Experimental Neurology 320 (2019) 112979

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research paper

Targeting high-mobility group box protein 1 (HMGB1) in pediatric traumatic brain injury: Chronic neuroinflammatory, behavioral, and epileptogenic consequences

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ARTICLE INFO

Keywords:
High-mobility group box protein-1 (HMGB1)
Glycyrrhizin
Inflammation
Post-traumatic epilepsy
Seizures
Pediatric
Neurotrauma
Traumatic brain injury
Behavior

ABSTRACT

High mobility group box protein-1 (HMGB1) has been implicated as a key mediator of neuroinflammation and neurodegeneration in a range of neurological conditions including traumatic brain injury (TBI) and epilepsy. To date, however, most studies have examined only acute outcomes, and the adult brain. We have recently demonstrated HMGB1 release after experimental TBI in the pediatric mouse. This study therefore examined the chronic consequences of acute HMGB1 inhibition in the same model, to test the hypothesis that HMGB1 is a pivotal mediator of neuropathological, neurobehavioral, and epilepsy outcomes in pediatric TBI. HMGB1 was inhibited by treatment with 50 mg/kg i.p. Glycyrrhizin (Gly), compared to vehicle controls, commencing 1 h prior to moderate TBI or sham surgery in post-natal day 21 mice. We first demonstrated that Gly reduced brain HMGB1 levels and brain edema at an acute time point of 3 days post-injury. Subsequent analysis over a chronic time course found that pediatric TBI resulted in short-term spatial memory and motor learning deficits alongside an apparent increase in hippocampal microglial reactivity, which was prevented in Gly-treated TBI mice. In contrast, Gly treatment did not reduce the severity of evoked seizures, the proportion of animals exhibiting chronic spontaneous seizure activity, or cortical tissue loss. Together, our findings contribute to a growing appreciation for HMGB1's role in neuropathology and associated behavioral outcomes after TBI. However, further work is needed to fully elucidate the contribution of HMGB1 to epileptogenesis in this context.

1. Introduction

Traumatic brain injury (TBI) is a major global public health problem and a leading cause of mortality and morbidity (Langlois, 2000), particularly in children under the age of 5 (Adelson et al., 2003; Thurman, 2016). Moderate and severe TBI in young children often has negative consequences across a wide spectrum of neurobehavioral functions, from sensorimotor to cognitive and psychosocial deficits (Anderson et al., 2005b; Anderson et al., 2012; Catroppa et al., 2012). Contributing to morbidity, post-traumatic epilepsy (PTE), defined as spontaneous, recurrent chronic seizures following a head injury, develops in an estimated 10–20% of children after severe TBI (Appleton and Demellweek, 2002b; Barlow et al., 2000; Chiaretti et al., 2000). Together, these chronic outcomes have a significant impact on a survivor's quality of life after childhood TBI (Anderson et al., 2010; Di Battista et al., 2012; Stancin et al., 2002).

Following the initial impact of a TBI, secondary injury mechanisms including excitotoxicity, oxidative stress, mitochondrial dysfunction, and neuroinflammation are initiated (Maas et al., 2008; Potts et al., 2006b; Schouten, 2007). Such neuropathological processes contribute

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https://doi.org/10.1016/j.expneurol.2019.112979
Received 28 March 2019; Received in revised form 29 May 2019
Available online 21 June 2019
0014-4886/

to the progression of secondary brain damage (Mazzola and Adelson, 2002), and are influenced by developmental age at the time of injury, with a younger brain typically showing increased vulnerability (Bittigau et al., 2003; Claus et al., 2010; Potts et al., 2006a). A complex milieu of inflammatory pathways are initiated within minutes after a TBI (Morganti-Kossmann et al., 2019), and increasing evidence implicates this neuroinflammatory response in epileptogenesis, the process by which PTE develops (Webster et al., 2017).

One of the key instigators and amplifiers of inflammation is high-mobility group box protein 1 (HMGB1), a DNA-binding protein that is both actively secreted by inflammatory cells and expelled by necrotic and injured cells (Scaffidi et al., 2002; Wang et al., 1999). Once released into the extracellular milieu, HMGB1 interacts with various cell-surface receptors to promote immune cell chemotaxis and pro-inflammatory cytokine release, including release of additional HMGB1 to create a self-perpetuating cycle (Bianchi and Manfredi, 2007; Harris et al., 2012). Consequently, HMGB1 has been implicated in promoting neuroinflammation, cerebral edema, and associated neurodegeneration after a range of experimental models of CNS insults (Gong et al., 2012; Kim et al., 2012; Li et al., 2019; Ohnishi et al., 2011; Okuma et al., 2012a; Sun et al., 2013). For example, in a fluid percussion model of TBI in adult rats, treatment with an anti-HMGB1 neutralizing monoclonal antibody was found to prevent HMGB1 translocation from the nucleus, preserve blood-brain barrier (BBB) integrity, and reduce edema (Okuma et al., 2012a). It is worth noting, however, that monoclonal antibodies have some limitations for clinical translation such as their potential for immunotoxicity and difficulties in crossing the BBB (Descotes, 2009). These previous studies have largely focused on adult animals, and acute outcome measures.

Accumulating evidence suggests that CNS injuries during early childhood can have differential consequences as a result of an age-dependent inflammatory response (Claus et al., 2010; Potts et al., 2006a; Webster et al., 2019). We have recently demonstrated an acute elevation in HMGB1 levels in serum after experimental TBI in pediatric mice, as well as localized perilesional release in brain tissue, to a greater extent in pediatric mice compared to in adult brain-injured mice (Webster et al., 2019). In children with TBI, increased HMGB1 in CSF is correlated with poor functional outcomes at 6 months (Au et al., 2012). Further, several studies have suggested that HMGB1 is a predictor of neuroinflammation and the extent of brain damage after prenatal and perinatal injuries (Chen et al., 2019; Zhang et al., 2016). These findings suggest that targeting HMGB1 may have great therapeutic potential in the immature injured brain.

In this study, we hypothesized that HMGB1 inhibition would yield neuroprotection after pediatric TBI, across a range of chronic neurological, neurobehavioral and neuropathological outcomes. In particular, we focused on the development of PTE, as previously characterized in this model (Semple et al., 2017b), based on emerging literature supporting a role of HMGB1 in the initiation and propagation of seizures (Paudel et al., 2019). Inhibition of HMGB1 was achieved by use of Glycyyrhizin (Gly), a naturally-occurring triterpene originating from liquorice root (*Glycyrrhiza glabra*) that binds directly to both box domains of the HMGB1 protein to inhibit its phosphorylation, translocation and secretion (Kim et al., 2012; Mollica et al., 2007). In comparison to many other approaches to inhibit HMGB1, Gly is reported to have few off-target effects, is already in clinical use for chronic hepatitis C, and is very well tolerated (Arase et al., 1997). Considering neurological conditions in which HMGB1 has been implicated, Gly treatment has been explored in adult experimental models of TBI, stroke, spinal cord injury, intracerebral hemorrhage and epilepsy (Gong et al., 2012; Kim et al., 2012; Li et al., 2015; Ohnishi et al., 2011; Okuma et al., 2014; Sun et al., 2013).

2. Methods

2.1. Mice

Male C57Bl/6 mice were obtained from the on-site breeding colony at the Florey Institute of Neuroscience and Mental Health animal facility in Melbourne, generated from breeding pairs purchased from the Animal Resource Centre (Perth, Australia). Mice were aged 3 weeks (p21–22) at the time of brain injury. All mice were group housed in specific pathogen free conditions under a 12 h light/dark cycle, with access to food and water ad libitum for the duration of the experiment. All experimental procedures were approved by the University of Melbourne Animal Ethics Committee (#15-016-UM) and conducted in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Experimental design

In the initial acute recovery study, a subset of mice subjected to experimental TBI were randomly assigned to treatment groups of vehicle (0.9% sterile saline) (*Veh*), Glycyrrhizin initiated post-injury (*Gly Post*), or Glycyrrhizin initiated prior to injury (*Gly Pre*). Gly (Sigma Aldrich) was administered at 50 mg/kg i.p. at 1 h, 6 h, 1 d and 2 d post-injury, with an additional 1 h pre-injury treatment for the *Gly Pre* group only (Fig. 1a). Mice were euthanised at 3 d post-injury and tissue collected fresh for edema analysis, snap frozen and homogenized for Western blot, or perfusion-fixed and sectioned for immunofluorescence.

In the subsequent chronic recovery study, mice were randomly assigned to TBI or sham surgery, then to receive either vehicle or Glycyrrhizin treatment, for a total of four groups: *Sham Veh, Sham Gly, TBI Veh*, or *TBI Gly*. Gly was administered at 50 mg/kg i.p. initiated at 1 h pre-injury, at 1 h, 6 h post-injury, then once daily for 7 additional days for a total treatment period of 1 week (Fig. 2a). We compared pre- versus post-injury treatment paradigms based upon extensive literature in other injury models (e.g. Kainic acid seizures, spinal cord ischemia, fluid percussion and weight drop TBI) in which some investigators have demonstrated efficacy when treatment is initiated prior to CNS injury, while others showed neuroprotection with post-injury treatment regimens (Gong et al., 2012; Gu et al., 2014; Luo et al., 2013; Luo et al., 2014; Okuma et al., 2014).

