1 (HO-1) catalyzes the rate-limiting reaction of heme breakdown, and may have both antioxidant and pro-oxidant effects. In prior studies, HO-1 overexpression protected astrocytes from heme-mediated injury *in vitro*. In the present study, we tested the hypothesis that selective astrocyte overexpression of HO-1 improves outcome after intracerebral hemorrhage (ICH).

Methods—Male and female transgenic mice overexpressing human HO-1 driven by the GFAP promoter (GFAP.HMOX1) and wild-type controls received striatal injections of autologous blood (25 μl). Blood-brain barrier disruption was assessed by Evans blue assay and striatal cell viability by MTT assay. Neurological deficits were quantified by digital analysis of spontaneous cage activity, adhesive removal, and elevated body swing tests.

Results—Mortality rate for wild-type mice was 34.8% and was similar for males and females; all GFAP.HMOX1 mice survived. Striatal Evans blue leakage at 24 hours was 23.4+/−3.2 ng in surviving WT mice, compared with 10.9+/−1.8 ng in transgenics. Peri-hematomal cell viability was reduced to 61±4% of contralateral at 3 days in WT mice, v. 80±4% in transgenics. Focal neurological deficits were significantly reduced in GFAP.HMOX1 mice, and spontaneous cage activity was increased.

Conclusions—Selective HO-1 overexpression in astrocytes reduces mortality, blood-brain barrier disruption, peri-hematomal cell injury, and neurological deficits in an autologous blood injection ICH model. Genetic or pharmacologic therapies that acutely increase astrocyte HO-1 may be beneficial after ICH.

Keywords

Blood-brain barrier; Heme Oxygenase; Preconditioning; Stroke

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Introduction

The heme oxygenases catalyze the breakdown of heme to carbon monoxide, ferrous iron, and biliverdin, which is subsequently reduced to bilirubin. The consequences of this reaction on outcome in hemorrhagic stroke models have been quite variable to date. Mice lacking heme oxygenase-1 (HO-1), the inducible isoform, sustained less injury and inflammation than their wild-type (WT) counterparts after collagenase-induced intracerebral hemorrhage (ICH).[1] Unconditional knockout of HO-2, the predominant neuronal isoform, had the opposite effect, but was somewhat protective in the blood injection ICH model.[2, 3] HO inhibitors, which have less utility in mechanistic studies due to their multiple off-target effects, have also been variably protective or deleterious.[4–6]

These conflicting reports may be reconciled by consideration of the diverse biological activity of heme breakdown products and the selective vulnerability of neurons to iron.[7] In cell culture, neuron loss after hemin or hemoglobin exposure is increased by HO-2 expression.[8, 9] This effect is mitigated by protein or low molecular weight iron chelators, resulting in net neuroprotection that may be mediated by the other reaction products.[3, 10, 11] Astrocytes, which are relatively resistant to acute iron toxicity due to ferritin induction,[12] are protected from hemin by both HO-1 and HO-2 in the absence of exogenous chelators.[13, 14] The effect of HO expression in any model may therefore depend on the cell population at greatest risk and the iron binding capacity of the cellular microenvironment.

These results suggest that nonselective targeting of HO expression or activity may not be an optimal therapeutic strategy. The efficacy of therapies that modulate HO in specific cell populations *in vivo* has not been defined. We have previously reported that increasing HO-1 expression in astrocytes was protective in a cell culture model of acute hemin toxicity.[15] In the present study, we tested this concept *in vivo*, using transgenic mice expressing human HO-1 controlled by the GFAP promoter and an established ICH model.

