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Toll like receptor 4 activation can be either detrimental or beneficial following mild repetitive traumatic brain injury depending on timing of activation

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ABSTRACT

A history of repeated concussion has been linked to the later development of neurodegeneration, which is associated with the accumulation of hyperphosphorylated tau and the development of behavioral deficits. However, the role that exogenous factors, such as immune activation, may play in the development of neurodegeneration following repeated mild traumatic brain injury (rmTBI) has not yet been explored. To investigate, male Sprague-Dawley rats were administered three mTBIs 5 days apart using the diffuse impact-acceleration model to generate ~100 G. Sham animals underwent surgery only. At 1 or 5 days following the last injury rats were given the TLR4 agonist, lipopolysaccharide (LPS, 0.1 mg/kg), or saline. TLR4 activation had differential effects following rmTBI depending on the timing of activation. When given at 1 day post-injury, LPS acutely activated microglia, but decreased production of pro-inflammatory cytokines like IL-6. This was associated with a reduction in neuronal injury, both acutely, with a restoration of levels of myelin basic protein (MBP), and chronically, preventing a loss of both MBP and PSD-95. Furthermore, these animals did not develop behavioral deficits with no changes in locomotion, anxiety, depressive-like behavior or cognition at 3 months post-injury. Conversely, when LPS was given at 5 days post-injury, it was associated acutely with an increase in pro-inflammatory cytokine production, with an exacerbation of neuronal damage and increased levels of aggregated and phosphorylated tau. At 3 months post-injury, there was a slight exacerbation of functional deficits, particularly in cognition and depressive-like behavior. This highlights the complexity of the immune response following rmTBI and the need to understand how a history of rmTBI interacts with environmental factors to influence the potential to develop later neurodegeneration.

1. Introduction

A history of concussion, particularly repeated injury, has been linked to an increased risk for the development of neurodegenerative diseases, with extensive studies on the effects of repeated impacts in NFL players in the USA finding the risk of dying from a neurodegenerative disease is 3 times higher than the general population (Lehman et al., 2012). Furthermore players with self-reported history of more than 3 concussions have a fivefold increased prevalence of mild cognitive impairment (Guskiewicz et al., 2005) and threefold increase in depressive-like behavior (Guskiewicz et al., 2007).

Of interest it has been suggested that repeated head impacts may be linked to the specific neurodegenerative disease, chronic traumatic encephalopathy (CTE). It is proposed that CTE generally develops in midlife, long after the initial injury, and has been linked to the development of clinical symptoms including memory disturbances, attention deficits and behavioral problems (McKee et al., 2009; Stern et al., 2011). It is characterized by widespread brain atrophy, beginning in the cortex and then progressing to the hippocampus, entorhinal cortex and amygdala (McKee et al., 2009), with the characteristic pathological feature of CTE being the accumulation of an abnormal protein- hyperphosphorylated tau-within the brain (Stern et al., 2011). These protein aggregations take the form of neurofibrillary tangles that are found intracellularly in the cytoplasm of neurons. Tau is a normal axonal protein that binds to microtubules, promoting microtubule assembly and stability. When tau becomes hyperphosphorylated, microtubules

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are disassembled, impairing axonal transport, which compromises neuronal functional and can ultimately lead to neuronal death (Ballatore et al., 2007). The detached tau is also prone to self-aggregation and polymerization, initially leading to the formation of tau oligomers, which then further aggregate into neurofibrillary tangles (Maeda et al., 2007), The exact mechanisms via which concussion may promote tau hyperphosphorylation, leading to later neurodegeneration, remain poorly understood.

It is also likely that external factors may play role in the disease process. Of particular interest is the role of activation of the innate immune receptor, toll like receptor (4). This receptor is known to be involved in the initial response to concussion, with levels of TLR4 mRNA increasing within hours following an insult (Mao et al., 2012), and can also be stimulated by a wide range of exogenous factors including infection, strenuous exercise, changes in diet and alcohol consumption (Bala et al., 2014; Chongwatpol et al., 2015; Couch et al., 2016; Erridge et al., 2007; Ghanim et al., 2009; Selkirk et al., 2008). TLR4 is a member of the TLR family that recognizes a diverse range of 'patterns' on exogenous and endogenous danger signals (Buchanan et al., 2010). Activation of the TLR4 signaling pathway leads to the robust and transient transcription of a myriad of pro-inflammatory factors, including cytokines (IL-1β, IL-6), chemokines and immune receptors (Buchanan et al., 2010), as a result of microglial activation. Chronic activation of microglia is a feature of CTE (Blaylock and Maroon, 2011), and it has been proposed that a chronic low grade inflammatory state within the brain promotes the development of other neurodegenerative diseases, like Alzheimer's Disease (AD) (Hensley, 2010). Previous research has shown that activation of the TLR4 signaling pathway is capable of exacerbating tau pathology in other tauopathies, such as AD (Kitazawa et al., 2005), and that activation of microglia is known to be associated with hyperphosphorylation of tau (Li et al., 2003). However, the effects of TLR4 activation following repeated mTBI on the later development of neurodegeneration have yet to be explored.