The dose and time of Gly treatments were chosen based on previous studies, in rodent models of kainic acid-induced seizures and intracerebral hemorrhage, in which this treatment regime results in reduced HMGB1 levels, edema and neuroprotection (Luo et al., 2013; Luo et al., 2014; Ohnishi et al., 2011). Gly has previously been reported to show full stability for at least 2 h after i.p. administration in rodents, with full metabolic breakdown by 12–24 h (Akao, 2000; Ploeger et al., 2001; Yamamura et al., 1995). Gly and its metabolite Glycyrrhetinic acid have been shown to cross the intact BBB in vivo (Tabuchi et al., 2012). While most previous studies utilizing Gly have considered only acute time points, we herein extended treatment until 7 days post-injury based on our understanding of the temporal nature of the neuroinflammatory response to TBI, as well as evidence of HMGB1 release from injured tissue until at least 3 days post-injury (Webster et al., 2019).

Mice were allowed to recover for a 3 m period before undergoing a battery of behavioral tests, analysis of spontaneous and evoked seizures as indicators of PTE, and then euthanasia by transcardial perfusion at 6 m post-injury, with tissue collected for histological and immunofluorescence analysis. All analyses were conducted by investigators blinded to group allocation.

2.3. Traumatic brain injury (TBI) model

Mice were randomly assigned to receive either a moderate TBI or a sham surgery. A controlled cortical impact was performed as previously

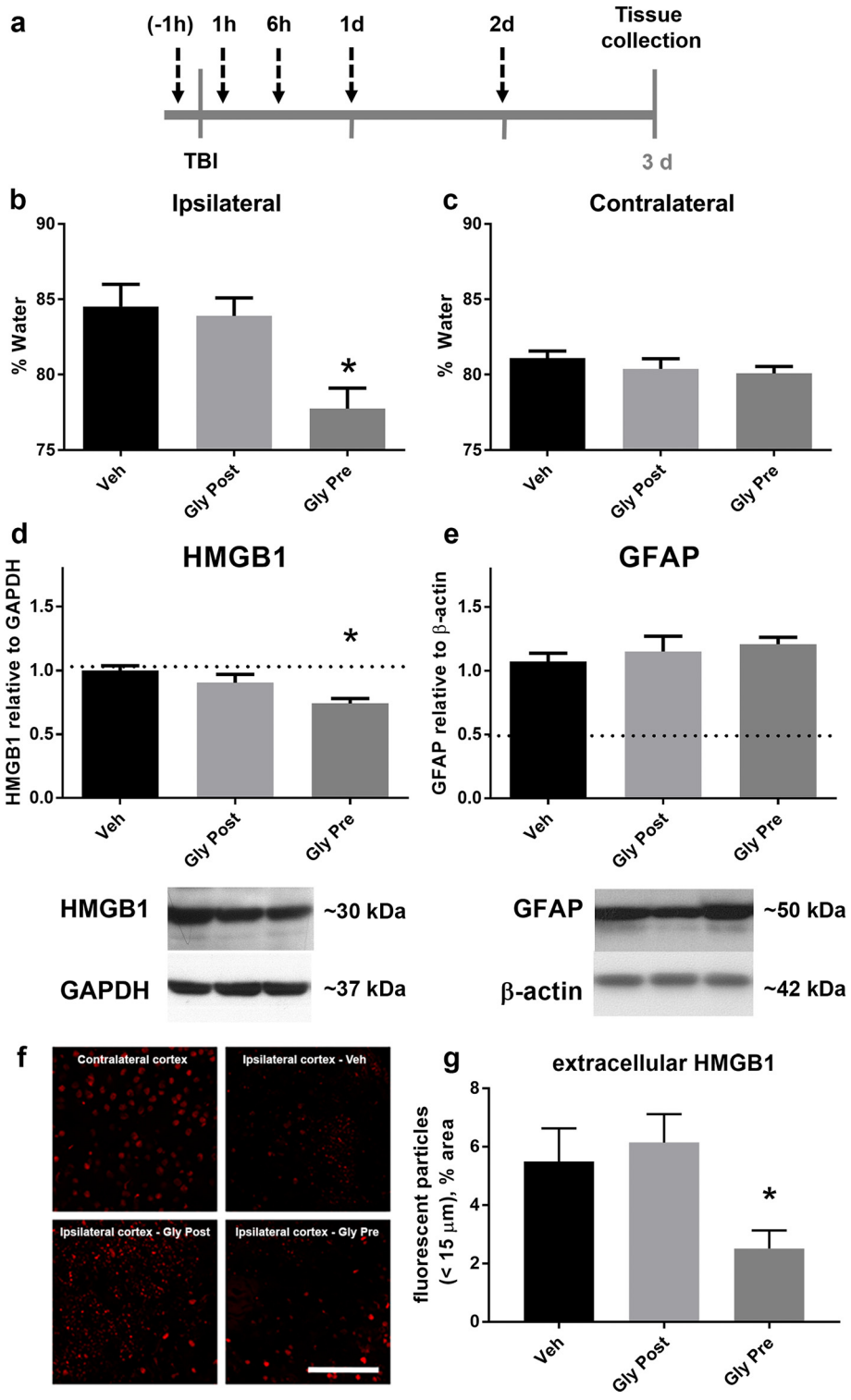

Fig. 1. Experimental timeline for pilot study, comparing treatment regimes in which Gly (50 mg/kg i.p.) was initiated either 1 h prior or 1 h after TBI in young mice, versus vehicle-treated TBI controls (a). All animals received additional treatments at 6 h, 1 d and 2 d post-injury before tissue collection at day 3. Quantification of percentage water content in tissue ipsilateral and contralateral to the injury (b, c) found that Gly ameliorated edema only when administered pre-injury (one-way ANOVA, $*p < .05$ post-hoc compared to vehicle; $n = 6–7$/group). Dotted line indicates mean level from naïve tissue samples. Western blotting (d, e) revealed a significant reduction of HMGB1 protein at 3 d post-injury in Gly Pre mice compared to vehicle treatment, but no such reduction in *Gly Post* mice (one-way ANOVA, $*p < .01$ post-hoc compared to vehicle; n = 6–7/group). Representative blots are illustrated below alongside loading controls GAPDH or β-actin (d, e), with molecular weights of detected bands as noted. Immunofluorescence staining for HMGB1 was also performed at 3 d post-injury (f, g), with quantification of extracellular HMGB1 revealing a localized reduction in the cortex of *Gly Pre* mice (one-way ANOVA, *p < .05 post-hoc compared to *Gly Post*). Data expressed relative to contralateral levels ($n = 5$/group).

described (Semple et al., 2017b; Tong et al., 2002), using a custom-built electrical cortical impactor fitted with a 3 mm tip (software controlled by LinMot-Talk 1100, Spreitenbach Switzerland) (Dent et al., 2015; Karve et al., 2016). Mice were positioned in a stereotaxic frame for all surgical procedures and administered s.c. 0.05 mg/kg buprenorphine for analgesia. Under isoflurane anesthesia, the skull was exposed by midline incision and a 4 mm diameter circular craniotomy located laterally over the parietal lobe, midway between Bregma and Lambda, was performed using a micro-drill to expose the intact dura of the brain. The injury was generated using a 3.0 mm convex tip, with a velocity of 4.5 m/s, and penetration depth of 1.5 mm for a sustained depression of 150 ms. These impact parameters typically result in moderate swelling and bleeding immediately following impact, which was observed in all injured mice. Following impact, the scalp was closed with sutures and each animal was administered 0.5 ml isotonic saline s.c. to prevent dehydration. Sham surgery consisted of all procedures with the exception of the actual traumatic impact. Following surgery, mice were individually housed in heat chambers and allowed to recover for 30–60 min before returning to group housing. Body weights were monitored daily for the first week after injury, then weekly thereafter.