Methods

Animals

Mice were transferred from the Schipper lab to Thomas Jefferson University where they were bred in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All protocols were approved by the local Institutional Animal Care and Use Committee. Mice expressing human HO-1 tagged with the Flag peptide in astrocytes (GFAP.HMOX1 mice, FVB background) were generated as previously described,[16] and were used when 3–6 months old. Although the transgene incorporates a tetracycline-controllable regulatory system (“TET-OFF”), tetracyclines were not administered due to their effects in stroke models. Genotype was verified by PCR using DNA obtained from tail clippings and the following primers:

HMOX1 Forward: 5′-CGG CTC ATG ATG TCT AGA TTA GA -3′

Reverse: 5′-AAT TAG AAT TCT CGC GCC CCC TA -3′

tTA (tetracycline activator) Forward: 5′-CGC TGA GGA TCC ATG GAC TAC AAA GAC GAT -3′

Reverse: 5′-CGT GCA TCT AGA TCA CAT GGC ATA AAG CCC -3′

ICH Model

Mice were randomly selected from WT and GFAP.HMOX1 cohorts of similar age, and were anesthetized with 2% isoflurane in oxygen delivered via a nose mask. Temperature was monitored with a rectal probe and maintained at 37±0.5°C with a heating lamp. The scalp was incised and a burr hole was made at the following stereotactic coordinates relative to bregma: 2.5 mm lateral, 0.5 mm rostral. Blood obtained from a tail vein was loaded into a Hamilton syringe with 28 gauge needle, which was inserted 3.0 mm below the skull surface. Injection rate was 1 μl/min using a pump (Harvard Apparatus, Holliston, MA); total injected volume was 25 μl. Ten minutes later, the needle was removed, skin was sutured, and the animal was allowed to recover in a warm environment. Mice were observed for signs of distress hourly for the first 4 h and then daily.

Injury Assessment—Outcome was assessed over 7 days after ICH by researchers blinded to genotype.

Blood-Brain Barrier Permeability Assay

Mice were treated with 4 ml/kg i.p. of 2% Evans blue in sterile saline. Three hours later, they were perfused with 50 ml PBS via left ventricular injection under isoflurane anesthesia, followed by cervical dislocation. Striata were removed, and Evans blue was extracted according to the method of Uyama et al.[17] Fluorescence (ex: 620 nm, em: 680 nm) of the extract solution was quantified, and striatal Evans blue content was interpolated from a standard curve.

MTT Assay

Perihematomal cell viability was quantified by MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay of striatal cell suspensions.[18] MTT, like the related TTC (2,3,5-triphenyltetrazolium chloride), is reduced by viable cells to a formazan dye; the product is readily extracted from cells and quantified by spectrophotometry. Tissue dissociation facilitates the delivery of an equal concentration of MTT to all cells. This approach is preferred to analysis of tetrazolium-stained tissue sections because experimental ICH does not produce a discrete infarct but rather a diffuse area of incomplete cell loss.[19]

Mice were euthanized by cervical dislocation after isoflurane anesthesia. Striata were removed by microdissection with Dumont forceps under a Stereomaster microscope (Fisher Scientific). Tissue was dissociated by trituration in 1 ml Hanks Balanced Salt Solution containing 27.8 mM glucose, 20.5 mM sucrose, and 4.2 mM sodium bicarbonate. The cell suspension was treated with 0.125 mg/ml MTT for 4 minutes in a 37°C water bath. Cells were then collected by centrifugation, and the formazan reaction product was extracted with 2 ml isopropanol. Absorbance (562 nm) of the solution obtained from each blood-injected striatum was normalized to that of the contralateral striatum to yield an index of cell viability. The close agreement of this method with perihematomal injury quantified by cell counts of striatal tissue sections has been established.[2, 18, 20]

Behavioral Outcome Measures

Functional outcome was assessed as previously described,[21] via: 1) quantification of spontaneous cage activity in 1 hour videos, using Homecage Scan (CleverSys., Reston, VA USA); 2) adhesive removal test; 3) elevated body swing test.

Immunoblotting and Immunostaining

The following antibodies were used: 1) Anti-HO-1, Enzo Life Sciences, Farmingdale, NY (Cat #ADI-SPA-895), 1:4000 dilution for immunoblotting and 1:250 for immunostaining; 2) Anti-GFAP, InVitrogen (Cat #130300), 1:1000 dilution; 3) Anti-flag M2 antibody, Sigma-Aldrich (Cat #F1804) 1:250 dilution. Methods have been detailed previously.[18, 20]

HO-1 ELISA

Mouse and human HO-1 were quantified in injected and contralateral striata using ELISA kits marketed by Enzo Life Sciences (Cat. #ADI-960-071 and ADI-EKS-800, respectively), following the manufacturer's instructions.

