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Full-length Article

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

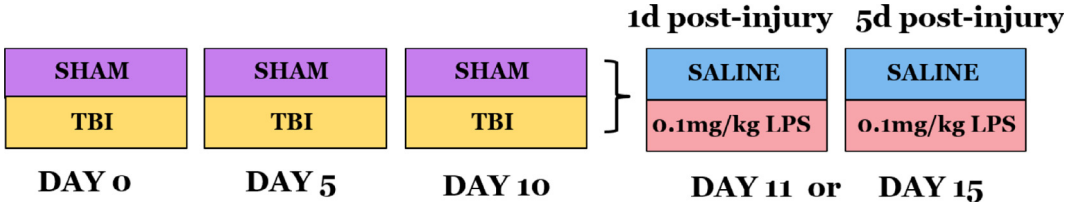

Fig. 1. Injury schedule.

percentage of total pixels within the field. For quantitation, a box (0.4 mm^2) was placed in four random locations within the hippocampus of each section and all immunoreactive cells with clear cell body morphology were counted by a blinded observer. Counts were performed twice and standard deviation between counts was typically <10%. For semi-quantification of the area occupied by microglia and astrocytes, the number of pixels above a set threshold value was determined and expressed as a percentage of total pixels within the field, within each of the boxes on which cell counts were performed, as previously described (Trapp et al., 2007).

3. Biochemical analysis

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

3.1. Western blot

Gel electrophoresis was performed using Bolt 4–12% Bis-Tris Plus gels (Life Technologies) with 50ug of protein loaded per well. Gels were run at 150V 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 (Life Technologies). Membranes were washed in 1X tris-buffered saline with tween (TBST) (3 washes × 5 min), stained with Ponceau S red solution (Fluka Analytical) (5 min) for protein visualization, and washed with distilled water until removal of Ponceau 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). Primary antibodies were used at individually optimized concentrations: rabbit anti-IkBα (1:500, 9242, Cell Signaling), mouse anti-STAT1 (1:250, 9532 Cell Signaling), mouse anti-pSTAT1 (1:250, 9167, Cell Signaling) mouse anti-post-synaptic density protein 95 (PSD95) (1:1000, ab18258, Abcam), rabbit anti-synaptophysin (1:1000, ab32127, Abcam), mouse anti-myelin basic protein (MBP) (1:250, ab62631, Abcam), mouse anti-neurofilament (1:300, ab24574, Abcam), tau AT180 (1:500, MN1040, Millipore), mouse Tau T22 oligomeric (1:250, ABN454, Millipore), mouse tau-5 (1:1000, 577801, Millipore) and the primary housekeeping antibody chicken anti-GAPDH (1:4000, ab83956, Abcam) or β-actin (1:400, ab134882, 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.

3.2. Multiplex assay

A Milliplex Mouse 9 plex cytokine kit (Millipore) was used to measure the concentration of 9 cytokines per sample (IL-1β, MCP-1, TNFα, IL-2, IL-6, IL-17a, IFNγ, G-CSF and IL-10). 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 TNFα, IL2 and IL10 fell below the detection range and were excluded from the final analysis.

4. 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 (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.

4.1. Open field

The open field test is a common measure of locomotor activity in rodents (Tatem et al., 2014).. It consists of a 1 m x 1 m box in which the animal is placed in the center and allowed to explore freely for five minutes, with the distance travelled calculated.

4.2. Elevated plus maze (anxiety)

The elevated plus maze is a common measure of anxiety in rodents (Walf and Frye, 2007) and is comprised of a cross shaped maze with two closed and two open arms. Rats are allowed to explore freely for 5 min, with rats exhibiting anxious behaviors preferring closed arms to the open.

4.3. Y maze (cognition)

The Y Maze assesses spatial and recognition memory in rodents, and utilizes the rodent's desire to explore new areas (Conrad et al., 1996). 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.

4.4. Barnes maze (cognition)

The Barnes maze is a commonly used test of learning and memory in rodents (Barnes, 1979). 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. 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.

4.5. 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 (Bogdanova et al., 2013).

5. Statistics

The acute phase of the study was evaluated using a two-way analysis of variance (ANOVA) followed by Holm-Sidak post hoc tests for multiple comparisons. In the chronic phase of the study, cognitive data was assessed via a repeated-measures two-way ANOVA, followed by Holm-Sidak post hoc tests, with all other

comparisons conducted using a one-way ANOVA followed by Holm-Sidak 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 Tukey's HSD 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.

6. Results

6.1. Acute Sickness response to LPS

There was a significant main effect for LPS administration ($F_{1,87} = 207.5$, $p < 0.01$), with all groups (sham, rmTBI 1d dosage and rmTBI 5d dosage) showing a significant reduction in weight compared to their respective saline controls ($p < 0.01$; Fig 2A). Of note, weight loss in response to LPS was significantly higher following rmTBI (18.6 ± 5.8 g in rmTBI 1d dosage and 18.9 ± 6.8 g in rmTBI 5d dosage animals) compared to sham:LPS animals (11.4 ± 7.6 g, $p < 0.01$). However, there was no main effect of LPS administration on distance travelled in the open field in either sham or rmTBI animals ($F_{1,38} = 0.6$; $p = 0.44$) at 24 h following the last LPS administration (Fig 2B).

6.2. Acute neuroinflammatory response

The effect of LPS on microglia was assessed morphologically by immunohistochemistry with the classic antibody specific for IBA-1 (Fig 3) and subsequently confirmed by enhanced expression of the activation marker Cd68 within the hippocampus (Fig 4). Acutely, a significant LPS/injury interaction was found in regards to number of IBA1 +ve cells ($p < 0.05$, $F_{2,18} = 6.52$, $p < 0.05$), with rmTBI 5d LPS treated rats showing a significant decrease in the number of IBA1 +ve cells compared to rmTBI 5d saline animals (32.3 ± 2.17 vs 39.2 ± 3.72 cells/mm^2) (Fig 2B). These results were supported by semi-quantification of the area occupied by IBA1 staining, with a significant injury/LPS administration interaction ($F_{2,18} = 13.92$, $p < 0.001$) and a significant main effect of both injury ($F_{2,18} = 13.23$, $p < 0.001$) and LPS administration ($F_{2,18} = 6.24$, $p < 0.05$) (Fig 2C). Post-hoc analysis found that in saline treated rats, a significant increase in IBA1 immunoreactive area was seen at 6 days following injury (in the rmTBI 5d saline animals) ($p < 0.01$), but not 2 d following injury (in the rmTBI 1d saline animals) ($p = 0.12$). Administration of LPS at 1d post injury significantly increased the area occupied by IBA1 immunoreactivity when compared to the rmTBI 1d saline treated rats (17.9 ± 3.4% vs 9.0 ± 3.4%; $p < 0.01$), suggesting increased branching. An opposite trend towards a decrease was seen in the rmTBI 5d LPS treated rats compared to rmTBI 5d saline rats (8.4 ± 1.7% vs 12.0 ± 1.1%; $p = 0.07$). A similar pattern was observed with CD68 immunostaining (Injury/LPS interaction $F_{2,18} = 18.22$, $p < 0.0001$), with a significant increase in the rmTBI 1d LPS treated rats versus rmTBI 1d saline (15.7 ± 8.1 vs 8.1 ± 2.6 cells/mm^2; $p < 0.01$), but a significant decrease in the rmTBI 5d LPS treated rats versus rmTBI 5d saline treated rats (8.5 ± 2.0 vs 18.5 ± 3.9 cells/mm^2; $p < 0.01$) (Fig 4B). Astrocytic activation was assessed by immunohistochemical analysis of GFAP (Fig 5). A significant main effect of injury was noted for both number of GFAP +ve cells ($F_{2,18} = 4.152$, $p < 0.05$; Fig 4B) and GFAP immunoreactive area ($F_{2,18} = 6.7$, $p < 0.01$; Fig4C), with rmTBI 1d saline animals having a significant increase in the area occupied by GFAP immunostaining compared to sham controls ($p < 0.05$). No additional effect of LPS administration was noted.

6.3. Analysis of acute cytokine response to injury and LPS administration

The acute response to injury and LPS administration was also evaluated by examining levels of six key inflammatory mediators: IL-1β, IL-6, IFNγ, IL-17, G-CSF and MCP-1 using a custom Multiplex array (Abacus) (Fig 6). A multivariate test (Hotelling's trace) demonstrated a significant main effect of group ($F_{30,\ 57} = 1.688$, $p < 0.05$). Individual two-way ANOVAs found a significant LPS administration x injury effect for IFNγ ($F_{2,18} = 6.7$, $p < 0.01$), IL-6 ($F_{2,18} = 7.8$, $p < 0.01$), G-CSF ($F_{2,19} = 13.05$, $p < 0.001$) and IL-17 ($F_{2,19} = 6.18$, $p < 0.01$), and a trend towards significance for IL-1β ($F_{2,18} = 3057$, $p = 0.07$). Post-hoc analysis revealed a consistent trend where LPS administration at 1d post-injury reduced pro-inflammatory cytokine expression (IL-6 $p < 0.05$, G-CSF $p < 0.01$, IFNγ $p = 0.05$, IL-17 $p = 0.09$ compared to rmTBI 1d saline animals) whilst LPS administration at 5d post-injury increased pro-inflammatory cytokine expression (IL-6 $p < 0.05$, G-CSF $p < 0.05$, IFNγ $p = 0.06$, IL-17 $p < 0.05$ compared to rmTBI 5d saline animals).

