Published in final edited form as:

Brain Res. 2016 March 15; 1635: 86–94. doi:10.1016/j.brainres.2015.12.060.

Hemoglobin-induced neuronal degeneration in the hippocampus after neonatal intraventricular hemorrhage

Thomas P. Garton^a, Yangdong He^a, Hugh J.L. Garton^a, Richard F. Keep^a, Guohua Xi^a, and Jennifer M. Strahle^{a,b,*}

^aDepartment of Neurosurgery, University of Michigan, Ann Arbor, MI, USA

^bDepartment of Neurological Surgery, St. Louis Children's Hospital, Washington University School of Medicine, St. Louis, MO, USA

Abstract

Neuronal degeneration following neonatal intraventricular hemorrhage (IVH) is incompletely understood. Understanding the mechanisms of degeneration and cell loss may point toward specific treatments to limit injury. We evaluated the role of hemoglobin (Hb) in cell death after intraventricular injection in neonatal rats. Hb was injected into the right lateral ventricle of post-natal day 7 rats. Rats exposed to anesthesia were used for controls. The CA-1 region of the hippocampus was analyzed via immunohistochemistry, hematoxylin and eosin (H&E) staining, Fluoro-Jade C staining, Western blots, and double-labeling stains. Compared to controls, intraventricular injection of Hb decreased hippocampal volume (27% decrease; $p < 0.05$), induced neuronal loss (31% loss; $p < 0.01$), and increased neuronal degeneration (2.7 fold increase; $p < 0.01$), which were all significantly reduced with the iron chelator, deferoxamine. Hb upregulated p-JNK (1.8 fold increase; $p < 0.05$) and increased expression of the Hb/haptoglobin endocytotic receptor CD163 in neurons in vivo and in vitro (cultured cortical neurons). Hb induced expression of the CD163 receptor, which co-localized with p-JNK in hippocampal neurons, suggesting a potential pathway by which Hb enters the neuron to result in cell death. There were no differences in neuronal loss or degenerating neurons in Hb-injected animals that developed hydrocephalus versus those that did not. Intraventricular injection of Hb causes hippocampal neuronal degeneration and cell loss and increases brain p-JNK levels. p-JNK co-localized with the Hb/haptoglobin receptor CD163, suggesting a novel pathway by which Hb enters the neuron after IVH to result in cell death.

Keywords

CD163; Deferoxamine; Hemoglobin; Hippocampus; Intraventricular hemorrhage; Neuron

1. Introduction

Intraventricular hemorrhage (IVH) is a significant source of mortality in preterm infants, resulting in infant death in about a quarter of cases (Murphy et al., 2002). IVH is associated

*Corresponding author. Tel.: +1 314 454 2810. strahlej@wustl.edu (J.M. Strahle).

Current Address: Department of Neurosurgery, One Children's Place, Suite 4S20, St. Louis, MO 63110-1077, USA.

with neuronal degeneration and cognitive dys-function (Georgiadis et al., 2008; Lewis and Bendersky, 1989). Because of its function in learning and memory, the hippocampus has been previously investigated as a target of injury-induced neuronal degeneration in the CA-1 through CA-4 and dentate gyrus regions (Ramani et al., 2013; Song et al., 2007; Zhang et al., 2015; Zlotnik et al., 2012). However, studies performed examining the effects of specific blood components in neonatal IVH-induced brain injury have been limited. Such studies would be beneficial, as they could reveal targets for therapeutic intervention.

One of the most abundant proteins in blood, hemoglobin (Hb), has been found to be toxic to cortical neurons in vitro (Regan and Panter, 1993), and direct injection of Hb into the hippocampus in adult rats induces neuronal death (Song et al., 2007). A recent finding suggests that intraventricular Hb induces cytotoxic markers in astrocytes and can cause significant cell death and inflammatory activation in the choroid plexus (Gram et al., 2013, 2014). It is known that the heme moiety released by Hb interacts with intracellular heme oxygenase (HO) proteins (such as HO-1 in macrophages and HO-2 in neurons), releasing bilirubin, carbon monoxide, and iron (Fe) cations able to participate in Fenton and Haber-Weiss reactions (Lee et al., 2010; Lok et al., 2011; Wagner et al., 2003; Wang and Dore, 2007). However, the mechanism for intracellular transit of the heme moiety into neurons is not fully understood. The main Hb receptor, CD163, has been thought to only be expressed in macrophages and monocytes (Polfliet et al., 2006). For this reason, it is not clear whether this HO-mediated mechanism is a direct actor in neuronal cell death, or if neuronal death is a bystander phenomenon from this process in other cells.

This study uses a neonatal rat IVH model to examine whether Hb can induce neuronal cell death in the hippocampus and the mechanisms involved. It particularly focuses on the role of Fe, CD163, and c-Jun N-terminal kinase (JNK).