Hemoglobin assay

Blood (10 μl) collected from a tail vein was added to 5 μl of 3.5% sodium citrate to prevent clotting. Cells were lysed by passage through 3 freeze-thaw cycles. After centrifugation to remove debris, hemoglobin concentration was determined by the method of Winterbourne.[22]

Statistical Analyses

Mortality data were analyzed with Fisher's exact test. Other data were analyzed with one-way ANOVA and the Bonferroni multiple comparisons test.

Results

A total of 139 mice were used in these experiments (71 males and 68 females). The presence of the HMOX1 and tTA genes in transgenic mice was confirmed by genotyping (Fig 1A). Immunoblotting demonstrated a moderate increase in striatal HO-1 expression in GFAP.HMOX1 mice compared with their WT counterparts (Fig. 1B). Double immunofluorescence staining indicated that striatal HO-1 expression was predominantly localized to GFAP+ astrocytes in both wild-type and transgenic mice (Fig 2). Increased HO-1 immunoreactivity was apparent in the latter group, particularly in proximity to microvessels. Mean blood hemoglobin concentration was very similar in WT and GFAP.HMOX1 mice (7.75±0.13 mM and 7.74±0.12 mM heme, respectively).

Reduced mortality in GFAP.HMOX1 mice

Sixteen of forty-six WT mice died within 24 hours of blood injection (Table 1), with similar mortality rates in males and females. No deaths were observed at later time points. All transgenic mice survived until completion of the experiment. Mortality reduction was significant for both males and females.

Astrocyte HO-1 overexpression reduces blood-brain barrier disruption

We have recently reported that systemic hemin therapy induces HO-1 overexpression in perivascular cells and reduces blood-brain barrier injury after experimental ICH.[21] Since astrocyte HO-1 overexpression is particularly prominent in GFAP.HMOX1 mice adjacent to vessels (Fig 2), blood-brain barrier integrity was assessed 24 hours after striatal blood injection. Evans blue leakage into the striatal parenchyma at this time point in GFAP.HMOX1 mice was less than half of that in WT mice (Fig. 3A).

Reduced peri-hematomal cell loss in GFAP.HMOX1 mice

Striatal blood injection resulted in loss of approximately 40% of cells in the ipsilateral striata of WT mice, with minimal change between days 3 and 7 (Fig. 3B). Striatal cell viability was significantly improved in transgenic mice ($p<0.05–0.01$ vs. WT controls).

Effect of ICH on HO-1 expression

Striatal HO-1 was quantified 7 hours after blood injection using ELISA kits specific for mouse or human HO-1. Consistent with prior observations,[23] native mouse HO-1 expression in injected striata was increased ~2.2-fold compared with contralateral in both WT and GFAP.HMOX1 mice (Fig. 4). Human HO-1 was expressed at a higher level in transgenics and was similar in injected and contralateral striata.

Effect of astrocyte HO-1 overexpression on behavioral outcome

Spontaneous cage activity prior to striatal blood injection was similar in WT and GFAP.HMOX1 mice. It was decreased by ~75% in WT mice 24 h after injection, compared with ~40% in transgenics ($p<0.05$, Fig. 5). A similar benefit at this early time point only was observed in the adhesive removal test ($p<0.001$); surviving WT mice rapidly regained function. A persistent deficit was observed in the elevated body swing test in WT mice, and benefit was noted in transgenics at a later time point.