6.4. Analysis of acute changes to the TLR4 signaling pathway in response to injury and LPS administration

Acute changes in signaling proteins involved in the TLR4 pathway were examined through determination of the levels of IκBa

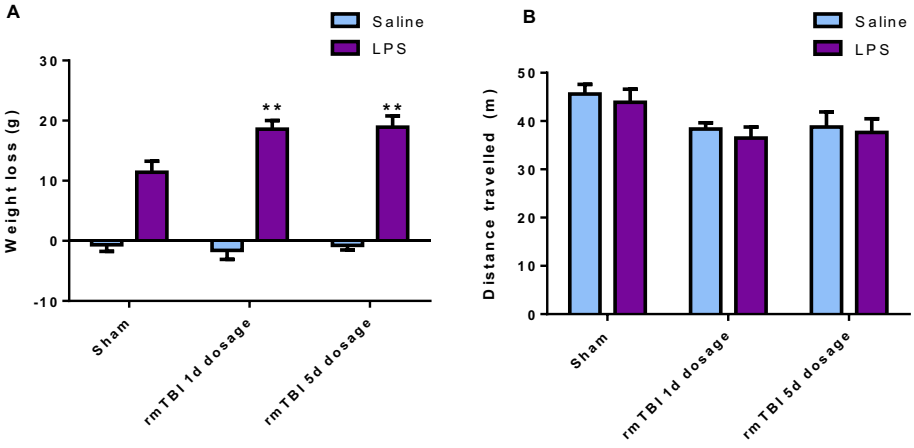

Fig. 2. Acute response to LPS administration as assessed by weight loss at 24 h post-dose (A) and distance travelled on the open field (B) (n = 8 per group, $^{**}p < 0.01$ compared to sham:LPS animals).

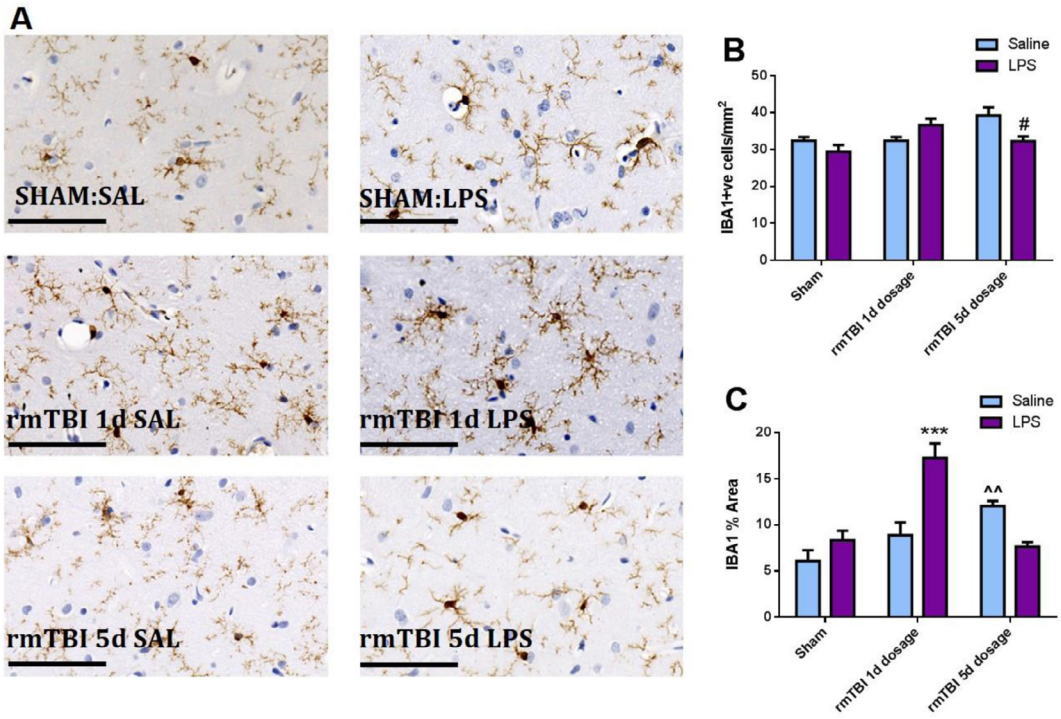

Fig. 3. Representative images of IBA1 staining within the hippocampus (A) with counts of the number of IBA1 +ve cells, as defined by clearly defined nucleus (B) and analysis of the % area stained by IBA1 in the hippocampus, as defined by the level of staining over a set threshold (C) at 24 h following LPS treatment. Although not statistically significant ($p = 0.07$), when given at 5d post-injury, LPS appeared to reduce the number of microglia, whereas LPS administration at 1d post-injury significantly increased the percent area occupied by IBA1, suggesting increased branching of microglia within the hippocampus when compared to saline control rmTBI rats at the same time point. ($n = 4$ per group, $^{\#}p < 0.05$ compared to rmTBI 5d saline treated animals $^{***}p < 0.001$ compared to rmTBI 1d saline treated rats, $^{\wedge\wedge}p < 0.01$ compared to sham:saline animals. Scale bar = 50 μm).

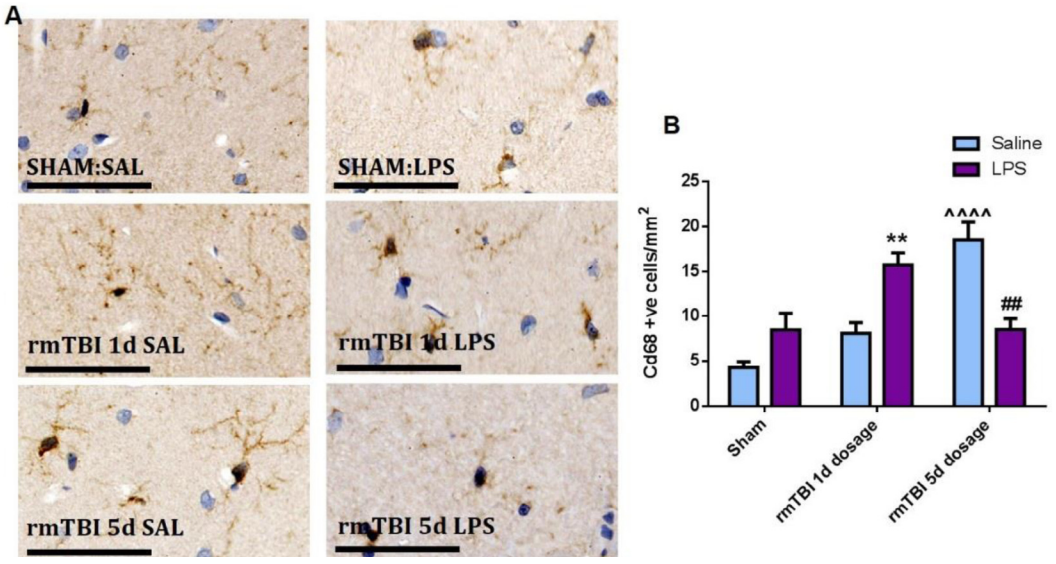

Fig. 4. Representative images of CD68 staining within the hippocampus (A) with counts of the number of CD68 +ve cells within the region (B). LPS administration at 1d post-injury increased the number of CD68 +ve cells within the hippocampus compared to saline treated rmTBI rats at the same time point. Conversely, LPS administration at 5d post-injury decreased the number of hippocampal CD68 +ve cells compared to saline-treated rmTBI rats at the same time point ($n = 4$ per group, $^{**}p < 0.01$ compared to rmTBI 1d saline treated rats, $^{\#\#}p < 0.01$ compared to rmTBI dD saline treated rats, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ compared to sham:saline rats. Scale bar = 50 μm).

and pSTAT1, to reflect activation of the Myd88 and TRIF pathways respectively (Fig 7). A significant effect of LPS administration ($p < 0.01$, $F_{2,18} = 13.27$) and injury ($p < 0.05$, $F_{1,18} = 4.82$) was noted in regards to expression of IκBa (with a trend towards an effect of injury Post-hoc analysis showed that in sham animals LPS administration significantly increased levels of IκBa (0.69 ± 0.15 vs 1.18 ± 0.36), an effect not seen in the injured animals at either 1 day (0.9 ± 0.25 vs 1.0 ± 0.15) or 5 day (0.41 ± 0.13 vs 0.42 ± 0.14) post-injury. In saline treated animals a significant reduction in IκBa levels was noted in the rmTBI 5D dosage group compared to the rmTBI 1D dosage group ($p < 0.05$), whilst both injury groups (1D LPS and 5D LPS treatment) had lower levels than

