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HIGH MOBILITY GROUP BOX PROTEIN-1 PROMOTES CEREBRAL EDEMA AFTER TRAUMATIC BRAIN INJURY VIA ACTIVATION OF TOLL-LIKE RECEPTOR 4

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Abstract

Traumatic brain injury (TBI) is a major cause of mortality and morbidity worldwide. Cerebral edema, a life-threatening medical complication, contributes to elevated intracranial pressure (ICP) and a poor clinical prognosis after TBI. Unfortunately, treatment options to reduce post-traumatic edema remain suboptimal, due in part, to a dearth of viable therapeutic targets. Herein, we tested the hypothesis that cerebral innate immune responses contribute to edema development after TBI. Our results demonstrate that high-mobility group box protein 1 (HMGB1) was released from necrotic neurons via a NR2B-mediated mechanism. HMGB1 was clinically associated with elevated ICP in patients and functionally promoted cerebral edema after TBI in mice. The detrimental effects of HMGB1 were mediated, at least in part, via activation of microglial toll-like receptor-4 (TLR4) and the subsequent expression of the astrocytic water channel, aquaporin-4 (AQP4). Genetic or pharmacological (VGX-1027) TLR4 inhibition attenuated the neuroinflammatory response and limited post-traumatic edema with a delayed, clinically implementable therapeutic window. Human and rodent tissue culture studies further defined the cellular mechanisms demonstrating neuronal HMGB1 initiates the microglial release of interleukin-6 (IL-6) in a TLR4 dependent mechanism. In turn, microglial IL-6 increased the astrocytic expression of AQP4. Taken together, these data implicate microglia as key mediators of post-traumatic brain edema and suggest HMGB1-TLR4 signaling promotes neurovascular dysfunction after TBI.

Keywords

neuroinflammation; DAMP; intracranial pressure; innate immunity; controlled cortical impact

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INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability, affecting over 1.7 million Americans annually (Nortje and Menon 2004). Preventative measures reduce the incidence and severity of TBI, yet one-third of hospitalized TBI patients die from secondary injuries that manifest in the hours and days after the initial traumatic event. Cerebral edema, the abnormal accumulation of fluid within the brain parenchyma, promotes elevated intracranial pressure (ICP), a life-threatening neurological complication. Sustained elevations in ICP typically peak within the first days after TBI, while the patient is under supervised medical care, and contribute to poor clinical outcomes (Levin et al. 1991; Saul and Ducker 1982). Unfortunately, neurosurgical approaches to manage elevated ICP remain limited and medical therapeutics are lacking due to the poorly defined mechanisms underlying edema development (Sahuquillo and Arikan 2006).

Cellular necrosis temporally correlates with immune activation and edema development after TBI (Czigner et al. 2007; Hayakata et al. 2004; Katayama and Kawamata 2003; Kawamata and Katayama 2006; Kawamata and Katayama 2007). Surgical excision of necrotic tissue reduced ICP and improved outcomes in neurotrauma patients; however, the mechanistic link between necrosis and secondary neurological injury remains poorly defined. Over-stimulation of N-methyl-D-aspartate receptors (NMDA-R) by elevated extracellular glutamate induces excitotoxicity, an important initiator of neuronal necrosis and edema after TBI (Ankarcrona et al. 1995). Similarly, persistent elevations in brain tissue and cerebrospinal fluid (CSF) glutamate concentrations correlate with injury severity in TBI patients (Dempsey et al. 2000; Faden et al. 1989; Narayan et al. 2002). Although NMDA-R antagonism reduced immune activation and attenuated neurological injury in rodent TBI models (Zhou et al. 2009), global NMDA-R antagonists were associated with poor therapeutic windows and clinically-intolerable side effects (Ikonomidou and Turski 2002). Coupled with recent evidence suggesting NMDA paradoxically improves long-term neurological and cognitive function after experimental TBI, elucidation of the detrimental mechanisms downstream from NMDA-R activation may provide novel therapeutic targets to improve outcomes after TBI.

Although an immunoprivileged organ, the brain can mount a cerebral innate immune reaction after infection or tissue injury to promote functional recovery/regeneration; however, innate immune effectors simultaneously induce a pro-inflammatory reaction, potentially exacerbating secondary injury and worsening outcomes. Damage-associated molecular pattern molecules (DAMPs) are multi-functional host proteins that are released after necrotic injury to initiate innate immune responses via activation of pattern-recognition receptors (PRR) (Iyer et al. 2009; Matzinger 1994; Won et al. 2006). One such DAMP, high-mobility group box protein 1 (HMGB1), is an evolutionarily conserved, non-histone DNA binding protein that passively translocates into the extracellular space following necrotic injury to activate PRRs, such as toll-like receptor 4 (TLR4).

We hypothesized that TLR4 mediates innate immune activation and edema development after TBI. A translational research approach, incorporating patient CSF, human tissue

culture, and pre-clinical TBI modeling was utilized to elucidate the complex role of HMGB1-TLR4 signaling in the development of secondary neurovascular injury after TBI.

MATERIALS AND METHODS

Supplies

Recombinant mouse IL-6 was purchased from Thermo Scientific (Rockford, IL). Recombinant human HMGB1, Ro25-6981, NVP-AAM077, and NMDA were purchased from Sigma-Aldrich (St. Louis, MO). VGX-1027 was purchased from Tocris (Ellisville, MO).

