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Injury timing alters metabolic, inflammatory and functional outcomes following repeated mild traumatic brain injury

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ABSTRACT

Repeated head injuries are a major public health concern both for athletes, and members of the police and armed forces. There is ample experimental and clinical evidence that there is a period of enhanced vulnerability to subsequent injury following head trauma. Injuries that occur close together in time produce greater cognitive, histological, and behavioral impairments than do injuries separated by a longer period. Traumatic brain injuries alter cerebral glucose metabolism and the resolution of altered glucose metabolism may signal the end of the period of greater vulnerability. Here, we injured mice either once or twice separated by three or 20 days. Repeated injuries that were separated by three days were associated with greater axonal degeneration, enhanced inflammatory responses, and poorer performance in a spatial learning and memory task. A single injury induced a transient but marked increase in local cerebral glucose utilization in the injured hippocampus and sensorimotor cortex, whereas a second injury, three days after the first, failed to induce an increase in glucose utilization at the same time point. In contrast, when the second injury occurred substantially later (20 days after the first injury), an increase in glucose utilization occurred that paralleled the increase observed following a single injury. The increased glucose utilization observed after a single injury appears to be an adaptive component of recovery, while mice with 2 injuries separated by three days were not able to mount this response, thus this second injury may have produced a significant energetic crisis such that energetic demands outstripped the ability of the damaged cells to utilize energy. These data strongly reinforce the idea that too rapid return to activity after a traumatic brain injury can induce permanent damage and disability, and that monitoring cerebral energy utilization may be a tool to determine when it is safe to return to the activity that caused the initial injury.

Introduction

Repeated head injuries are a major public health concern both for youth and adult athletes, as well as members of the police and armed forces. In the US alone, each year approximately 1.4 million people are hospitalized with a traumatic brain injury (Langlois et al., 2006). The Centers for Disease Control have estimated that there are likely an additional 3.8 million untreated concussions and traumatic brain injuries in the US annually (Navarro, 2011). There is an innate conflict between an institutional desire to return individuals to the playing field or to duty following a TBI, and the need to protect these individuals from the catastrophic brain damage that can result from subsequent head injuries. Among American college football players, three or more concussions triple the likelihood that they will suffer a concussion subsequently. Indeed, among players that experience two separate concussions, over 90% will experience both concussions within 10 days of each other and often will exhibit a longer duration and intensity of symptoms (Thomas et al., 2011). In some rare conditions, a repeated TBI close in time can induce severe and uncontrolled cerebral edema, termed second impact syndrome, that can have catastrophic outcomes including death (Kelly et al., 1991; McCrory and Berkovic, 1998).

There is mounting evidence that there is a period of enhanced vulnerability to subsequent injury following a TBI. Injuries that occur close together in time produce greater cognitive, histological, and behavioral impairments than do injuries separated by a longer period and this phenomenon has been reported in both clinical populations and experimental animals (Laurer et al., 2001; Longhi et al., 2005; Meehan et al., 2012; Prins et al., 2013; Silverberg et al., 2013). For instance, repeated injuries separated by three but not seven days exacerbate cognitive dysfunction following TBI (Longhi et al., 2005). Therefore, understanding both the mechanism for this period of enhanced vulnerability and identifying a clear marker to determine when it has resolved is of paramount importance.

The pathophysiology of TBI is complex and varies according to the type, severity and location of the injury, as well as age, comorbidities, and genetic background of the patient (Cuthbert et al., 2011; Darrah et al., 2013; Graham et al., 1995; Maas et al., 2008; Mosenthal et al.,

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2002; Robertson et al., 2011; Thompson et al., 2006; Zaloshnja et al., 2008). However, one common thread that runs through TBI research is that injuries induce a metabolic crisis in the nervous system wherein energetic requirements to maintain membrane potentials, ion balances, and neurotransmitter buffering coincide with dysregulation of cerebral blood flow and a reduced capacity to utilize energy (Glenn et al., 2003; Hovda et al., 1995; Jaggi et al., 1990; Katayama et al., 1990). Maintaining positive energy flow to the injured CNS is of utmost importance, as a failure to maintain neuronal membrane potentials would result in rapid osmotic swelling, cerebral edema, and cell death in affected cells.

Many studies have reported, in both humans and experimental animals, that glucose utilization rises acutely after injury and then enters a period of prolonged metabolic depression. The initial hypermetabolism is apparently the response to the release of excitatory amino acids immediately following injury and may be necessary to recover some aspects of homeostasis in the injured brain (Glenn et al., 2003). The return of cognitive and executive function correlates strongly with the restoration of normal glucose metabolism in both humans and animals (Ip et al., 2003; Lin et al., 2012). However, to our knowledge only one study has examined the effects of a repeated traumatic brain injury occurring during the state of altered cerebral glucose metabolism, on functional and histological outcomes, as well as how a repeated injury alters subsequent glucose utilization dynamics (Prins et al., 2013) although other reports of changes in cerebral energy metabolism and mitochondrial function have been reported (Tavazzi et al., 2007; Vagnozzi et al., 2007). We hypothesized that a repeated injury during a period of dysregulated cerebral glucose utilization (e.g. three days after an injury as has been previously reported (Yoshino et al., 1991)) would lead to poorer functional and histological outcomes, and exacerbate inflammatory responses in part by altering subsequent cerebral glucose regulation.

Materials and methods

Adult male Swiss Webster mice were purchased from Charles River Laboratories (Wilmington, MA). Upon arrival in our laboratory, mice were housed five per cage and maintained in a 14:10 light–dark cycle. Mice had ad libitum access to food (Harlan Teklad #8640) and filtered tap water. All experimental conditions were approved by the Ohio State University Institutional Lab Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines.

Injuries

Single and repeated impact acceleration injuries were induced with a modified version of the Marmarou weight drop device (Biegon et al., 2004; Marmarou et al., 1994; Zohar et al., 2003) to produce the following experimental groups: 1) sham injured (sham), 2) injured once (1INJ), 3) injured twice three days apart (2Inj(3)), or 4) injured twice 20 days apart (2Inj(20)) (see Fig. 1 for a detailed experimental time course). Mice were anesthetized with isoflurane vapors and their skulls exposed. A plastic plunger was placed onto the surface of the skull overlying the left cortex just posterior to the bregma and lateral to the midline. A weight (36.73 g) was dropped from a height of 15 cm that impacted the plunger, which was in direct contact with the skull. Mice were then inspected for skull fractures, the skin sutured and monitored for return of consciousness. Mice with skull fractures were removed from subsequent analysis.