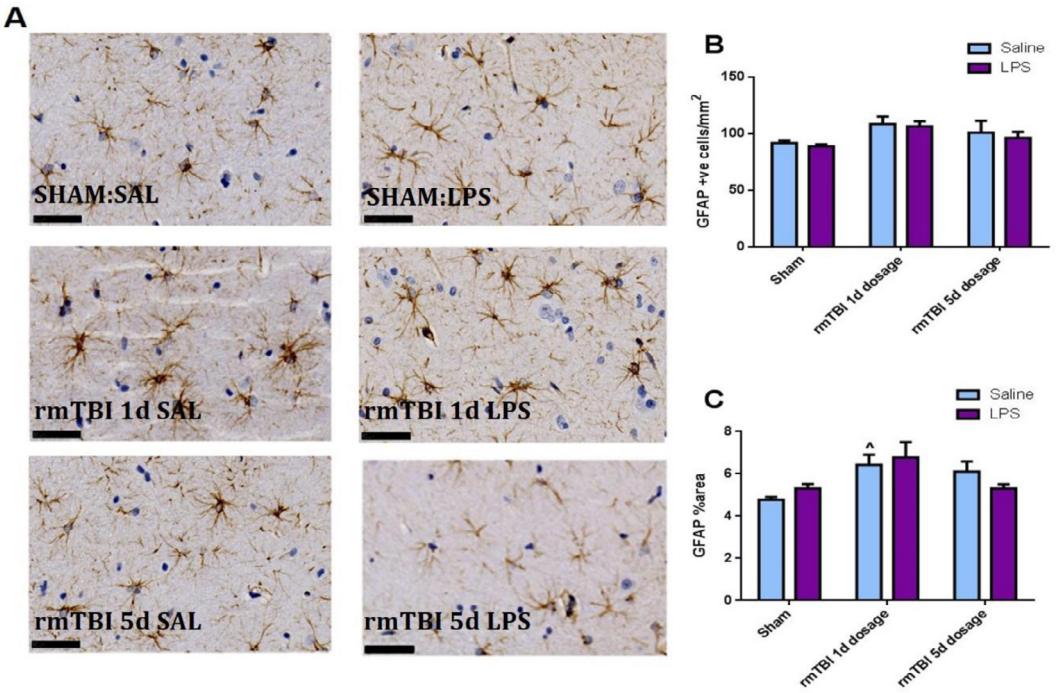

Fig. 5. Representative images of GFAP staining within the hippocampus (A) with counts of the number of GFAP +ve cells, those that had clearly defined cell bodies surrounded by GFAP +ve processes (B), as well as calculation of the GFAP +ve immunoreactive area, as determined by the number of pixels over a set threshold (C). (n = 4 per group, $^{\wedge}p < 0.05$ compared to sham: saline treated rats. Scale bar = 50 μm).

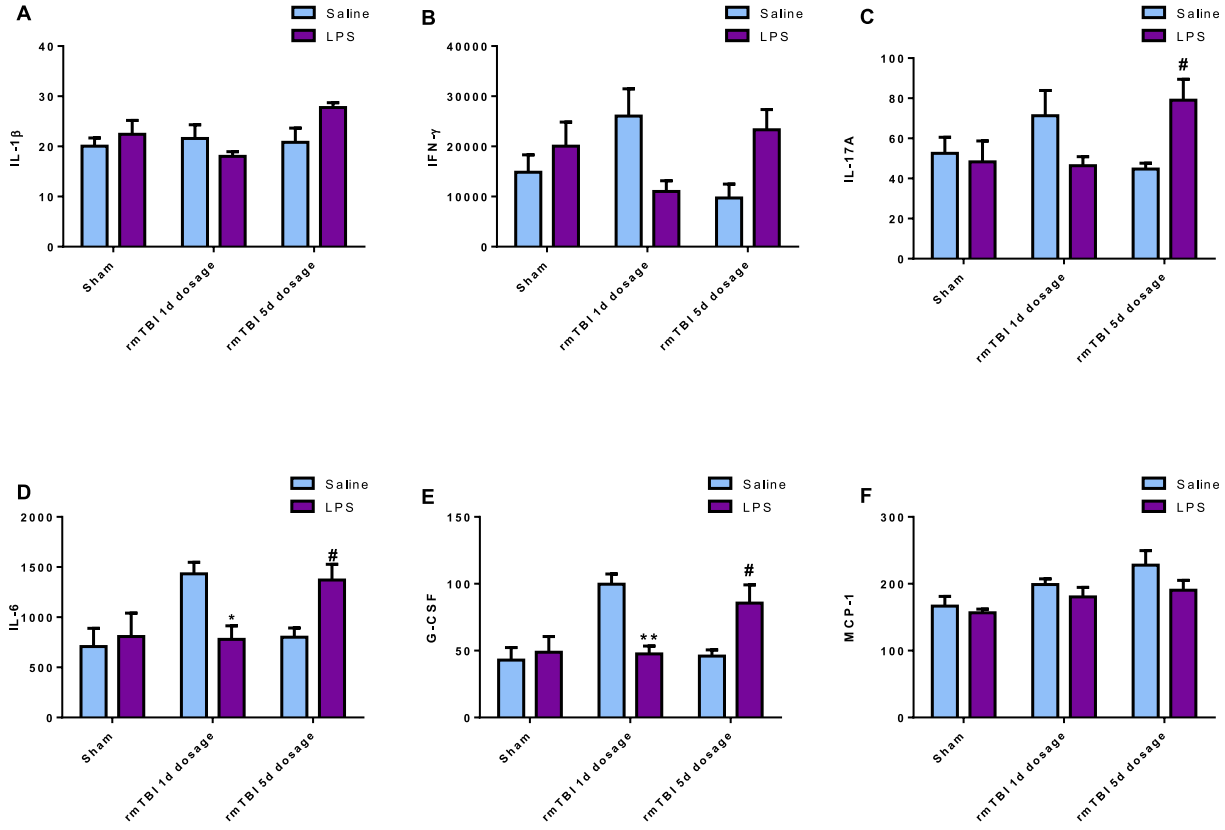

Fig. 6. Analysis of the cytokine response to injury and LPS administration through Multiplex analysis of IL-1β (A), IFN-γ (B), IL-17A (C), IL-6 (D), G-CSF (E) and MCP-1 (F) at 24 h following LPS administration. (n = 3–5 per group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared to rmTBI 1D saline treated animals, $^{\#}p < 0.05$ compared to rmTBI 5d saline treated animals.

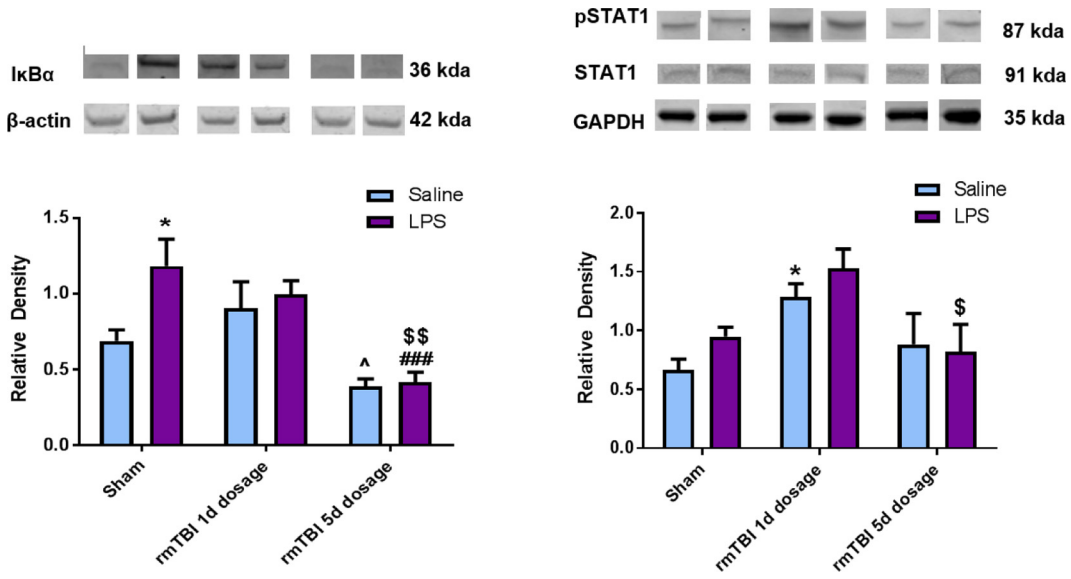

Fig. 7. Evaluation of the effects of LPS administration following rmTBI on levels of IκBa (A) and pSTAT1 (B) acutely (n = 4 per group, $^{*}p < 0.05$ compared to sham:saline, $^{\wedge}p < 0.05$ compared to rmTBI 1D saline, $^{\#\#\#}p < 0.001$ compared to sham:LPS and $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ compared to rmTBI 1D LPS animals).

sham-LPS animals ($p < 0.001$ and $p < 0.01$ respectively). The expression of pSTAT1 was examined relative to STAT1, with a significant effect of injury noted ($p < 0.01$ $F_{2,18} = 8.11$) Post-hoc analysis showed a significant increase in levels of pSTAT1 in rmTBI 1D saline animals compared to shams ($p < 0.05$), with lower levels in LPS 5D treated rmTBI animals than LPS 1D treated rmTBI animals ($p < 0.05$)

6.5. Chronic neuroinflammatory response

The neuroinflammatory response was also assessed at 3 months post-injury. Within the hippocampus, no difference in the number of IBA +ve cells was noted between the groups (Fig 8B), but analysis of IBA1 immunoreactive area via a one-way ANOVA identified a significant injury effect ($F_{4,14} = 7.816$, $p < 0.05$) (Fig 8C). Post hoc analysis showed that rmTBI saline animals had a significantly greater area of IBA1 immunoreactivity compared to rmTBI 5d LPS treated animals (8 ± 0.2% vs 5.5 ± 0.9%, $p < 0.05$). These results were supported with analysis of CD68 immunohistochemistry (Fig 9) similarly revealing a significant injury effect ($F_{4,14} = 4.6$, $p < 0.05$), which appeared to relate to the higher levels of CD68 +ve cells in rmTBI saline treated animals compared to all other groups (42.4 ± 19.4 cells/mm^2 in rmTBI saline treated animals vs 20.5 ± 2.3 and 18.2 ± 7.1 cells/mm^2 in sham:saline and sham:LPS