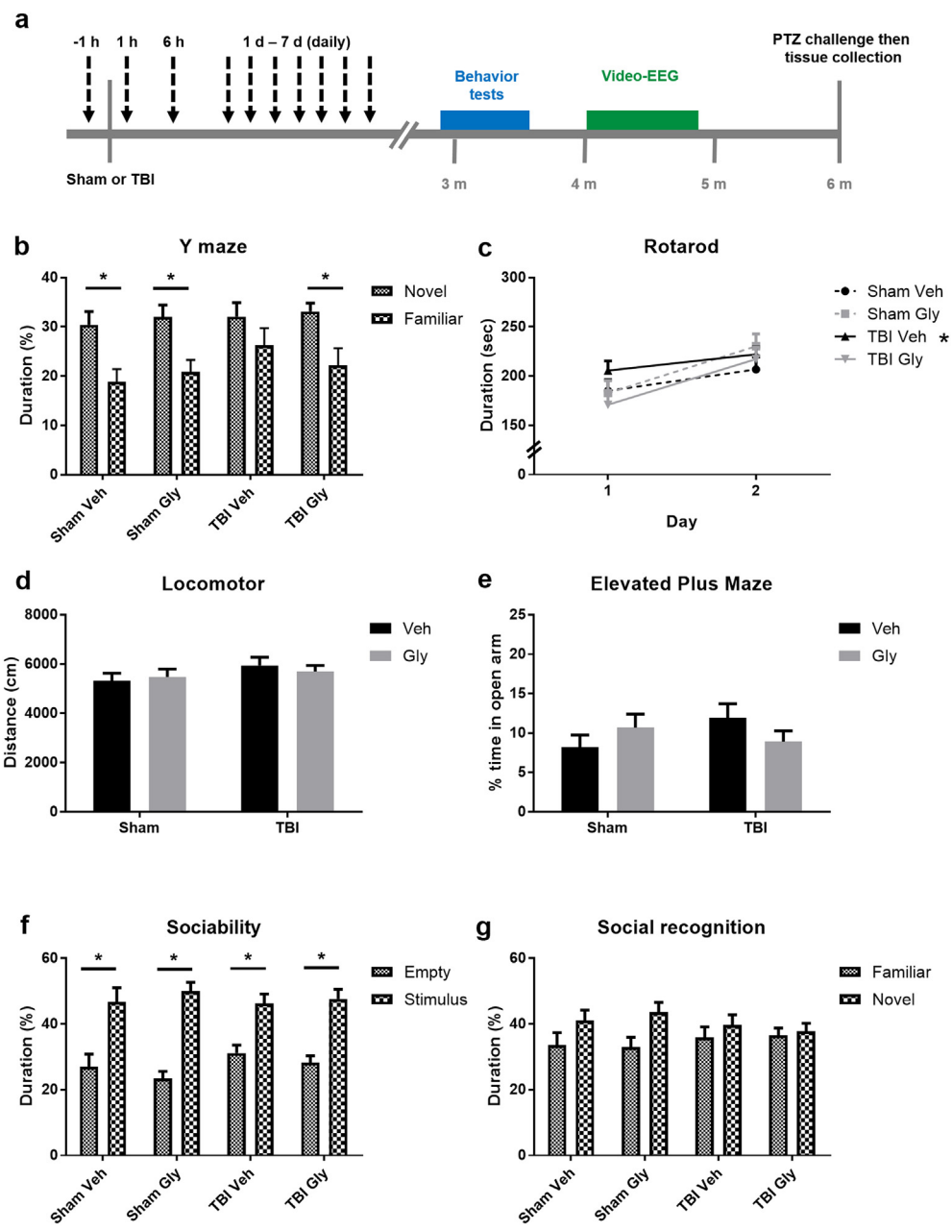

Fig. 2. Experimental timeline for chronic time course. Gly (50 mg/kg i.p.) or vehicle was administered 1 h prior to TBI or sham surgery, then 1 h, 6 h and daily thereafter until 7 d (a). Behavior was assessed at 3 m post-injury. The Y maze revealed a short-term spatial memory deficit in TBI Veh mice but not *TBI Gly* mice or sham mice (b; *p < .05, 2-way ANOVA post-hoc). The rotarod detected an improvement in motor performance between d 1 and 2, in all groups except *TBI Veh* (c; *p < .05 post-hoc). No effects of TBI or Gly treatment were observed in tests for locomotor activity (d), anxiety-like behavior (e), sociability (f) or social recognition (g).

Some mice displayed acute seizures within 1 h post-injury. Six animals died within this period (< 1 h post-injury), likely attributed to these acute seizures, and were not included in any analyses. Two *TBI Veh* and one *TBI Gly* mice survived their acute seizure and were included in subsequent analyses as they were not observed to have any subsequent seizures within the first week, nor significantly greater weight loss compared to animals that did not exhibit an acute seizure.

2.4. Edema analysis

From the acute study (Fig. 1), the contralateral and ipsilateral brain hemispheres (excluding the pericontusional cortex) were collected from $n = 6–7$/group and weighed immediately ('wet weight'). Tissue was dried in a 100 °C oven overnight then weighed again ('dry weight') for the calculation of the percentage water content (Sun et al., 2017).

2.5. Western blot

From the acute study, the ipsilateral pericontusional cortex was snap frozen upon collection (n = 6–7/group) and then manually homogenized followed by incubation in RIPA buffer containing protease and phosphatase inhibitors (Sigma Aldrich) for 10 min (Alcaraz et al., 1990). Total protein content in the supernatant was quantified by BCA assay (Pierce BCA quantification kit), and all samples were diluted to 1 mg/ml. Ten μg of protein per sample was combined with loading dye then loaded onto a 7.5% polyacrylamide gel, for separation by SDS-PAGE using a Bio-Rad Mini-Protean apparatus. Samples were then transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% skim powdered milk and incubated with one of the following primary antibodies: anti-HMGB1 (#ab18256 Abcam, 1:1000 for 24 h), anti-GFAP (#Z033401-2, Dako 1:10,000, for 24 h) and anti-CD68 (#ab31630 Abcam 1:1000, for 7 d). Membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (#ab97051, Abcam, 1:60000 for 1 h) or rabbit anti-goat IgG

(#P0449 Dako, 1:2000 for 1.5 h) secondary antibodies. Band density was quantified using NIH ImageJ software, with values normalized to levels of house-keeping protein GAPDH (#sc-48166, Santa Cruz, 1:1000) or β-actin (#ab8227, Abcam, 1:1000). Values from naïve age-matched mice, collected from a previous study, are indicated graphically for reference by a dotted line.

2.6. Acute HMGB1 immunofluorescence

To examine localized acute release of HMGB1 from neurons into the extracellular space after pediatric TBI, a subset of mice ($n = 5$/group) were perfusion-fixed at 3 days post-injury, paraffin-embedded and sectioned for staining. Mice were euthanized by overdose with sodium pentobarbitone for transcardial perfusion with 0.9% saline followed by 4% PFA. Brains were extracted whole and post-fixed overnight in 4% PFA, then transferred to a 70% ethanol solution for 3–7 days and then embedded in paraffin. Coronal sections were collected to span the parietal lobes (Bregma −0.05 mm to −0.35 mm, 7 μm thick) by the Biomedical Histology Facility at the University of Melbourne. Two sections per brain (280 μm apart) containing the core of the brain lesion were dewaxed by immersion in xylene and decreasing ethanol concentrations, followed by microwave antigen retrieval in 10 mM citrate buffer containing 0.5% Tween-20. Sections were blocked with 10% normal donkey serum (NDS) and 0.1% Triton-X100 in phosphate buffered saline (PBS), then incubated in a 5% NDS/0.1% Triton-X100/PBS solution overnight at 4 °C with a rabbit polyclonal anti-HMGB1 antibody (#ab18256, Abcam, 1:1000). On day 2, a 1 h room-temperature incubation with donkey anti-rabbit AF 594 (Invitrogen) was followed by incubated with 0.3% Sudan Black to reduce tissue auto-fluorescence, the nuclear counterstaining with DAPI (4′, 6-diamidino-2-phenylindole), and cover-slipping with Dako fluorescent mounting medium.

Extracellular HMGB1 protein was quantified as previously described (Webster et al., 2019). In brief, 20 x images were captured using a Nikon Ti-Eclipse microscope and CMOS Andor Zyla camera, of the cortical lesion core and equivalent region from the contralateral neocortex, at a set exposure time. Images were calibrated then thresholded in ImageJ to a predetermined limit to create a mask. Using the analyze particle function, quantification of fluorescence was limited to particles 0–15 μm diameter to omit HMGB1 nuclear staining. The percent fluorescent area was expressed relative to the contralateral cortex (baseline) fluorescence for each section.

2.7. Behavior testing

Behavior tests were performed ~3 months post-injury, in a dedicated mouse behavioral suite, staggered over four cohorts with final $n = 12$–15 per group. One *Sham Veh* mouse had aberrant behavior during the testing (unusual inactivity) and was excluded from all analyses due to inability to perform. Mice were separated into individual cages during the week prior to testing, then acclimatized to the testing room for at least 30 min prior to the testing.

Locomotor activity was assessed in individual Activity Monitor cells (Med Associates, Inc.) for a 30 min period (Semple et al., 2017b). Mice were allowed free exploration of the activity cell arena, and total distance travelled was tracked as a measure of general activity. Anxiety-like behavior was evaluated using an Elevated Plus Maze, based on the predisposition of mice to avoid open spaces (Semple et al., 2015b; Shultz et al., 2014). Automated tracking software (TopScan, Clever Sys) recorded distance moved and time spent in the open versus closed arms. Short term spatial recognition and learning was assessed with the Y maze, in two phases; a 15 min habituation phase, during which the mouse could access the home and one other arm of the maze; and a 5 min test phase, conducted after a 30 min rest period, in which the mouse had access to all three arms of the maze. During this test phase, the percent duration spent in the familiar versus novel arms was quantified. An accelerating rotarod was used to assess general motor function, coordination and motor learning, in four trials across 2 consecutive days, with acceleration from 4 to 40 rpm over 4 min (Semple et al., 2015b). Social behavior was assessed in the three-chamber social approach test (Semple et al., 2015b). The task consists of three consecutive 10 min stages; (1) habituation; (2) social preference – the choice between a stimulus mouse versus empty cage; and (3) social recognition/memory – the choice between a now-familiar stimulus mouse versus a novel stimulus. Activity of the test animal was recorded via TopScan tracking software, and analysis performed on percent time spent in each of the chambers.