2. Results

2.1. Neuronal loss in the CA-1 region of the hippocampus

Hb-injected rats had significantly lower cell counts in the ipsilateral CA-1 region of the hippocampus than anesthesia-only controls as quantified via H&E staining (280 ± 29 vs. 391 ± 25 neurons/mm, respectively; $p < 0.01$; Fig. 1A). Hippocampal neuronal loss was also seen where Hb-injected rats had significantly fewer NeuN-positive cells than anesthesia-only control rats (238 ± 35 vs. 344 ± 50 neurons/mm, respectively; $p < 0.01$; Fig. 1B). Using Fluoro-Jade C (FJC) staining, we found that Hb injection significantly increased the number of degenerating neurons in the hippocampus compared to controls (250 ± 32 vs. 121 ± 23 neurons/mm, respectively; $p < 0.01$; Fig. 1C). Bilateral hippocampal volume was assessed with magnetic resonance imaging (MRI), which demonstrated that Hb-injected rats had smaller hippocampal volumes than controls (23.3 ± 4.4 vs. 31.9 ± 3.5 mm³, respectively; $p < 0.05$; Fig. 1D). Importantly, the reduction of hippocampal volume was not specific to the side of the injection. There was no significant difference in volume between the ipsi- and contralateral hippocampi.

2.2. Hydrocephalus does not affect neuronal loss in the hippocampus

We have previously reported that intraventricular injection of Hb in neonatal rats can induce hydrocephalus (Strahle et al., 2014). To investigate whether the presence of hydrocephalus was a confounder, animals were categorized by whether or not they developed hydrocephalus, defined as the presence of a total ventricular volume larger than the mean of all artificial cerebrospinal fluid (aCSF)-injected rats plus 3 standard deviations. Four Hb-injected rats randomly selected from each group (animals that developed hydrocephalus and animals that did not) were analyzed for ipsilateral CA-1 hippocampal cell and neuronal counts via H&E staining and immunohistochemistry for NeuN, and neuronal degeneration via FJC staining. There was no significant difference in the number of hippocampal neurons or neuronal degeneration between the two groups (H&E: hydrocephalus vs. no hydrocephalus; 275 ± 30 vs. 284 ± 32 cells/mm, respectively; $p=0.322$. NeuN: 229 ± 33 vs. 257 ± 41 cells/mm, respectively; $p=0.177$. FJC: 257 ± 40 vs. 244 ± 25 cells/mm, respectively; $p=0.219$.) (Fig. 2).

2.3. Deferoxamine (DFX) attenuates Hb-induced neuronal damage

Treatment with the iron chelator DFX attenuated hippocampal cell loss and, specifically, hippocampal neuronal loss. Thus Hb-injected rats treated with DFX had significantly higher cell counts in the hippocampus than Hb-injected rats treated with vehicle (341 ± 17 vs. 259 ± 20 cells/mm, respectively; $p<0.05$; Fig. 3A) as well as higher neuronal counts (294 ± 21 vs. 238 ± 35 neurons/mm, respectively; $p<0.05$; Fig. 3B). Treatment with DFX also attenuated the Hb-induced neuronal degeneration detected by FJC staining (173 ± 16 vs. 250 ± 32 neurons/mm in vehicle-treated rats; $p<0.01$; Fig. 3C). Finally, DFX treatment also attenuated the Hb-induced loss of hippocampal volume (29.5 ± 3.3 vs. 23.0 ± 0.9 mm³ in Hb + vehicle; $p<0.05$; Fig. 3D).

2.4. Activated JNK and CD163 are upregulated in the hippocampus after Hb injection

Western blot analysis demonstrated a significant increase in the expression of activated JNK after intraventricular Hb injection. The ratio of phosphorylated or activated JNK to dephosphorylated or deactivated JNK was increased 1.8 fold ($p<0.05$; Fig. 4A and C). This suggests a potential Hb-induced pathway responsible for neuronal cell death found in IVH. CD163, the Hb/haptoglobin receptor, was also found to be upregulated 1.5 fold after Hb injection compared to controls ($p<0.05$; Fig. 4B).

2.5. Activated JNK co-localizes to the Hb receptor CD163

Given the extracellular nature of the Hb injection and the increase in intracellular JNK activation, we investigated the effects of Hb injection on expression of the Hb scavenger/receptor CD163. Double-labeling of CD163 and activated phosphorylated JNK (p-JNK) demonstrated high levels of co-localization in the hippocampus of Hb-injected brains compared to controls (Fig. 5A), suggesting that endocytosis of Hb via CD163 could trigger the JNK cascade.

2.6. CD163 is expressed on neurons after Hb injection

Visually, co-localization between CD163 and p-JNK appeared to occur on hippocampal neurons. Using double-labeling between the neuronal marker, neuron-specific enolase (NSE), and CD163, we found moderate levels of CD163 expression on hippocampal neurons in Hb-injected animals (Fig. 6). CD163 expression on hippocampal neurons is a novel finding.