2-Deoxyglucose autoradiography

We performed 2DG autoradiography in order to assess dynamic changes in central glucose metabolism following single or repeated injuries. Mice underwent either a single injury, repeated injuries separated by three days, or repeated injuries with a 20 day period between them. SHAM injured mice were collected at each of the injury time points.

Mice were injected with five microcuries of ^{14}C labeled 2-deoxyglucose (American Radiolabeled Chemicals, St. Louis MO) suspended in 0.1 ml sterile saline. Forty-five minutes after the injection, mice were rapidly decapitated and the brains removed and quickly frozen in dry ice-cooled isopentane. Brains were then cut at 25 μm on a cryostat and thaw mounted onto charged slides. Radioactivity was visualized by placing the slides on a phosphor-imaging screen (GE) for approximately 72 h with a ^{14}C standard slide (American Radio Labeled Chemicals). The imaging screen was scanned on a Typhoon imager (Kodak Model S0320) set to a 50-μm resolution. The slides were then counterstained with cresyl violet and photographed. The photographs of the cresyl-stained sections were then overlain over the autoradiography images in Photoshop (Adobe Corporation, San Jose CA) in order to assure accurate anatomical densitometry measurements. Densitometry measurements were taken on the hemispheres both ipsilateral and contralateral to the injury in the sensorimotor cortex, hippocampal CA1, CA2, and CA3 fields, the dentate gyrus, and thalamus. A standard curve was generated by assessing the density of phosphor staining from the standard slide and interpolated using the Rodbard equation in ImageJ (NIH).

Real time PCR

Animals for gene expression analysis were treated as described above and then rapidly decapitated 24 h after the final injury. The brains were removed using aseptic techniques and stored in RNAlater RNA stabilization solution (Ambion, Austin, TX) overnight at 4 °C. Sections of the forebrain surrounding the lesion site were dissected out, homogenized and RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's protocol. Extracted RNA was suspended in 30 μl RNase-free water and RNA concentration was determined by spectrophotometer (Nanodrop-1000, Nanodrop

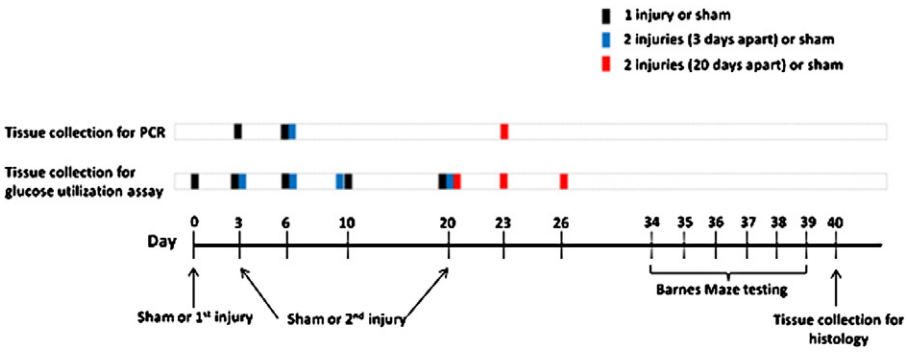

Fig. 1. Experimental timeline. Animals were subjected to either a single sham or a TBI procedure, or were re-injured either 3 or 20 days later. Tissue collection time points for each group are shown for PCR, the glucose utilization assay, and histology (FluoroJade C, silver stain, IBA-1 and GFAP). The Barnes maze behavioral assay of learning and memory was conducted beginning 34 days after the first sham or injury procedure (in order to accommodate a 2-week recovery period for the 2Inj(20d) animals).

Technologies, Wilmington, DE). All RNA samples were stored at −80 °C until further analysis. cDNA was created via reverse transcription of 2 μg of RNA from each sample with MMLV Reverse Transcriptase. Relative gene expression analysis was conducted using qPCR. A TaqMan 18S Ribosomal RNA primer and probe set (labeled with VIC fluorescent dye; Applied Biosystems, Foster City, CA) were used as the control gene for relative quantification. Pre-made primer probe kits for all genes were purchased from Applied Biosystems. Amplification was performed on an ABI 7900 Sequencing Detection System by using Taqman® Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Relative gene expression of individual samples run in duplicate was calculated by comparison to a relative standard curve.

Tissue processing

Fluoro Jade C histochemistry. Briefly slides were dried at room temperature, immersed in a basic ethanol solution (80% containing 1% sodium hydroxide) and then rinsed in 70% ethanol and distilled water. Slides were then treated with potassium permanganate (.06% in dH20) for 10 min, rinsed with water and then incubated in Fluoro Jade C (0.0001% in a 1% acetic acid solution) and then rinsed in dH_20 and thoroughly dried on a slide-warmer, cleared in xylene and coverslipped with DPX (Sigma).

In order to assess degeneration of neuronal processes, we performed silver staining using a NeuroSilver Kit (FD NeuroTechnologies, Columbia MD) according to the manufacturer's instructions. Briefly, mice were overdosed with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde following injury. Sections were cut at 60 μm on a cryostat, stained with the silver staining kit, mounted on gel covered slides and then coverslipped with permount.

Microglial activation and astrocytic reactivity were assessed with IBA-1 and GFAP immunohistochemistry, respectively. Briefly, mice were transcardially perfused with 4% paraformaldehyde 40 days after the initial injury. The brains were removed, cryoprotected in sucrose solution, and frozen on crushed dry ice. Brains were then cut into 16-μm sections and mounted on charged slides. The tissue was washed, blocked in bovine serum albumin, incubated with the IBA-1 (Wako USA, Richmond VA) or GFAP (Dako North America, Carpinteria, CA) antibodies, washed and treated with a biotinylated secondary antibody, and then visualized with ABC and DAB (Vector Laboratories, Burlingame, CA).

Spatial learning and memory

In order to assess the consequences of the timing of repeated injuries on spatial learning and memory performance, mice in all four experimental groups underwent testing on the Barnes maze beginning on day 34 after an initial injury (or sham). This time point allows mice in all groups a period of at least two weeks to recover following injury (Fig. 1).

The Barnes maze is a brightly lit circular arena (36 in. in diameter) with 18 evenly spaced holes, one leading to a dark box and the others blocked off with black inserts. On the first day, animals were acclimated to the maze; mice were then guided from the center of the maze to the target hole. Mice then underwent five days of training consisting of three consecutive 60-sec trials separated by brief returns to their home cages. One day after the last training trial, animals were given a 60-sec probe trial in which the escape box was blocked off. Latency to find the target hole was recorded daily and time spent in the quadrant that formerly contained the target was recorded during the probe trial.

