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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

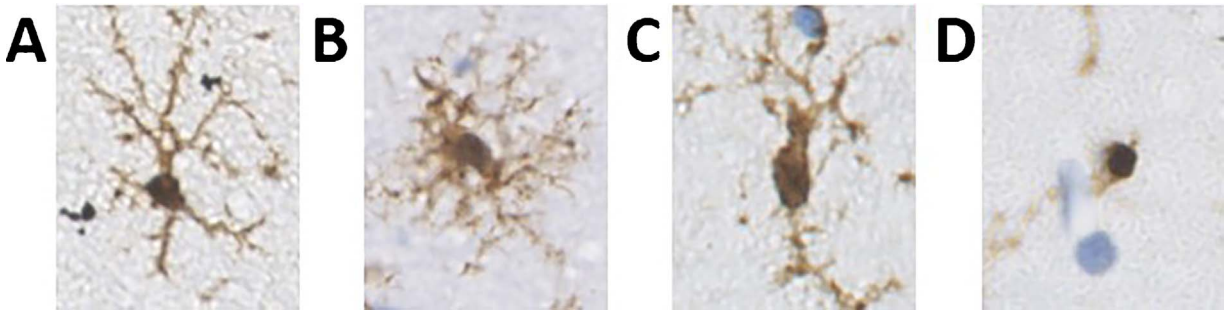

Fig. 1. Morphological classification of microglia activation states. Resting microglia were classified as those that were ramified with fine processes and smaller bodies (A). Activated microglia were counted as those that were either hyper-ramified with an enlarged cell body and thicker processes (B) or those that had an enlarged cell body with a reduction in process number (C). Macrophage-like cells were assessed as those that had no processes (D).

and all chronic tissue slides were performed simultaneously with the DAB reaction developed for the exactly the same length of time (7 min). Slides were washed to remove excess DAB and lightly counterstained with haematoxylin, dehydrated and mounted with DePeX from histolene. Negative control sections, in which no primary antibodies were added, were developed at the same time.

Slides were digitally scanned using a Nanozoomer, viewed with the associated NDP view software, with images exported for analysis with Image J [35,36]. For quantitation, the area of hippocampus within each section was determined. For IBA1 all immunoreactive cells with clear cell body morphology were counted by a blinded observer, with all digital slides labelled with a code unrelated to the treatment group. Counts were performed twice and standard deviation between counts were < 10%. In addition the morphology of each microglia was assessed and characterized as one of three morphologies: ramified (small cell body with multiple fine processes), active (either hyper-ramified with an enlarged cell body or an enlarged cell body with a reduction in process number) or macrophage (rounded, no processes) (Fig. 1) [37]. The proportion of each subtype of microglia was then calculated for each animal. For analysis of CD68 staining, all cells with clear nuclear staining within the hippocampus were counted, with this then expressed as CD68 + ve cell/mm^2.

2.4. Biochemical analysis

Rats were terminally anaesthetized with isoflurane prior to transcardial perfusion with saline. The brains were removed and the hippocampus separated and snap frozen. Protein was then extracted in standard RIPA buffer, with protein concentration estimated with a Pierce BCA Protein Assay (Thermoscientific).

2.4.1. Western blot

Gel electrophoresis was performed using Bolt 4–12% Bis-Tris Plus gels (Life Technologies) with 50 µg of protein loaded per well. Gels were run at 150 V for 30–45 min, depending on the molecular weight of the protein of interest, and transferred to a PVDF membrane using the iBlot 2 Dry Blotting System in accordance with the manufacturer's instructions (Life Technologies). Membranes were washed in 1X tris-buffered saline with tween (TBST) (3 washes × 5 min), and stained with Ponceau S red solution (Fluka Analytical) (5 min) for protein visualization. Following visual inspection to ensure equal protein loading in each well, membranes were washed with distilled water until removal of Ponceau S had been achieved. Membranes were incubated for 2.5 h with primary and secondary antibodies (1:3000, Li-Cor) in 1X iBind solution using the iBind Western System (Life Technologies), in accordance with the manufacturer's instructions. Primary and secondary antibodies were used at individually optimized concentrations: mouse anti-post-synaptic density protein 95 (PSD-95) (1:1000, ab18258, Abcam), mouse anti-myelin basic protein (MBP) (1:250, ab62631, Abcam) and the primary housekeeping antibody chicken anti-GAPDH (1:4000, ab83956, Abcam). Western blots were imaged using an Odyssey Infrared Imaging System (model 9120; software version 3.0.21) (LI-COR, Inc.) at a resolution of 169 µm. Analysis was performed using ImageJ version 1.49 and Image Studio Lite version 5.2. The same control sample was run on each gel, with expression of protein normalized to the housekeeper and to this loading control. Analysis was done by a blinded observer with all protein extraction samples labelled with a code for loading onto gels, which was unbroken on completion of analysis.

2.4.2. Multiplex assay

To measure cytokine levels acutely (IL-1β, MCP-1, TNFα, IL-2, IL-6, IL-17, IFNγ, G-CSF and IL-10), a Milliplex Mouse 9-plex cytokine kit (Millipore) was used. Samples were loaded onto 96 well plates in triplicates and run in accordance to manufacturer's instructions. Plates were read using a Magpix Luminex multiplex array (Abacus-ALS, Queensland) and data expressed as pg/ml of concentration. Experimental data was calibrated against standard curves of all 9 cytokines, which were fitted using a 5 parameter log fit through Analyst software (Millipore, Australia). The values for IFNγ fell below the detection range and were excluded from the final analysis.