2.7. CD163 mRNA upregulated in vitro in response to Hb

To confirm Hb-induced CD163 expression in neurons, primary cultured neocortical neurons were treated with either vehicle or 20 μ M Hb. At 24 h, Hb significantly induced CD163 mRNA compared to vehicle-treated cells (1.3 ± 0.2 fold; $p < 0.05$).

3. Discussion

In neonatal humans, IVH originates from the germinal matrix, a subventricular structure replete with arterioles that involute late in gestation. IVH causes significant morbidity and mortality (Heron et al., 2010) and is strongly associated with cognitive disability. The hippocampus plays a critical role in memory and learning and is intimately associated with the ventricular system, making it a potential site of IVH-induced injury. Despite this, there have been few studies investigating hippocampal injury after IVH in neonatal animal models.

In our study, Hb caused significant degeneration of hippocampal neurons compared to controls, as shown by H&E staining, immunohistochemistry, and FJC staining for neuronal degeneration. The FJC stain is specific to neurons, omitting even degenerating microglia, and is thus preferable over TUNEL staining (Eyüpoglu et al., 2003) for evaluation of degenerating neuronal cells. MRI quantification showed hippocampal tissue loss concordant with the loss of neurons. The decrease in hippocampal volume was bilateral, indicating that the hippocampal atrophy was not a result of direct trauma. The hippocampal atrophy did not appear to be confounded by the presence of hydrocephalus, as neuronal cell counts were not significantly different between animals with hydrocephalus and those without. Thus, we conclude that the hippocampal neuronal cell death seen is caused specifically by the free Hb injected and not by either injection trauma or hydrocephalus-induced damage.

One possible mechanism by which Hb causes neuronal death is via the ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}) cations transported within the heme core of the protein. Fe^{2+} can induce oxidative stress due to its ability to participate in Fenton and Haber-Weiss reactions, which produce free radicals and reactive oxygen species (Collard, 2009). To assess whether the reactive Fe cations within Hb contribute to the damage seen in this study, we treated Hb-injected rats with a Fe^{3+} chelator, DFX. DFX has been extensively investigated as a possible treatment for intracerebral hemorrhage (Cui et al., 2015). Treatment with DFX attenuated the adverse effects of the Hb injection on the hippocampus. Cell and neuronal counts both increased with respect to vehicle-treated animals, neuronal degeneration decreased, and hippocampal volume increased. This neuronal rescue was not complete, as neuronal cell counts for the DFX-treated animals were still less than anesthesia-only controls ($p < 0.05$;

data not shown). This may reflect the ability of DFX to chelate Fe^{3+} but not Fe^{2+} . It may also suggest that while Fe plays a key role in Hb-induced neuronal death, other components of Hb or perhaps previous intermediate molecules in the Hb-degradation pathway cause damage as well. This deserves further investigation. Fe within Hb may play a significant role in neuronal degeneration following IVH, however, acknowledging that DFX also has other neuroprotective roles; eg, reducing inflammation and apoptotic activity, attenuating edema, and reducing post-IVH hydrocephalus (Meng et al., 2015; Nakamura et al., 2004; Papazisis et al., 2008; Song et al., 2007; Strahle et al., 2014).

Potential lipopolysaccharide (LPS) contamination of the injected hemoglobin was tested using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, Rockford, IL) and a LPS level of 0.064 EU/mg hemoglobin was detected. It should be noted that to induce neuroinflammation in neonatal rats, an intracerebroventricular LPS dose of 3 ug (30 EU) has been described (Wang et al., 2014), i.e. 500 fold greater than the potential LPS contamination of the hemoglobin.

DFX has recently been reported to downregulate expression of p-JNK in the basal ganglia linking JNK to Fe (Ni et al., 2015). JNK is a member of the MAPK signaling pathway that is activated by cytokines and environmental stress (Weston and Davis, 2007). JNK's primary role is that of dead/survival signaling. It has been shown to play roles in apoptotic pathways via Fas, p53, c-Myc, Bax, and caspase cascades (Chen, 2012; Davis, 2000; Dhanasekaran and Reddy, 2008; Papadakis et al., 2006). In our study, Hb-injected animals displayed elevated levels of p-JNK expression without significant change in total JNK (T-JNK) expression, indicating that Hb specifically induces the activation of JNK. This suggests a mechanism through which Hb can induce neuronal death.