Controlled cortical impact

The Committee on Animal Use for Research and Education at the Medical College of Georgia approved all animal studies, in compliance with NIH guidelines. Adult male CD-1 (Charles River, Wilmington, MA), C3H/OuJ (wild-type), or C3H/HeJ (TLR4 mutant) (Jackson Laboratories) mice were anesthetized with xylazine (8 mg/kg)/ketamine (60 mg/kg) and subjected to a sham injury or controlled cortical impact, per our laboratory (Kimbler et al. 2012; Laird et al. 2010). Briefly, mice were placed in a stereotaxic frame (Amscien Instruments, Richmond, VA) and a 3.5 mm craniotomy was made in the right parietal bone midway between bregma and lambda with the medial edge 1 mm lateral to the midline, leaving the dura intact. Mice were impacted at 4.5 m/s with a 20 ms dwell time and 1 mm depression using a 3 mm diameter convex tip, mimicking a moderate TBI. Sham-operated mice underwent the identical surgical procedures, but were not impacted. The incision was closed with VetBond and mice were allowed to recover. Body temperature was maintained at 37°C using a small animal temperature controller throughout all procedures (Kopf Instruments, Tujunga, CA, USA). Food and water were provided *ad libitum*.

Treatments

For acute drug administration studies, 100 µL placebo (phosphate-buffered saline, PBS) or VGX-1027 (VGX; 25–100 mg/kg; Tocris, Ballwin, MO), a highly selective TLR4 antagonist, was administered via the tail vein 15 minutes prior to or up to 8 hours after TBI. 50 mg/kg VGX was determined to be optimal for TLR4 inhibition in dose finding studies (data not shown) and was used throughout these studies. Doses and intraperitoneal route of administration (100 µL volume/mouse) for the selective NMDA-R type 2A (NR2A) antagonist, 5 mg/kg NVP-AAM077, and the NMDA-R type 2B (NR2B) antagonist, 6 mg/kg Ro25-6981, were consistent with previous reports in the literature showing biological activity within the brain (Anastasio et al. 2009; Chaperon et al. 2003; Fox et al. 2006). All drug treatments were well tolerated and differences in locomotor activity and body weights were not observed, as compared to placebo-treatment mice. For tissue culture studies, 400 nM NVP-AAM077, or 500 nM Ro-25-6981 were used to inhibit NR2A and NR2B, respectively, as compared to vehicle-treated cultures. These concentrations are in line with previous reports (Malherbe et al. 2003; Milnerwood et al. 2012; Yang et al. 2012) and were the lowest effective concentrations at preventing NMDA toxicity in cortical neurons (data not shown).

Assessment of cerebral edema

Brain water content (BWC), a sensitive measure of cerebral edema, was quantified using the wet-dry method, as detailed by our group (Kimble et al. 2012; Laird et al. 2010). At 24h post-injury, a time-point associated with significant edema formation after experimental TBI (Laird et al. 2010), BWC was estimated in 3 mm coronal sections of the ipsilateral cortex (or corresponding contralateral cortex), centered upon the impact site. Tissue was immediately weighed (wet weight), then dehydrated at 65°C. The sample was reweighed 48h later to obtain a dry weight. The percentage of tissue water content was calculated using the following formula: $BWC = [(wet\ weight) - (dry\ weight) / wet\ weight] * 100$. Non-invasive determination of brain edema was performed using a horizontal 7.0T BioSpec MRI spectrometer (Bruker Instruments) equipped with a 8.9 cm micro-imaging gradient insert (100 gauss/cm), as detailed previously by our laboratory (Kimble et al. 2012).

Patient CSF collection

CSF was obtained from 26 consecutive adult TBI (Glasgow Coma Scale 3T-12T) or 9 normal pressure hydrocephalus (NPH) patients requiring CSF diversion in the Department of Neurosurgery at the Medical College of Georgia. Patient specimens were collected from adults (age 18–73) without regard for age, race, gender, or socioeconomic status. NPH patients served as an ideal control population as these patients exhibit significant edema without a traumatic event. The Institutional Review Board at Georgia Regents University approved all studies. HMGB1 levels in CSF were determined by quantitative Western blotting.

Cell culture

CD-1 Mouse cortical neurons (99% purity) and astrocytes (>97% purity) were cultured exactly as described by our laboratory (Sukumari-Ramesh et al. 2010). Primary human microglia or astrocytes were purchased from ScienCell and cultured per manufacturer's recommended protocols. C8-B4 and EOC.20 microglial cells were obtained from ATCC (Manassas, VA) and cultured per recommended protocols. Cell viability was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay or by lactate dehydrogenase (LDH) release assays.

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from cell/tissue cultures or from the pericontusional cortex (or from the corresponding contralateral cortex) using a SV RNA Isolation kit (Promega). qRT-PCR was performed on a Cepheid SmartCycler II using the Superscript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen), as detailed by our group (Laird et al. 2010; Wakade et al. 2009). Primers utilized were as follows: TLR4: (FP 5'-GCTTTCACCTCTGCCTTCAC-3'; RP 5'-GAAACTGCCATGTTTGAGCA-3'), AQP4: (FP 5'-CGGTTTCATGGAAACCTCACT-3'; RP 5'-CATGCTGGCTCCGGTATAAT-3'), IL-6: (FP 5'-AGTTGCCTTCTTGGGACTGA-3'; RP 5'-TCCACGATTTCCAGAGAAC-3'), and RPS3: (FP 5'-AATGAACCGAAGCACACCATA-3'; RP 5'-ATCAGAGAGTTGACCGCAGTT-3'). Product specificity was confirmed by melting curve analysis and visualization of single,

appropriately sized band on a 2% agarose gel. Gene expression levels were quantified using a cDNA standard curve and data were normalized to RPS3, a housekeeping gene that was unaffected by TBI (Laird et al. 2010). Data are expressed as fold change versus placebo treatment.