Discussion

This study provides the first evidence that selective over-expression of HO-1 in astrocytes is beneficial after ICH. The GFAP.HMOX1 transgene reduced mortality, an endpoint of obvious clinical relevance but an uncommon outcome measure in rodent ICH studies. Unequal mortality in WT and transgenic groups may have confounded the results of other planned outcome measures, since the most severely injured mice in only the transgenic group survived for later testing. While this bias would be predicted to diminish the observed benefit of astrocyte HO-1 overexpression, GFAP.HMOX1 mice nevertheless sustained significantly less blood-brain barrier injury, peri-hematomal cell loss, and sensorimotor deficits than surviving WT mice.

These observations are consistent with prior reports that peripheral hemin injection and ischemic preconditioning are protective in ICH models.[21, 24] Both of these therapies increase CNS HO-1 expression, but also have pleiotropic effects on signaling pathways and gene expression that limit their utility as mechanistic probes. The present findings provide more specific evidence that HO-1 overexpression *per se* is sufficient to manifest protection.

It is noteworthy that Zeynalov et al. observed that the benefit of ischemic preconditioning in a permanent middle cerebral artery occlusion model was lost in HO-1 knockout mice, consistent with an effect that also requires HO-1 expression.[25]

Astrocytes play a key role in the development and maintenance of the blood-brain barrier in the rodent CNS.[26] Experimental evidence suggests that HO-1 expression may be essential for optimizing this function in an oxidative environment.[27, 28] Alfieri et al.[29] have recently reported that preconditioning stimuli increase HO-1 expression primarily in perivascular astrocytes, associated with preservation of barrier function in a rat transient middle cerebral artery occlusion model. Consistent with this observation, Evans blue leakage into the brain parenchyma was decreased by about half in GFAP.HMOX1 mice, which overexpress HO-1 prominently in perivascular astrocytes. These results add to the growing body of evidence that HO-1 improves microvascular function after a wide variety of acute injuries, including cardiac,[30] liver,[31] and bowel[32] ischemia-reperfusion, hemorrhagic shock,[33] seizures,[34] and sickle cell disease.[35]

The protective effect of astrocyte HO-1 overexpression after ICH observed in 3–6 month old GFAP.HMOX1 mice contrasts with the pathology that develops with aging in these transgenic animals. At 48 weeks, they exhibit behavioral disturbances characterized by hyperkinesia and impaired prepulse inhibition, associated with glial iron deposition, oxidative mitochondrial injury, macroautophagy and neuritic degeneration.[16, 36] The adverse effect of this transgene in older mice suggests that therapies producing a long-term increase in astrocyte HO-1 expression may be ineffective or poorly tolerated. It also highlights the need to test therapies that increase astrocyte HO-1 in older animals.

Striatal blood injection is usually a nonlethal event in rodents. In prior studies from this laboratory, mortality in Swiss-Webster or C57BL/6 × 129 mice has ranged from 0–4%, which is comparable to that reported in multiple other studies.[2, 21, 37, 38] The 34% mortality rate in WT FVB mice was unexpected and highlights the prominent influence of strain on vulnerability to hemorrhagic CNS injury. FVB mice have been used infrequently in acute CNS injury models, but reported results have been largely consistent with the present observations. Compared with C57BL/6 and other strains, FVB mice sustained more hippocampal neuron loss and mortality after injection of glutamate receptor agonists.[39, 40] Consistent with increased vulnerability to excitotoxic stress, they also sustained larger infarcts and increased mortality after striatal endothelin injection combined with common carotid artery occlusion and/or L-NAME injection,[41] and increased lesion size after spinal cord crush injury.[42] Excitotoxicity has also been implicated in early injury after ICH,[43, 44] and may be exacerbated by the effects of thrombin on glutamate release[45, 46] and glutamate receptor responses.[47] Further investigation of the mechanisms mediating early death of FVB mice after ICH is an important topic for future investigation. The injury produced by technically-feasible blood injection volumes in this strain may accurately reflect the injury severity of clinical ICH, which also has a ~34% in-hospital mortality in the United States.[48] A moderate mortality rate facilitates its use as a primary outcome measure. Although most rodent studies are not designed to quantify the effect of genetic modifications or pharmacotherapies on mortality, this hard endpoint may be more predictive of clinical efficacy than surrogate injury markers.[49]