Moreover, we have identified a pathway through which p-JNK may be influenced by Hb – CD163. CD163 is a primary Hb receptor previously thought to only be present in macrophages (Kristiansen et al., 2001). It has also been found on circulating monocytes (Galea et al., 2012). Its ability to clear Hb from the extracellular space has linked it to anti-inflammatory pathways (Moestrup and Møller, 2004; Schaer et al., 2006, 2007; Van Gorp et al., 2010). CD163 primarily acts as a receptor for Hb already bound to the scavenger haptoglobin, but it has the ability to endocytose even unbound Hb, though with lower affinity (Galea et al., 2012). Endocytosis of Hb would allow it to more effectively influence and upregulate the JNK apoptotic cascade. Indeed, in our study, co-localization of CD163 to activated JNK supports the hypothesis that JNK activation and its apoptotic actions are linked to Hb uptake into cells. Specifically, we found that p-JNK and CD163 appeared to be co-localized on neurons. Upon further investigation, we co-localized CD163 to neuronal marker NSE in Hb-injected animals. CD163 has long been viewed as a marker for macrophages or monocytes, and it has not been observed in neurons prior to this study. The expression of CD163 has been shown to be increased in close proximity to brain lesions, either suggesting an increase in presence of CD163-expressing macrophages in the area, or potentially an induction of CD163 expression on neurons (Holfelder et al., 2011). In the current study, we found increased CD163 mRNA expression in cortical neurons in vitro after Hb exposure. This suggests that CD163 can and does express on neuronal membranes if extracellular Hb concentrations are sufficiently high. It is possible that without sufficient

levels of plasma Hb, CD163 would be in its natural state of non-expression. However, during IVH, large amounts of Hb enter the ventricles, as simulated by the Hb injection in our model, and it could be sufficient to induce CD163 expression on the neuronal cell membrane. Indeed, previous assertions that CD163 solely expressed on monocytes/macrophages were made under investigation of models that did not simulate IVH and thus, didn't account for raised extracellular Hb (Law et al., 1993; Polfliet et al., 2006). The role of CD163 in Hb-uptake into neurons and Fe overload, JNK activation and cell death and its response to DFX treatment, in addition to long-term neurologic outcome merits further investigation.

4. Conclusion

We have identified that Hb may be a significant contributor to neuronal degeneration in the neonatal hippocampus after IVH. Further, we have found that hippocampal degeneration was associated with expression of the Hb receptor CD163 and activation of JNK in hippocampal neurons, and the degeneration was reduced by DFX, indicating a potential role of Fe. These results suggest that some Hb released after IVH is taken up into neurons, causing increased cellular Fe, JNK activation, and cell death.

5. Experimental procedures

5.1. Neonatal rat model

Postnatal day 7 Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were kept in a 12-hr light/12-hr dark environment and given ad libitum access to water (mother rats) and maternal milk. Animals underwent lateral intraventricular injection under isoflurane (3.0% induction, 1.5% maintenance) or pentobarbital (40–50 mg/kg). Animals were returned to the cage with their mother and allowed to recover. All intraventricular injection volumes were 20 μ L. Animals in the experimental groups were injected with aCSF ($n=10$), 50 mg/mL bovine Hb (MP Biomedicals, Santa Ana, CA; $n=4$ for Western blot), and 150 mg/mL bovine Hb (MP Biomedicals; $n=8$ for histological analysis). Control groups underwent anesthesia alone ($n=9$). Animals were sacrificed at either 24 (for Western blot) or 72 h (for staining). Animal use protocols were approved by our institution's Committee on the Use and Care of Animals. Details of the experimental protocol have previously been described (Strahle et al., 2014).

5.2. DFX treatment

Eight additional animals underwent lateral ventricular injection of Hb (150 mg/mL) and were then administered intraperitoneal injections of either vehicle (sterile water or saline; $n=4$), or DFX ($n=4$) 2 h following the intraventricular injection, and then twice a day for 24 h. No difference was observed between the animals treated with sterile water-derived vehicle and saline-derived vehicle. The concentration of the injected solution was 50 mg/mL and the total amount given was 100 mg/kg (40 μ L for a 20-g rat).

5.3. Cell culture

Primary neuronal cultures were obtained from embryonic day-17 Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). Cultures were prepared according to a previously described procedure with some modifications (Jiang et al., 2002). Briefly, the cerebral cortex was dissected, stripped of meninges, and dissociated by a combination of 0.5% trypsin digestion and mechanical trituration. The dissociated cell suspensions were seeded into poly-L-lysine pre-coated 6-well plates at a density of 600,000/cm². Cells were grown in neurobasal medium with 2% B27, 0.5 mM glutamine, and 1% antibiotic-antimycotic, and maintained in a humidified incubator at 37 °C with 5% CO₂. Half of the cultured media was changed every 3–4 days. Neurons were used for experiments after 7 days. Neurons were treated with either vehicle or human Hb (20 µM; Sigma-Aldrich, St. Louis, MO). Cells were collected 24 h later for real-time PCR.