Finally, spatial learning and memory was evaluated using the Morris Water Maze (MWM), as previously described (Semple et al., 2017b), comprised of six trials/day for 5 consecutive days. Days 1 and 2 involved a 'visible platform,' whereby the frequently-relocated target platform was raised above the water surface and clearly identified with a flag. Days 3–5 involved a 'hidden platform,' in which the platform remained in one location but was hidden below the water surface. The task requires the use of external spatial cues to locate the platform. Both distance travelled and duration to reach the platform were analyzed from automated video tracking, with a maximal duration of 60 s allowed per trial. At the end of day 5, the platform was removed completely, and a 'probe trial' was performed to assess spatial memory retention as the amount of time spent in the target quadrant (where the platform previously was).

2.8. Video-EEG for seizure detection

After behavioral analysis, between 4 and 5 months post-injury, mice were implanted with extradural electroencephalographic (EEG) electrodes for subsequent video-EEG recordings. Three custom-made stainless steel electrodes (9 mm screws soldered to a gold-tipped plug) were screwed into the skull under isoflurane anesthesia, and fixed into place with dental acrylic cement (Semple et al., 2017b). Electrodes were positioned over the parietal lobe contralateral to the craniotomy site (the recording electrode), or over the ipsilateral and contralateral frontal lobes (reference and ground electrodes) (Fig. 5a). One mouse died during implantation, and three mice were excluded due to dislodgement of the electrode cap during continuous EEG recording. Thus, the final group numbers for seizure analysis were *Sham Veh* $n = 13$, *Sham Gly* n = 13, *TBI Veh* n = 13, *TBI Gly* $n = 14$.

After a one week recovery period, mice were connected via tether to the EEG recording system, alongside simultaneous video-recording via a digital camera accompanied by an infrared light for both night and day recordings. EEG data was captured and analyzed using Profusion EEG software (Compumedics v. 5.0). Between 3 and 5 mice were recording simultaneously, being connected to the system for 48 h periods (followed by a 48 h break) for a total of 6–8 d of recording per mouse. A two-way ANOVA showed no difference in average total of recording days as a result of injury ($F_{1,49} = 0.36$, $p = .5522$) or treatment ($F_{1,49} = 1.40$, $p = .2429$), nor an injury x treatment interaction ($F_{1,49} = 0.013$, $p = .9105$). For analysis, entire data files were manually reviewed and scored by investigators blinded to group allocation. An electrographic event (EEG seizure) was defined as a high-amplitude (at least 3× baseline) rhythmic discharge that represented an evolving, oscillatory, and atypical EEG pattern, lasting longer than 5 s, accompanied by associated behavioral disturbance from video recording (Bolkvadze and Pitkanen, 2012; Liu et al., 2016; Semple et al., 2017b). Seizures per animal per group were quantified, along with the average length of seizure per animal.

Finally, at 6 m post-injury, mice were challenged with the pro-convulsive drug pentylenetetrazol (PTZ; Sigma-Aldrich), to induce evoked seizures as an indicator of underlying network excitability. 30 mg/kg i.p. was administered, a dose that induces only a minor, sub-convulsive response in naïve or sham mice (Bao et al., 2011; Nehlig and Pereira de Vasconcelos, 1996; Semple et al., 2017b). Individual mice were placed in a standard cage for PTZ administration, and responses

were recorded over a 30 min period via video-EEG. The number of epileptiform events was quantified, defined as high amplitude (> 2 x baseline) abnormal EEG events. Seizures were further defined as epileptiform events lasting > 5 s duration.

Immediately following completion of the PTZ recording period, mice were euthanized by overdose with sodium pentobarbitone for transcardial perfusion, followed by processing of brain tissue for sectioning, as described above for the 3 day time point.

2.9. Chronic glial immunofluorescence

Immunofluorescence staining for glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor protein (Iba1) was used to detect reactive astrocytes and microglia, respectively, at 6 m post-TBI. Five evenly-spaced sections per brain (280 μm apart) were dewaxed and blocked as described above for the HMGB1 immunofluorescence, then incubated in a 5% NDS/0.1% Triton-X100/PBS solution overnight at 4 °C with primary antibodies as follows: goat polyclonal anti-Iba1 (#ab5076 Abcam, 1:750), or mouse monoclonal anti-GFAP (#MAB360 Millipore, 1:1000). Primary antibodies were detected on day 2 by a 1 h room-temperature incubation with donkey anti-goat IgG AF 488 (for Iba1), or donkey anti-mouse IgG AF 488 (for GFAP; Invitrogen). Finally, sections were incubated with 0.3% Sudan Black to reduce tissue autofluorescence, nuclear counterstained with DAPI, then cover-slipped with Dako fluorescent mounting medium.

Photomicrographs were captured of GFAP and Iba1 stained sections at 10 x magnification using an Olympus IX-81 fluorescent microscope, under fixed exposure times (60 ms for GFAP and 290 ms for Iba1). ROIs were the ipsilateral lesion cortex (encompassing the most evident region of tissue damage from the cortical surface; only intact tissue included), the perilesional cortex (one field of view lateral), and the ipsilateral hippocampus DG and CA3 regions (defined by their neuroanatomical structures from DAPI staining). Staining intensity of intact tissue was quantified using ImageJ (http://imagej.nih.gov/ij/; National Institutes of Health), and values were expressed per brain as ipsilateral/contralateral intensity.

2.10. Volumetric analysis

Five sections per brain (280 μm apart), adjacent to those used for immunofluorescence, were allocated for hematoxylin and eosin (H&E) staining for volumetric estimation of the cortex and hippocampus at 6 m post-TBI. Staining and imaging was performed by the Monash Histology Platform, Alfred Research and Medical Education Precinct, Monash University at 40× magnification on an Aperio Scanscope AT Turbo. The images were then exported to ImageJ, and dorsal cortex and hippocampal volumes were estimated via the Cavalieri method following measurement of tissue areas on each by an investigator blinded to group allocation. Measurements were restricted to the dorsal hemispheres, defined by an inferior horizontal boundary line perpendicular to the most ventral point of the third ventricle at the midline (Semple et al., 2015a). Estimations of area were expressed as ipsilateral area/contralateral area.

2.11. Statistical analysis

Data analysis was performed using GraphPad Prism version 6.07. Data are expressed as group means ± SEM. Differences among groups for each age group was analyzed by one-way or two-way ANOVA with post-hoc tests following detection of significant main effects, as well as in instances where an a priori decision was made to directly compare between specific groups of interest (e.g. arm preference in the Y-maze, and chamber preference in the three-chamber test, within each group). Repeated measures were used for data collected across a time course (e.g. rotarod and Morris water maze). Statistical significance was defined as $p < .05$. Data that failed normality tests were instead analyzed by non-parametric tests. Percentage of animals per group that exhibited seizures were compared by Fisher's exact test.

3. Results

3.1. Gly treatment initiated prior to, but not after injury, reduced acute HMGB1 levels and edema

We firstly sought to compare the acute effects of Gly treatment, initiated either 1 h before or 1 h after pediatric TBI, in terms of neuropathology including brain edema, HMGB1 release, and neuroinflammation from tissue collected at 3 d post-injury (Fig. 1a). Edema was quantified as the water content of brain samples collected ipsilateral and contralateral to the injury site (Fig. 1b, c). This method detected a reduction in edema ipsilateral to the injury in *Gly Pre* mice compared to vehicle-treated controls (one-way ANOVA $F_{2,17} = 7.961$, $p = .0036$; post-hoc $p < .05$), but not in *Gly Post* mice. There were no differences in water content in the contralateral hemisphere, confirming the focal nature of the injury ($p > .05$).

HMGB1 levels in the injured cortex were quantified by Western blot at 3 d post-injury (Fig. 1d), revealing an overall reduction of HMGB1 protein in the TBI *Gly Pre* mice compared to TBI vehicle-treated mice ($F_{2,17} = 6.49$, $p = .008$). This was confirmed by immunofluorescence staining for HMGB1, to more closely examine extracellular release of the protein (Fig. 1f, g). Quantification of extracellular HMGB1 (defined as fluorescent particles outside of cell nuclei, i.e. < 15 μm diameter) in the injured cortex confirmed a reduction in HMGB1 release in *Gly Pre* mice (one-way ANOVA, $F_{2,\ 12} = 4.28$, $p = .04$; post-hoc $p < .05$ *Gly Pre* versus *Gly Post*).

Surprisingly, this observed reduction in HMGB1 did not translate into reduced neuroinflammation, at least at this time point, with no differences in the level of GFAP protein in *Gly Pre* versus *Gly Post* or vehicle-treated mice (Fig. 1e; $F_{2,17} = 0.59$, $p = .57$). Similarly, no differences in CD68, an indicator of macrophage-like cells, were observed between groups ($F_{2,17} = 1.7$, $p = .21$; data not shown).