HO-1 expression is increased in microglia after ICH.[50] The effect of microglial HO-1 on hemorrhagic CNS injury is unknown, but prior results suggest that it may be deleterious. Wang and Doré reported that microglial activation and perihematomal free radical levels were reduced in unconditional HO-1 knockout mice, consistent with a pro-inflammatory effect.[1] This was associated with reduced mean lesion volume and early neurological deficits. The specific contribution of microglial HO-1 to ICH-related injury could be determined in future experiments using transgenic mice selectively overexpressing human HO-1 in the microglial compartment.

HO-1 is readily induced in cultured astrocytes, which are then robustly protected against heme-mediated injury.[51] The translational potential of upregulating astrocyte HO-1 after ICH remains undefined but initial reports suggest feasibility. Systemic therapy with the nonselective inducers sulforaphane and hemin increased HO-1 in perivascular cells including astrocytes in naïve and injured rodent brains.[21, 29] This resulted in improvement in barrier function and neurobehavioral outcome, with clinically relevant time windows in TBI and ICH models.[21, 52] The present results provide a rationale for the testing of genetic and pharmacologic therapies that more specifically enhance HO-1 expression or activity in astrocytes after acute hemorrhagic injuries.

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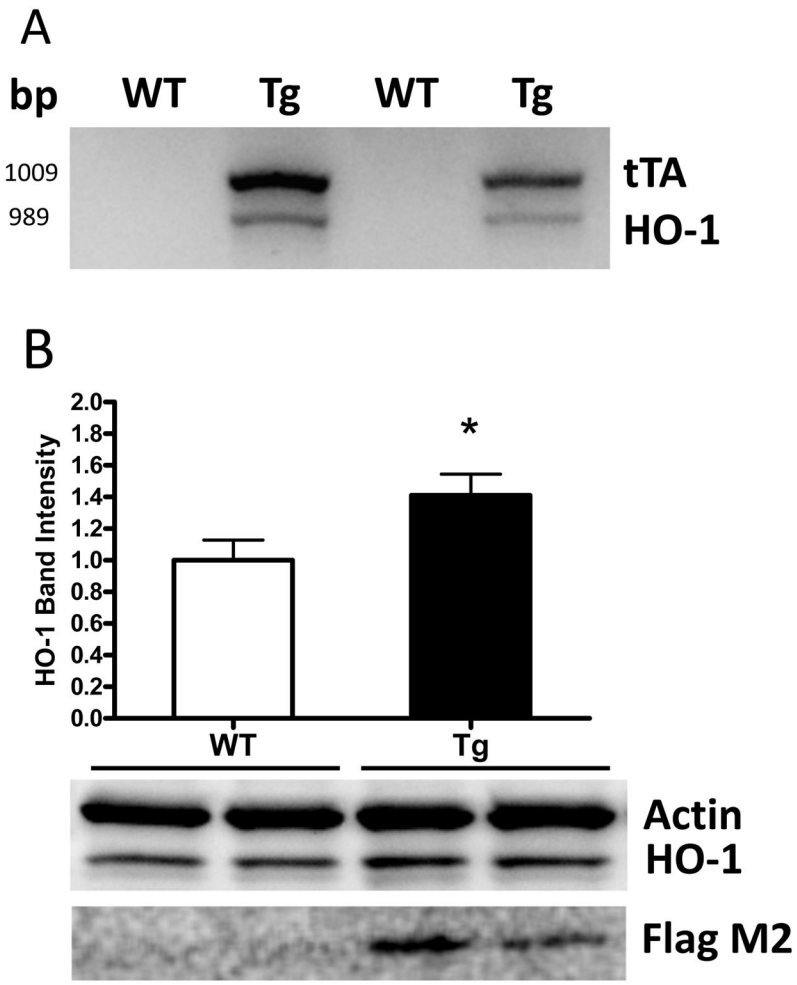

Figure 1.