Microscopy

Fluoro Jade positive neurons were counted in the injured and contralateral cortex and hippocampus. Quantification of microglial (IBA-1 positive cells) and astrocytic (GFAP positive cells) expression was conducted as previously reported (Neigh et al., 2009), with minor modifications. Activation was scored in the following regions: sensorimotor cortex, hippocampus proper, piriform cortex, and thalamus, separately in the ipsilateral and contralateral hemispheres. The degree of activation (qualitatively determined by both the number and morphology of the glial cells) was given a score of 0–3, where 0 = no glial activation, 1 = mild activation, 2 = pronounced activation, and 3 = pronounced activation throughout the region. In addition, a second score was assigned using the same criteria, however this second score took into account the global degree of activation in each hemisphere. The scores were summed for each animal.

Similarly, silver-staining intensity was assessed by qualitatively assessing axonal staining in the corpus callosum and other forebrain white matter tracts by a trained observer blinded to the experimental conditions (Namjoshi et al., 2013). Multiple sections (at least three) were observed at 10× magnification. The degree of staining was then scored on a 5-point scale (0 = minimal staining, 5 = dark staining throughout white matter tracts and the corpus callosum) for both the ipsilateral and contralateral hemispheres. An additional blinded observer independently re-scored a subset of the sections, without prior knowledge of the score and inter-rater reliability was very high >0.9.

Data analysis

All parametric data (gene expression, Barnes maze, and autoradiography) were analyzed with one-way analysis of variance and significant differences were further analyzed with Least Significant Differences multiple comparisons test in SPSS. Nonparametric data including the qualitative assessments of the silver staining, IBA-1 and GFAP staining were analyzed with Kruskal–Wallis tests for independent samples followed by nonparametric multiple comparison tests. Differences were considered statistically significant if $p < 0.05$.

Results

^{14}C-2-Deoxyglucose autoradiography

mTBI induced a biphasic change in glucose uptake as measured by ^{14}C 2-deoxyglucose autoradiography. Following a single injury, glucose uptake rose in the sensorimotor cortex and CA1 field of the hippocampus, and six days after injury uptake returned to baseline levels (Figs. 2A–B). By ten days after injury there were no significant differences between animals that had undergone a single injury and sham animals. In another group, we re-injured mice three days after the initial injury (2Inj(3d)); prior to the resolution of cerebral hyperglycolysis. In contrast to the single injury mice, the 2Inj(3d) groups exhibit no significant changes in glucose utilization at any time point analyzed following repeated injuries. Finally, if the injuries were spaced 20 days apart (2Inj(20d)), well after the resolution of increased glucose utilization, an increase in glucose utilization occurred six days after the second injury that closely paralleled the changes that were observed six days after a single injury. Glucose utilization data for the ipsilateral (Table 1) and contralateral (Table 2) regions indicated that, although the specific timing of the changes in glucose utilization varied, in no case did two injuries separated by three days induce a statistically significant increase in glucose utilization. SHAM injured mice were collected at each of the injury time points and were later collapsed into a single SHAM group as there were no statistical changes across time in these animals.

We next measured gene expression for the glucose transporter proteins 1 and 3 (*Slc2a1* and *Slc2a3* respectively) with qPCR. mRNA expression levels for both genes were unaltered by injury ($p > 0.05$ in both cases; Fig. 2C).