Based on this evidence, that the *Gly Pre* treatment regime was sufficient to reduce HMGB1 levels and acute edema, we proceeded with a long-term experiment using this same treatment regime, extended over the first week post-injury, to next examine the chronic consequences of HMGB1 inhibition on functional and neuropathological outcomes after pediatric TBI.

3.2. Gly treatment selectively prevented spatial and motor learning deficits

Mice underwent pediatric TBI or sham surgery at p21, and were randomized to receive either Gly or vehicle treatment commencing 1 h prior to injury, then at 1 h, 6 h and then daily post-injury for 7 d, for a total of 4 groups: *Sham Veh*, *Sham Gly*, *TBI Veh* and *TBI Gly*. At 3 m post-injury, all mice underwent a battery of neurobehavioral assays for cognitive, sensorimotor and psychosocial functioning (Fig. 2a).

In the Y-maze (Fig. 2b), overall there was a main effect preference for maze arm (2-way ANOVA, $F_{1,51} = 22.18$, $p < .0001$). Bonferroni's multiple comparisons test revealed this preference for the novel arm over the familiar arm to be present for all groups (*Sham Veh, Sham Gly* and *TBI Gly*; $p < .05$) except for the *TBI Veh* mice (n.s.). This suggests a TBI-related deficit in short-term spatial memory which appears to be prevented by Gly treatment. In the accelerating rotarod task (Fig. 2c), there was an overall main effect of time (2-way RM ANOVA, $F_{1,53} = 63.26$, $p < .0001$) and a time x group interaction ($F_{3,53} = 3.86$, $p = .01$), with Bonferroni's test revealing that all groups (*Sham Veh, Sham Gly* and *TBI Gly*; $p < .05$) except *TBI Veh* mice (n.s.) demonstrated an improvement in performance between d 1 and d 2. This suggests a TBI-related motor learning deficit which appears to be prevented by Gly treatment.

Neither of the above-mentioned tasks were confounded by gross sensorimotor deficits, as all mice showed comparable general activity

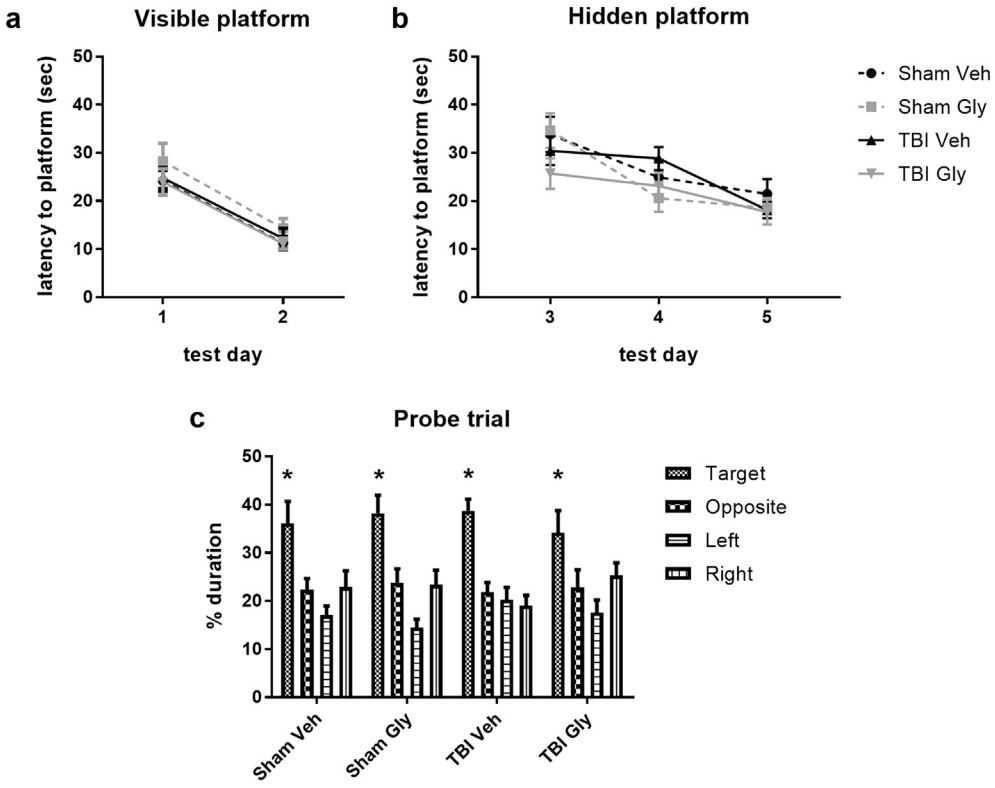

Fig. 3. Spatial learning and memory evaluated in the MWM test. In the visible platform stage (a), all groups showed learning of the task and a comparable latency to reach the platform. In the hidden platform stage (b), both TBI groups failed to show an improvement (reduced latency) between days 3 and 4 (2-way ANOVA post-hoc, n.s.), whereas performance did improve with time in both sham groups (post-hoc, $p < .05$). In the probe trial (c), all groups showed a preference for the target quadrant (*$p < .05$, one-way ANOVA post-hoc).

(i.e. distance travelled in a locomotor arena) (2-way ANOVA n.s.; Fig. 2d). Similarly, all mice spent a similar duration in the open arms of the Elevated Plus Maze (Fig. 2e), indicating no change in anxiety as a consequence of either pediatric TBI or Gly treatment (2-way ANOVA, n.s.). Social behaviors were assessed in the three-chamber social approach task, which revealed normal sociability (a preference for the stimulus mouse over an empty chamber) in all groups (*$p < .05$, 2-way ANOVA post-hoc; Fig. 2f). In a subsequent stage of the task, which assesses for social recognition memory, none of the groups showed a significant preference of the novel mouse over the now-familiar mouse (Fig. 2g).

Finally, we performed the MWM test to evaluate spatial learning and memory. In the visible platform stage (Fig. 3a), all groups showed a reduction in latency to reach the platform on day 2 versus day 1 (2-way RM ANOVA, effect of time $F_{1,52} = 121.0$, $p < .0001$) but no group differences, indicating comparable learning of the task. When the platform was then hidden from view (Fig. 3b), to assess spatial memory, we again saw an overall effect of time ($F_{2,104} = 32.73$, $p < .0001$) as well as a significant interaction between time and group ($F_{6,104} = 2.22$, $p = .0473$). Post-hoc analyses revealed that both sham groups showed a reduced latency to the platform between days 3 to 4 ($p < .05$), whereas neither of the TBI groups showed an improvement at this time (n.s.), indicating a TBI-deficit in this task. However, no effects of Gly treatment were detected. At the end of test day 5, the platform was removed from the pool and mice underwent a 60 s probe trial to evaluate memory retention (Fig. 3c). In this task, all groups showed a preference for the target quadrant (where the platform was previously located) compared to other quadrants of the pool, indicating intact memory retention (one-way ANOVAs; *Sham Veh* $F_{3,44} = 6.37$, $p = .0011$; *Sham Gly* $F_{3,52} = 11.22$, $p < .0001$; *TBI Veh* $F_{3,56} = 16.51$, $p < .0001$; *TBI Gly* $F_{3,44} = 3.96$, $p = .0138$; Tukey's post-hoc, $p < .05$ for target vs. at least one other quadrant).

3.3. Experimental pediatric TBI results in a small percentage of mice developing epilepsy

The development of chronic spontaneous seizures, an indicator of PTE, was evaluated by continuous video-EEG at 5–6 months post-injury following implantation of subdural EEG electrodes (Fig. 4a). An average of 7 days (range: 6–8) of 24/7 continuous video-EEG recordings were obtained for analysis per mouse (Table 1), the duration of which did not differ between the groups (2-way ANOVA, $F_{1,49} = 0.013$, $p = .9105$). Only three post-TBI mice developed spontaneous seizures (2 *TBI Veh* and 1 *TBI Gly*), associated with behavioral disturbances including clonic contractions observed primarily as freezing behavior, repetitive head nodding, tail stiffening and loss of balance. The proportion of epileptic animals did not differ between the treatment groups (Fisher's exact test: $p = .58$). Interestingly, these mice were not the same animals that were seen to have acute post-injury seizures immediately after surgery. No spontaneous seizures were observed in sham animals. Comparisons between *TBI Veh* and *TBI Gly* are problematic due to the low overall number of animals displaying spontaneous seizures, such that we were underpowered to detect statistical significance. However, we did note that the total number of seizures and average duration of seizures both seemed to be higher in *TBI Veh* mice compared to *TBI Gly*, hinting at a potential anti-epileptogenic effect of *Gly*. Further studies with adequate statistical power to account for the low incidence of spontaneous seizures, or alternative experimental approaches, are required to draw such a conclusion.