2.5. Statistics

Behavioral analysis and molecular analysis, excluding the multiplex data, was analyzed via two-way analysis of variance (ANOVA), followed by Bonferonni post-hoc tests for multiple comparisons. Multiplex data for inflammatory cytokines was analyzed using a multivariate analysis of variance (MANOVA). Following a significant multivariate test (Hotelling's trace), individual two-way ANOVAs were conducted for each marker to probe the LPS/injury interaction, with Bonferonni post-hoc tests used for multiple comparisons. A p value of less than 0.05 was considered significant. All graphical data are presented as mean ± SEM.

3. Results

3.1. Behavior

The behavioral response to mTBI and LPS administration was assessed at 3 months post-injury. General locomotor activity was analyzed via the open field (Fig. 2A), with no effect of either injury ($F_{1,35}$ = 2.4, p = 0.13) or LPS administration noted ($F_{1,35}$ = 0.19, p = 0.67). Anxiety like behavior was assessed via the EPM, with a significant effect of both injury ($F_{1,35}$ = 14.53, p < 0.001) and LPS administration ($F_{1,35}$ = 9.0, p < 0.02) seen. mTBI animals treated with LPS spent significantly more time in the open arm compared to saline treated mTBI animals (105.6 ± 35.9 vs 62.4 ± 16.3 s, p < 0.05) and both the sham groups (sham:saline: 41.6 ± 27.6, p < 0.001; sham:LPS 54.8 ± 25.5 s, p < 0.01). In the FST, a