Western blotting

Whole cell lysates were prepared from 3 mm coronal sections centered upon the site of impact. A 1-mm micropunch was collected from the pericontusional cortex or from the corresponding contralateral hemisphere. Tissue was placed in complete RIPA buffer, sonicated, and centrifuged for 10 minutes at 14,000×g at 4°C. For conditioned media or human studies, cell culture supernatants or patient CSF were concentrated using Millipore Amicon ultracentrifugal filters prior to quantification. Protein concentrations were quantified using a BCA protein assay kit (Pierce, Rockford, IL). 30 µg of protein were resolved on a 4–20% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. Blots were incubated overnight at 4°C in primary antibody [(1:200 anti-TLR), (1:200 anti-AQP4 antibody), (1:100 anti-HMGB1, (1:500 anti-NSE), (1:1000 anti-β-actin), (1 µg/mL HMGB1, Abcam #ab18256), or (1:2000 anti-β actin, Abcam) followed by a 2h incubation with an Alexa Fluor-tagged secondary antibody at room temperature, per our laboratory (Laird et al. 2010; Sukumari-Ramesh et al. 2010). Blots were visualized using a Li-Cor Odyssey near-infrared imaging system and densitometry analysis was performed using Quantity One software (Bio-Rad, Foster City, CA).

Enzyme-linked immunoassay (EIA)

Interleukin-6 (IL-6) levels were quantified by human or mouse IL-6 EIA kits (Invitrogen), using manufacturer's recommended protocols.

Immunohistochemistry

Deeply anesthetized mice were perfused with saline, followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed overnight in paraformaldehyde followed by cryoprotection with 30% sucrose (pH 7.4) until brains permeated. Serial coronal sections (12 µM) were prepared from the pericontusional cortex using a cryostat microtome (Leica, Wetzlar, Germany) and directly mounted onto glass slides. For comparisons, sections from sham-operated mice were prepared from tissue located directly below the craniectomy (e.g. same anatomical brain region). Sections were incubated at room temperature with 10% normal donkey serum in phosphate-buffered saline containing 0.4% Triton X-100 for 1 h, followed by incubation with the primary antibody [TLR4 (1:200; Santa Cruz), AQP4 (1:100, Santa Cruz), HMGB1 (1:100, Abcam), Iba1 (1:100, Wako), or NeuN (1:500, Millipore)] overnight at 4°C. Sections were then washed and incubated with the appropriate Alexa Fluor-tagged secondary antibody. Omission of primary antibody served as a negative control.

Confocal microscopy

Immunofluorescence was determined using a LSM510 Meta confocal laser microscope (Carl Zeiss), as described by our group (Sukumari-Ramesh et al. 2012). Cellular co-localization was determined in Z-stack mode using 63X oil immersion Neofluor objective (NA 1.3) with the image size set at 512×512 pixels. The following excitation lasers/emission filters settings were used for various chromophores: argon2 laser was used for Alexa Fluor 488, with excitation maxima at 490 nm and emission in the range 505–530 nm. A HeNe1 laser was used for Alexa Fluor 594 with excitation maxima at 543 nm and emission in the range 568–615 nm. Z-stacks (20 optical slices) were collected at optimal pinhole diameter at 12-bit pixel depth and converted into three-dimensional projection images using LSM510 Meta imaging software.

Statistical analysis

Multi-group comparisons were made using a one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's post-hoc test. Results are expressed as mean $\pm$ SEM. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Inhibition of the NMDA-R NR2 subunit reduces HMGB1 release and cerebral edema

Excitotoxicity is an essential initiating factor of secondary neurological injury. To establish whether NR2 mediates neuronal injury and the development of cerebral edema, NR2 selective inhibitors were employed after a moderate TBI in mice. Edema was increased in the ipsilateral cortex after TBI ($83.3 \pm 0.5\%$ brain water content; BWC) as compared to sham ($78.4 \pm 1.0\%$, $p < 0.01$ vs. TBI). Pretreatment with 5 mg/kg NVP-AAM077, a selective NR2A antagonist, did not reduce edema ($84.4 \pm 1.7\%$) whereas 6 mg/kg Ro25-6981, a selective NR2B antagonist, significantly attenuated edema ($80.3 \pm 0.5\%$; $p < 0.01$ vs. TBI) at 24h post-TBI, a time point associated with peak cellular edema in this model (Figure 1A).