(A) PCR genotyping products demonstrating presence of Flag-HMOX1 (HO-1) and tTa genes in GFAP.HMOX1 mice. (B) Immunoblots of striatal lysates of GFAP.HMOX1 transgenic (Tg) mice and WT controls, demonstrating increased HO-1 in transgenics; expression of transgene is confirmed by immunoreactivity to Flag peptide. Bar order correlates with lane order. *P<0.05 vs. WT, 6/condition.

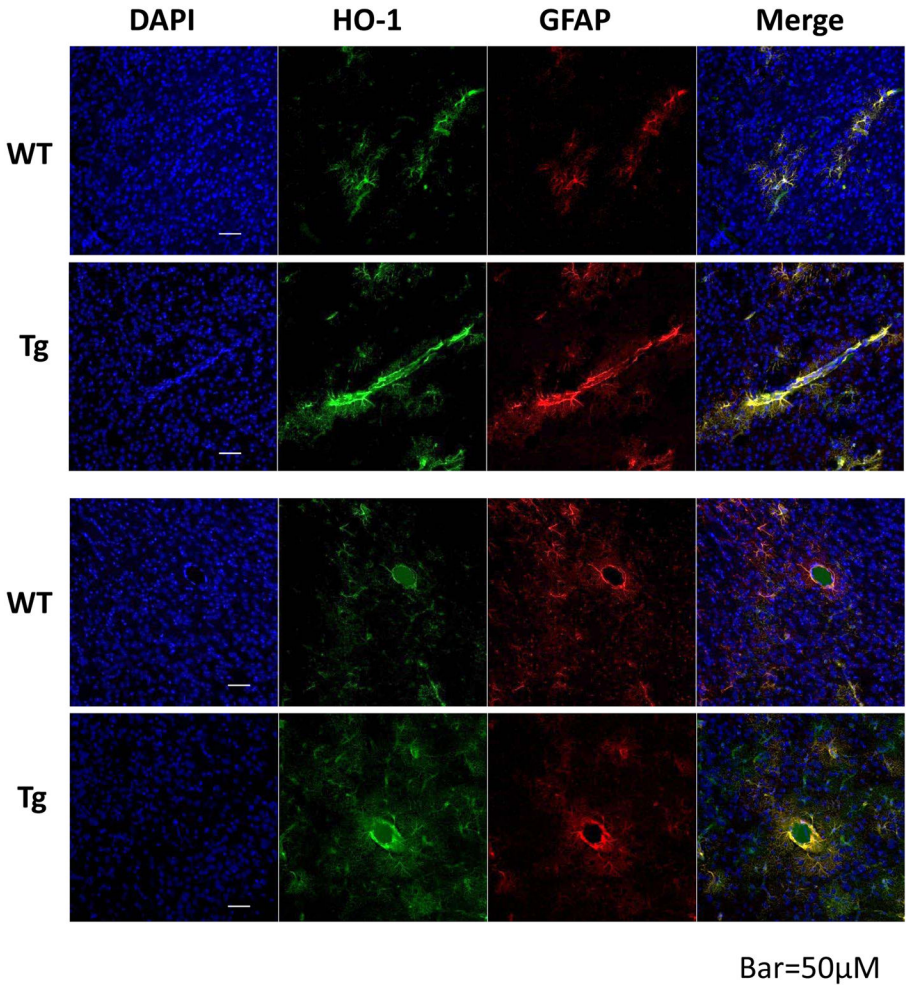

Figure 2.

Increased striatal HO-1 expression in GFAP.HMOX1 (Tg) mice. Sections were immunostained with antibodies to HO-1 and GFAP. Increased HO-1 expression is apparent in GFAP.HMOX1 mice, particularly in perivascular astrocytes.