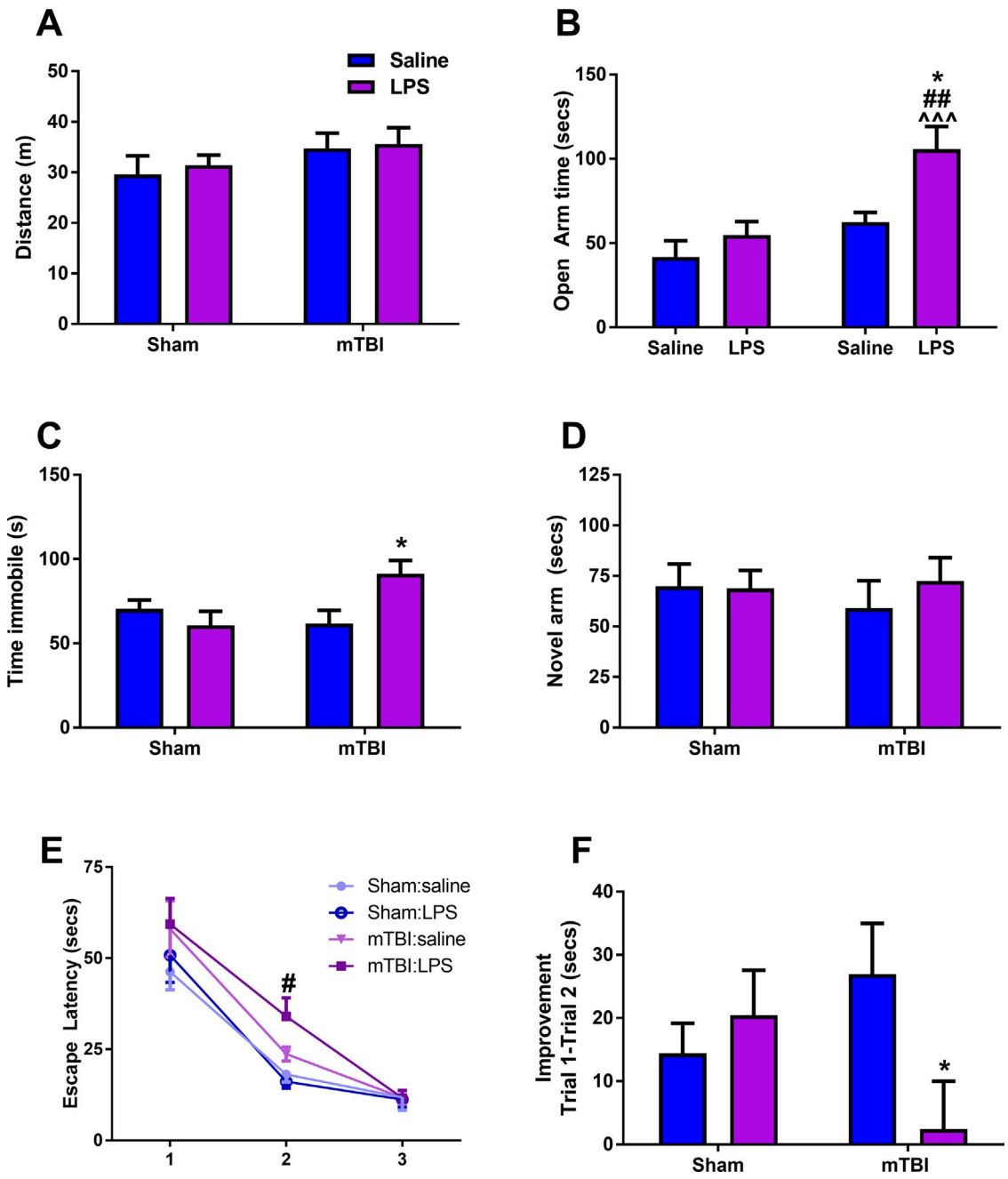

Fig. 2. Behavior was analyzed at 3 months post-injury. General locomotor activity was determined via distance travelled on the open field (A), anxiety-like behavior as time in the open arm on the EPM (B), depressive-like behavior as time spent immobile on the FST (C) and cognition as time spent in the novel arm on the Y-maze (D) and performance on the Barnes Maze (E-F), including time to find the escape box over 3 training days (E) and improvement in time finding the location of a new escape box over two trials (F). (n = 8–11 per group, ^^^p < 0.01 compared to sham:saline animals, ##p < 0.01, #p < 0.05 compared to sham:LPS animals, *p < 0.05 compared to mTBI:saline animals).

measure of depressive-like behavior, a significant main interaction ($F_{1,35}$ = 7.3, p < 0.05) was noted, driven by an increase in time spent immobile in the LPS treated mTBI animals as compared to saline treated mTBI animals (91.3 ± 22.1 vs 61.7 ± 23.5 s, p < 0.05). No effects of injury ($F_{1,35}$ = 0.1, p = 0.75) or LPS administration were seen in the Y Maze ($F_{1,35}$ = 0.3, p = 0.59). However, in the Barnes Maze, a significant main effect of treatment ($F_{(6,105)}$ = 2.8, p < 0.05) and time ($F_{6,105}$ = 88.5, p < 0.0001) were seen. On the first day of training, no difference was seen between sham or mTBI animals, regardless of treatment. However, on day 2, mTBI: LPS treated rats had a significantly higher escape latency than sham: LPS treated rats (34.0 ± 13.5 vs 16.1 ± 5.6 s, p < 0.05), with a trend towards an increase compared to sham:saline animals (18.1 ± 6.0, p = 0.09). No difference was noted between mTBI saline treated animals (23.71 ± 5.38 s) and either of the sham groups (sham:saline p = 0.56, sham:LPS p = 0.64). By day 3, all animals were behaving similarly, with no differences between groups. Indeed, all groups showed a significant difference between day 1 and day 3 of training on the Barnes Maze (p < 0.001). A probe trial was conducted on Day 5 to assess cognitive flexibility in terms of the ability to learn a new location for the escape box over two trials, with a significant difference seen between performance in the two trials ($F_{(1,72)}$ = 4.93, p < 0.05). Post-hoc analysis found that the mTBI:LPS treated rats failed to show an improvement in performance over the two trials, as calculated as the time to find the new location on trial 1 minus the time taken on trial 2. Indeed mTBI:LPS animals were significantly different from mTBI:saline

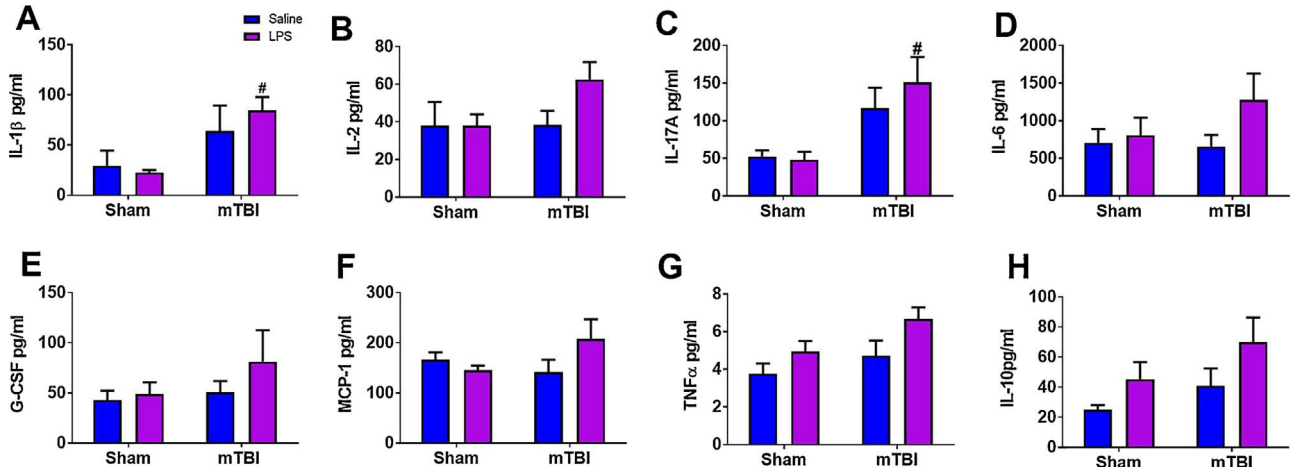

Fig. 3. Analysis of the cytokine response to injury and LPS administration through Multiplex analysis of IL-1β (A), IL-2 (B), IL-17A (C), IL-6 (D), IL-10 (D), G-CSF (F), MCP-1 (G) and TNFα (H) at 24 h following LPS administration. (n = 4 per group, #p < 0.05 compared to sham:LPS animals).

animals (improvement of 2.5 ± 21.4 vs 26.9 ± 24.2 s, p < 0.05).