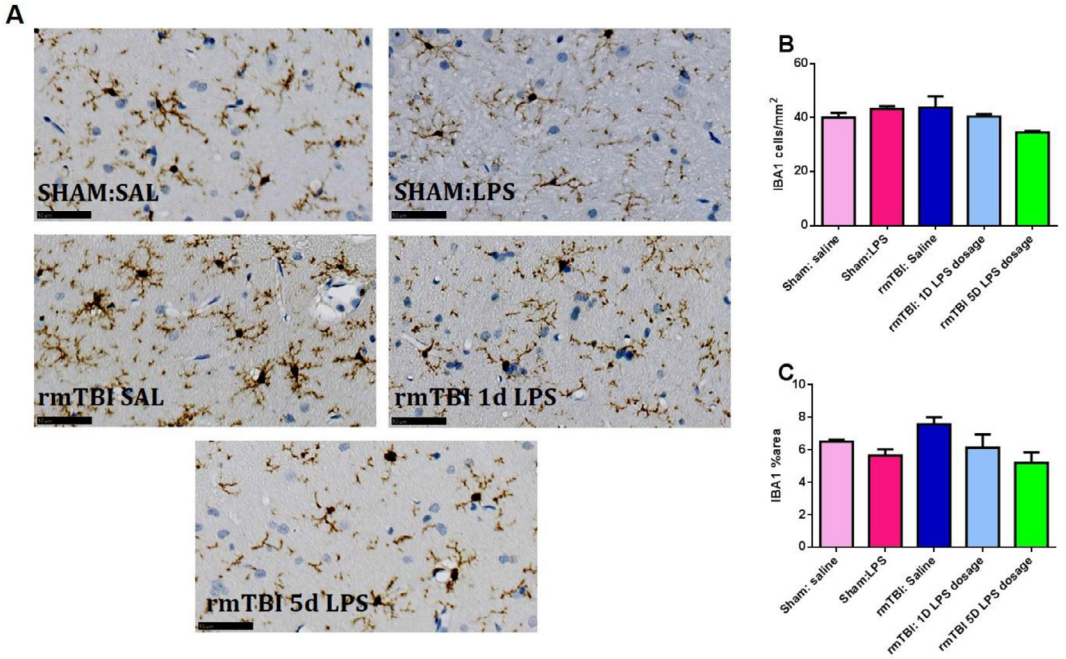

Fig. 8. Representative images of IBA1 staining within the hippocampus (A), with counts of the number of IBA1 +ve cells, as defined as those with a clearly defined cell body (B) and analysis of the% area stained by IBA1, as determined by the number of pixels over a set threshold (C) at 3 months following LPS treatment. rmTBI saline animals had a significantly greater area of IBA1 immunoreactivity compared to rmTBI 5d LPS treated animals (n = 4 per group, $^{\wedge}p < 0.05$ compared to rmTBI saline animals, scale bar = 50 μm).

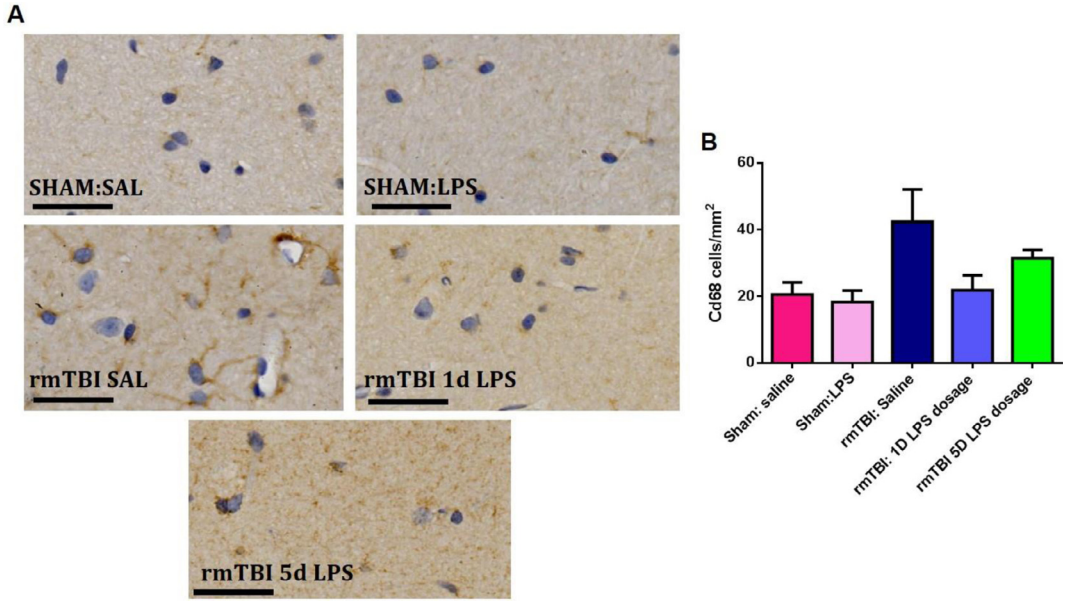

Fig. 9. Representative images of Cd68 staining within the hippocampus (A), with counts of the number of CD68 +ve cells (B). (n = 4 per group, scale bar = 50 μm).

animals respectively) (Fig 9B). No significant differences were noted in the astrocytic response within the hippocampus at 3 months post-injury, although there was a trend towards an injury effect on the number of GFAP +ve cells ($F_{4,14} = 3.188$, $p = 0.09$), which appeared to relate to an increase within the rmTBI saline treated animals (Fig 10).

6.6. Effect of LPS administration on tau phosphorylation and aggregation

The effect of injury and LPS administration on tau phosphorylation was examined by examining the expression of AT180 relative to total tau (Fig 11). Acutely, a significant main effect of LPS administration was noted ($F_{2,16} = 13.56$, $p < 0.001$), with post hoc analysis revealing that rmTBI 5d LPS treated animals had significantly higher AT180:tau:5 levels than all other groups ($p < 0.05$). By 3 months a significant group effect was still noted ($F_{4,20} = 3.5$, $p < 0.05$), with increased levels in rmTBI saline animals (1.74 ± 0.3) compared to sham:saline (0.97 ± 0.4, $p = 0.08$) and sham:LPS animals ($p = 0.83 \pm 0.3$, $p < 0.05$), which was prevented when LPS was administered at 1d post-injury (0.7 ± 0.3, $p < 0.05$), but not 5d post-injury (1.3 ± 0.7). Aggregation of tau was also evaluated, via analysis of levels of oligomeric tau. Acutely a significant effect of LPS administration was noted ($F_{2,16} = 14.97$, $p < 0.001$),

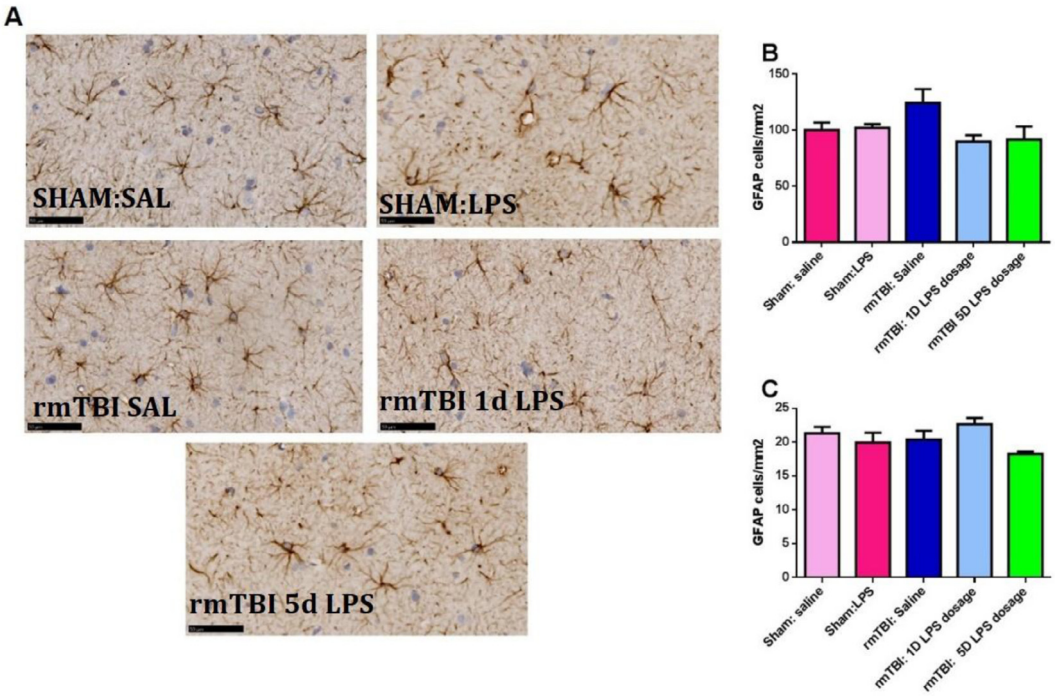

Fig. 10. Representative images of GFAP staining within the hippocampus (A) with counts of the number of GFAP +ve cells, as in those that had clearly defined cell bodies surrounded by GFAP +ve processes (B), as well as calculation of the GFAP +ve immunoreactive area, as determined by the number of pixels over a set threshold (C) at 3 months post-injury. (n = 4 per group, scale bar = 50 μm).