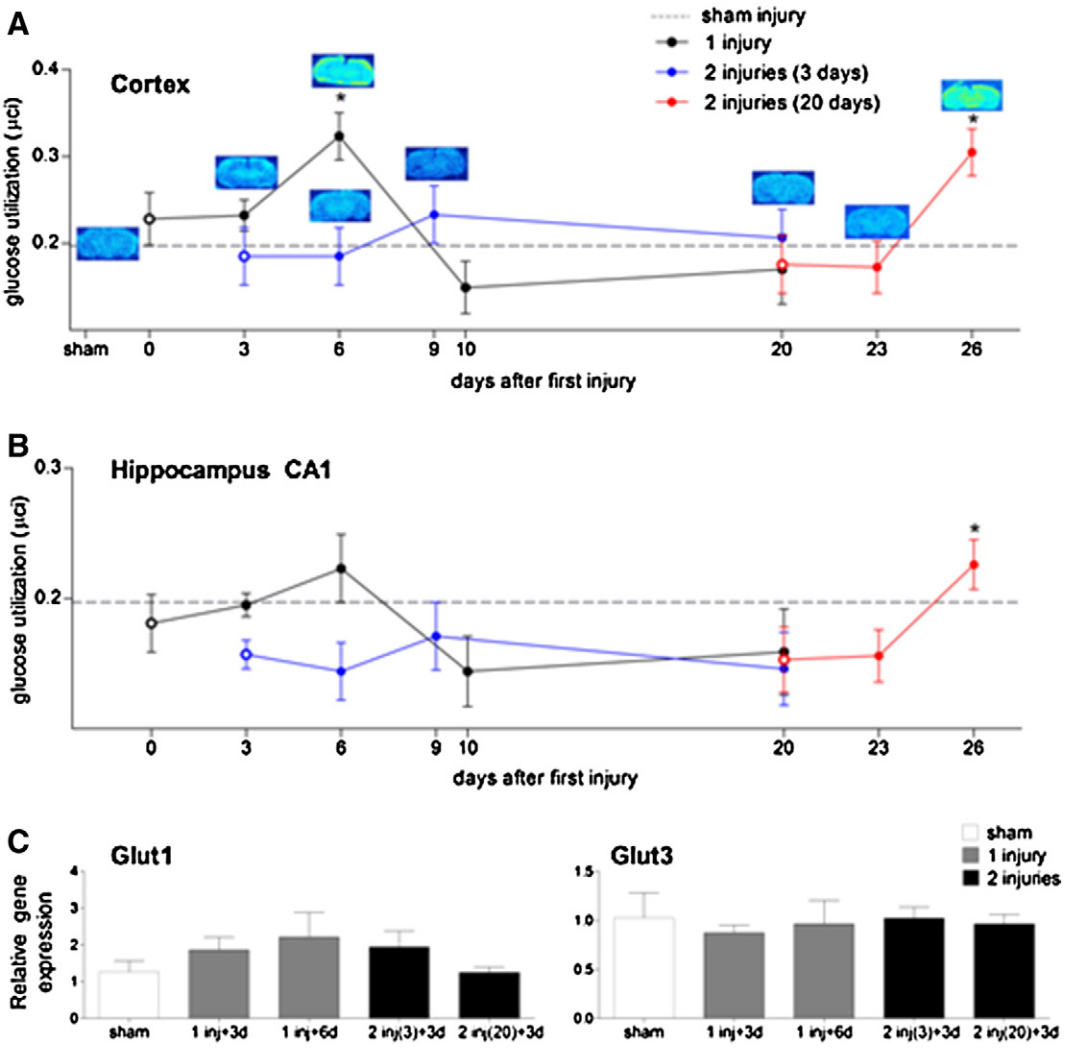

Fig. 2. Time course of cerebral glucose utilization following single or repeated traumatic brain injuries. Cerebral glucose utilization, shown as the mean microcuries (µci ± SEM), in the A) cortex and B) hippocampus CA1 following sham, single, or repeated injury. C) Gene expression of the glucose transporters *GLUT1* and *GLUT3* was not significantly altered by the injury condition 24 h after the final injury or SHAM procedure. *Significantly different from sham animals ($p < 0.05$). For glucose utilization studies n = 5–9/time point for injured animals and n = 24 SHAM animals, for gene expression n = 5–10/group.

Table 1
Glucose utilization in the ipsilateral hemisphere, represented as the mean microcuries (±SEM). An asterisk (*) denotes significant difference ($p < 0.05$) from sham group n = 5–9/time point for injured animals and n = 24 SHAM animals.

Days after injury								
Group	Sham	0	3	6	10	20	23	26
CA2	0.16(0.01)							
1 injury		0.17(0.02)	0.19(0.01)	0.20(0.02)	0.12(0.03)	0.16(0.03)		
2 injuries (3 days)			0.17(0.02)	0.15(0.02)	0.20(0.03)	0.15(0.03)		
2 injuries (20 days)						0.15(0.02)	0.17(0.03)	0.23(0.02)*
CA3	0.16(0.01)							
1 injury		0.18(0.02)	0.17(0.01)	0.19(0.02)	0.12(0.02)	0.15(0.02)		
2 injuries (3 days)			0.16(0.02)	0.13(0.02)	0.16(0.03)	0.14(0.02)		
2 injuries (20 days)						0.13(0.02)	0.15(0.02)	0.22(0.02)*
Dentate gyrus	0.16(0.01)							
1 injury		0.20(0.01)	0.19(0.01)*	0.20(0.02)*	0.13(0.02)	0.14(0.03)		
2 injuries (3 days)			0.17(0.01)	0.18(0.02)	0.20(0.04)	0.15(0.03)		
2 injuries (20 days)						0.16(0.01)	0.16(0.03)	0.22(0.02)*
Thalamus	0.19(0.01)							
1 injury		0.23(0.03)	0.24(0.02)*	0.25(0.03)	0.13(0.03)	0.16(0.02)		
2 injuries (3 days)			0.24(0.03)	0.19(0.03)	0.27(0.04)*	0.21(0.04)		
2 injuries (20 days)						0.20(0.04)	0.22(0.04)	0.31(0.03)*

Table 2
Glucose utilization in the contralateral hemisphere, represented as the mean microcuries (±SEM). An asterisk (*) denotes a significant difference ($p < 0.05$) from sham group, n = 5–9/time point for injured animals and n = 24 SHAM animals.

Days after injury								
Group	Sham	0	3	6	10	20	23	26
Cortex	0.22(0.01)							
1 injury		0.24(0.02)	0.24(0.01)	0.34(0.04)*	0.15(0.03)*	0.16(0.03)		
2 injuries (3 days)			0.24(0.03)	0.19(0.03)	0.20(0.03)	0.16(0.03)		
2 injuries (20 days)						0.19(0.02)	0.20(0.03)	0.31(0.03)*
CA1	0.17(0.01)							
1 injury		0.17(0.01)	0.20(0.01)	0.20(0.02)	0.12(0.02)*	0.18(0.03)		
2 injuries (3 days)			0.17(0.02)	0.15(0.01)	0.16(.03)	0.16(.03)		
2 injuries (20 days)						0.14(0.02)	0.16(0.03)	0.22(0.02)*
CA2	0.20(0.01)							
1 injury		0.17(0.01)	0.19(0.01)	0.21(0.02)	0.18(0.03)	0.21(0.04)		
2 injuries (3 days)			0.17(0.02)	0.15(0.02)	0.19(0.03)	0.16(0.02)		
2 injuries (20 days)						0.15(0.02)	0.17(0.03)	0.25(0.02)
CA3	0.20(0.01)							
1 injury		0.17(0.02)	0.18(0.01)	0.19(0.02)	0.18(0.02)	0.21(0.04)		
2 injuries (3 days)			0.17(0.01)	0.13(0.02)*	0.17(0.02)	0.14(0.02)*		
2 injuries (20 days)						0.12(0.01)*	0.15(0.03)	0.22(0.02)
Dentate gyrus	0.20(0.01)							
1 injury		0.18(0.02)	0.21(0.01)	0.20(0.02)	0.19(0.05)	0.21(0.04)		
2 injuries (3 days)			0.18(0.02)	0.17(0.03)	0.20(0.04)	0.21(0.04)		
2 injuries (20 days)						0.14(0.02)	0.17(.03)	0.23(0.02)
Thalamus	0.24(0.02)							
1 injury		0.23(0.03)	0.25(0.02)	0.28(0.03)	0.18(0.03)	0.21(0.04)		
2 injuries (3 days)			0.27(0.02)	0.20(0.03)	0.28(0.04)	0.26(0.04)		
2 injuries (20 days)						0.22(0.03)	0.23(0.05)	0.32(0.03)

Neuronal degeneration

There were virtually no Fluoro Jade positive cells in any condition (data not shown) although there was significant evidence of degenerating axons, which were further assessed with silver staining. Silver staining analysis indicated that 2Inj(3d) had significantly greater axonal degeneration (Kruskal–Wallis with pairwise comparisons; $p < 0.001$; Fig. 3) than either of the injury groups or sham animals.

Glial activation

Microglial activation assessed after the conclusion of behavioral testing indicated that both single and repeated injuries similarly increased microglial activation in the ipsilateral piriform and sensorimotor cortices, hippocampus, and thalamus relative to sham treated animals (Kruskal–Wallis with pairwise comparisons; $p < 0.001$; Fig. 4 and Table 3). In contrast, the contralateral hemisphere exhibited increased microglial activation only in the 2Inj(20d) group ($p < 0.005$; Table 3).

Astrocyte staining was significantly elevated following repeated injuries in the ipsilateral sensorimotor cortex, piriform cortex, hippocampus and thalamus (Kruskal–Wallis with pairwise comparisons; $p < 0.05$; Fig. 4 and Table 3). There was no statistical elevation of GFAP staining in the contralateral hemisphere of any group.