Consistent with these data, Ro25-6981 reversed NMDA-induced excitotoxicity (NMDA + Ro: $95.4 \pm 9.8\%$ viability vs. NMDA: $49.8 \pm 12.2\%$; $p < 0.05$ vs. NMDA; not different from vehicle) in mouse cortical neurons, which express both NR2A and NR2B (Sheng et al. 1994). Conversely, NVP-AAM077 had no significant effect on cytotoxicity, as compared to NMDA-treated cultures (NMDA+NVP: $41.9 \pm 7.6\%$) (Figure 1B). Parallel to these findings, NMDA increased the extracellular content of HMGB1 ($326.0 \pm 4.1\%$ of HMGB1 levels in vehicle-treated cultures; $p < 0.01$) and decreased intracellular HMGB1 content by 21% (Figure 1C). In line with the viability data, Ro25-6981 ($175.0 \pm 2.7\%$ of vehicle; $p < 0.05$ vs. NMDA), but not NVP-AAM077 ($303.0 \pm 3.7\%$ of vehicle; not different from NMDA group), reduced the extracellular accumulation of HMGB1 after NMDA treatment (Figure 1C).

Immunoreactivity for HMGB1 was predominantly neuronal either under basal conditions or within the contralateral cortex after TBI, although 10–20% of immunopositive cells morphologically resembled glia (Figure 1D). Consistent with widespread neuronal necrosis, decreased expression of NeuN, a nuclear neuronal marker, mirrored a reduction in HMGB1 immunoreactivity throughout the contused cortex. Conversely, HMGB1 immunoreactivity

persisted in NeuN⁺ cells after TBI, suggestive of neuronal-specific release of HMGB1 into the extracellular space after TBI.

The functional importance of HMGB1 release after TBI was next assessed. HMGB1^{-/-} mice are non-viable; however, the triterpene compound, glycyrrhizic acid (GLY) neutralizes HMGB1 activity and permits functional testing after TBI (Mollica et al. 2007). GLY (600 mg/kg, i.p.) significantly reduced cerebral edema ($83.3 \pm 0.5\%$ BWC in TBI vs. $80.9 \pm 0.6\%$ in TBI+GLY; $p < 0.05$) (Figure 1E), indicative of a deleterious role for HMGB1 release after TBI. Consistent with this assertion, we correlated CSF concentrations of HMGB1 with poor neurological outcomes in subarachnoid hemorrhage patients (King et al. 2011). Taken together, NR2B-dependent excitotoxicity may initiate HMGB1 release and subsequent neurovascular injury.

Elevated HMGB1 in the CSF of TBI patients

HMGB1 was undetectable or lowly expressed within CSF from all nine normal pressure hydrocephalus control patients studied. In contrast, HMGB1 was elevated within CSF of 26 consecutive moderate/severe (Glasgow Coma Scale 3T-12T) TBI patients undergoing extraventricular drainage for elevated ICP, with highest expression detected over the first 72h (Figure 2). These findings suggest HMGB1 release was induced by trauma, rather than occurring as a secondary consequence of increased brain water. Notably, the temporal pattern of HMGB1 expression within CSF mirrored the presence of neuron-specific enolase (NSE), an intracellular, neuronal-specific marker. These findings are consistent with the release of HMGB1 from injured neurons following TBI.

Inhibition of microglial TLR4 reduces cerebral edema with an extended therapeutic window

TLR4, a transmembrane PRR, is a putative HMGB1 receptor (Hayakata et al. 2004). Increased TLR4 gene (1.4 ± 0.1 -fold increase; $p < 0.05$ vs. sham) and protein (3.0 ± 0.3 -fold; $p < 0.05$ vs. sham) expression were observed within the ipsilateral cortex between 6–24h after TBI (Figure 3A), mirroring the temporal pattern of edema development in this TBI model. As microglial activation paralleled edema development after closed head injury (Czigner et al. 2007), it is noteworthy that TLR4 expression was restricted to Iba1⁺ microglia/macrophages within the peri-contusional cortex (Figure 3B). In line with a detrimental role for TLR4 activation after TBI, BWC and edema volume were attenuated in C3H/HeJ mice, which contain a non-functional TLR4 gene (BWC: $80.1 \pm 0.8\%$; edema volume: 14.7 ± 1.3 mm³; $p < 0.05$ vs. sham), as compared to C3H/OuJ strain control mice after TBI (BWC: $82.5 \pm 0.9\%$; edema volume: 22.9 ± 1.8 mm³, $p < 0.05$ vs. sham) (Figure 4A, B). Similarly, pharmacological inhibition of TLR4 using a 4h post-treatment with 50 mg/kg VGX-1027 (VGX) significantly attenuated edema (TBI $83.9 \pm 0.5\%$ BWC vs. TBI+VGX: $81.9 \pm 0.5\%$; $p < 0.05$). In contrast, neither pre-treatment nor a 1h post-treatment with VGX effectively attenuated post-traumatic edema (data not shown). The beneficial effects on edema were lost after an 8h post-treatment (Figure 4C).

Cellular edema is associated with the expression of the astrocytic water channel, aquaporin-4 (AQP4) after TBI. Consistent with this notion, pericontusional AQP4

expression was reduced in C3H/HeJ mice (1.7 ± 0.3 -fold increase vs. sham; $p < 0.05$), as compared to C3H/OuJ mice (3.4 ± 0.3 -fold increase vs. sham) (Figure 4D). Similarly, 4h post-treatment with VGX attenuated post-traumatic AQP4 expression (1.6 ± 0.1 -fold increase vs. 2.2 ± 0.2 -fold increase after TBI; $p < 0.05$ vs. TBI) (Figure 4E), implicating TLR4 as a therapeutic target to modulate AQP4 expression.