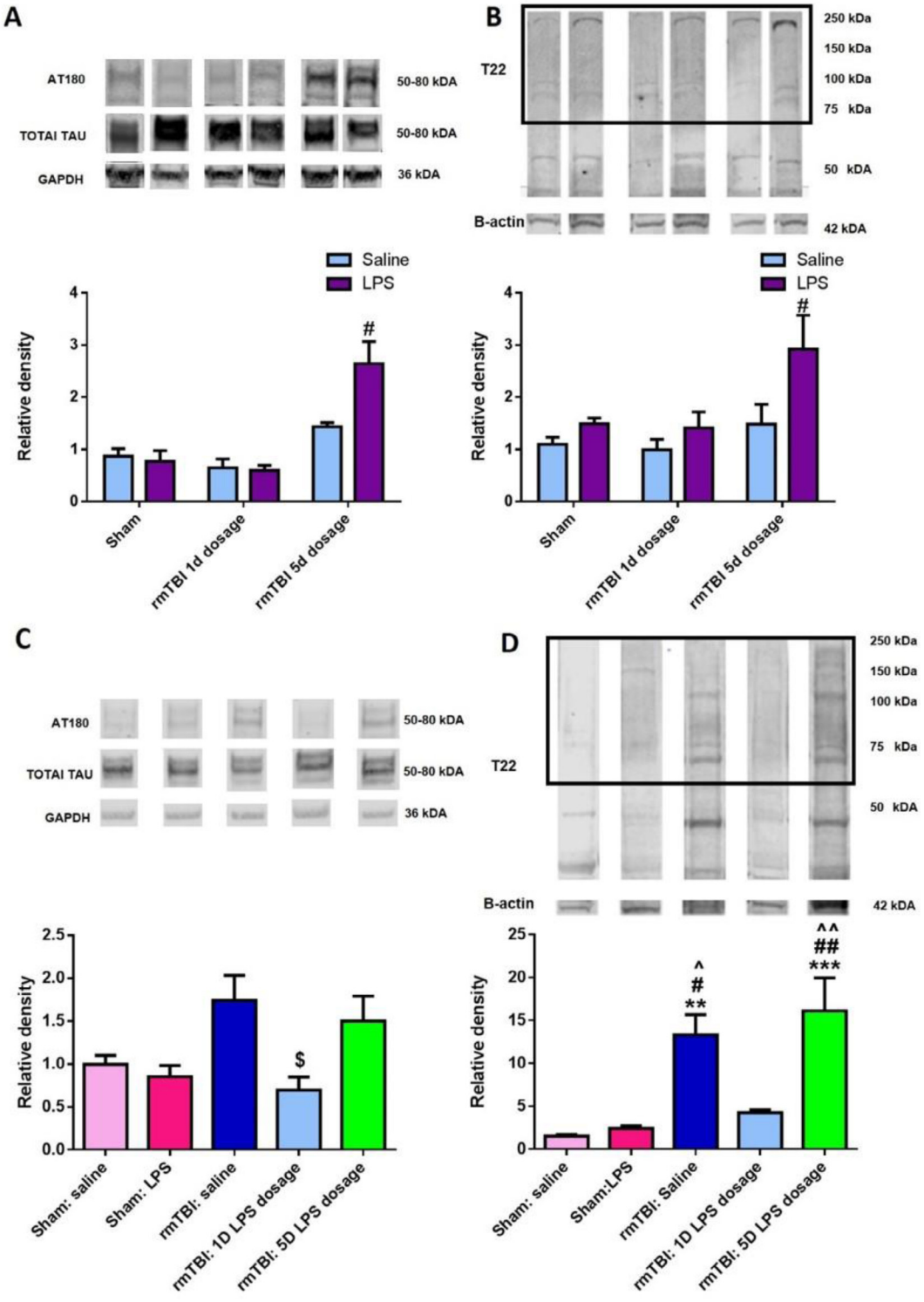

Fig. 11. Evaluation of the effects of LPS administration following rmTBI on markers of tau phosphorylation and aggregation both acutely (A, B) and chronically (C, D). LPS administration at 5 d post-injury increased tau phosphorylation and aggregation acutely. (A–D n = 4 per group, E-H sham:saline and sham:LPS n = 6, rmTBI:saline, rmTBI 1D LPS and rmTBI 5d LPS n = 4–5 per group, A and B $^{\#}p < 0.05$ compared to rmTBI:5d saline animals, C–D $^{\$}p < 0.05$ compared to rmTBI: saline and rmTBI: 5d LPS dosage animals, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to sham:saline animals $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to sham:LPS animals, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ compared to rmTBI: 1d LPS dosage animals).

with post hoc analysis finding that LPS administration at 5d post-injury significantly increased levels of oligomeric tau compared to saline treated animals at the same time-point (2.92 ± 1.3 vs 1.49 ± 0.67). A significant group effect was also noted at 3 months post-injury, ($F_{4,20} = 6.08$, $p < 0.001$), with rmTBI saline (13.3 ± 4.7) and rmTBI 5D LPS animals (15.9 ± 8.0) having significantly higher levels than all other groups (sham:saline 1.5 ± 0.4, sham:LPS 2.4 ± 0.6 and rmTBI 1D LPS animals 4.6 ± 0.6).

6.7. Effect of LPS administration of neuronal injury

Western blot analysis was used to investigate protein levels of a number of markers of neuronal injury within the hippocampus (Fig. 12). Synaptophysin, a presynaptic vesicular protein representing an index of synapse formation, was unaltered acutely by either injury ($p = 0.84$) or LPS treatment ($p = 0.95$) (Fig 12A). However, by 3 months post-injury, there was a significant injury effect ($F_{4,20} = 4.2$, $p < 0.05$) with post hoc analysis showing that rmTBI animals that were treated with LPS at 5 d post-injury had significantly less synaptophysin immunoreactivity than both sham groups (0.16 ± 0.04 vs 0.82 ± 0.39 and 0.80 ± 0.30 in sham:saline and sham:LPS animal respectively, $p < 0.05$) (Fig 10E). Similarly, PSD-95, a post-synaptic scaffolding protein, was also found to be unaffected acutely by either injury ($p = 0.45$) or LPS administration ($p = 0.33$) (Fig 10B). By 3 months post-injury, a difference between groups was noted ($F_{4,20} = 3.7$, $p < 0.05$), with both rmTBI saline treated rats (0.76 ± 0.46) and rmTBI 5d LPS treated rats (0.90 ± 0.47) appearing to have a lower relative expression of PSD-95 than sham animals. (1.47 ± 0.32 and 1.31 ± 0.29 for sham-saline and sham-LPS respectively), which was not evident in rmTBI 1d LPS animals (1.62 ± 0.50, $p < 0.05$ compared to rmTBI saline animals) (Fig 12F).

Axonal damage was evaluated by examining the expression of NFL and MBP. MBP is essential for normal myelination, and acutely a significant injury x LPS administration effect was observed acutely ($F_{2,17} = 5.813$, $p < 0.05$) (Fig 12C). Post-hoc analysis found that LPS when given at 1d post-injury led to a significant increase in expression of MBP ($p < 0.01$), whilst LPS when given at 5d post-injury led to a significant decrease in expression of MBP ($p < 0.05$) when compared to their relative saline rmTBI animals at 24 h following LPS administration. At 3 months post-injury, a significant injury effect was still apparent ($F_{4,20} = 11.97$, $p < 0.001$), with relative expression of MBP significantly lower in the rmTBI: saline (0.61 ± 0.20) and 5d LPS treated rmTBI animals (0.33 ± 0.20) when compared to sham:saline (0.94 ± 0.15) and sham:LPS animals (0.95 ± 0.13) (Fig 10G). Notably, rmTBI 1d LPS treated rats showed an increase in MBP immunoreactivity (1.26 ± 0.14) when compared to the other two injured groups, suggesting preservation of myelin ($p < 0.0001$). When evaluating NFL, there was a significant interaction acutely between injury and LPS administration ($F_{2,17} = 6.0$, $p < 0.05$), with a significant main effect of injury ($F_{2,17} = 4.3$, $p < 0.05$) (Fig 12D). Indeed, a significant increase was seen in relative expression of NFL at 48 h following injury in the rmTBI 1d saline group when compared to sham:saline animals ($p < 0.05$) (1.24 ± 0.08 vs 0.63 ± 0.31), which had returned to sham level by 6 d following injury (rmTBI 5D saline group, 0.83 ± 0.44). LPS treatment at 1d post-injury prevented the increase in NFL, with levels comparable to that seen in shams, whilst when LPS was given at 5d post-injury, a significant increase in NFL immunoreactivity was noted when compared to rmTBI 5d saline animals (1.55 ± 0.51 vs 0.83 ± 0.44) and both sham groups ($p < 0.05$). By 3 months post-injury, there were no differences in NFL levels between groups ($p = 0.99$) (Fig 12H).

6.8. Behavior

The behavioral response to rmTBI and LPS administration was assessed at 3 months post-injury. General locomotor activity was analyzed via the open field (Fig 13A), with a significant overall group effect found ($F_{4,36} = 5.5$, $p < 0.01$), with post hoc analysis showing that rmTBI was associated with a hyper-locomotive activity in the open field, with rmTBI:saline rats traversing 39.7 ± 5.2 m compared to 33.8 ± 4.2 m in sham:saline rats ($p < 0.05$) and 32.0 ± 6.6 m in sham:LPS rats ($p = 0.06$). A similar hyperactivity was seen in rmTBI 5d LPS treated rats ($p < 0.05$ compared to sham:saline; 39.7 ± 2.8 m, $p = 0.09$ compared to sham:LPS). However this effect was negated in rmTBI 1d LPS treated rats, who were no different to shams (32.2 ± 3.9 m).