5.4. Real-time quantitative PCR

In the in vitro studies, cell medium was removed and plates were washed 3 times with PBS. Cells were quickly scraped and collected by centrifugation at 4 °C, then stored at –80 °C. Total RNA was extracted from the cultured cells with Trizol reagent (Gibco BRL, Grand Island, NY), and 1 µg RNA was digested with deoxyribonuclease I (DNaseI, amplification grade; Gibco BRL). Complimentary DNA was synthesized by reverse transcription using the digested 1-µg RNA (11 µL) with 14 µL reaction buffer (Perkin Elmer, Foster City, CA) containing dNTP (dATP, dCTP, dGTP, and dTTP), 25 mmol/L MgCl₂, 10 × PCR buffer II, Random Hexamer Primer, Rnase inhibitor, and MuL V reverse transcriptase. The reaction was performed at 42 °C for 30 min and terminated at 99 °C after 5 min. Diethyl pyrocarbonate water (75 µL) was added to dilute the complimentary DNA to 100 µL and stored at –20 °C for later use.

Real-time quantitative PCR was performed with SYBR green (Qiagen, Germantown, MD) as a double-strand DNA-specific dye in an Eppendorf Mastercycler ep realplex unit (Eppendorf North America Inc., Hauppauge, NY). The CD163 primers were designed from known sequences of rat CD163-mRNA (Gene-Bank no. NM_001107887.1) searched by PrimerQuest (Integrated DNA Technologies Inc., Coralville, IA). The primers were: rat CD163 5'-GCTGAAATCCTCGGGTTGGCATTT –3' (forward primer) and 5'-AGCGTGAAGATGTAGCTGTGGTCA –3' (reverse primer). A housekeeping gene, GAPDH, served as a control. The primers were: 5'-CCGTGCCAAGATGAAATTGGCTGT-3' (forward) and 5'-TGTGCATATGTGCGTGTGTGTGTG-3' (reverse). PCR reaction was run in triplicate on 96-well plates with a total volume of 20 µL per well using 2.5 × SYBR Green universal master mix. Cycling conditions were 2 min at 95 °C, 30 sec at 95 °C, 30 s at 60 °C, 1 min at 72 °C, 40 cycles, and a melting-curve program (60 °C to 95 °C with warming of 1.75 °C per min). The relative quantification analysis module was used to compare expression levels of a target gene. The expression levels were calculated by using the C_T method (Livak and Schmittgen, 2001). With this method, we had a value equal to 1 when no change in relative expression occurred between untreated and treated samples. We defined over-expression as $2^{-C_T} > 1$ and under-expression as $2^{-C_T} < 1$.

5.5. MRI volume measurements

At 24 h after intraventricular injection, all rats used in this study underwent T2-weighted MRI. The sequences used have been previously described (Strahle et al., 2014). Bilateral dorsal and ventral horns of the hippocampus, as well as the ventricular systems (lateral ventricles, temporal horn, aqueduct, and 4th ventricles) were outlined using NIH Image J software (<http://rsbweb.nih.gov/ij/index.html>) and volumes calculated according to protocols published previously (Strahle et al., 2014; Wolf et al., 2002).

5.6. Western blot analysis

The ipsilateral hippocampus of anesthesia-only controls ($n=3$) and Hb-injected rats ($n=4$) was investigated. Fifty micrograms of protein from each sample was denatured at 95 °C for 5 min and then loaded onto columns in a 4% stacking/12% polyacrylamide gel and separated at constant 30 mA. Gels were transferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature or overnight at 4 °C. Membranes were blocked with 5% carnation milk in TBST for 1 h. Primary antibodies included polyclonal rabbit anti-p-SAPK/JNK (#9252 S; Cell Signaling Technology Inc.), monoclonal mouse anti-rat CD163 (MCA342R; AbD Serotec, Raleigh, NC), and monoclonal rabbit anti- β -Actin (#4970L; Cell Signaling Technology Inc.). Secondary antibodies were goat anti-mouse IgG (H+L)-HRP conjugate (#170-6516; Bio-Rad Laboratories, Hercules, CA) and goat anti-rabbit IgG (H+L)-HRP conjugate (#170-6515; Bio-Rad Laboratories). Membranes were developed with Lumi-Light Western blotting substrate (Roche, Indianapolis, IN) and visualized in a FluorChemM imager (Protein Simple, Santa Clara, CA). Protein band signals were analyzed using Image J software.

5.7. Immunohistochemistry/H&E staining

Experimental groups consisted of anesthesia-only controls ($n=4$) and Hb-injected 150 mg/mL ($n=8$), Hb+vehicle ($n=4$), and Hb+DFX ($n=4$) rats. For immunohistochemistry, slides were dried and incubated at room temperature in 0.01 M PBS, pH 7.4, with 0.3% Triton 100 (Sigma-Aldrich) for 15 min, washed with PBS, and then incubated at room temperature with 1:10 horse serum in PBS for 30 min. Slides were then incubated in either 1:400 monoclonal mouse anti-NeuN (MAB377; EMD Millipore Corp.) or 1:300 monoclonal mouse anti-rat CD163 (MCA342R; AbD Serotec) overnight at 4 °C. Slides were washed in PBS and incubated in a 2:1 PBS/methanol mixture with 0.3% H₂O₂ for 20 min. After being washed in PBS again, slides were incubated in 1:500 horse anti-mouse secondary antibody for 90 min. Slides were washed in PBS before being incubated for 1–3 min in 3,3'-diaminobenzidine-4HCl (Liquid DAB Substrate Kit; Invitrogen, Carlsbad, CA), dehydrated, and cover-slipped with Permount mounting medium (Fisher Scientific, Waltham, MA).