Inflammatory responses

qPCR was used to assess inflammatory responses to single and repeated mTBI. *Interleukin-1* gene expression was altered by injury ($F_{4,26} = 3.97$, $p < 0.05$; Fig. 5A) such that 2Inj(3d) exhibited higher gene expression than all other groups ($p < 0.05$ in all cases). Similarly, *tumor necrosis factor* gene expression was altered across groups ($F_{4,27} = 3.46$, $p < 0.05$; Fig. 5B), and again this was mediated by higher TNF gene expression in the 2Inj(3d) group relative to all other groups ($p < 0.05$ in all cases). *Itgam* gene expression, a gene that encodes a cell surface marker expressed on microglia, was also different across groups ($F_{4,22} = 3.29$, $p < 0.05$; Fig. 5C) with elevated gene expression in both the 2Inj(3d) and 2Inj(20d) mice relative to sham animals ($p < 0.05$). Finally, *glial fibrillary acidic protein* gene expression was altered by injury ($F_{4,23} = 5.98$, $p < 0.01$; Fig. 5D) such that both the 2Inj(3d) and 2Inj(20d) groups exhibited increased gene expression relative to sham animals ($p < 0.05$).

Spatial learning and memory

Barnes maze testing was performed to assess spatial learning and memory following single or repeated mTBI. All groups learned the task, as evidenced by a reduction in latency to find the escape hole across trials ($F_{4,116} = 113.17$, $p < 0.0001$). However, by days four ($F_{3,29} = 4.56$, $p < 0.01$) and five ($F_{3,29} = 3.10$, $p < 0.05$) of training there were significant differences among groups in the latency to find the escape hole such that 2Inj(D3) mice were impaired relative to all other groups (Fig. 6A; $p < 0.05$). In the probe trial following the final training session, all mice spent significantly more time in the quadrant of the maze that had contained the target hole; however, there was a significant difference among groups ($F_{3,34} = 3.37$, $p < 0.05$) such that 2Inj(3d) mice spent significantly less time in the target quadrant than all other groups (Fig. 6B; $p < 0.05$ in all cases).

Discussion

Traumatic brain injuries often cluster together in time such that injuries render individuals temporarily more susceptible to poor outcomes following subsequent injury. In this study, we utilized a modified Marmarou weight drop device (Biegon et al., 2004; Marmarou et al., 1994; Zohar et al., 2003) in mice and report that inflammatory responses, axonal degeneration, and spatial learning and memory are worse in injuries that are close together in time. Here, mice underwent single or repeated injuries with either a 3- or a 20-day delay, and exhibited divergent histological, metabolic, and functional differences. A single injury induced a transient but marked increase in local cerebral glucose utilization in the injured hippocampus and sensorimotor cortex, whereas a second injury, three days after the first, rapidly reduced glucose utilization and failed to induce an increase six days later as occurred following a sham or 20 day repeated injury. Moreover, repeated injuries that were separated by three days were associated with greater axonal degeneration, enhanced inflammatory responses, and poorer

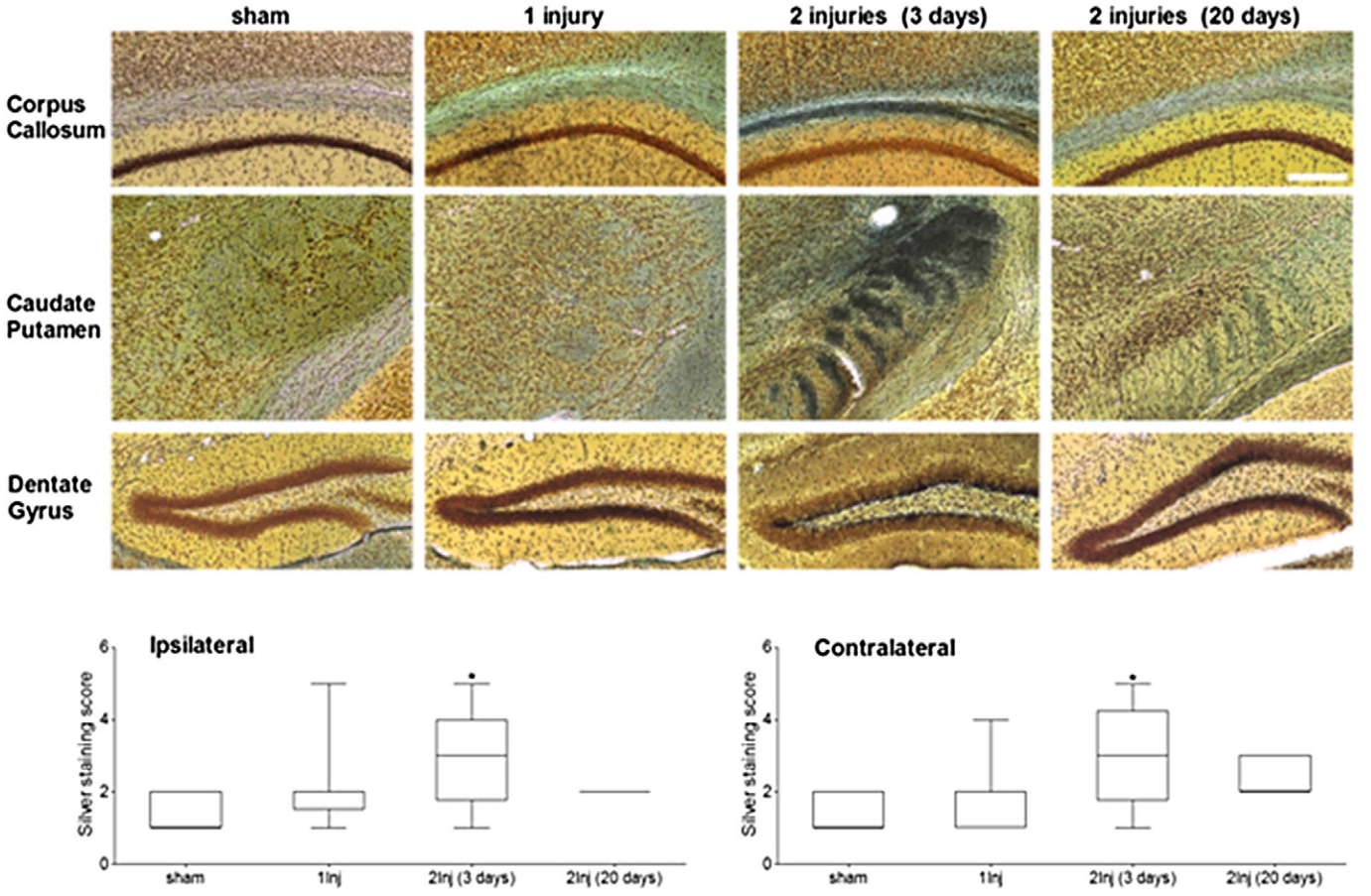

Fig. 3. TBI-induced axonal degeneration. Repeated TBIs (occurring 3 days apart) cause a significant and widespread axonal degeneration as assessed via silver staining. Axonal degeneration was particularly pronounced in the 2Inj(3d) corpus callosum, caudate putamen, and hippocampus dentate gyrus forty days after the initial injury. Scale bar = 200 μm. *Significantly different from sham animals ($p < 0.05$), n = 6–14/group.