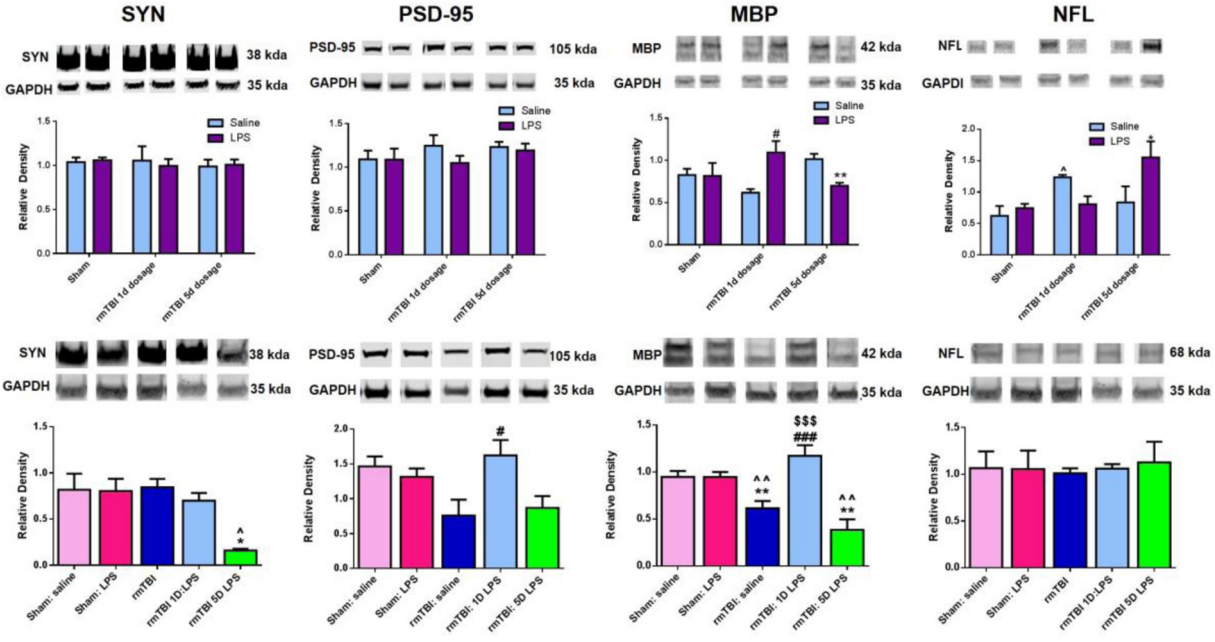

Fig. 12. Evaluation of the effects of LPS administration following rmTBI on the expression of markers of neuronal injury acutely (A–D) and chronically (E–H). LPS administration at 5 d post-injury appeared to increase axonal injury acutely and lead to chronic losses in synaptic integrity and myelin. Conversely, LPS administration at 1d post-injury appeared to be protective, preventing accumulation of NFL acutely and preserving myelination and synaptic integrity chronically. (A–D n = 4 per group, E–H sham:saline and sham:LPS n = 6, rmTBI:saline, rmTBI dD LPS and rmTBI 5d LPS n = 4–5 per group, A–D $^{\wedge}p < 0.05$ compared to sham:saline animals, $^{*}p < 0.05$, $^{**}p < 0.01$ compared to rmTBI 5d saline animals, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared to rmTBI saline animals; E–H $^{\#}p < 0.05$ compared to rmTBI saline animals, $^{\wedge\wedge}p < 0.01$, $^{\wedge}p < 0.05$ compared to sham:saline, $^{**}p < 0.01$, $^{*}p < 0.05$ compared to sham:LPS animals, $^{\$\$\$}p < 0.001$ compared to rmTBI 5D saline animal).

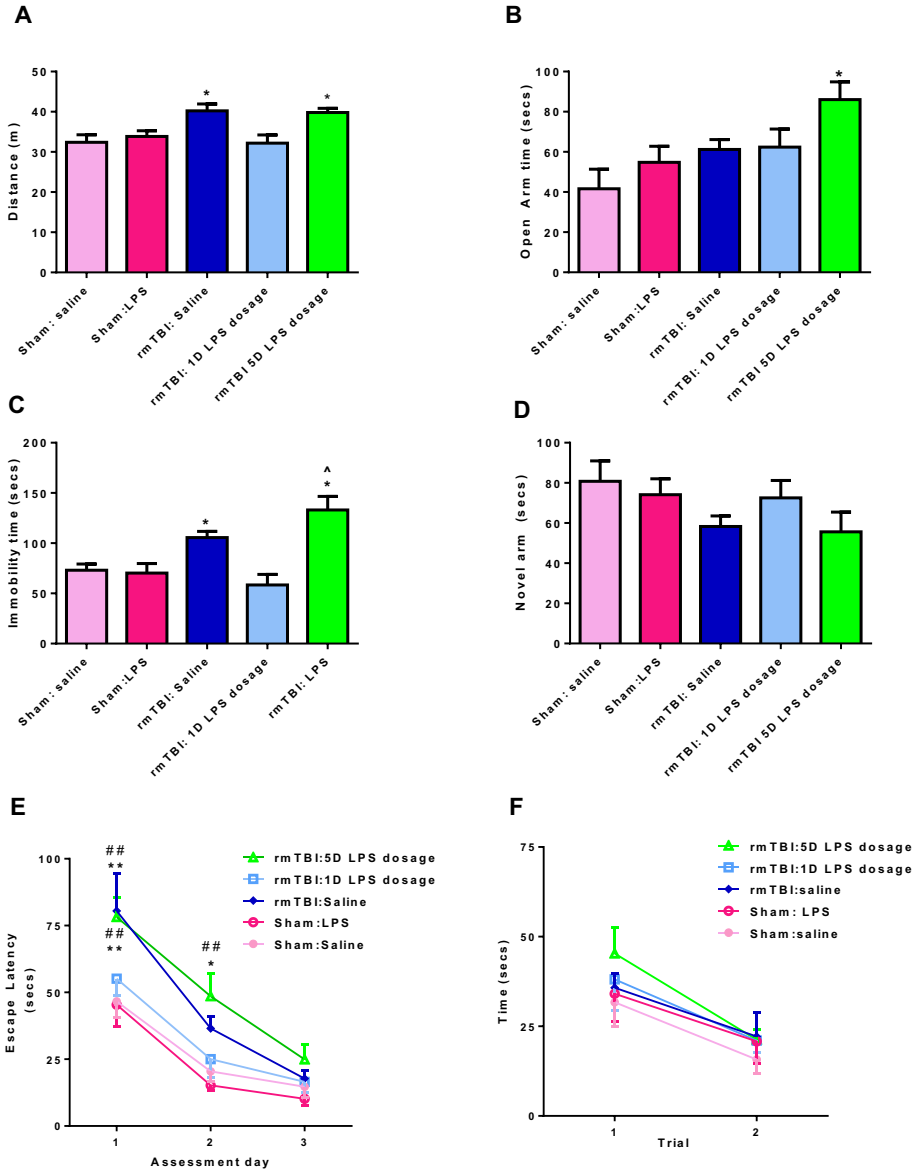

Fig. 13. Behavior was analyzed at 3 months post-injury. General locomotor activity was determined via distance travelled on the open field (A), anxiety-like behavior as time in the open arm on the EPM (B), depressive-like behavior as time spent immobile on the FST (C) and cognition as time spent in the open arm on the Y-maze (D) and the Barnes Maze, including time to find the escape box over 3 training days (E) and time spent finding the location of a new escape box over two days (F). (n = 8–10 per group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared to sham:saline animals, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to sham LPS animals, $^{\wedge}p < 0.05$ compared to rmTBI 1d saline animals).

The EPM was used to evaluate anxiety-like behavior (Fig 13B), with anxious rats spending less time in the open arm. An overall significant group effect was noted ($F_{4,36} = 3.6$, $p < 0.05$), with rmTBI 5d LPS treated animals exhibiting decreased anxious behavior with significantly more time spent in the open arm compared to sham: saline animals ($p < 0.05$) and a trend towards significance compared to the sham:LPS animals ($p = 0.08$). No difference was noted between the rmTBI:saline and rmTBI 1d LPS treated animals and the sham groups.

Depressive-like behavior was assessed as immobility time within the FST (Fig 12C). Analysis via one-way ANOVA identified a significant group effect ($F_{4,37} = 9.5$, $p < 0.0001$). No differences were noted between the sham:saline (73.0 ± 16.6secs) and the sham:LPS animals (69.7 ± 33.0 s). However animals in the rmTBI: saline group had a significant increase in depressive-like behavior compared to shams ($p < 0.05$; 101.2 ± 15.8 s), which was prevented with administration of LPS at 1d following the last injury (58.9 ± 28.1 s). Conversely, administration of LPS at 5d post-injury appeared to exacerbate depressive-like behavior further (133.1 ± 37.9 s), with these animals significantly more depressed than rmTBI 1d LPS treated animals, as well as shams ($p < 0.05$).

A one-way ANOVA found no significant differences in time spent in the novel arm within the Y Maze ($p = 0.19$) (Fig 13D). In contrast, on the Barnes Maze, a main effect of injury ($F_{4,40} = 7.56$, $p < 0.001$) and time ($F_{2,80} = 63.9$, $p < 0.0001$) were seen. rmTBI:saline and rmTBI:5d LPS treated animals were significantly impaired in their ability to find the location of the escape box when compared to sham:saline and sham:LPS animals ($p < 0.05$) (Fig 11E). On the first training day, sham:saline rats took an average of 46.1 ± 17.1 s to locate the escape box, with sham:LPS performing in a similar manner (45.4 ± 17.1 s). This is contrasted by the rmTBI:saline animals (80.5 ± 44.2 s) and rmTBI:5d LPS animals (78.1 ± 20.5 s), whilst rmTBI:1d LPS animals were similar to the shams (55.13 ± 19.8 s). By the second day of training, only the rmTBI 5d LPS animals were significantly different to shams ($p < 0.05$; 48.6 ± 24.3 s compared to 20.5 ± 10.7 s and 15.3 ± 6.2 s

in sham:saline and sham:LPS groups respectively), with rmTBI: saline animals finding the escape box in 36.5 ± 14.1 s and rmTBI 1d animals in 25.0 ± 20.8 s. By Day 3 of training, no significant differences were noted between the groups. 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 no significant differences seen between groups ($p = 0.8$) (Fig 13F).

