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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

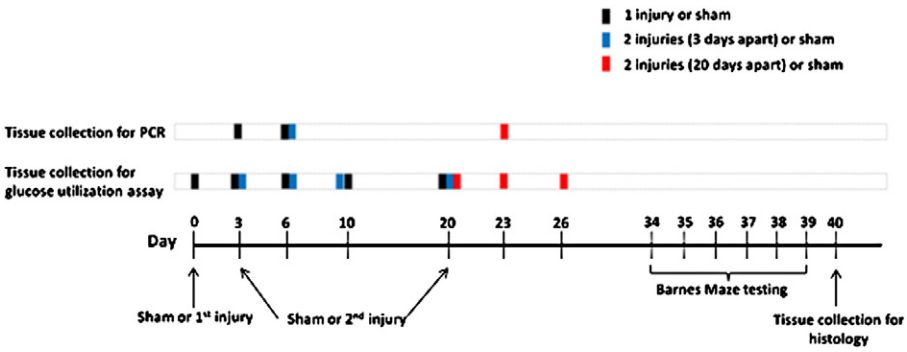

Fig. 1. Experimental timeline. Animals were subjected to either a single sham or a TBI procedure, or were re-injured either 3 or 20 days later. Tissue collection time points for each group are shown for PCR, the glucose utilization assay, and histology (FluoroJade C, silver stain, IBA-1 and GFAP). The 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).