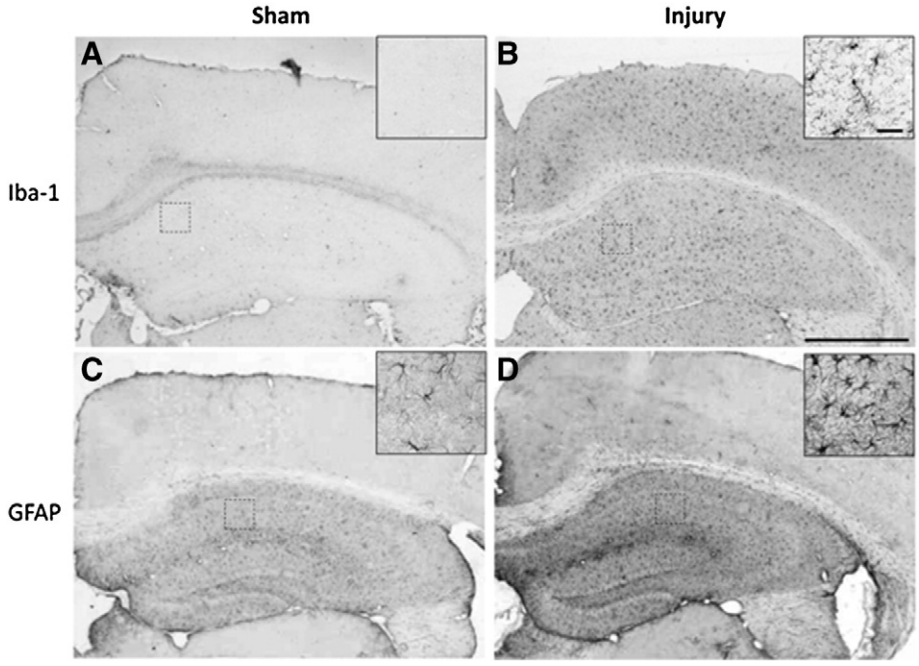

Fig. 4. TBI-induced glial reactivity. Microglial activation was evident in all injury groups, regardless of the number and timing of the injuries. Shown are representative photomicrographs of IBA-1 positive microglia from A) sham and B) injured hippocampus and overlying cortex, and GFAP positive astrocytes in the hippocampus and overlying cortex from C) sham and D) repeatedly injured mice. Inset scale bar = 20 μm, low magnification scale bar = 400 μm, n = 10–16/group for injured animals and n = 22 SHAM animals. Glial staining occurred on tissue collected 40 days after the initial injury.

Table 3
Glial reactivity after mild traumatic brain injury. Qualitative assessment of IBA-1 positive microglia and GFAP positive astrocytes in the brain after traumatic brain injury or sham surgery, data are presented as mean (±SEM). An asterisk (*) denotes a significant difference ($p < 0.05$) from 'sham' group, (†) denotes a significant difference from '1 injury' group, n = 10–16/group for injured animals and n = 22 SHAM animals. Glial staining occurred on tissue collected 40 days after the initial injury.

Ipsilateral hemisphere					Contralateral hemisphere			
Condition	Sensorimotor cortex	Hippocampus	Piriform cortex	Thalamus	Sensorimotor cortex	Hippocampus	Piriform cortex	Thalamus
IBA-1 (microglia)								
Sham	1.60 (0.24)	1.47 (0.22)	1.73 (0.27)	1.13 (0.22)	1.13 (0.19)	1.27 (0.21)	1.80 (0.27)	0.93 (0.15)
1 injury	3.00 (0.35)*	2.87 (0.34)*	3.13 (0.32)*	2.53 (0.31)*	2.27 (0.33)	2.27 (0.33)	2.40 (0.32)	1.93 (0.34)
2 injuries (3 days)	3.38 (0.33)*	3.13 (0.24)*	2.88 (0.29)	2.38 (0.29)*	2.44 (0.26)	2.44 (0.26)	2.63 (0.22)	1.81 (0.23)
2 injuries (20 days)	3.50 (0.18)*	3.60 (0.27)*	3.50 (0.27)*	3.00 (0.30)*	3.60 (0.27)*†	3.60 (0.27)*	3.50 (0.27)*	3.00 (0.30)*
GFAP (astrocytes)								
Sham	1.32 (0.15)	2.41 (0.19)	1.68 (0.20)	1.09 (0.11)	1.05 (0.08)	2.41 (0.18)	1.32 (0.17)	1.00 (0.07)
1 injury	2.20 (0.32)	2.93 (0.15)	2.07 (0.18)	1.27 (0.15)	1.00 (0.10)	2.53 (0.17)	1.53 (0.16)	0.93 (0.07)
2 injuries (3 days)	2.63 (0.38)*	3.38 (0.26)*	2.63 (0.26)*	1.56 (0.18)	1.43 (0.26)	2.81 (0.21)	2.00 (0.24)	1.19 (0.10)
2 injuries (20 days)	2.90 (0.48)*	3.30 (0.26)	2.80 (0.36)*	2.10 (0.31)*	1.70 (0.34)	3.00 (0.21)	1.90 (0.23)	1.30 (0.21)

performance in a spatial learning and memory task. The three day time point that we selected for the early injuries in this study corresponds to a period of depressed glucose utilization in previous studies (Yoshino et al., 1991). If the increased glucose utilization observed after a single injury is an adaptive component of recovery, and the 2Inj(3d) mice are not able to mount this response, then this second injury may produce a significant energetic crisis such that energetic demands outstrip the ability of the damaged cells to utilize energy. Taken together these data indicate that animals that do not mount an increase in glucose utilization following mTBI exhibit poorer outcomes than those that do.