3.2. Acute cytokine response

The acute response to injury and LPS administration was evaluated by examining levels of eight key inflammatory mediators: IL-1β, IL-6, IL-2, IL-17, G-CSF, TNFα, IL-10 and MCP-1 using a custom Multiplex array (Abacus) (Fig. 3). A multivariate test (Pillai's trace) demonstrated a significant main effect of group ($F_{27,111}$ = 3.0, p < 0.05). Individual two-way ANOVAs found a significant main effect of LPS administration for IL-1β ($F_{1,12}$ = 10.98, p < 0.01) and IL-17 ($F_{1,12}$ = 13.55, p < 0.01). Post-hoc analysis found that, for IL-1β and IL-17, mTBI animals treated with LPS had significantly higher levels of these pro-inflammatory cytokines than sham:LPS animals (p < 0.05). There was no difference between mTBI:saline treated animals and either of the sham controls (p > 0.05).

3.3. Neuroinflammatory response

The effect of mTBI and subsequent LPS administration on microglia/macrophage number within the hippocampus was assessed by immunohistochemistry with the classic antibody specific for IBA-1 (Fig. 4) and CD68 (Fig. 5). Acutely, a significant effect of injury was seen on the number of IBA1 + ve cells ($F_{1,12}$ = 35.3, p < 0.001), with both mTBI groups having significantly higher numbers of IBA1 + ve cells compared to their respective shams (sham:saline, 32.0 ± 1.7 vs mTBI:saline, 42.5 ± 1.3 cells/mm^2, p < 0.001; sham:LPS, 29.4 ± 3.2 vs mTBI:LPS, 38.6 ± 3.9, p < 0.05). Analysis of microglia phenotype, found an increase in the % of activated microglia following injury (sham:saline 9.81 ± 2.69; sham:LPS 7.58 ± 2.16 vs mTBI:saline 20.57 ± 7.97; mTBI:LPS 22.69 ± 5.28%), but no effect on the number of macrophage-like cells (1.32 ± 1.93, 2.6 ± 1.15, 1.96 ± 3.19, 2.9 ± 3.0 respectively) (Fig. 4C). Indeed a two-way ANOVA comparing the%activated microglia found a significant effect of injury ($F_{1,12}$ = 26.78, p < 0.001), with the mTBI:saline and mTBI:LPS animals both significantly different from sham:saline and sham:LPS animals (p < 0.05), but not each other.

By 3 months post-injury, there was a significant main effect of LPS administration ($F_{1,14}$ = 7.9, p < 0.05), but not injury ($F_{1,14}$ = 0.6, p = 0.44) on the number of IBA1 + ve cells, with post-hoc analysis showing that mTBI:LPS animals had higher numbers of IBA1 + ve cells than mTBI:saline animals (36.4 ± 4.1 vs 45.9 ± 3.0 cells/mm^2, p < 0.05). Analysis of the morphology of these IBA1 + ve cells found that the mTBI:LPS animals had a higher number of activated microglia than all other groups (33.43 ± 10.89 vs 18.66 ± 5.9, 17.10 ± 9.98 and 17.12 ± 6.15 in the sham:saline, sham:LPS and mTBI:saline groups respectively). A two-way ANOVA of the % activated microglia found a significant interaction ($F_{1,14}$ = 4.95, p < 0.05), with a trend towards a significant effect of injury ($F_{1,14}$ = 3.38 p = 0.09) and LPS administration ($F_{1,14}$ = 3.37, p = 0.09). No difference between the % of macrophage like cells was found between any of the groups.

Positive CD68 nuclear staining was also assessed to confirm whether injury with LPS administration had an effect on phagocytic activity of microglia/macrophages. As seen with the lack of phenotypic changes to a macrophage-like appearance with the IBA1 staining, no significant differences in the number of CD68 + ve cells was seen either acutely (p = 0.89) or chronically (p = 0.88), with only a few scattered cells seen associated with blood vessels in any of the animals regardless of injury or LPS administration (Fig. 5).

3.4. Neuronal injury

Western blot analysis was used to investigate the effects of injury and LPS administration on the relative expression of proteins related to synapses, myelination and axonal structure (Fig. 6) and chronically (Fig. 7). A main injury effect on levels of PSD-95, a post-synaptic scaffolding protein was seen acutely ($F_{(1,12)}$ = 14.7, p < 0.01), although there was no main effect of LPS administration ($F_{(1,12)}$ = 0.90, p = 0.36). Post-hoc analysis found a significant increase in PSD-95 levels between the mTBI:saline animals and sham:saline animals (1.82 ± 0.47 vs 1.09 ± 0.20, p < 0.05), with a trend towards a difference between mTBI:LPS and sham:LPS animals (1.53 ± 0.27 vs 1.08 ± 0.26, p = 0.05). By 3 months post-injury, a significant interaction between LPS and injury was noted ($F_{(1,17)}$ = 6.2, p < 0.05), with a significant main effect of LPS administration ($F_{(1,17)}$ = 6.7, p < 0.05). Post-hoc analysis found a significant decrease in the relative density of PSD-95 in mTBI:LPS animals compared to mTBI saline animals (0.88 ± 0.2 vs 1.30 ± 0.26, p < 0.05), although no difference was seen between these animals and shams (sham:saline 1.13 ± 0.15, p = 0.15; sham:LPS 1.12 ± 0.12, p = 0.16). MBP which is essential for normal myelination, and the key cytoskeletal element, NFL were examined to determine the effect of injury and LPS administration on proteins associated with axonal structure No effect on the levels of MBP was seen either acutely ($F_{(1,12)}$ = 0.4, p = 0.52) or chronically ($F_{(1,17)}$ = 0.4, p = 0.55). In contrast, for NFL, acutely, a significant effect of injury was seen ($F_{(1,12)}$ = 10.03, p < 0.01), with mTBI:saline animals having a higher relative density of NFL than their shams (1.13 ± 0.45 vs 0.51 ± 0.2), with no further significant effect of LPS administration ($F_{(1,12)}$ = 0.01 p = 0.9). By 3 months post-injury, no effect of either injury ($F_{(1,17)}$ = 0.04, p = 0.84) or LPS