7. Discussion

A history of repeated mild head impacts has been linked to the later development of neurodegeneration, which is associated with the accumulation of hyperphosphorylated tau and the development of behavioral deficits (McKee et al., 2009; Shultz et al., 2012a). However, the role that exogenous factors, such as immune activation, may play in the development of neurodegeneration following rmTBI had not yet been explored. In this study, it was found that there were differential effects of TLR4 activation induced via a peripheral injection of low dose LPS, following rmTBI, depending on the timing of activation. When given at 1d 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 and chronically, as seen by a restoration of levels of MBP, and both MBP and PSD-95, respectively. Furthermore, these animals did not develop behavioral deficits, with performances similar to shams on the open field, EPM, FST and Barnes Maze, reflecting no changes in locomotion, anxiety, depressive-like behavior or cognition. Conversely, when LPS was given at 5d post-injury, there appeared to be a failure of microglia to respond appropriately, with an apparent decrease in the number of activated microglia, as assessed via CD68 staining, but an increase in pro-inflammatory cytokine production. This was associated 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 study provides further support that repeated mild head impacts can have long-term detrimental outcomes, with rmTBI animals exhibiting cognitive impairment and depressive-like behavior at 3 months post-injury. This is consistent with previous reports that retired NFL 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). Although the mechanism behind how repeated mild head impacts may lead to later neurodegeneration and tau accumulation is not yet known, one hypothesis is the induction of a persistent inflammatory response. Indeed, in the present study, rmTBI animals had increased levels of activated microglia at 3 months post-injury. This is similar to reports from other animal models of repeated mild head injury, with Mouzon et al., finding microglial and astrocytic activation up to 12 months post-injury (Mouzon et al., 2012). Moreover, clinical findings have shown that retired National Football League players have increased microglial activation in the supramarginal gyrus and right amygdala compared to age matched healthy controls (Coughlin et al., 2015), with the presence of activated microglia also reported in several case reports of CTE (Goldstein et al., 2012; McKee et al., 2010; Saing et al., 2012).

These persistently activated microglia can release cytokines, chemokines and other neurotoxic chemicals (e.g. IL-1β, TNFα, superoxide radicals, nitric oxide) that can drive ongoing neuronal injury (Faden and Loane, 2015; Faden et al., 2016). Acutely, the only marker of neuronal injury appeared to be an increase in NFL levels at 48 h following rmTBI. This is in contrast to earlier reports following single moderate-severe TBI, where NFL levels typically decrease acutely following injury due to calpain mediated breakdown. (Huh et al., 2002; Posmantur et al., 1994). In contrast, in rmTBI, the increase in NFL at 48 h may reflect a rebound reparative response, as NFL levels have been shown to increase within the CSF and serum following concussion, indicating that axonal injury does occur acutely (Zetterberg et al., 2013). Nonetheless, further research would be needed to confirm this. Despite minimal evidence of acute neuronal damage within the hippocampus, ongoing neuronal injury was seen within this study, with rmTBI animals showing a progressive loss of MBP, in line with the myelin loss reported in clinical cases of CTE (McKee et al., 2009).

In addition, the persistent inflammation may explain the increase in levels of tau phosphorylation within the hippocampus at 3 months post-injury in the rmTBI animals, as hypothesized by a recent comprehensive review of the literature in this area (Collins-Praino and Corrigan, 2017). Chronic activation of the innate immune system has been shown to exacerbate tau phosphorylation in animal models of tauopathy (Kitazawa et al., 2005; Lee et al., 2010; Li et al., 2003; Sy et al., 2011). In the current study, levels of tau phosphorylation were not noted in the first week following injury, in contrast to reports from some other models (Tan et al., 2016). This may relate to the region studied,, with the cortex typically showing higher levels early after injury (McAteer et al., 2016; Tan et al., 2016) due to the concentration of mechanical force in this region (Cloots et al., 2008). Indeed, in CTE, involvement of the hippocampus with tau pathology is seen in later stages of the disease (Stage III), with earlier stages typically only involving the cortex (McKee et al., 2013).

Nonetheless, given the evidence that neuroinflammation can play a role in the development of neurodegeneration following rmTBI, this study sought to investigate the effect of modulating the immune response in the acute phase following injury. A low dose of the classical TLR4 agonist, LPS (0.1 mg/kg) was chosen to reflect levels of LPS that would be seen with low grade systemic inflammation (Chongwatpol et al., 2015; Couch et al., 2016), although modest increases of LPS can also be induced systemically in a number of other situations, including ingestion of high fat meals (Erridge et al., 2007; Ghanim et al., 2009), with severe exertion (Selkirk et al., 2008), and acute alcohol consumption (Bala et al., 2014). This dose of LPS had minimal effects in sham animals, with evidence of weight loss at 24 h post-dose, but no effect on locomotor activity or neuroinflammation within the hippocampus. Indeed previous reports have shown that a similar dose of LPS rapidly increases systemic cytokine production within 3 h of administration, but this returns to baseline by 24 h (Teeling, 2007), with no evidence of microglial activation or behavioral deficits in naïve mice at 24 h post-dose (Couch et al., 2016). Conversely, when given on a background of pre-existing inflammation induced after rmTBI in this study, LPS had profound effects, with dosage at 1d post-injury protective, and dosage at 5d post-injury exacerbating injury.

The failure to augment the inflammatory response with LPS at 1d post-injury may reflect the development of endotoxin tolerance. Endotoxin tolerance describes the phenomenon whereby repeated activation of the immune system can lead to a transient decrease in sensitivity to subsequent immune challenges, seen as a reduction in the production of pro-inflammatory mediators (Mendez et al., 1999). The concussive injury would have provided the setting for pre-existing immune activation. A concussive insult activates the immune response, with the release of damage-associated molecules (DAMPs) from disrupted cells and extracellular matrix activating the TLR4 signaling pathway (Koedel et al., 2007). Indeed, Mao et al., showed that a moderate concussive brain injury increases hippocampal TLR4 within 4 h following injury (Mao et al., 2012), with increases in the levels of pro-inflammatory cytokines, like IL1β and TNFα, reported at 3–6 h following

concussive insults (Khuman et al., 2011; Perez-Polo et al., 2015). The magnitude of this inflammatory response is correlated with injury severity (Kinoshita et al., 2002), but is greatly amplified in animals receiving multiple injuries compared to a single injury (Aungst et al., 2014; Faden et al., 2016). In the present study, rmTBI animals still had significantly elevated levels of the pro-inflammatory cytokines IL1β, IFNγ and IL17 at 48 h following the last impact, providing evidence of immune activation following rmTBI.

When LPS was administered during this heightened inflammatory phase at 1d post-injury, it had protective effects. Of note, recent research has indicated that endotoxin tolerance, rather than reflecting a failure to respond to subsequent stimuli, is the result of differential expression of the two TLR4 pathways (Biswas et al., 2007; Piao et al., 2009; Vartanian et al., 2011). The TLR4 signal is relayed through two adaptor proteins, Myd88 and TRIF, with the MyD88 pathway leading to NF-κB activation and subsequent pro-inflammatory cytokine production, whilst the TRIF pathway promotes activation of the interferon regulatory factor IRF3, leading to the production of anti-inflammatory IFN-β (Buchanan et al., 2010). The role of these two signaling pathways in the differing response to LPS was indirectly examined within this study through evaluation of levels of IKBα and pSTAT-3 at 24 h post-LPS dose. IκBα is a key mediator of the Myd88 dependent NFκB pathway, with its degradation allowing NFκB to move from the cytoplasm to the nucleus and to target key target pro-inflammatory genes (Oeckinghaus and Ghosh, 2009). Following LPS administration a rapid degradation of IKBα is observed within 15–30 min, which corresponds with NFκB activation and induction of pro-inflammatory cytokine production (Cruz et al., 2001; Reis et al., 2011). A potential mechanism driving endotoxin tolerance has been suggested to be elevation of the levels of IKBα (Dobrovolskaia et al., 2003), as seen in endotoxin tolerant human monocytes (Chen et al., 2009). This concurs with our findings where LPS administration in shams caused an increase in IκBα levels at 24 h following exposure, with a trend towards an increases seen in rmTBI animals at 48 h following last injury. This would then act to reduce the ability of NFκB to translocate to the nucleus with a subsequent immune stimulus (Cohen-Lahav et al., 2006), as concurs with the reduction of pro-inflammatory cytokine production seen in LPS treated animals at 1D following rmTBI. It should be noted that LPS stimulation in rmTBI animals did not produce a further change in IκBα levels, suggesting that there is a limit on this potential protective mechanism. Of note by 6 days post-injury (rmTBI 5D saline animals) levels of IκBα were reduced, which could allow greater amounts of NFκB activation, in line with the exacerbated inflammatory response seen to LPS administration.