A wealth of research has shown that cerebral glucose utilization changes following traumatic brain injuries in both humans and rats (Bergsneider et al., 1997; Prins et al., 2013; Yoshino et al., 1991). Importantly, many studies have reported a rapid (minutes to hours) increase in glucose utilization after an injury followed by a prolonged period of reduced uptake. Early increases in glucose utilization are a predictor of positive outcomes in some clinical studies and have been conceptualized as a homeostatic response to cope with disruptions in ion balance and excess glutamate release (Hovda et al., 1990). In the current study, we report that glucose utilization does not change statistically after a single injury until six days later, when uptake markedly increases. Further, we measured cerebral glucose utilization out to 20 days after an initial injury and never found evidence of statistically depressed glucose uptake although the values were numerically lower.

Maintaining a positive energy balance following TBI is critically important to prevent inflammation and subsequent axonal disconnections. Importantly, axon damage from this type of injury is rarely the result of the shearing forces associated with the initial impact (Adams et al., 1982; Christman et al., 1994). In fact, subsequent energetic failure, mitochondrial dysfunction, cytoskeletal damage, and other secondary responses significantly contribute to axonal degeneration (Johnson et al., 2013; Maxwell et al., 2003; Okonkwo and Povlishock, 1999). After TBI, there is a significant increase in intracellular calcium in axons that can activate proteases such as calpain and calcineurin that can in turn induce serious damage to axonal structures and potentiate energy failure (McGinn et al., 2009; Singleton et al., 2001; Wolf et al., 2001). Further, mitochondria appear to buffer extracellular calcium but can experience transition pore opening leading to reduced energy production, oxidative stress, further calcium release, and finally axonal inflammation and disconnection (Lifshitz et al., 2004; Okonkwo and Povlishock, 1999). ATP-dependent calcium pumps and sodium potassium co-transporters are necessary to restore ionic balance but require large amounts of energy at a time when energy production is impaired (Limbrick et al., 1995; Sun et al., 2008; Wolf et al., 2001). Despite these pathological processes occurring in the traumatized brain, many mTBI motor and cognitive impairments are relatively mild and spontaneously resolve over time (Anderson et al., 2000; Demeurisse, 2000; Schmidt et al., 1999). However, if a subsequent injury occurs during

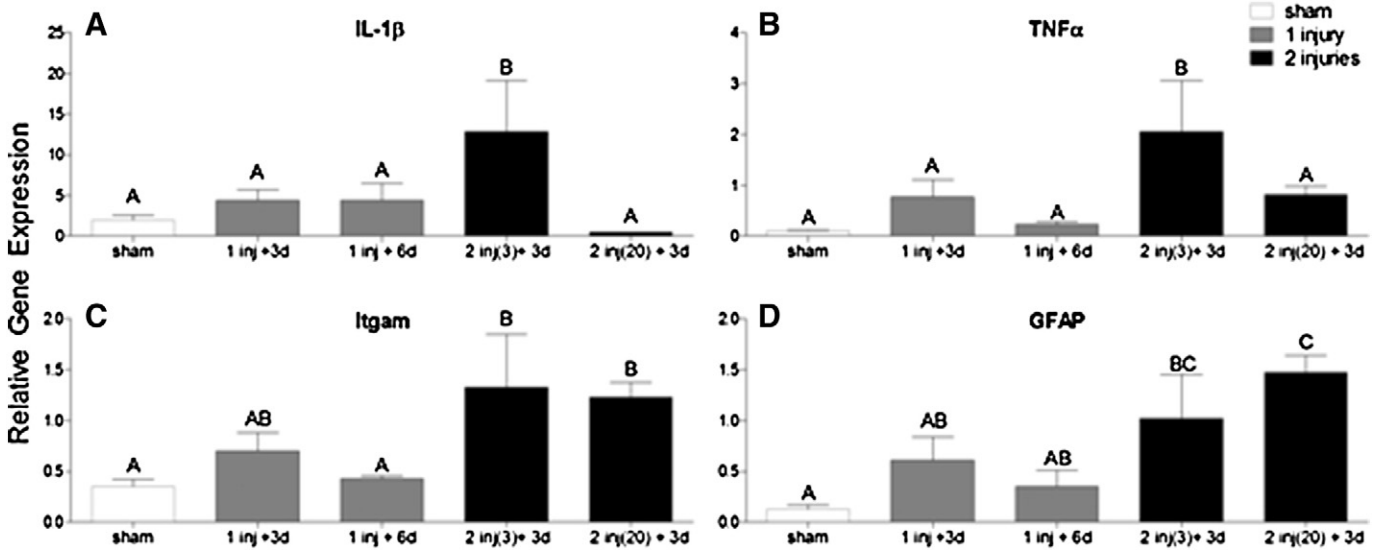

Fig. 5. Gene expression of neuroinflammation markers following TBI. mRNA expression is represented relative to *18S* rRNA (mean ± SEM). Gene expression of proinflammatory cytokines A) *IL-1β* and B) *TNFα* and glial markers C) *Itgam* and D) *GFAP* is significantly altered by TBI, and is exacerbated by a repeated injury 24 h after the final injury or SHAM procedure. Bars that do not share the same letter are significantly different from each other ($p < 0.05$), n = 5–10/group.