Next, all mice were administered a low dose of the pro-convulsive drug PTZ (30 mg/kg PTZ i.p.) to evaluate their evoked seizure response, to assess changes in seizure threshold as a surrogate indicator of an increased likelihood to develop PTE. Epileptiform events were quantified from EEG and found to be increased in TBI mice compared to shams (2-way ANOVA $F_{1,47} = 12.28$, $p = .001$), indicating increased brain excitability at 6 months post-injury (Fig. 4b). However, no effect of Gly treatment was observed ($F_{1,47} = 0.38$, $p = .5385$).

No differences were detected between groups in the latency to the first epileptiform event or behavioral abnormality (data not shown). A

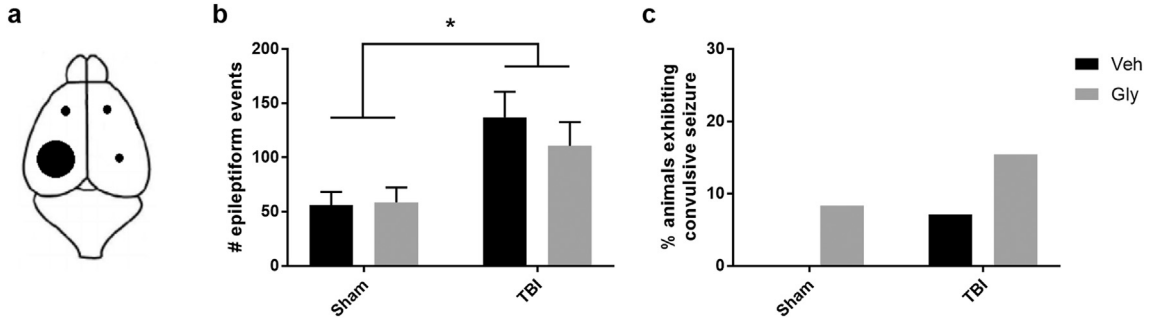

Fig. 4. PTZ-evoked seizure response at 6 months post-TBI. Electrode placement is illustrated in (a), with the recording electrode in the parietal lobe contralateral to the craniotomy/injury site (large black circle), and ground and reference electrodes in the frontal lobes. TBI mice exhibited an increase in epileptiform events in response to PTZ compared to sham controls (2-way ANOVA, $p < .05$) (b), while the percentage of mice that progressed to a convulsive seizure did not differ between groups (c).

Table 1
Spontaneous seizures detected by video-EEG at 5 months post-surgery.

	% mice with seizures	Total # seizures	Average duration (s)	Average recorded duration, days
Sham Veh	0% (0/13)	0	0	7.189 (0.231)
Sham Gly	0% (0/13)	0	0	7.522 (0.284)
TBI Veh	15.38% (2/13)	7	29	7.064 (0.217)
TBI Gly	7.14% (1/14)	2	19	7.339 (0.284)

Fraction in parentheses in first column indicates the number of mice with seizures relative to the total number of mice per group. Number in parentheses in last column indicates SEM.

small percentage of mice advanced to a full tonic-clonic convulsive seizure in response to PTZ (Fig. 4c), which was not statistically different between TBI groups (Fisher's exact test, $p > .9999$).

3.4. Gly treatment modulates chronic hippocampal microglial reactivity

Mice were euthanized at 6 months post-injury, and brains collected to evaluate chronic neuropathology neuroinflammation. TBI mice exhibited an increase in GFAP immunofluorescence staining compared to sham controls in the lesioned cortex (Fig. 5a, b; 2-way ANOVA, $F_{1,20} = 14.18$, $p = .0012$), indicating chronic astrogliosis after injury, which was not influenced by Gly treatment ($F_{1,20} = 0.06$, $p = .8165$). This was region-specific, as no group differences were observed for GFAP reactivity in the hippocampus CA3 (Fig. 5c; 2-way ANOVA n.s.).

Iba1 reactivity was also quantified as an indicator of microgliosis at this time point (Fig. 5d). In contrast to GFAP, Iba1 levels were similarly low in all groups in the lesioned cortex (Fig. 5e; 2-way ANOVA n.s.). However, in the hippocampus CA3 (Fig. 5f), we observed a strong trend towards an injury effect ($F_{1,20} = 4.166$, $p = .0547$), and a significant effect of Gly treatment ($F_{1,20} = 5.92$, $p < .05$). While there was no significant injury x treatment interaction ($F_{1,20} = 2.91$, $p = .1035$), this finding suggests region-specific, low but persistent microglial activation in the hippocampus that appears to be influenced by acute Gly treatment. As with GFAP analysis, no differences were seen between the groups in the perilesional cortex (lateral to the lesion core) or hippocampus DG (not shown).

3.5. Gly treatment did not affect chronic neuropathology

H&E staining was used to assess the extent of chronic neuropathology at 6 months post-injury. Quantification of regional volumes revealed an injury-dependent loss of cortical tissue in TBI mice compared to sham controls (Fig. 6a, 2-way ANOVA $F_{1,20} = 23.7$, $p < .0001$), which was not influenced by Gly treatment ($F_{1,20} = 0.09$, $p = .7664$). There were no differences between any of the groups in hippocampal volumes (Fig. 6b), suggesting that the injury parameters (e.g. impactor depth) were insufficient to induce significant tissue loss in this region.

4. Discussion

HMGB1 has been implicated as a key mediator of neuroinflammation and neurodegeneration in a range of CNS conditions. In the adult injured brain, antagonism of HMGB1 has been reported to reduce edema, BBB permeability, glial reactivity, cognitive and motor deficits, across an acute post-injury time course (Okuma et al., 2014; Okuma et al., 2012b; Okuma et al., 2019; Pang et al., 2016; Yang et al., 2018). In the current study, we have examined for the first time the effect of HMGB1 inhibition on chronic outcomes after experimental TBI of the pediatric brain, building upon our recent findings of HMGB1 release in the brain and serum in this model (Webster et al., 2019).

We first demonstrate that Gly treatment commencing prior to TBI was able to reduce brain HMGB1 levels (both locally and globally) alongside brain edema, whereas treatment commenced 1 h post-TBI failed to have such effects. Pursing the Gly pre-treatment paradigm, our chronic time course over a 6 month period found that pediatric TBI resulted in short-term spatial memory and motor learning deficits which were prevented in *TBI Gly* mice. This corresponded with an apparent reduction in chronic hippocampal microgliosis. In contrast, while TBI induced an increase in evoked seizures compared to sham controls, and extensive cortical tissue loss, Gly treatment did not influence these outcomes.

Our focus on the pediatric brain is justified for several reasons. Firstly, young children have a high incidence of TBI, and show particular vulnerability to poor long-term outcomes post-TBI (Anderson et al., 2005b). Secondly, accumulating evidence indicates that the neuroinflammatory response to a TBI differs as a consequence of age-at-insult, whereby the younger brain may show exacerbated leukocyte infiltration, BBB dysfunction and cytokine production compared to after a comparable insult in adulthood (Anthony et al., 1998; Anthony et al., 1997; Claus et al., 2010; Potts et al., 2006a; Webster et al., 2019). Such distinctions may account for differential responses to potential therapeutics, such that one cannot simply assume that a novel pharmacological agent that yields neuroprotection in the adult brain will have a comparable effect in the immature brain (Potts et al., 2006a). Thirdly, HMGB1 may be of particular importance in the pediatric injured brain, due to developmental changes in basal expression of both HMGB1 as well as one of its receptors TLR4, which peaks during early post-natal life (Enokido et al., 2008; Kaul et al., 2012). Finally, high levels of HMGB1 in the CSF after TBI in pediatric patients has been reported to correlate with unfavorable Glasgow Outcome Scores at 6 months post-injury, implicating HMGB1 as a mediator of chronic outcomes, and suggesting utility as a predictive biomarker in this population (Au et al., 2012).