Double-labeling was performed in a similar fashion with a few differences. Slides were dried and then incubated with 15% normal goat serum and 15% normal horse serum in 2% BSA in 0.1% saponin PBS for 30 min at 37 °C. Slides were then incubated overnight with 1:300 monoclonal mouse anti-rat CD163 (MCA342R; AbD Serotec) in 0.1% saponin PBS. The following day this procedure was repeated with the exception that the slides were washed with PBS prior to blocking, and that the primary antibody was 1:300 polyclonal rabbit anti-p-SAPK/JNK (#9252S; Cell Signaling Technology) or 1:80 rabbit anti-NSE

(AbD Serotec) overnight. Slides were then washed in PBS, blocked with the same blocking solution, and then incubated with FITC horse anti-mouse secondary antibody in 0.1% saponin PBS, washed in PBS, and incubated in Rhodamine goat anti-rabbit secondary antibody in 0.1% saponin PBS. Finally, the slides were washed in PBS for 2 h before imaging with a fluorescence microscope.

Slides were also prepared for H&E staining. Experimental groups included the following: anesthesia control ($n=4$), Hb 150 mg/mL ($n=8$), Hb+vehicle ($n=4$), and Hb+DFX ($n=4$) treatment. Slides were prepared as for immunohistochemistry, dried, lightly stained with hematoxylin (10 s), and washed under running tap water for 2 min. Next, they were counter-stained with eosin for 10 s and washed again. Slides were then dehydrated and cover-slipped.

5.8. FJC staining

Specimens were prepared for FJC staining as for immunohistochemistry and dried for 30 min before being washed in a basic alcohol solution consisting of 1% NaOH in 80% ethanol. They were then washed in 70% ethanol before being rinsed in water for 2 min. Slides were then incubated in a 0.06% potassium permanganate solution for 10 min, rinsed in dH₂O, and incubated in 0.0001% FJC stock solution. Next, the slides were rinsed in dH₂O, cleared in xylene, and cover-slipped. Pictures were taken immediately using a fluorescent microscope.

5.9. Hippocampal cell-counting

Cell-counting was performed on immunohistochemistry and H&E- and FJC-stained slides. Individual cells were manually counted along the ipsilateral CA-1 region of the hippocampus at 40 $\times$ magnification. We expressed these results as the number of cells per unit length of the pyramidal cell layer in mm. Five different images of the CA-1 region were quantified per animal and the average used.

5.10. Statistical analysis

Data are represented as mean $\pm$ standard deviation. Two group comparisons were carried out using the Mann-Whitney U test, while multi-group comparisons were analyzed using the Kruskal-Wallis test. A p -value less than 0.05 was considered significant.

Acknowledgments

This study was supported by funding from NIH Grants NS073959, NS079157, NS007222, and NS090925 as well as the Pediatric Hydrocephalus Foundation. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Abbreviations

aCSF	artificial cerebrospinal fluid
DFX	deferoxamine
Fe	iron
Fe²⁺	ferrous iron

Fe³⁺	ferric iron
FJC	Fluoro-Jade C
Hb	hemoglobin
H&E	hematoxylin and eosin
IVH	intraventricular hemorrhage
JNK	c-Jun N-terminal kinase
MRI	magnetic resonance imaging
NSE	neuron-specific enolase
p-JNK	phosphorylated c-Jun N-terminal kinase
T-JNK	total c-Jun N-terminal kinas