Neuro-immune signaling mediates astrocytic AQP4 expression

The differential localization of TLR4 (microglia) and AQP4 (astrocytes) suggested cellular crosstalk may culminate in edema formation after TBI. Peri-contusional mRNA expression of IL-6, an immune modulator implicated in elevated ICP in TBI patients, was significantly increased (17.7 ± 3.2 -fold increase; $p < 0.01$ vs. sham) in C3H/OuJ mice (Figure 5A) as compared to C3H/HeJ mice (9.1 ± 2.5 -fold increase vs. sham, $p < 0.01$; $p < 0.05$ vs. C3H/OuJ TBI). Similarly, IL-6 protein expression was higher in C3H/OuJ mice, as compared to C3H/HeJ mice after TBI (995.8 ± 178.2 pg/ml in C3H/OuJ vs. 540.8 ± 53.44 pg/ml in C3H/HeJ; $p < 0.01$) after TBI (Figure 5B).

Further mechanistic delineation was provided by tissue culture studies showing conditioned media (CM) collected from NMDA-treated neurons increased the expression and release of IL-6 from C8-B4 microglia or from primary human microglia at 6h (Figure 6A, C). In contrast, CM collected from NMDA-treated neurons did not induce IL-6 release from EOC. 20 microglia, which are derived from C3H/HeJ mice and lack functional TLR4 (Figure 6B). In line with these observations, treatment with GLY (data not shown), immunoneutralization of HMGB1 activity in neuronal-CM (Figure 6A–C), or pharmacological inhibition of microglial TLR4 similarly reduced IL-6 release from C8-B4 microglial cells (Figure 7A) or from primary human microglia (Figure 7B) after treatment with neuronal-CM. Pre-treatment of neurons with concentrations of Ro25-6981 that attenuated excitotoxicity and HMGB1 release (Figure 1), limited IL-6 release by NMDA-treated neuronal-CM whereas pre-treatment with NVP-AAM077 had no significant effect (Figure 8A, B). Furthermore, either recombinant HMGB1 or lipopolysaccharide (LPS; specific exogenous TLR4 agonist) increased microglial IL-6 release in a TLR4-dependent manner, mimicking the effect of CM derived from necrotic neurons (Figure 9A). Notably, the ability of recombinant HMGB1 to stimulate IL-6 release was not due to endotoxin contamination, as co-addition of polymixin B had no inhibitory effect (data not shown). Consistent with these data, the TLR4 antagonist, VGX, reversed the stimulatory effect of both LPS and HMGB1 on IL-6 release in primary human microglia (Figure 9B).

Finally, addition of CM from HMGB1-stimulated microglia increased AQP4 expression in primary astrocytes, as compared to vehicle-stimulated microglial-CM (Figure 10A). Notably, IL-6 immunoneutralization within microglial-CM attenuated the AQP4 induction whereas exogenous IL-6 promoted AQP4 protein expression (Figure 10A, B). Together, these data implicate neuronal NR2B activation as an initiating factor in microglial activation after TBI.

DISCUSSION

Sustained elevations in ICP typically peak within the first days after TBI, while the patient is in the neuro-intensive care unit, and induces tissue hypoperfusion, brain herniation, and a poor clinical prognosis (Alberico et al. 1987; Miller et al. 1977). Unfortunately, efficacious treatment options to reduce mortality and improve long-term outcomes following TBI are lacking and represent a major unmet clinical need. The data presented herein identify a coordinated, innate immune signaling pathway involving neuronal-microglial-astrocytic communication and culminating in the development of cerebral edema.

Massive cellular necrosis correlates with the formation of cerebral edema after TBI. Excision of necrotic brain tissue alleviates elevated ICP and improves patient outcomes, suggesting a factor(s) downstream of necrotic cell death may initiate a detrimental signaling cascade after TBI. Over activation of NMDA-R due to excess glutamate release is widely recognized as an important cause of neuronal cell death, neuroinflammatory activation, and the development of secondary brain injury after stroke and TBI (Faden et al. 1989; Palmer et al. 1993); however, global NMDA-R antagonists are clinically associated with intolerable side effects, poor drug efficacy, narrow therapeutic windows, and interference with synaptic transmission (Ikonomidou and Turski 2002). Thus, elucidation of the mechanisms linking glutamate excitotoxicity with secondary neurological injury may provide therapeutic opportunities to improve outcomes without adversely altering normal brain function.

NMDA-R exist as a tetrameric protein receptor complex comprised of two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits that determine the biophysical properties (Cull-Candy and Leszkiewicz 2004). Interestingly, recent evidence suggests NMDA-R activation may induce either neuroprotection or neurodegeneration after TBI (Ankarcrona et al. 1995; Biegon et al. 2004). NR2A and NR2B are the predominant subunits within the adult forebrain; thus, we postulated the identity of the NR2 subunit might explain the paradoxical roles of NMDA-R after TBI. Consistent with this assertion, Ro25-6981, a highly selective NR2B antagonist, reduced brain edema whereas NVP-AAM077, a selective NR2A antagonist, was without effect. Consistent with a detrimental effect of NR2B activation, Ro25-6981 also attenuated neuronal excitotoxicity and reduced HMGB1 release in cultured neurons.