In contrast, endotoxin tolerance has also been proposed to be caused due to a switching from the Myd88 to the TRIF pathway. Activation of TLR4 via LPS in macrophages initially produces large quantities of TNFα, but this is markedly reduced on re-exposure to LPS (Fan and Cook, 2004; Liew et al., 2005), with an enhancement of IFNβ production during secondary exposure (Broad et al., 2007), suggesting of a switching from activation of the Myd88 to the TRIF pathway. Here phosphorylation of STAT-1 was used as an indirect measure of TRIF pathway activation, as its phosphorylation induced by IFNβ (Biswas et al., 2007). *In vitro* studies have shown that STAT-1 phosphorylation occurs slightly later following LPS exposure than NFκB activation, peaking at 4 h post-injury and then falling steadily over the next 20 h (Sikorski et al., 2011). However, with re-exposure to an immune stimulus this STAT-1 phosphorylation occurs earlier, at 30–60 min, with tolerized cells showing an increase in STAT1 phosphorylation basally (Biswas et al., 2007). This is similar to our finding that rmTBI 1D saline treated animals had higher levels of pSTAT1 than shams, an effect that had dissipated in the rmTBI 5D saline treated animals. This is line with the fact that the period of endotoxin tolerance is transient (Chu et al., 2016). Although no direct effect of LPS was noted, this may relate to the time-point, in 24 h post-administration, given that as described earlier many of these signaling changes occur within hours of exposure. Nonetheless the higher baseline levels of psTAT1, accompanied by the reduced expression of pro-inflammatory mediators would support a predominance of TRIF over Myd88 expression in response to LPS at the 1D time-point, an effect no longer seen at 5D post-injury.

A potential effect of enhanced TRIF signaling, may be a shift from a M1-like phenotype to an M2-phenotype in human microglial cultures (Tarassishin et al., 2011), as this is enhanced by IRF3 expression. Indeed, in organotypic hippocampal slice cultures, re-exposure to LPS promoted microglial polarization towards an M2-like phenotype (Ajmone-Cat et al., 2013). Activated microglia have phenotypic sub-populations with different molecular signatures of gene expression, with M1 microglia promoting a classic pro-inflammatory state, while M2 microglia are important for tissue remodeling and suppress the inflammatory response (Colton, 2009; David and Kroner, 2011). Disproportionate M1-like activation has been proposed as a potential mechanism that may explain the link between TBI and later neurodegeneration, as it hampers repair and allows tissue damage to persist for years after the initial insult (Bigler, 2013). Although not directly assessed, the evidence from the present study suggests that LPS administration at 1d following rmTBI may have promoted a switch from an M1 to an M2 activation like state. Following LPS administration at 1d, there is evidence of acute microglial activation seen at 24 h post-dose through enhanced expression of IBA1 and CD68, which are not specific for either activation state (Walker and Lue, 2015). Interestingly, this increase in microglial expression was associated with a decrease in pro-inflammatory cytokine levels. This was accompanied by a decrease in axonal injury acutely, with preservation of levels of MBP and a prevention of the increase in NFL. Furthermore this change in the inflammatory environment within the brain had long-lasting effects, with 1d LPS treated animals demonstrating no evidence of neuronal injury or functional deficits at 3 months post-injury. This may relate to the protective effects of M2 microglia, as they can ensheath neurons to provide trophic support under damaging conditions (Streit, 2002) and have been shown to drive oligodendrocyte differentiation to support remyelination of damaged axons (Miron et al., 2013).

Neuroprotective effects of low-dose LPS associated with the development of endotoxin tolerance have been most thoroughly characterized when low dose LPS is given prior to a central nervous system insult, primarily stroke (Ahmed et al., 2000; Bastide et al., 2003; Rosenzweig et al., 2004). However Chen et al., found that LPS treatment prior to an aseptic cryogenic lesion reduced lesion volume and decreased cell death, with this attributed to a shift towards an M2 phenotype within microglia (Chen et al., 2012). A few studies have also investigated the effects of post-injury immune activation. Bingham et al., found that, following an *in vitro* scratch model of TBI, cells immediately exposed to LPS following the insult showed a reduction in cell death, with an accompanying significant increase in the levels of anti-inflammatory IL-10 (Bingham et al., 2011). Following an inflammatory insult to the brain induced by an intrastriatal injection of IL-1β, administration of 0.05 mg/kg of LPS intranasally at 2 h post-insult led to a reduction in systemic pro-inflammatory cytokines (TNFα and IFNγ), with a reduction in activated microglia as measured via ED1 immunostaining within the brain (Bingham et al., 2013). Similarly, LPS administered 2 h after a spinal cord contusion injury reduced leukocyte recruitment to the injured area (Davis et al., 2005). It is unclear why these studies found a decrease in resident

microglial activation, in contrast to the present study, but it may relate to differences in the timing of administration, the dose and the different nature of the CNS insult. Indeed, administration of a higher dose of LPS (4 mg/kg) immediately after a weight drop TBI increased pro-inflammatory cytokine production with an accompanied increase in cell death (Hang et al., 2004). Similar results were seen with administration of IL-1β (20 μg/kg or 40 μg/kg) following fluid percussion injury which worsened motor outcome and increased contusion volume (Utagawa et al., 2008) and a severe peripheral inflammatory insult in a tibial fracture exacerbates neuroinflammation, with a worsening of edema and motor performance post-TBI (Shultz et al., 2015). Thus it is evident that induction of more significant levels of inflammation post-TBI can have detrimental effects.

We also found that LPS administration at 5 d post-rmTBI exacerbated neuronal damage and functional deficits. It is known that the period of endotoxin tolerance is transient. For example, within neuron-glia cultures, two LPS treatments spaced 18 h apart showed evidence of endotoxin tolerance on the second exposure, with production of less TNFα, but when treatment was delayed to 30 h, high levels of TNFα were again produced upon re-exposure (Chu et al., 2016). Here, it appears that, at 5d post-injury, a traditional pro-inflammatory response was triggered in response to LPS administration, given the increase in pro-inflammatory cytokine production (IL-6, IFNγ and IL-17). However, this was not accompanied by evidence of microglial activation, with the number of IBA1 +ve cells actually decreasing in these animals. This could potentially represent the development of microglial immunosenescence, caused by repeated injury forcing microglia to undergo repeated bouts of division. Streit et al., showed that a repeated cranial nerve crush model markedly reduced microglial proliferation compared to a single insult, a hallmark of cell senescence (Streit, 2006). Immunosenescence describes the aging of immune cells, resulting in changes in cell morphology, function and behavior, leading to irreversible growth arrest (Rawji et al., 2016). Despite having fewer cells, these microglia have a more pro-inflammatory phenotype, with microglia senescence characterized by an inability to shift from a pro-inflammatory to an anti-inflammatory phenotype (Caldeira et al., 2014). Furthermore they show impaired phagocytic capacity which could facilitate the accumulation of pathological proteins and debris (Streit and Xue, 2012). It has been suggested that activated microglia express CD68 in response to phagocytic activity (Graeber et al., 1998), which may provide an explanation for the lack of CD68 +ve cells in animals in the present study. Nonetheless, further studies are needed to examine the specific morphology of these microglia, in order to investigate whether immunosenescence is present, with senescent microglia characterized by a lack of branching, twisting of processes and increased cell soma size (Streit and Xue, 2012).

Regardless of the mechanism, it is evident that LPS, when given at 5d post-injury, enhances pro-inflammatory cytokine production 24 h post-dose, which is associated with increased neuronal damage- with loss of myelin and increased levels of NFL. This is consistent with previous work showing that augmenting the inflammatory response is associated with an increase in axonal injury (Hellewell et al., 2010), and loss of myelin (Johnson et al., 2013). Of note, the 5d LPS administration had long-term detrimental effects, with a progressive decrease in synaptophysin levels at 3 months post-injury, indicating loss of synaptic integrity, an effect not seen in rmTBI:saline animals, with a slight exacerbation of behavioral deficits. The exact mechanism driving this is unknown, as there was no evidence of microglial or astrocytic activation histologically at 3 months post-injury, but further studies would be needed to investigate the function of these cells to determine whether they were still contributing to a pro-inflammatory environment, given the role that chronic inflammation plays in the development of neurodegeneration (Faden and Loane, 2015; Faden et al., 2016).

The development of neurodegeneration at 3 months post-injury in the rmTBI 5d LPS animals was not accompanied by a further increase in phosphorylated or aggregated tau when compared to rmTBI:saline animals. At 24 h after dosage, these animals had an acute increase in levels of phosphorylated tau (AT180) and oligomeric tau (T22), as would be expected given the evidence that induction of the inflammatory response facilitates tau phosphorylation (Kitazawa et al., 2005; Lee et al., 2010; Li et al., 2003; Sy et al., 2011), but this did not persist to 3 months post-injury, with these animals having similar levels of AT180 immunoreactivity to shams. This may reflect larger amounts of damage within the hippocampus, with less neurons available to contain phosphorylated tau, or may reflect phosphorylation at different sites, with tau containing 80 potential serine/threonine and 5 potential tyrosine sites on its longest isoform (Tau_{441}), with phosphorylation occurring at at least 30 of them (Mendoza et al., 2013). Another possibility is a change in the conformation status of tau with recent work by Kondo et al., demonstrating an increase of cis p-tau, but not trans-p tau, acutely after injury, with cis p-tau associated with neuronal injury (Kondo et al., 2015). Intruigingly there are case reports in the literature of repeat concussion cases showing later neuronal loss within the hippocampus without appreciable tau pathology (McKee et al., 2009), suggesting in a subset of cases the appearance of tau pathology and neurodegeneration can be separate processes. Further investigation will be needed to investigate other tau phosphorylation markers apart from AT180 to determine whether any alterations in tau pathology are caused by LPS administration at 5d following rmTBI. Further, given the relative increase in oligomeric tau seen acutely but not chronically in these animals, it would be worthwhile investigating whether these animals had more extensive spread of the disease. It has been suggested that tau oligomers may act as templates for the misfolding of native tau, thereby seeding the spread of the toxic forms of the protein (Gerson and Kayed, 2013), along connection pathways.

In summary, this study found that activation of TLR4 following rmTBI had differential effects depending on the timing of activation. When administered at 1d post-injury it was neuroprotective, whereas at 5d post-injury it exacerbated injury, both acutely and chronically. 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.

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