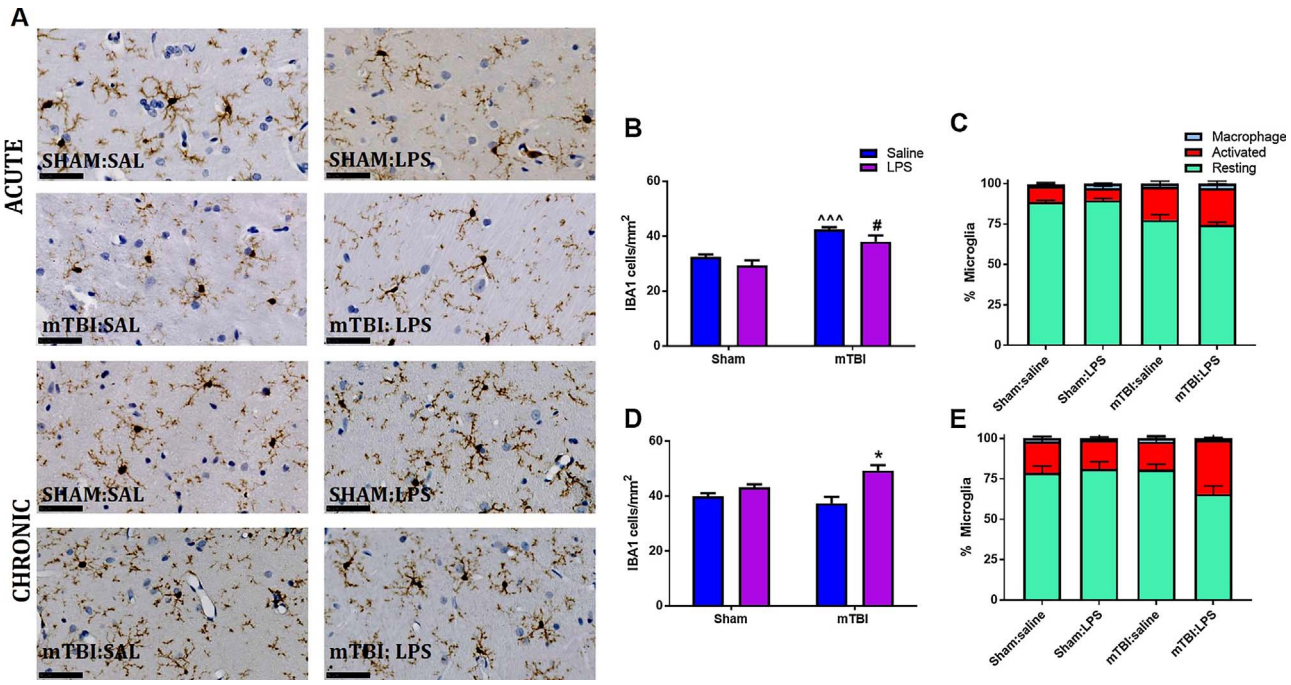

Fig. 4. Representative images of IBA1 immunohistochemistry within the hippocampus (A), with counts of the number of IBA1 +ve cells at 24 h post-LPS (B) and at 3 months post-administration (D) and assessment in the changes in the proportions of microglial morphologies acutely (C) and chronically (E). (Scale bar = 50 μm; 24 h: n = 4 per group, 3 months n = 4–5 per group, ^^^p < 0.001 compared to sham:saline, #p < 0.05 compared to sham:LPS, *p < 0.05 compared to mTBI:saline).