HMGB1 exhibited a predominant neuronal localization within the mouse cerebral cortex. Notably, decreased immunoreactivity for HMGB1 was temporally correlated with the loss of NeuN within the peri-contusional cortex, suggesting HMGB1 was released by injured neurons into the extracellular space after TBI. This possibility was further supported by the parallel accumulation of HMGB1 and NSE within the CSF of neurotrauma patients. These findings are potentially significant as acute elevations (<72h post-TBI) of NSE within CSF strongly correlated with deterioration to brain death in severe TBI patients (Bohmer et al. 2011). Consistent with this assertion, we first demonstrated that CSF content of HMGB1 correlated with poor neurological outcomes in subarachnoid hemorrhage patients (King et al. 2011). Finally, we identified a functional role for HMGB1 release following studies showing that the glycyrrhizic acid (GLY), an HMGB1 neutralizing compound used in the clinical management of liver disease (Mollica et al. 2007; Orlent et al. 2006), reduced brain

edema after TBI. Together, these findings support the notion that activation of NR2B promotes neuronal injury and increases neuronal HMGB1 release to augment brain swelling, although the mechanisms remain unstudied.

HMGB1 is a prototypical DAMP, activating the innate immune system after tissue injury. TLR4, an important component of the innate immune system, is a putative receptor for HMGB1 (Parker et al. 2007), although a functional role for TLR4 after TBI remains unstudied. A time dependent increase in microglial TLR4 expression occurred within the pericontusional cortex, exhibiting both a temporal and spatial overlap with edema development. This localization pattern is consistent with a report suggesting HMGB1 activated microglia, the resident cerebral innate immune cells, and induced progressive neurodegeneration (Gao et al. 2011). Coupled with reports correlating immune activation with the development of post-traumatic edema, our data suggest the release of neuronal HMGB1 may induce microglial activation.

Functionally, C3H/HeJ mice, which harbor a point mutation in the TLR4 gene, exhibited a reduction in post-traumatic edema, as compared to C3H/OuJ strain control mice. Although interesting in pre-clinical settings, genetic inhibition possesses limited translational value. VGX-1027 (VGX), an orally active, small molecule TLR4 inhibitor, is in Phase I clinical trials for the treatment of inflammatory diseases (Stojanovic et al. 2007). Whereas pre-treatment or a 1h post-treatment with 50 mg/kg VGX was ineffective, a 4h post-treatment significantly attenuated edema. This clinically-implementable therapeutic window corresponded to the delayed induction of TLR4 at 6h post-TBI and was consistent with peak serum concentrations at 2h after intraperitoneal administration of VGX (Stosic-Grujicic et al. 2007).

Both vasogenic and cellular edema increase brain swelling (Klatzo 1967); however, recent work suggests cellular edema predominates in the acute phase after TBI (Unterberg et al. 2004; Youn et al. 2006). Astrocytic swelling, a characteristic feature of cellular edema, commences within hours of TBI in humans (Ito et al. 1996; Kimelberg 1992; Narayan et al. 2002) and is associated with increased expression of AQP4 water channels (Badaut et al. 2002; Gunnarson et al. 2008; Nase et al. 2008). The roles of AQP4 remain controversial (Papadopoulos et al. 2004; Yang et al. 2008), yet AQP4-deficient mice exhibited less edema and reduced mortality after ischemic stroke or acute water intoxication (Manley et al. 2000). Unfortunately, antagonists of AQP4 do not currently exist; thus, an improved mechanistic understanding of AQP4 regulation may provide a target to limit edema development. Recent work from our laboratory identified curcumin, a non-specific TLR4 inhibitor (Youn et al. 2006), reduced AQP4 expression and cerebral edema after TBI (Laird et al. 2010). Consistent with this finding and in line with a previously unexplored regulatory role for TLR4 activation in AQP4 expression, C3H/HeJ mice exhibited attenuated AQP4 expression within the peri-contusional cortex, as compared to C3H/OuJ mice. This finding was further supported by the observation that a 4h post-treatment with 50 mg/kg VGX, a dose that limited cerebral edema, similarly reduced AQP4 expression. These data raise the possibility that TLR4 inhibition reduces edema via a reduction in AQP4 expression; however, we cannot exclude the possibility that TLR4 activation directly promotes cell swelling, which in

turn, leads to subsequent AQP4 expression. This important question will be the subject of future work by our laboratory.

Inhibition of TLR4 attenuated the expression of AQP4, an astrocytic water channel implicated in the development of cellular edema, although the mechanism whereby microglia communicate with astrocytes remained unresolved. Elevated CSF and serum concentrations of interleukin-6 (IL-6), a microglial-derived cytokine, strongly correlated with neuronal injury and elevated ICP after TBI in humans (Hergenroeder et al.; Pleines et al. 2001). In the present study, TBI-induced IL-6 gene and protein expression were attenuated in C3H/HeJ mice, as compared to C3H/OuJ strain control mice. Together, our pre-clinical modeling and patient-derived data suggested neuronal-microglial-astrocytic signaling mediates edema development after brain injury; however, to better define the cellular mechanisms, we next employed tissue culture modeling experiments.