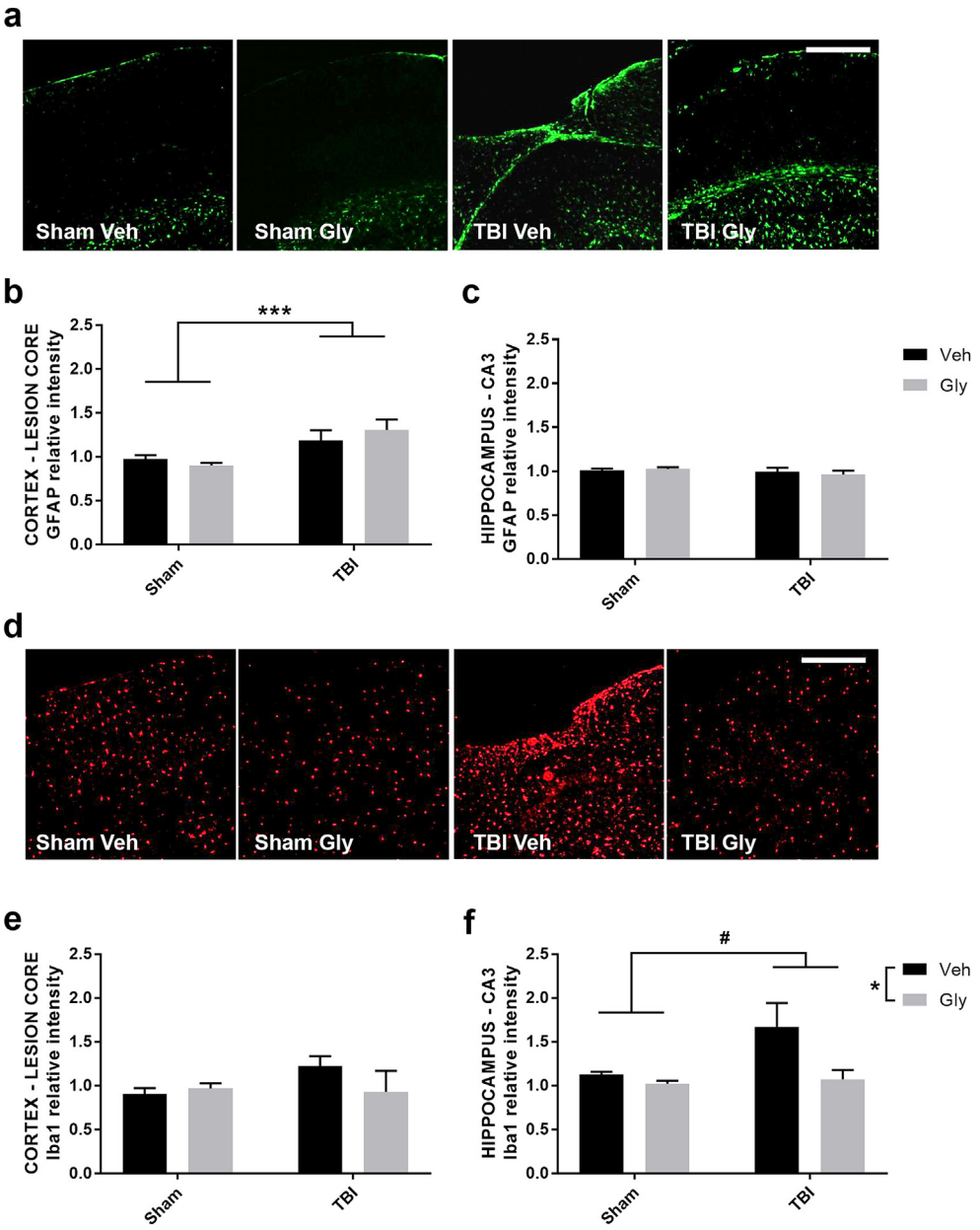

Fig. 5. Chronic gliosis after pediatric TBI. GFAP-positive astrocytes (a) were quantified by fluorescent intensity in the cortex (b) and hippocampus (c), revealing persistent astrogliosis in the cortex compared to sham controls (2-way ANOVA, $***p < .0005$). In contrast, immunoreactivity for Iba1-positive microglia (d) trended towards being elevated in the hippocampus at this time (f; $\#p = .055$), and Iba1 intensity was higher in Gly-treated compared to Veh-treated mice ($*p < .05$). Scale bar = 100 μm.

4.1. Gly inhibition of HMGB1 – acute outcomes

The dose and timing of Gly treatment were chosen based on several previous studies, in rodent models of kainic acid-induced seizures and intracerebral hemorrhage, in which this treatment regime results in reduced HMGB1 levels, reduced edema, and neuroprotection We chose a dose of 50 mg/kg i.p. based on reports of good bioavailability for rodent brain tissue; although several studies have reported neuroprotection with doses as low as 10 mg/kg (Akman et al., 2015; Ploeger et al., 2001).

We first confirmed that Gly reduced brain HMGB1 levels compared to vehicle treatment. Secondly, we demonstrated that Gly reduced acute brain edema ipsilateral to the injury, a common consequence of TBI and hallmark of the neuroinflammatory response (Fig. 1). These findings are consistent with previous studies in adult TBI and intracerebral hemorrhage (Ohnishi et al., 2011; Ohnishi et al., 2014; Yang et al., 2018).

Strikingly, these effects were restricted to mice that received a first dose of Gly at 1 h prior to injury, in addition to post-injury treatment, whereas mice that did not receive the pre-injury dose were comparable to vehicle-treated TBI mice. While this finding is somewhat disheartening from the perspective of future clinical translation of pharmaceuticals targeting HMGB1 (treatment commencing prior to a TBI is probably not feasible!), it does provide some insight into mechanisms by which HGMB1 influences cerebral edema – this effect must be occurring in the very acute stage after pediatric TBI, within the first 1 h. HMGB1 may promote edema via direct and indirect effects on vascular endothelial cells and pericytes to increase BBB permeability, a major cause of edema formation (Liu et al., 2007; Okuma et al., 2012a; Zhang et al., 2011).

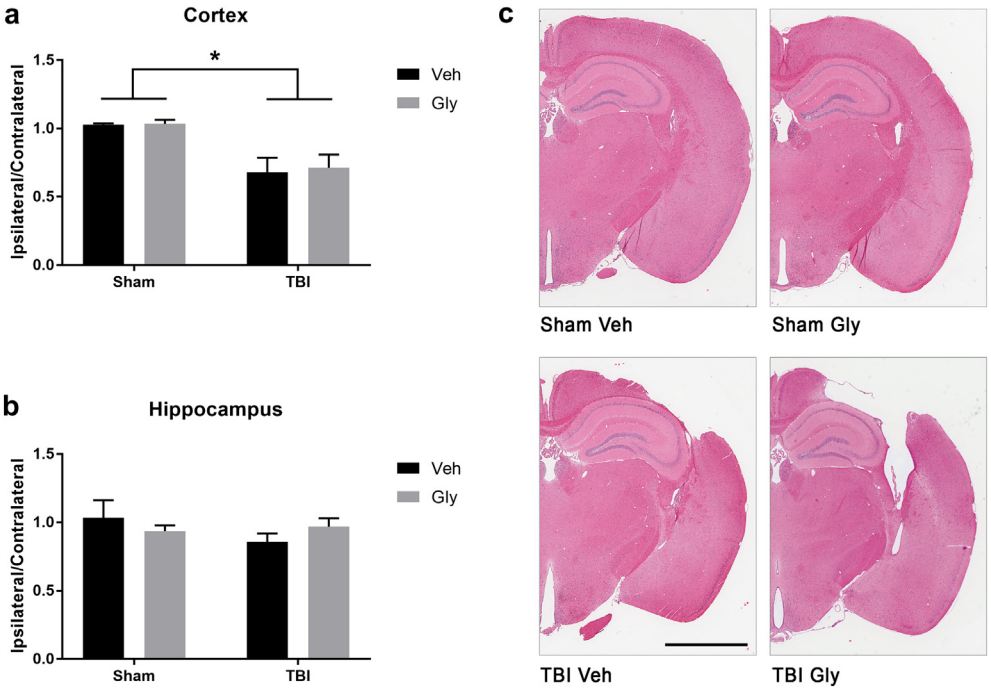

Fig. 6. Chronic tissue loss after pediatric TBI was not prevented by Gly treatment. The volume of the dorsal cortex (a) and hippocampus (b) were quantified at 6 months post-injury. Cortical tissue loss is evident after pediatric TBI in both Veh and Gly-treated groups (2-way ANOVA, *$p < .05$). Representative H&E-stained sections from each group are shown (c). Scale bar = 2000 μm.

4.2. Gly inhibition of HGMB1 - chronic outcomes

Our study primarily focused on chronic outcomes after pediatric TBI, based on the belief that it is crucial to understand how acute inhibition of HMGB1 manifests over time, as many of the implications of early-life brain injuries are seen months to years after the initial insult (Anderson et al., 2005a; Levi et al., 1991; Lewin et al., 1979). These can include behavioral and cognitive deficits, and an increased risk of developing PTE (Riazi et al., 2010; Schoderboeck et al., 2009). Increased acute or prolonged inflammation has been linked to worsening these long-term outcomes in experimental models (Chhor et al., 2017; David et al., 2009; Riazi et al., 2010).

Our findings, of TBI-induced deficits in vehicle-treated mice in the Y-maze and rotarod, suggest chronic dysfunction in short-term spatial memory and motor learning abilities after pediatric TBI, consistent with previous reports (Kamper et al., 2013; Semple et al., 2017a). In contrast, *TBI Gly* mice were not different from sham controls, indicating that Gly treatment either prevented or ameliorated these deficits. These cognitive effects were quite subtle, as they were not detected in the MWM test for longer-term spatial learning and memory. However, our findings generally align with those from adult TBI models, whereby Gly or other methods to block HMGB1 have also yielded improvements in cognitive and/or sensorimotor dysfunction (Okuma et al., 2014; Okuma et al., 2012b; Okuma et al., 2019). This neuroprotective effect may be either direct, via HMGB1's reported actions on TLR4 in long-term potentiation and memory function (Costello et al., 2011; Jacewicz et al., 2009; Mazarati et al., 2011; Tarr et al., 2011); or indirect, via the modulation of chronic gliosis which can influence neurobehavioral outcomes (Briones et al., 2013; Faden et al., 2016; Osier et al., 2015).