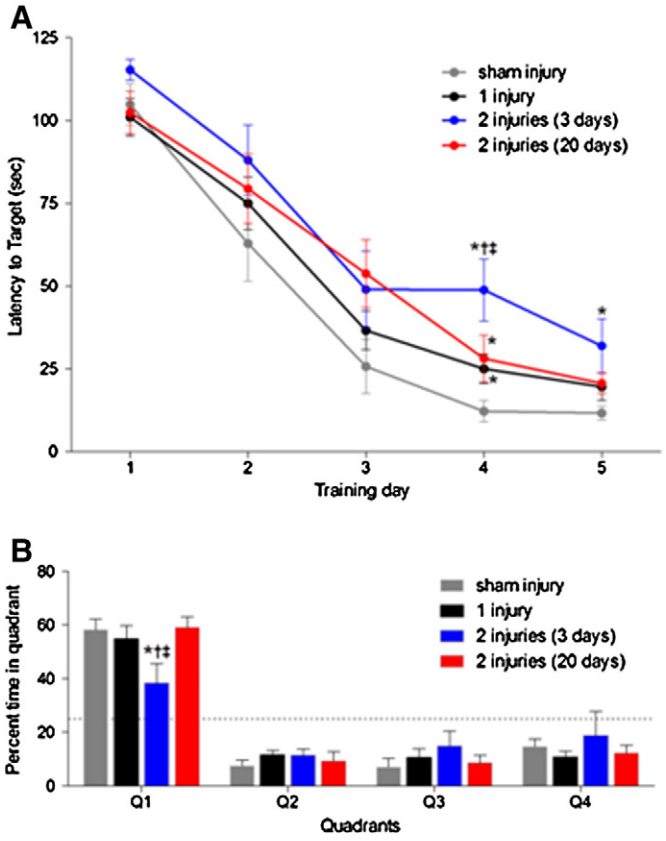

Fig. 6. Learning and memory deficits following TBI. Learning and memory were assessed via Barnes maze testing 34 days after the initial injury. Shown are A) latencies to finding the escape (mean seconds ± SEM) across five trial days and B) percent time (mean ± SEM) spent in each quadrant following the removal of the escape box (Q1 = target quadrant). *Significantly different from sham animals, †significantly different from single injury animals, ‡significantly different from repeated injury animals (20 days apart) ($p < 0.05$), n = 8–10/group.

this critical period of energetic impairment, an energetic bottleneck occurs that in turn allows increased inflammatory responses, greater axonal degeneration, and results in poorer functional outcomes (Laurer et al., 2001; Meehan et al., 2012; Prins et al., 2010; Tavazzi et al., 2007; Vagnozzi et al., 2005).

The specific molecular mediators underlying TBI-induced changes in cerebral glucose metabolism are not fully understood, though many studies have reported that failure of the cerebral vasculature to respond to the increased energetic demands after injury contribute to this phenomenon (Bouma and Muizelaar, 1992; Enevoldsen and Jensen, 1978; Yamakami and McIntosh, 1991). Increasing cerebral blood flow after injury reduces the negative metabolic and functional consequences of TBI (Harris et al., 2012). Future studies will be necessary to determine whether cerebral blood flow is negatively impacted by injuries closer together in time versus injuries separated by a longer delay as this may explain the abolition of enhanced glucose utilization in the 2Inj(3d) group. Further, some studies have reported upregulation of the glucose transporter proteins responsible for glucose movement across the blood–brain barrier (glut1) and into neurons (glut3) (Hamlin et al., 2001) following severe TBI but we find no difference in the mRNA expression of either gene in the injured hemisphere in our milder version.

Our glucose utilization data differ from many of the extant reports in two ways, 1) we report a significant delay prior to increased glucose utilization and 2) we do not report a reduction in glucose utilization following the initial increase. The lack of depression in glucose regulation is not unprecedented, as a similar phenomenon has been reported in juvenile rats (Appelberg et al., 2009; Babikian et al., 2010). To our knowledge this is the first report of cerebral glucose utilization in mice following experimental head injury and the changes we report may reflect species differences. Further, repeating the injury three days after an initial injury completely prevented the enhanced glucose uptake observed in the single injury group at the same time point, indicating that our methodology is sensitive to bidirectional shifts in glucose utilization.

In the current study, both single and repeated injuries resulted in increases in microglial staining throughout the forebrain while only repeated injuries increased GFAP immunoreactivity in the ipsilateral hemisphere. However, proinflammatory cytokine gene expression and markers of glial proliferation were significantly elevated only in the 2Inj(3d) group. The dissociation of gene expression from morphology of the glia is consistent with previous observations that histological evidence of glial activation can last for months while the active period of gene expression can be much shorter ranging from hours to a few days (Gehrmann et al., 1991; Taupin et al., 1993). Further, IBA-1 + resident brain microglia are not the sole source of proinflammatory cytokine expression though they are the predominant source early after injury. An additional complication of this approach is that mice in the 2Inj(20d) group survived nearly three weeks longer after their initial injury than the 2Inj(3d) group, suggesting that direct comparison of microglial morphology may be complicated by the time since the initial injury. More importantly, the injury used in the current study did not induce significant increases in *IL-1* or *TNF* gene expression unless it occurred close in time to a previous injury. Inflammatory responses may be exacerbated during this time period for a number of non-mutually exclusive reasons including 1) that the injury occurred during a period of low grade but acute inflammation and 2) the energetic crisis may have reduced the ability of the affected cells to remove cellular debris and normalize membrane potentials and ionic balance. Conversely, inflammation can potentiate the excitotoxic effects of glutamate and excess calcium leading in turn to greater inflammation (Fogal et al., 2005; Fogal and Hewett, 2008). Future studies will assess whether protein concentrations of these cytokines are also elevated, and the role of alterations in inflammatory responses as mediators of the increased period of vulnerability.

Functionally, mice that underwent a closed head injury exhibited poorer acquisition of the Barnes maze paradigms. However, there was a significantly greater impairment of performance during both the acquisition and probe trials of mice in the 2Inj(3d) group relative to all others. This is consistent with previous literature on repeated closed head injuries in mice (Creeley et al., 2004; Laurer et al., 2001; Longhi et al., 2005; Meehan et al., 2012). This occurs despite the fact that the behavioral testing for the 2Inj(3d) group occurred following a recovery period that was nearly three weeks longer than the 2Inj(20d) group; indicating that the timing of injuries in relation to each other (and not to the timing of the learning and memory test) is the key factor in behavioral outcomes. Further, as there is some spontaneous recovery of function in many TBI models, it remains possible that the performance would have been worse if tested closer to the injuries.

In conclusion, the data presented here add to, and extend, the growing body of literature linking metabolic dysfunction after mild traumatic brain injury to a period of enhanced vulnerability to subsequent injuries. Mice undergoing mild closed head injuries three days apart exhibited greater inflammation, proinflammatory cytokine gene expression, and axonal degeneration as well as functional deficits compared to mice that underwent either a single injury or repeated injuries separated by 20 days. Further, although single injury and 2Inj(20d) mice developed a significant increase in glucose uptake 6 days after the final injury, this did not occur in the 2Inj(3d) group, an effect that may be indicative of a severe metabolic crisis in the injured CNS. Future studies will be necessary to determine the precise mediators of metabolic dysfunction and causally link this phenomenon to poor outcomes in this model. The specific time points that we have investigated here may not correspond directly to either the most common interval

between injuries or the length of the period of enhanced vulnerability to subsequent injury in humans, but provide additional evidence that this period of enhanced vulnerability both functionally and metabolically does exist. Finally, these data strongly reinforce the public health concern that too rapid return to activity after a traumatic brain injury can induce permanent damage and disability. If we can design interventions that can begin to normalize metabolism after TBI, it may well prevent the most serious consequences of both single and repeated TBI.

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