REFERENCES

- Chen F. JNK-induced apoptosis, compensatory growth, and cancer stem cells. *Cancer Res.* 2012; 72:379–386. [PubMed: 22253282]
- Collard KJ. Iron homeostasis in the neonate. *Pediatrics.* 2009; 123:1208–1216. [PubMed: 19336381]
- Cui HJ, et al. Efficacy of deferoxamine in animal models of intracerebral hemorrhage: a systematic review and stratified meta-analysis. *PLoS One.* 2015; 10:e0127256. [PubMed: 26000830]
- Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell.* 2000; 103:239–252. [PubMed: 11057897]
- Dhanasekaran DN, Reddy EP. JNK signaling in apoptosis. *Oncogene.* 2008; 27:6245–6251. [PubMed: 18931691]
- Eyüpoglu IY, et al. Identification of neuronal cell death in a model of degeneration in the hippocampus. *Brain Res. Brain Res. Protoc.* 2003; 11:1–8. [PubMed: 12697257]
- Galea J, et al. The intrathecal CD163-haptoglobin-hemoglobin scavenging system in subarachnoid hemorrhage. *J. Neurochem.* 2012; 121:785–792. [PubMed: 22380637]
- Georgiadis P, et al. Characterization of acute brain injuries and neurobehavioral profiles in a rabbit model of germinal matrix hemorrhage. *Stroke.* 2008; 39:3378–3388. [PubMed: 18845808]
- Gram M, et al. Hemoglobin induces inflammation after preterm intraventricular hemorrhage by methemoglobin formation. *J. Neuroinflamm.* 2013; 10(100):1–13.
- Gram M, et al. Extracellular hemoglobin-mediator of inflammation and cell death in the choroid plexus following preterm intraventricular hemorrhage. *J. Neuroinflamm.* 2014; 11(200):1–15.
- Heron M, et al. Annual summary of vital statistics: 2007. *Pediatrics.* 2010; 125:4–15. [PubMed: 20026491]
- Holfelder K, et al. De novo expression of the hemoglobin scavenger receptor CD163 by activated microglia is not associated with hemorrhages in human brain lesions. *Histol. Histopathol.* 2011; 26:1007–1017. [PubMed: 21692033]
- Jiang Y, et al. Thrombin-receptor activation and thrombin-induced brain tolerance. *J. Cereb. Blood Flow Metab.* 2002; 22:404–410. [PubMed: 11919511]
- Kristiansen M, et al. Identification of the haemoglobin scavenger receptor. *Nature.* 2001; 409:198–201. [PubMed: 11196644]
- Law SK, et al. A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. *Eur. J. Immunol.* 1993; 23:2320–2325. [PubMed: 8370408]
- Lee JY, et al. Hemoglobin and iron handling in brain after subarachnoid hemorrhage and the effect of deferoxamine on early brain injury. *J. Cereb. Blood Flow Metab.* 2010; 30:1793–1803. [PubMed: 20736956]

- Lewis M, Bendersky M. Cognitive and motor differences among low birth weight infants: impact of intraventricular hemorrhage, medical risk, and social class. *Pediatrics*. 1989; 83:187–192. [PubMed: 2913549]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Lok J, et al. Intracranial hemorrhage: mechanisms of secondary brain injury. *Acta Neurochir. Suppl*. 2011; 111:63–69. [PubMed: 21725733]
- Meng H, et al. Deferoxamine alleviates chronic hydrocephalus after intraventricular hemorrhage through iron chelation and Wnt1/Wnt3a inhibition. *Brain Res*. 2015; 1602:44–52. [PubMed: 25152462]
- Moestrup SK, Møller HJ. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann. Med*. 2004; 36:347–354. [PubMed: 15478309]
- Murphy BP, et al. Posthaemorrhagic ventricular dilatation in the premature infant: natural history and predictors of outcome. *Arch. Dis. Child. Fetal Neonatal Ed*. 2002; 87:F37–F41. [PubMed: 12091289]
- Nakamura T, et al. Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. *J. Neurosurgery*. 2004; 100:672–678.
- Ni W, Okauchi M, Hatakeyama T, Gu Y, Keep RF, Xi G, Hua Y. Deferoxamine reduces intracerebral hemorrhage-induced white matter damage in aged rats. *Exp. Neurol*. 2015; 272:128–134. Epub 2015 March. <http://dx.doi.org/10.1016/j.expneurol.2015.02.035>. [PubMed: 25749188]
- Papadakis ES, et al. The regulation of Bax by c-Jun N-terminal protein kinase (JNK) is a prerequisite to the mitochondrial-induced apoptotic pathway. *FEBS Lett*. 2006; 580:1320–1326. [PubMed: 16458303]
- Papazisis G, et al. Deferoxamine decreases the excitatory amino acid levels and improves the histological outcome in the hippocampus of neonatal rats after hypoxia-ischemia. *Pharmacol. Res*. 2008; 57:73–78. [PubMed: 18243015]
- Polfliet MMJ, et al. The rat macrophage scavenger receptor CD163: expression, regulation and role in inflammatory mediator production. *Immunobiology*. 2006; 211:419–425. [PubMed: 16920481]
- Ramani M, et al. Neurodevelopmental impairment following neonatal hyperoxia in the mouse. *Neurobiol. Dis*. 2013; 50:69–75. [PubMed: 23064437]
- Regan RF, Panter SS. Neurotoxicity of hemoglobin in cortical cell culture. *Neurosci. Lett*. 1993; 153:219–222. [PubMed: 8327197]
- Schaer CA, et al. Constitutive endocytosis of CD163 mediates hemoglobin-heme uptake and determines the noninflammatory and protective transcriptional response of macrophages to hemoglobin. *Circ. Res*. 2006; 99:943–950. [PubMed: 17008602]
- Schaer DJ, et al. Gating the radical hemoglobin to macrophages: the anti-inflammatory role of CD163, a scavenger receptor. *Antioxid. Redox Signal*. 2007; 9:991–999. [PubMed: 17508920]
- Song S, et al. A new hippocampal model for examining intracerebral hemorrhage-related neuronal death: effects of deferoxamine on hemoglobin-induced neuronal death. *Stroke*. 2007; 38:2861–2863. [PubMed: 17761912]
- Strahle JM, et al. Role of hemoglobin and iron in hydrocephalus after neonatal intraventricular hemorrhage. *Neurosurgery*. 2014; 75:696–706. [PubMed: 25121790]
- Van Gorp H, et al. Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. *Mol. Immunol*. 2010; 47:1650–1660. [PubMed: 20299103]
- Wagner KR, et al. Heme and iron metabolism: role in cerebral hemorrhage. *J. Cereb. Blood Flow Metab*. 2003; 23:629–652. [PubMed: 12796711]
- Wang J, Dore S. Heme oxygenase-1 exacerbates early brain injury after intracerebral haemorrhage. *Brain*. 2007; 130:1643–1652. [PubMed: 17525142]
- Wang P, You SW, Yang YJ, et al. Systemic injection of low-dose lipopolysaccharide fails to break down the blood-brain barrier or activate the TLR4-MyD88 pathway in neonatal rat brain. *Int. J. Mol. Sci*. 2014; 15(6):10101–10115. [PubMed: 24905408]
- Weston CR, Davis RJ. The JNK signal transduction pathway. *Curr. Opin. Cell Biol*. 2007; 19:142–149. [PubMed: 17303404]