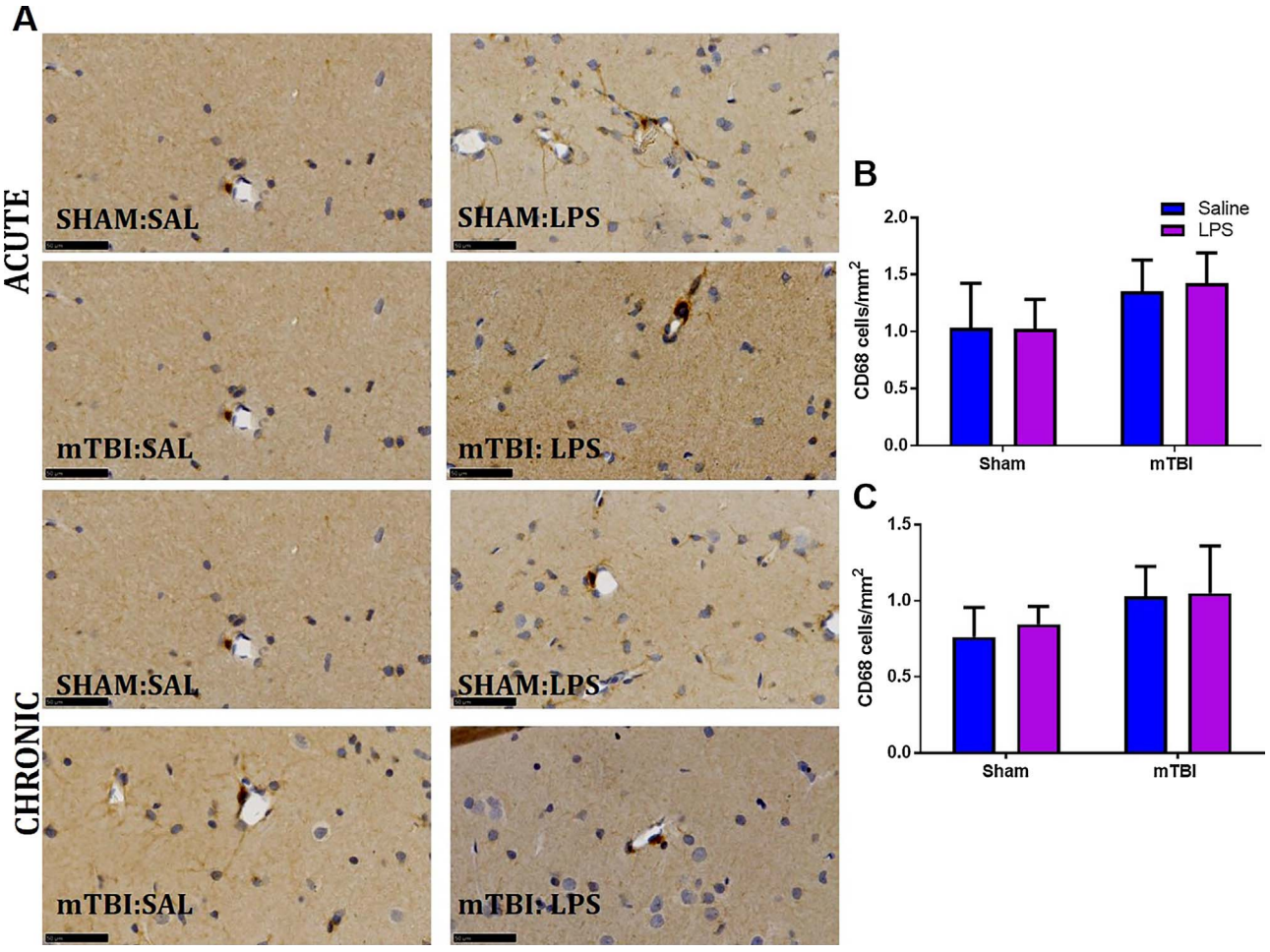

Fig. 5. Representative images of CD68 immunohistochemistry within the hippocampus (A), with counts of the number of IBA1 +ve cells at 24 h post-LPS (B) and at 3 months post-administration (C). (Scale bar = 50 μm; 24 hrs: n = 4 per group, 3 months n = 4–5 per group).

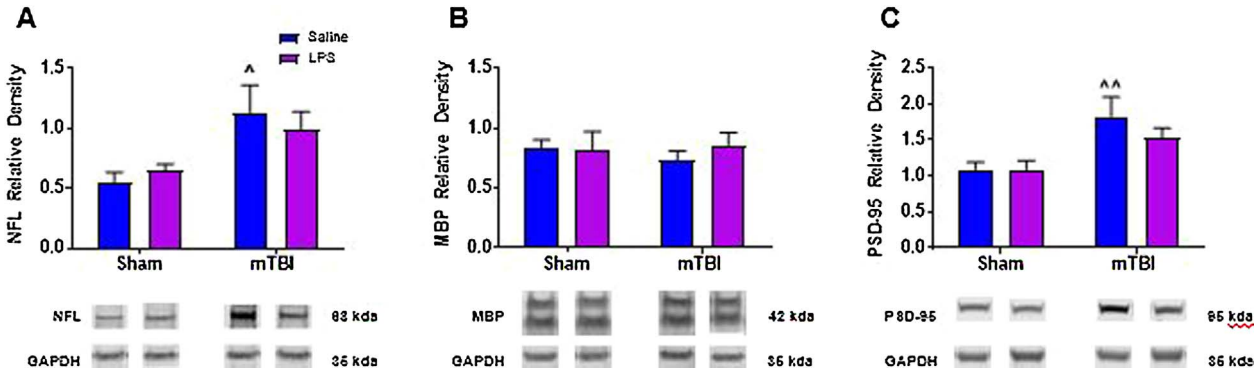

Fig. 6. Evaluation of the effects of LPS administration following mTBI on the expression of NFL (A), MBP (B) and PSD-95 (C) acutely. (n = 4 per group, ^p < 0.05, ^^p < 0.01 compared to sham:saline).

administration ($F_{(1,17)} = 0.01$, $p = 0.91$) on NFL was seen.

