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Research report

The effect of an acute systemic inflammatory insult on the chronic effects of a single mild traumatic brain injury

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ABSTRACT

A small but significant proportion of mild traumatic brain injury (mTBI) sufferers will report persistent symptoms, including depression, anxiety and cognitive deficits, in the months, or even years, following the initial event. This is known as post-concussion syndrome and its pathogenesis is not yet known. This study sought to investigate the role of a peripheral inflammatory insult in the development of ongoing behavioral symptoms following a mTBI. To investigate, male Sprague-Dawley rats were administered a single mTBI using the diffuse impact-acceleration model to generate ~100 G of force. Sham animals underwent surgery only. At 5 days following surgery, rats were given either the TLR4 agonist, lipopolysaccharide (LPS, 0.1 mg/kg), or saline via an intraperitoneal injection. mTBI animals showed an exaggerated response to LPS, with an increase in the expression of pro-inflammatory cytokines within the hippocampus at 24 h post-dose, an effect not seen in sham animals. This was associated with the development of persistent behavioral deficits in the mTBI:LPS animals at 3 months post-injury. These behavioral deficits consisted of increased time spent immobile on the forced swim-test, indicative of depressive like behavior, impaired cognitive performance on the Barnes Maze and decreased anxiety on the Elevated Plus Maze. In contrast, animals administered mTBI alone had no deficits. This study provides evidence that a peripheral inflammatory stimulus can facilitate ongoing symptoms following a mTBI. As such this provides a basis for further exploration of exogenous factors which promote immune system activation as potential targets for intervention to allow the resolution of symptoms following a mTBI.

1. Introduction

Mild traumatic brain injury (mTBI) is one of the most prevalent neurological conditions, with estimates that roughly 42 million people worldwide each year may suffer a mTBI [1]. Indeed, over 80% of all TBIs are classified as mild injuries [2,3], the result of a non-penetrating direct or indirect blow to the head, accompanied by loss of consciousness for less than 30 min and/or alterations to mental state [4].

In 10–20% of individuals, symptoms may persist for a number of weeks, months or even years following a mTBI [5,6]. This constellation of symptoms occurring after a mTBI, encompassing headaches, dizziness, fatigue, cognitive impairment and neuropsychiatric symptoms, such as irritability and reduced tolerance to stress, is known as post-concussion syndrome (PCS) [7,8]. The pathogenesis of why these persistent symptoms occur in a minority of sufferers remains unclear, with a number of proposed hypotheses, including underlying biopsychosocial factors [9], persistent abnormalities in brain functional connectivity [10] or lower pre-injury cognitive reserve [11]. Recently, it has been suggested that development of persistent inflammation may also play a key role [12]. Indeed, increased levels of circulating pro-inflammatory cytokines are linked to the development of a number of symptoms described in PCS, such as cognitive impairment, depression and fatigue [13–15]. In support of this, Su et al. found that patients with higher serum C-reactive protein, indicative of systemic inflammation, were more likely to have persistent psychological symptoms and cognitive impairment at 3 months following a mTBI [16].

It is known that even a mTBI elicits a neuroinflammatory response, with acute activation of microglia and astrocytes [17,18] and increased expression of inflammatory cytokines and chemokines, both systemically and within the brain itself [19,20]. Although this typically resolves within weeks [18], a prior neuroinflammatory stimulus can cause an exaggerated response to other inflammatory stimuli, including peripheral inflammatory insults [21], with this phenomenon known as microglial priming [22]. Microglial priming is seen as a higher baseline expression of inflammatory mediators, a lower threshold of activation and an exaggerated response following activation [21]. This concept has been demonstrated following a moderate, diffuse TBI in mice, whereby a peripheral immune challenge at 1 month post-injury acutely

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worsened memory consolidation on the Barnes Maze [22] and enhanced depressive-like behavior on the tail suspension test [23]. Similarly following a severe fluid percussion injury administration of IL-1β (20 μg/kg or 40 μg/kg), worsened motor outcome with an increase in contusion volume at 3 days post-injury. Inflammatory insults may also prime the response to TBI, with a severe peripheral inflammatory insult in a tibial fracture administered immediately prior to a mild diffuse TBI in mice exacerbating neuroinflammation, with a worsening of motor performance at 30 days post-TBI [23]. However, the effect of a mild peripheral immune stimulus acutely following a single diffuse mTBI on the development of persistent behavioral symptoms has not yet been examined.