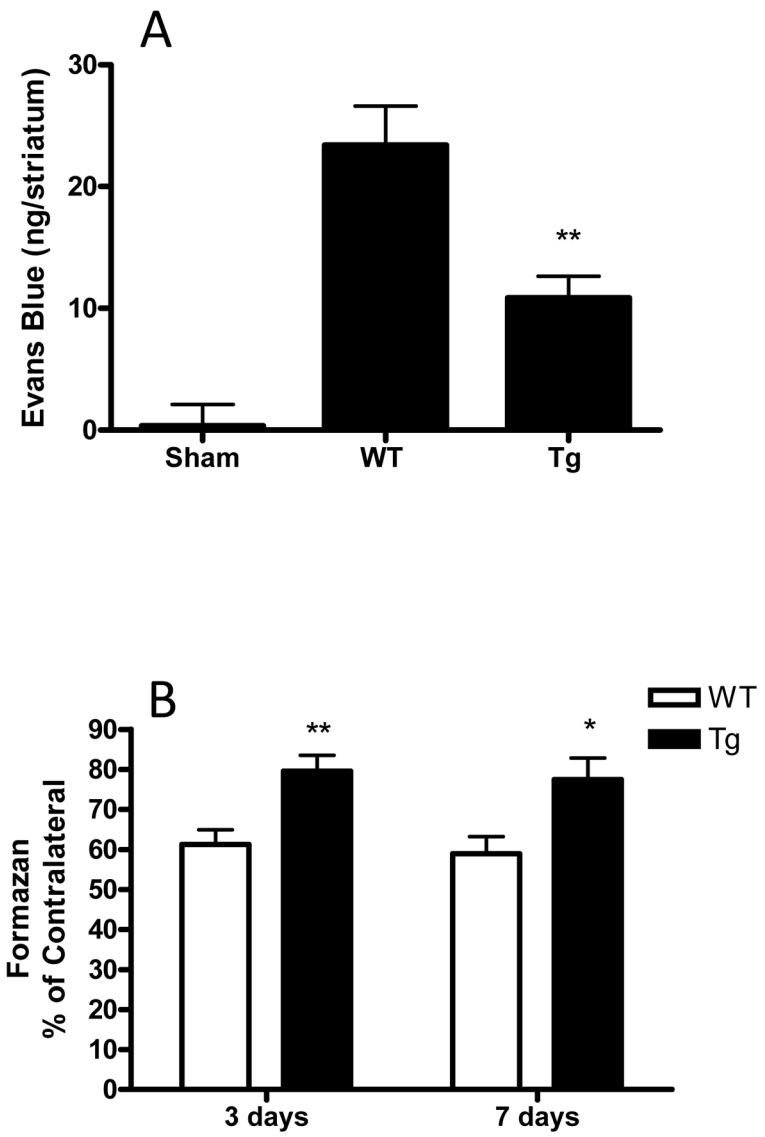

Figure 3.

Astrocyte HO-1 overexpression reduces blood-brain barrier breakdown and perihematomal cell death after ICH. (A) Evans blue leakage into the striatum was quantified 24 hours after ICH, 7–8/condition; (B) Striatal cell viability was quantified 3 and 7 days by MTT assay, 5–12/condition. *P<0.05, **P<0.01 vs. WT.

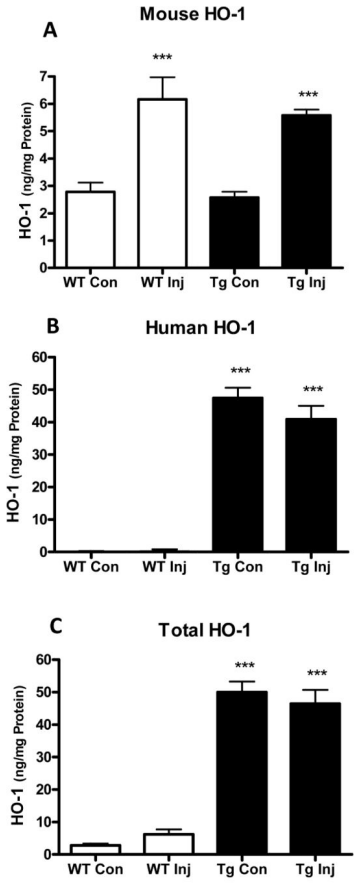

Figure 4.