4. Discussion

Following a mTBI, a proportion of patients will develop persistent symptoms, including irritability, memory dysfunction and difficulty concentrating. This study sought to investigate the role that inflammation could play in the development of these symptoms. It was found that a single systemic immune stimulus induced by a peripheral injection of low dose LPS at 5D following a single mTBI was sufficient to induce chronic behavioral changes at 3 months following injury. mTBI animals treated with LPS showed increased depressive-like behavior on the FST, decreased anxiety on the EPM and decreased cognitive flexibility on the Barnes Maze. This was associated with an exaggerated response acutely to LPS administration, with significantly increased levels of pro-inflammatory cytokines, IL1β and IL-17A at 24 h post-dose, an effect not seen in either shams or saline-treated mTBI animals. Furthermore, LPS administration following mTBI was sufficient to drive the development of a persistent neuroinflammatory state seen as an increased number of microglia at 3 months post-injury in the mTBI:LPS, but not mTBI:saline, animals, accompanied by a reduction in the levels of the synaptic marker, PSD-95.

In this study, the model of mild diffuse-impact acceleration was insufficient to produce long-term behavioral deficits at 3 months post-injury alone. In previous studies, functional deficits following a single mTBI, such as impaired cognition, have been most robustly reported at 1 month post-injury [18,38,39], although there have been reported deficits out to 3 months post-injury, including cognitive deficits on the MWM [40,41] and increased depressive-like behavior on the FST [42]. Failure to see behavioral change following mTBI alone in this study could indicate that the tests used here were not sensitive enough to detect subtle deficits, or that the underlying injury model was milder than those previously reported. Nonetheless, there was evidence of an acute response to injury, with increased microglial numbers within the hippocampus at 6D post-injury, although this was not associated with significant increases in levels of pro-inflammatory cytokines and was resolved by 3 months post-injury. This is in line with previous reports that following mTBI, levels of cytokines rapidly increase within the first 3–6 h [43], but rapidly return towards baseline by 24 h post-injury [44]. Conversely, alterations in glial reactivity may increase in the week following injury before resolving [45]. For example, using a model of mild lateral fluid percussion injury (mLFP), Shultz et al. found that microglial and astrocytic reactivity was increased at 4 days post-injury, but had returned to baseline by 1 month [18,46], in line with the reports of increased microglia at 6D post-injury that had resolved by 3 months within this study. As the severity of the injury increases there appears to be an increased likelihood of persistent neuroinflammatory changes, with, for example Fenn et al. reporting the presence of persistently activated microglia within the hippocampus and parietal cortex following a moderate midline FP injury [47]. Nonetheless the acute neuroinflammatory response observed in the current study was accompanied by acute alterations in structural integrity, with increased expression of the cytoskeletal protein, NFL, and the dendritic protein, PSD-95, at 6D post-injury that had resolved by 3 months post-injury. This may indicate a rebound reparative response, with NFL levels shown to increase within the CSF and serum following concussion [48]. A similar pattern has been shown with PSD-95, with decreases found 24 h following a mild closed head impact in mice, that had rebounded to a 12% increase compared to shams at 3 days post-injury [49]. This suggests that following mTBI there are temporary structural changes that then activate endogenous repair processes.

In contrast to the short-lived acute effects of a single mTBI alone, administration of LPS at 5D post-injury was sufficient to induce a chronic neuroinflammatory response, with associated behavioral

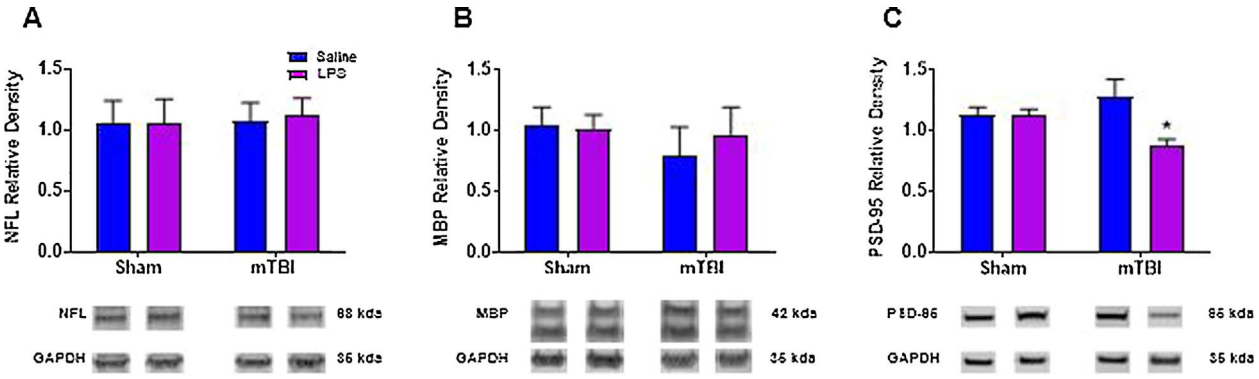

Fig. 7. Evaluation of the effects of LPS administration following mTBI on the expression of NFL (A), MBP (B) and PSD-95 (C) chronically. (n = 4–6 per group, *p < 0.05 compared to mTBI:saline).

deficits, at 3 months post-injury. The timing and dosage of LPS were as per our previous publication in a model of repeated mTBI [50] and reflects levels of LPS that would be seen with low grade systemic inflammation [25,26]. It should be noted that similar increases in circulating levels of LPS can also be induced in a number of other situations including ingestion of high fat meals [51,52], with severe exertion [53], and acute alcohol consumption [54] and thus this may have implications for management of the recovery from a single mTBI. The mildness of this peripheral inflammatory insult can be seen in the response to LPS in sham animals, where no change in cytokine levels seen at 24 h post-dose. This is in line with a study by Teeling et al. where a similar dose of LPS induces cytokine production within 3 h of administration, with return to baseline by 24 h [55].