2. Methods

All studies were performed within the guidelines established by the National Health and Medical Research Committee of Australia and were approved by the Animal Ethics Committee of the University of Adelaide. Male Sprague Dawley rats (10–12 weeks) were housed in a controlled temperature environment under a 12 h light/dark cycle with uninterrupted access to food and water. Rats were randomly allocated to receive either sham surgery or a single mTBI, using the modified version of the Marmarou impact-acceleration model to deliver ~100 G of force [24]. At 5 days following injury, animals were randomly allocated to receive either 0.1 mg/kg of LPS (E coli 055:B5) or an equal volume of saline via intraperitoneal injection, with the administrator blinded to the treatment. This LPS administration was as per previous studies, which indicated that this dosing protocol generates a low grade systemic inflammatory response [25,26]. To study the acute effects of LPS, at 24 h following LPS administration, animals were sacrificed and the brains were removed for either immunohistochemical (n = 4 per group) or biochemical analysis (n = 4 per group). In order to examine whether LPS administration had long-term effects, another group of animals underwent a behavioral battery at 3 months post-injury (sham:saline, sham:LPS n = 11; mTBI n = 9 and mTBI:LPS n = 8) prior to being sacrificed, with half allocated to immunohistochemical analysis (sham:saline, sham:LPS n = 5, mTBI, mTBI:LPS n = 4) and half to molecular analysis (sham:saline, sham:LPS n = 6, mTBI n = 5, mTBI:LPS n = 4). It should be noted that the last component of the behavioral battery is the forced-swim test (FST). As such animals were sacrificed at 24 h following this test, to ensure no adverse effects of the stress of the test on neuropathological measurements. Previous studies have shown that serum corticosterone levels peak at 30 min post-FST exposure and return to control level by 2 h [27].

2.1. Rodent model of TBI

Male, Sprague-Dawley rats (350–400 g) were injured using the diffuse impact-acceleration model of brain injury, which has been extensively used in our laboratory for a number of years and is well characterized in terms of metabolic, histologic and neurologic outcomes [28,29]. Animals are placed on a 10 cm thick foam cushion, and a 450 g weight is dropped from 1 m onto a steel disc affixed to the rat's skull. This produces an acceleration/deceleration injury that is typical of a mild head injury. Following injury, the skin overlying the injury site is stapled and the rats are returned to their home cage. Temperature is maintained throughout all procedures using a water-heated thermostatically controlled heating pad. Sham control animals undergo surgery, but do not receive an impact.

2.2. Functional outcome assessment

A behavioral battery was performed at three months post-injury with animals tested daily in order from the least to the most stressful test. This consisted of the Open Field (Day 90), Elevated Plus Maze (EPM) (Day 91), Y Maze (Day 92), Barnes Maze (Days 93–95 & Day 97) and the forced swim test (FST) (Day 98). All testing was analyzed via Anymaze™ software.

2.2.1. Open field (baseline locomotion)

The open field test consists of a 1 m × 1 m box in which the animal is placed in the centre and allowed to explore freely for five minutes, with the distance travelled calculated. This is a common measure of locomotor activity in rodents [30].

2.2.2. Elevated plus maze (anxiety)

The EPM is a cross shaped maze with two closed and two open arms and is used to evaluate anxiety in rodents [31]. Rats are allowed to explore the maze for 5 min, with rats exhibiting anxious behaviors preferring to spend more time in the closed arms than the open arms.

2.2.3. Y maze (cognition)

The Y Maze assesses spatial and recognition memory in rodents [32]. Three arms are arbitrarily assigned as start, novel and other arms and are randomly alternated between animals. The rat is first introduced into the maze with the novel arm blocked off and allowed to freely explore for three mins. One hour after initial exposure, the rat is reintroduced into the maze with all three arms open and allowed to explore freely for three min. Unimpaired animals will spend more time in the novel arm compared to cognitively impaired animals.

2.2.4. Barnes maze (cognition)

The Barnes maze is a commonly used test of learning and memory in rodents [33]. It consists of a circular maze 1.2 m in diameter with 18 escape holes placed around the circumference with an escape box located underneath one of the holes. Rats are placed in the centre of the maze and the time taken to find the escape box determined. During the acquisition phase, each rat is given 2 trials a day for 3 days. Following a rest day, a probe trial is conducted where the box is moved 90° from original position to assess cognitive flexibility in terms of the ability of the animal to learn the new location of the escape box.

2.2.5. Forced swim test (depressive-like behavior)

Animals are placed in a plastic cylinder filled with water (20–24 °C) to a depth of 30 cm for 6 min. Amount of time spent immobile is then used as a reflection of behavioral despair and helplessness, a rodent analogue of depressive-like behavior [34].

2.3. Immunohistochemistry

Rats were terminally anaesthetized with isoflurane and transcardially perfused with 10% formalin at either 24 h following the LPS dose or 24 h following completion of the behavioral battery at 3 months post-injury. Three hippocampal sections per brain, 5 μm thick, were collected at 250 μm intervals, representing the region from Bregma −2.5 to −4 mm. Slides were then stained with the microglial/macrophage marker IBA1 (1:1000, Wako Pure Chemical Industries) or the activated microglia/macrophage marker (1:500, Abcam). Slides were first dewaxed and dehydrated with endogenous peroxidase activity blocked by incubation with 0.5% hydrogen peroxide in methanol for 30 min. Slides were then washed in 2 × 3 min in phosphate buffered saline (PBS) before antigen retrieval retrieved by heating at close to boiling point for 10 min in citrate retrieval buffer. Once the slides had cooled below 40 °C they were washed with PBS before being blocked with 3% normal horse serum in PBS for 30 min. The primary antibody was applied to the slides which were left to incubate overnight. The next day slides were washed in 2 × 3 min of PBS before an anti-rabbit IgG (IBA1) or anti-mouse (CD68) biotintylated antibody was added for 30 min. After a further PBS wash, slides were incubated with streptavidin peroxidase conjugate for 60 min followed by another rinse with PBS. The immunocomplex was then visualised with precipitation of DAB (Sigma D-5637) in the presence of hydrogen peroxide. All acute