- Wolf OT, et al. Volumetric measurement of the hippocampus, the anterior cingulate cortex, and the retrosplenial granular cortex of the rat using structural MRI. *Brain Res. Brain Res. Protoc.* 2002; 10:41–46. [PubMed: 12379436]
- Zhang P, et al. Cerebral hypoxia-ischemia increases toll-like receptor 2 and 4 expression in the hippocampus of neonatal rats. *Brain Dev.* 2015; 37:747–752. [PubMed: 25530127]
- Zlotnik A, et al. Effect of glutamate and blood glutamate scavengers oxaloacetate and pyruvate on neurological outcome and pathohistology of the hippocampus after traumatic brain injury in rats. *Anesthesiology.* 2012; 116:73–83. [PubMed: 22129535]

Fig. 1.

Representative images showing that intraventricular injection of Hb caused neuronal loss in the ipsilateral hippocampal CA-1 region at 72 h post-injection. (A) H&E staining (40× magnification; scale bars=20 μm), (B) immunohistochemistry for NeuN (40× magnification; scale bars=20 μm), (C) FJC staining of neuronal degeneration (40× magnification; scale bars=20 μm), and (D) MRI assessment of total hippocampal volume. Areas measured are outlined in yellow. (E) Quantification of the respective staining and hippocampal volume (* $p < 0.01$ vs. control group; # $p < 0.05$ vs. control group).

Fig. 2.

(A) Representative H&E staining, NeuN immunohistochemistry, and FJC staining of the ipsilateral hippocampus in rats that did and did not develop hydrocephalus (hydro) after intraventricular Hb injection (40× magnification; scale bars=20 μm). (B) Quantification of the numbers of cells, neurons, and degenerating neurons. None of the differences between the group with and the group without hydrocephalus were significant at the $p<0.05$ level.

Fig. 3.

Representative images showing the effects of systemic DFX treatment on Hb-induced hippocampal damage. (A) H&E staining (40× magnification; scale bars=20 μm), (B) immunohistochemistry for NeuN (40× magnification; scale bars=20 μm), (C) FJC staining of neuronal degeneration (40× magnification; scale bars=20 μm), and (D) MRI assessment of hippocampal volume (areas measured are outlined in yellow). (E) Quantification of the respective staining (* $p < 0.05$ vs. Hb+Vehicle).

Fig. 4.

Effect of intraventricular Hb injection on JNK activation and CD163 expression in the hippocampus. (A) Representative Western blot for p-JNK and T-JNK. Lanes 1–3 (controls); lanes 4–6 (Hb-injected animals). (B) Representative Western blot for CD163 with β -actin as the control. Lanes 1–3 (controls); lanes 4–6 (Hb-injected animals). (C and D) Bar graphs depicting quantification of p-JNK to T-JNK and CD163 to β -actin ratios (* $p < 0.05$ vs. control).

Fig. 5.
Co-localization of p-JNK with CD163 in the hippocampus was particularly evident after intraventricular Hb injection (40× magnification; scale bars=20 μ m).

Fig. 6.

Neuronal expression of CD163 in the hippocampus after intraventricular Hb injection. (A) Immunohistochemistry for CD163 in negative (–) control, anesthesia-only control, and Hb-injected brains. Hb injection was accompanied by a marked increase in CD163 (+) cells in the hippocampus compared to controls (40× magnification; scale bars=20 μm). (B) Double-labeling of CD163 (in red) and NSE (in green) showed co-localization of CD163 and NSE after Hb injection, indicating neuronal expression of CD163 (40× magnification; scale bars=20 μm).