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 3 mTBIs (rmTBI), spaced 5 days apart, using the modified version of the Marmarou impact-acceleration model to deliver ~100 G of force (McAteer et al., 2016). This time interval between injuries and number of injuries has been described as optimal to produce cumulative long-term functional deficits after rmTBI (Shultz et al., 2012b). At either 1 or 5 days following the last-injury, rmTBI 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 (Fig 1). This dosage was based on previous studies showing that this dose of LPS was sufficient to generate a low grade systemic inflammatory response (Chongwatpol et al., 2015; Couch et al., 2016). As no effect of timing of LPS dosage was seen in sham animals, half the group received LPS at 1d and the other at 5d post-surgery, with these animals combined as the Sham-LPS group. To study the acute effects of LPS, 24 h following LPS administration, animals underwent the open field test prior to being sacrificed and the brains were removed for either immunohistochemical (n = 4 per group) or biochemical analysis (n = 4 per groups) as detailed below. In order to examine whether LPS administration had long-term effects, animals underwent a behavioral battery at 3 months post-injury prior to being sacrificed, with half allocated to immunohistochemical analysis (n = 5–6 per group) and half to biochemical analysis (n = 4 per group).

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 (Corrigan et al., 2012; Heath and Vink, 1995). To deliver rmTBI, the weight is dropped onto the steel disc from 1 m on days 0, 5 and 10. A 10 cm thick foam cushion decelerates the head after impact, thus producing an acceleration/deceleration injury that is typical of a mild head injury. After injury, the skin overlying the injury site is sutured 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. This rat model of rmTBI is known to promote the accumulation of hyperphosphorylated tau both acutely and chronically following TBI (McAteer et al., 2016).

2.2. Immunohistochemistry

Rats were terminally anaesthetized with isoflurane and transcardially perfused with 10% formalin. 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 for markers of neuroinflammation (GFAP 1:40,000, MO782 Dako; IBA1 1:1000, 019–19741, Wako Pure Chemical Industries; CD68 1:500 Abcam). Following dewaxing, endogenous peroxidases were blocked with methanol/hydrogen peroxide (0.5%), followed by antigen retrieval in citrate buffer. Sections were then incubated with 30% normal horse serum for 1 h, prior to incubation overnight at room temperature with the specific primary antibody. The next day, the appropriate biotinylated secondary antibody (1:250, Vector) was applied for 30 min, followed by streptavidin horseradish peroxidase for 60 min, with the bound antibody detected with 3,3-diaminobenzidine tetrahydrochloride (Sigma). Sections were counterstained with hematoxylin. Slides were digitally scanned using a Nanozoomer, viewed with the associated NDP view software, with images exported for analysis with Image J (Corrigan et al., 2011, 2014). In ImageJ, the number of pixels above a set threshold value was determined and expressed as a

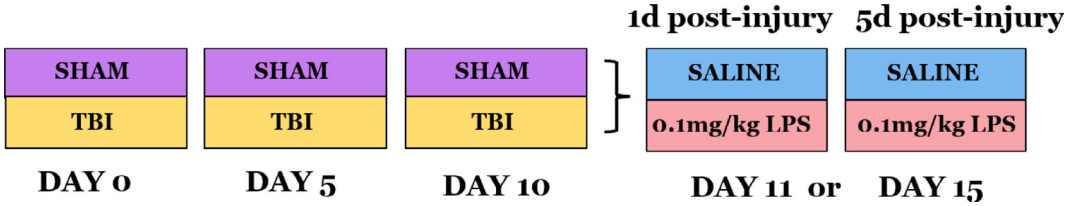

Fig. 1. Injury schedule.