Microglial activation is an important initial response to neuronal injury (Lai and Todd 2008; Nimmerjahn et al. 2005; Schafer et al. 2013). Our data in a pre-clinical TBI model suggest the NR2B-dependent induction of neuronal excitotoxicity induces HMGB1 release. Interestingly, conditioned media collected from NMDA-stimulated mouse cortical neurons increased the release of IL-6 from C8-B4 microglia or from primary human microglia. Notably, no IL-6 stimulation was observed in EOC.20 microglia, which are derived from C3H/HeJ mice and lack a functional TLR4 gene. Immunoneutralization of HMGB1 within neuronal-conditioned media completely blocked the stimulatory activity with respect to IL-6 release, implicating HMGB1 as a key mediatory factor and suggesting a possible role for TLR4 in this effect. Subsequently, the addition of VGX to neuronal-conditioned media also reversed the IL-6 stimulatory activity. Direct addition of either lipopolysaccharide (LPS), a specific TLR4 agonist, or recombinant HMGB1 induced TLR4-dependent IL-6 release from C8-B4 microglia and primary human microglia, but not from EOC.20 cells, further indicating that neuronal HMGB1 may be essential to trigger microglial IL-6 release. Finally, we observed that conditioned media from HMGB1 treated microglial stimulated AQP4 expression in primary human astrocytes via a IL-6 dependent mechanism.

In summary, we identified a novel role for HMGB1-TLR4 signaling in the regulation of AQP4 and in the promotion of cerebral edema, a primary cause of patient mortality after brain injury (see Figure 11 for summary mechanism). We implicated NR2B is an important initiating factor in promoting cerebral innate immune responses, linking acute excitotoxicity with secondary neurological injury. Finally, we identified a mechanism whereby microglial-astrocytic interactions increase acute neurovascular injury, using pre-clinical modeling and human tissue cultures. Taken together, these translational research findings provide an important mechanistic framework for future therapeutic development.

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Figure 1. NR2 inhibition reduces HMGB1 release and cerebral edema

(A) Administration of the NR2B antagonist, Ro25-6981 (Ro; 6 mg/kg, i.p.), 15 minutes prior to TBI attenuated cerebral edema at 24h post-TBI in mice. Pre-treatment with the selective NR2A antagonist, NVP-AAM007 (NVP; 5 mg/kg, i.p) did not reduce edema, as assessed by brain water content. Edema was quantified in the ipsilateral (black bars) and contralateral cortices (grey bars). (B) Mouse cortical neurons were treated with NVP (400 nM) or Ro (500 nM) in the presence of NMDA (50 μ M) + 10 μ M glycine for 20 minutes. Cell viability was assessed 24h later. (C) Mouse cortical neurons were treated as in (B, C). Whole cell lysates and conditioned media (6 pooled wells/sample) were collected for Western blotting of HMGB1 (left panel). Densitometric quantification of HMGB1 was performed in cellular lysates (black bars) and in conditioned media (grey bars) (right panels). (D) Cellular localization of HMGB1 (red) and NeuN (green), a neuron-specific nuclear marker, in the mouse brain using confocal microscopy. NeuN and HMGB1 were co-localized throughout the cortex of sham mice (top panels). In contrast, immunoreactivity of both NeuN and HMGB1 were decreased in the pericontusional cortex by 24h post-TBI (middle panels) whereas the contralateral cortex after TBI appeared as in sham mice (bottom panels). A high magnification image (right panel), further demonstrates the loss of HMGB1 immunoreactivity in NeuN⁺ neurons after TBI. (E) Glycyrrhizic acid (GLY; 600 mg/kg, i.p., 15 minute pre-treatment) reduced brain water content at 24h post-TBI in the ipsilateral cortex (black bars), without affecting edema in the contralateral cortex. For all animal studies, data are mean $\pm$ SEM from 6–8 mice/group. For cell culture studies, data are mean $\pm$ SEM from at least three independent cultures (n=6 wells/group in each trial). Data were analyzed by One-way ANOVA. *p<0.05, **p<0.01.

DAYS POST-TBI	HMGB1 (ng/mL)
Non-Traumatic Control	0.80 ± 0.51
Day 1	6.31 ± 1.16
Day 2	4.77 ± 1.34
Day 3	4.13 ± 0.90
Days 4-7	2.71 ± 1.08

Figure 2. Elevated HMGB1 in the CSF of TBI patients

CT scan (left panel) and temporal pattern of HMGB1 and NSE, a neuron-specific marker, content in the CSF (right panel) from a representative TBI patient (19/M, motor vehicle accident), as assessed by Western blotting. HMGB1 was detected in the CSF of 26 consecutive moderate/severe TBI patients. In contrast, HMGB1 was not observed in CSF from any of the 9 normal pressure hydrocephalus control patients.

Figure 3. Microglial TLR4 expression after TBI

Temporal pattern of TLR4 (A) mRNA and protein expression in the pericontusional cortex after TBI. qRT-PCR data from sham-operated (black bars) or TBI (grey bars) mice were normalized to RPS3, a housekeeping gene that was unaffected by TBI. A representative Western blot of TLR4 expression is depicted in the lower panel from pooled samples. (B) TLR4 (red) co-localizes with the microglial/macrophage specific marker, Iba1 (green), as assessed by confocal microscopy. Merged images are shown in the right panels. Data were analyzed in sham-operated mice or in the ipsilateral (IPSI) or contralateral (CONTRA) cortices at 24h post-TBI. Data are representative of 6 mice/group. Scale bar = 20 μ m.