Conversely, when given on a background of pre-existing inflammation induced by a mTBI, LPS administration was sufficient to induce a pro-inflammatory response that persisted to 24 h post-dose, with significant increases in levels of IL-1β and IL-17 were seen within the hippocampus. The source of these increased pro-inflammatory cytokines cannot be confirmed within this study, as this could be related either to resident immune cells or infiltration of peripheral immune cells. It has been well described that peripheral inflammation can promote neuroinflammation via a number of routes including circumventricular organs, vagal afferents and the brain endothelium [56], as well as increased trafficking of peripheral immune cells, predominantly monocytes [57]. Nonetheless, the presence of pro-inflammatory cytokines is consistent with the theory that a single mTBI is sufficient to prime microglia, a phenomenon whereby an exaggerated inflammatory response is seen following immune activation [58]. Indeed Fenn et al. and Muccigrosso et al. demonstrated the ability of mTBI to prime microglia, with a midline moderate FP injury leading to an exaggerated inflammatory response to an intraperitoneal injection of low dose LPS (0.33 mg/kg) at 1 month post-injury, with increased expression of IL1β and TNFα [47,59]. This was associated with acute behavioral symptoms, with increased time spent immobile on the tail suspension test and decreased sucrose preference, indicative of depressive-like behavior [47,59]. Furthermore, mice developed cognitive deficits, as seen as increased escape latency on the Barnes Maze [59]. These studies demonstrated the acute effects of a systemic inflammatory insult at a delayed time point following TBI, whereas here we describe chronic deficits seen at 3 months post-administration. It should be noted that both LPS and saline treated animals had an increase acutely in the number of activated microglia, although only in the LPS treated animals was this associated with increased pro-inflammatory cytokine production. This may relate to a differential proportion of M1:M2 microglia in these groups with activated microglia having phenotypic sub-populations with different molecular signatures of gene expression. M1 microglia promote a classic pro-inflammatory state, while M2 microglia are important for tissue remodeling and suppress the inflammatory response [60,61]. This distinction cannot be made on morphology alone and would require further investigation.

Nonetheless, in the current study, animals administered LPS at 5 days post-injury showed evidence of persistent neuroinflammation, with an increase in microglia numbers within the hippocampus at 3 months injury, with a trend towards an increased percentage of activated microglia. This was accompanied by a loss of the synaptic protein, PSD-95. Indeed, a persistent inflammatory response can have toxic effects on neurons through mechanisms such as oxidative stress, apoptosis, and excitotoxicity [62]. PSD-95 is postsynaptic membrane protein that is found adjacent to the presynaptic sites of neurotransmitter release [63] and is thought to be involved in a number of important functions including synaptogenesis, synaptic plasticity and the processes of learning and memory [64,65]. Of note following a focal controlled cortical impact delayed loss of PSD-95 within the hippocampus, corresponded with the development of cognitive deficits in the novel object recognition task [66]. This provides a potential link between the delayed loss of PSD-95 in the mTBI:LPS animals seen here and the development of deficits in spatial learning. Indeed the hippocampus was examined here as it is the key region associated with cognition, with disruption to this area suggest to underlie many of the chronic symptoms seen both clinically [67] and experimentally [68] following a mTBI. Indeed a single mTBI has been shown to lead to long-term changes in both memory function and hippocampal structure [69]. Furthermore inflammatory changes have been demonstrated acutely following injury within the hippocampus [68].

In addition to cognitive deficits mTBI:LPS animals showed increased time spent immobile on the FST, and increased time spent in the open arm of the EPM. Although traditionally increased time spent in the open arm of the EPM is seen as indicative of decreased anxiety [31,70], it could also be related to disinhibition or increased impulsivity [71–73]. Indeed, similar findings have previously been reported acutely following mTBI [18], suggesting that this may be a particular behavioral phenotype seen in rodents following this type of insult. Future studies could incorporate specific behavioral paradigms that assess impulsivity such as the stop-signal reaction time [74] and the go/no-go tasks [75], which both require animals to stop a response when a certain cue is presented, acting as a measure of inhibitory control [76]. Nonetheless, the constellation of behavioral symptoms seen here are in line with those reported to be clinically associated with PCS, which encompasses impulsivity, depression and cognitive impairment [77]. This supports the theory that induction of a persistent inflammatory state may facilitate the development of ongoing behavioral symptoms in some individuals following mTBI, which can be precipitated by exposure to a subsequent immune challenge.

In summary, this study found that a peripheral immune stimulus in the subacute stage following a mTBI was sufficient to promote the development of persistent behavioral symptoms at 3 months post-injury. This highlights a potential mechanism that may drive ongoing symptomology in a proportion of mTBI sufferers and the need to better understand how mTBI can interact with other inflammatory insults.

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