At 6 months post-injury, we detected an effect of Gly treatment on region-specific microglial activation in the hippocampus. This is consistent with several other studies demonstrating that direct inhibition or downregulation of HMGB1 reduces activation of microglia after various neuropathologies including ischemia and kainic acid seizures (Gao et al., 2018; Kim et al., 2006; Luo et al., 2013). Long-term microglial activation has been reported in patient cohorts after TBI, similar to that seen here, in terms of not being in the focal lesion area but rather in subcortical structures away from the lesion site (Ramlackhansingh et al., 2011). Gly appeared to reduce or prevent this response, which may account for the observed behavioral changes. Of note, the effect of Gly on gliosis was restricted to Iba1+ microglia, not GFAP+ astrocytes, which persisted in the injured cortex to 6 months. This is in contrast to some previous evidence from adult models that HMGB1 release from astrocytes is associated with neurological consequences (Hayakawa et al., 2012; Kim et al., 2008; Passalacqua et al., 1998; Yang et al., 2012). Our findings suggest that, at least in the context of pediatric TBI, acute HMGB1 inhibition does not influence either acute or chronic astrogliosis.

4.3. HMGB1 and post-traumatic epileptogenesis

Accumulating evidence implicates HMGB1 in seizure onset (ictogenesis) and epilepsy development (epileptogenesis) (Paudel et al., 2019). For example, elevated levels of HMGB1 have been found in epileptogenic tissue resected during surgery (Crespel et al., 2002), while studies in adult rodents have shown that inhibiting HMGB1 reduced seizure pathology and progression to epilepsy, particularly in acquired epilepsy models (Iori et al., 2013; Maroso et al., 2010; Maroso et al., 2011). The mechanism by which HMGB1 promotes neuronal hyperexcitability is proposed to be via NMDA receptor potentiation in hippocampal neurons (Balosso et al., 2014). This mechanism is likely exacerbated in the post-injury CNS, whereby injury-induced production of free radicals and reactive oxygen species promotes the oxidation of HMGB1 to consequently augment NMDA functionality, thus promoting seizure susceptibility (Balosso et al., 2014).

While epileptogenesis after a brain insult is a chronic process ongoing over many months (Webster et al., 2017), the process can begin as early as the day that a TBI occurs (Annegers et al., 1980). This suggests that even very acute manipulation of the inflammatory response may yield long-term modulation of epileptogenesis. Indeed, a recent study from our laboratory demonstrated that acute inhibition of interleukin-1 receptor signaling over the first week post-injury was sufficient to reduce the incidence and severity of chronic PTZ-evoked seizures and associated behavioral deficits after pediatric TBI (Semple et al., 2017b). This evidence supports our evolving theory that inflammatory cascades initiated during the acute post-injury period can influence the reorganization of neuronal circuitry after TBI to influence the likelihood of late post-injury seizures and/or seizure susceptibility.

Here, while we confirmed that pediatric TBI resulted in chronic susceptibility to PTZ-evoked seizures and the emergence of spontaneous seizures in a subset of mice, as recently established by our group (Semple et al., 2017b), acute Gly treatment did not ameliorate this response. As PTE is notoriously intractable to pharmacological intervention (Diaz-Arrastia et al., 2000), this negative finding may reflect HMGB1 having a less pivotal role in epileptogenesis in this context compared to in other epilepsy models in which targeting HMGB1 was shown to interfere with seizure development. Indeed, HMGB1 is just one factor in the complex milieu of pro-seizure molecules released into the extracellular environment in response to a brain insult, such that other players may compensate for the inhibition of HMGB1 in our model system.

Alternatively, due to the small proportion of mice that developed spontaneous seizures (15% of all TBI mice), we were ultimately underpowered to detect a potential statistically significant difference between *TBI Veh* and *TBI Gly* mice in these measures. Based on this incidence, power calculations determined that a sample size of 235 mice per TBI group would be needed to accurately detect potential differences in this paradigm. As such, we cannot completely reject our hypothesis that Gly may influence seizure outcomes, and further studies are needed to fully understand the relationship between HMGB1 and post-traumatic epileptogenesis. Other studies in adult TBI models have found variable rates of PTE from 9 to 50%, positively correlated with increasing severity of injury and dependent upon the time post-injury when assessed (Guo et al., 2013; Hunt et al., 2009b; Kharatishvili et al., 2006; Raible et al., 2015). In pediatric TBI, we have recently reported that ~80% of TBI mice had at least one spontaneous seizure within a 7 day recording window, at 5–6 months post-injury (Semple et al., 2017b). The difference between the current study and our recent one is most likely attributed to differing depths of the injury induced, being 1.5 mm ('moderate' severity) herein compared to 1.73 mm ('severe') as used previously. Here, we chose a reduced depth to avoid overt mechanical damage to the hippocampus as seen previously, to allow for investigation of changes in hippocampal circuitry after TBI (ongoing work).

In the context of what has been done in adult models (Guo et al., 2013; Hunt et al., 2009a; Hunt et al., 2010), the incidence of PTE seen here is consistent with an injury that is considered moderate in nature. Together with clinical evidence (Kieslich et al., 2001), these data reiterate that the development of spontaneous chronic seizures is dependent upon injury severity and/or depth; and in particular, involvement of the hippocampus, a region known for its importance in seizure generation and propagation (Bolkvadze and Pitkanen, 2012; Heinemann et al., 2012). Similarly, social behavior deficits that have previously been reported by our group and others after severe pediatric TBI (Bajwa et al., 2016; Semple et al., 2012; Semple et al., 2014) were not observed in the current cohort, supporting neuroanatomical evidence that the hippocampus as also crucial for social interest and recognition (Bannerman et al., 2001; Maaswinkel et al., 1996).

4.4. Implications and limitations

Despite several previous studies demonstrating a role for HMGB1 in neuroinflammation and neurodegeneration after TBI, these have all been conducted in adult animals, and the majority have focused solely on acute outcomes. This is problematic when considering the clinical scenario of pediatric TBI, whereby age at the time of injury may be a factor that influences outcomes, and secondary damage progression including the process of epileptogenesis develops over a chronic time course (Anderson et al., 2005a; Keret et al., 2017). Further, although HMGB1 has been implicated in epilepsy and TBI independently, to our knowledge, no studies to date have considered a potential role of HMGB1 in epilepsy as a consequence of brain injury. Our study is therefore novel on several fronts.

This study has demonstrated that while acute treatment of Gly after pediatric TBI can confer some neuroprotection long-term, particularly in relation to microglial activation and associated cognitive deficits, Gly did not influence the extent of the cortical tissue atrophy, nor did is modulate seizure susceptibility. While it is unclear whether these mixed outcomes are attributed to the chronic time point at which we evaluated outcomes, or are a product of a unique response of the pediatric brain, this study highlights the importance of evaluating potential therapeutic interventions at different developmental stages to account for potential age-specific consequences. Pediatric TBI is a chronic condition, whereby comorbidities of neurobehavioral dysfunction and PTE are known to develop and evolve over time (Appleton and Demellweek, 2002a; Babikian et al., 2015; Christensen et al., 2009). It is therefore imperative that we extend our studies of potential therapeutic interventions, such as inhibitors of HMGB1, to a chronic and clinically-relevant time course as we have done in these experiments.

While our study failed to demonstrate a central role for HMGB1 in post-traumatic epileptogenesis in the pediatric brain, abundant evidence from other model systems continue to identify this inflammatory mediator as pro-ictogenic and pro-epileptogenic. Due to the frequently resistant nature of PTE to pharmacological interventions, a polypharmacy, multifaceted approach is most likely required in order to prevent or reduce chronic post-traumatic seizures (Keret et al., 2017; Larkin et al., 2016; Semah et al., 1998). Other novel approaches such as antagonism of the interleukin-1 receptor (Semple et al., 2017b), may prove to have enhanced neuroprotective and anti-epileptic potential when used in conjunction with agents targeting HMGB1, due to different targets but somewhat overlapping downstream targets and consequences (Sha et al., 2008). Further, in addition to Gly, many other compounds have been identified for their ability to bind and inhibit HMGB1, including synthetic molecules, HMGB1-specific antibodies, peptides and proteins, as well as bent DNA-based duplexes (Musumeci et al., 2014). While we chose Gly for this study based upon previous evidence that it readily crossed the BBB and afforded neuroprotection in the adult injured brain, to our knowledge, a direct comparison between HMGB1-targeting compounds has not been conducted to determine whether some are superior to others for brain pathologies. As we did not compare different doses of Gly, it is also unclear whether an alternative dosing regimen may have been more optimal for our model.

5. Conclusion

This study is the first to examine the effect of HMGB1 inhibition in the pediatric injured brain, and across a chronic time course. Our findings contribute to a growing appreciation for HMGB1's role in neuropathology and associated behavioral outcomes after TBI. However, further work needs to be conducted to fully elucidate the contribution of HMGB1 to epileptogenesis in this context.

Acknowledgements

The authors acknowledge funding support from the National Health and Medical Research Council of Australia (NHMRC) in the form of Project Grants and Career Development Fellowships to BDS and SRS. The authors also thank the Biomedical Histology Facility at the University of Melbourne, and Monash Histology Platform, Monash University at the Alfred Hospital.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2019.112979.

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