Striatal HO-1 expression after ICH. Bars indicate mean mouse (A), human (B) and total (C) HO-1 in injected (Inj) and contralateral (Con) striata of WT and GFAP.HMOX1 (Tg) mice (±S.E.M., 6/condition) 7 hours after striatal blood injection. ***P < 0.001 vs. contralateral expression (A) or corresponding WT condition (B,C).

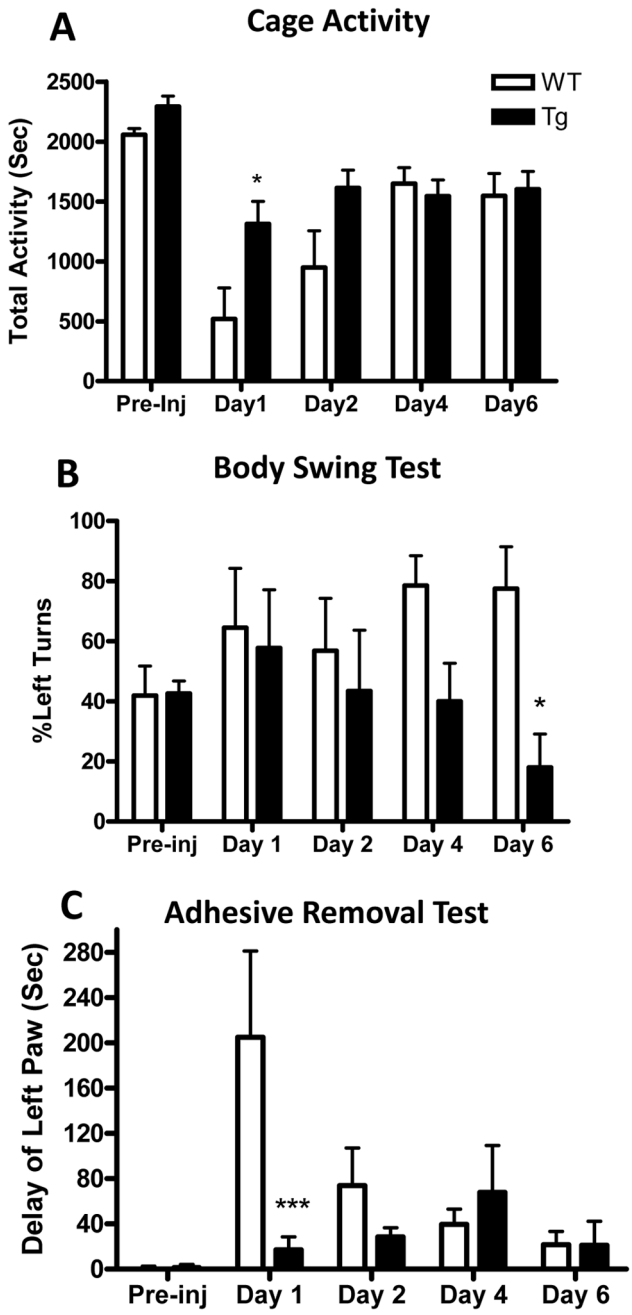

Figure 5.

Astrocyte HO-1 overexpression reduces neurological deficits. Bars represent mean values (±SEM, 5–7/condition) at indicated days after striatal blood injection for: A) activity time (sum of seconds walking, feeding, vertical hanging, rearing and jumping during 1 hour recording); B) percentage left swings; C) left minus right adhesive removal times.

Table 1

Astrocyte HO-1 overexpression reduces mortality after ICH. Mortality rates in wild type (WT) and GFAP.HMOX1 mice (Tg HO-1) after striatal injection of 25 μl autologous blood. All deaths occurred within 24 hours.

	WT		Tg HO-1		
	Mortality	**N**	**Mortality**	**N**	**P value**
Male	30.4%	23	0	22	0.0092
Female	39.1%	23	0	21	0.0016
Both	34.8%	46	0	43	<0.0001