Figure 4. TLR4 activation promotes brain edema

(A) Brain water content was assessed in the pericontusional cortex of C3H/OuJ (wild-type) or C3H/HeJ (TLR4 mutant) mice at 24h post-TBI (grey bars). Basal brain water content was not different between the strains in sham-operated mice (black bars). (B) T2-weighted (T2W) images were collected at 24h post-TBI from C3H/OuJ or C3H/HeJ mice. A representative serial brain scan from each genotype is depicted (left panels). Edema volume was quantified and represented as mean ± SEM. For panel A&B, n=10 mice/group. (C) A 4h post-treatment (4h Post-Tx) with the selective TLR4 antagonist, VGX-1027 (VGX; 12.5–50 mg/kg, i.p.), reduced brain water content in the ipsilateral (black bars) cortex at 24h post-TBI. In contrast, VGX did not significantly affect edema under basal conditions or in the contralateral cortex (grey bars). (D) Expression of AQP4 following TBI in C3H/OuJ and C3H/HeJ mice, as depicted in a representative Western blot (left panel). β-actin served as a loading control and was used to normalize Western blotting data (right panel). *p<0.05, ***p<0.001 vs. sham. (E) Post-treatment with VGX (50 mg/kg, i.p.) reduced post-traumatic AQP4 protein expression by Western blotting. A representative blot is provided (top panel) with quantified group data. *p<0.05. For all studies, data are mean ± SEM from 10–12 mice/group.

Figure 5. TLR4-dependent cortical IL-6 production after TBI

Quantification of peri-contusional IL-6 (A) gene and (B) protein expression in C3H/OuJ and C3H/HeJ mice at 12h post-TBI, as assessed by qRT-PCR and EIA, respectively. qRT-PCR data were normalized to RPS3. EIA data are expressed as pg/mL. Data are mean $\pm$ SEM from 8 mice/group. * $p < 0.05$, ** $p < 0.01$.

Figure 6. Neuronal HMGB1 release induces TLR4-dependent IL-6 production in microglia
Release of IL-6 by (A) mouse C8-B4 microglial cells, (B) EOC.20 microglial cells, or (C) by primary human microglia following stimulation with serum free control media (VEH) or with neuronal conditioned media (neuronal-CM) collected 24h after treatment with vehicle or 50 μ M NMDA. NMDA-treated neuronal-CM increased IL-6 release, as assessed by EIA, in C8-B4 cells and in primary human microglia, but not in EOC.20 microglia that lack a functional TLR4. Antibody immunoneutralization of HMGB1 (HMGB1 nAb) within neuronal-CM eliminated the stimulatory effect of NMDA treated neurons on microglia IL-6 production. Data are mean $\pm$ SEM from at least three independent experiments (n=6/experiment; *p<0.05).

Figure 7. VGX-1027 attenuates the stimulatory effect of neurons on microglial IL-6 release
 Treatment with VGX-1027 (VGX; 25 μ M) eliminated the stimulatory effect of NMDA-treated neuronal-CM in (A) mouse C8-B4 microglial cells and (B) in primary human microglia. Data are mean $\pm$ SEM from at least three independent experiments (n=6/experiment; *p<0.05).

Figure 8. Ro25-6981 attenuates the stimulatory effect of neurons on microglial IL-6 release NR2B antagonism attenuates the release of IL-6 by (A) mouse C8-B4 microglial cells or (B) primary human microglia following stimulation with serum-free control media (VEH) or with neuronal conditioned media (neuronal-CM) collected 24h after treatment with vehicle or 50 μ M NMDA. Selective NR2B inhibition with 500 nM Ro25-6981 at the time of neuronal NMDA-treatment attenuates IL-6 release whereas NR2A inhibition with 400 nM NVP-AAM077 was ineffective. Data are mean $\pm$ SEM from three independent experiments (n=6/experiment; *p<0.05).

Figure 9. Activation of TLR4 induces IL-6 release from microglia

(A) The exogenous TLR4 agonist, lipopolysaccharide (LPS), or recombinant HMGB1 (100 ng/mL) stimulated IL-6 release from C8-B4 microglial cells (top, left panel) or from primary human microglia (bottom panel). In contrast, neither LPS nor HMGB1 induced IL-6 release in EOC.20 microglia (top, right panel). (B) VGX-1027 (VGX; 25 μ M; grey bars), attenuated the stimulatory effect of either LPS or HMGB1 treatment on IL-6 release in primary human microglia. Data are mean $\pm$ SEM from three independent experiments (n=6/experiment; *p<0.05).

Figure 10. Microglial derived IL-6 stimulates AQP4 expression in human astrocytes

(A) Conditioned media from primary human microglia (microglial-CM) after HMGB1 treatment stimulated AQP4 expression in primary human astrocytes after a 12h treatment, as compared to vehicle-treated cultures. Microglia were treated for with 100 ng/ml HMGB1 for 20 minutes followed by a wash out. Defined serum free media was then used for the collection of microglial-CM. Immunoneutralization of IL-6 within HMGB1-stimulated microglial-CM eliminated the stimulatory effect on AQP4 expression in primary human astrocytes. (B) Recombinant IL-6 stimulated AQP4 protein expression, as assessed by Western blotting, in primary astrocyte cultures. * $p < 0.05$ vs. other groups. Data are mean $\pm$ SEM from five independent experiments ($n=3$ /experiment).

Figure 11. Summary diagram of HMGB1-TLR4 signaling in the development of cerebral edema following TBI

A detailed description of the mechanism is provided in the Discussion.