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Research report

Evaluation of early chronic functional outcomes and their relationship to pre-frontal cortex and hippocampal pathology following moderate-severe traumatic brain injury

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ABSTRACT

TBI is a significant risk factor for the development of dementia, with the interaction between structural damage from TBI and neuroinflammation potentially driving this relationship. This study investigated the early chronic post-TBI neuroinflammatory response and its relationship to both neurodegenerative pathology and functional impairment up to 3 months post-injury. Sprague-Dawley rats underwent either sham surgery or the Marmarou model of diffuse moderate-severe TBI. At 1-month and 3-months post-injury, a functional battery encompassing motor function, depressive-like behaviour, anxiety and cognition was performed. Western blot and immunohistochemical analysis assessed a range of inflammatory, neurodegenerative and oxidative stress markers. At both 1 and 3-months post injury, depressive-like behaviour was significantly increased in TBI animals, with TBI animals also exhibiting impaired cognitive flexibility at 3 months, although learning and memory remained intact. This was accompanied by a significant decrease in markers of synaptic integrity and astrocytic and microglia number within the pre-frontal cortex at 1-month post-injury, although this resolved by 3-months post-injury. In contrast, minimal pathology was evident within the hippocampus at 1 month, with only a decrease in neurofilament-light seen at 3 months post-injury. Thus, following a moderate-severe diffuse injury, the pre-frontal cortex is most vulnerable to early neuro-structural changes. While these changes are resolved at 3 months post-injury, future studies should investigate whether they re-emerge or progress to other areas, such as the hippocampus, at later time points, which could predispose individuals to the development of dementia.

1. Introduction

Traumatic brain injury (TBI) represents one of the leading causes of mortality and disability worldwide. The Centre of Disease Control and Prevention stated that, in 2010 alone, there were approximately 2.5 million emergency department visits, hospitalisations and deaths due to TBI [1,2]. There is increasing evidence to suggest that neuronal injury is ongoing following a TBI [3–5], and that moderate-severe TBI may lead to progressive neurodegeneration, such as dementia and associated cognitive and behavioural deficits. Population based studies following patients with moderate-severe TBI showed these functional deficits persisting years later, even after motor function recovery [6–8]. An Australian health survey of TBI cases reported an overall decrease in mental health quality and elevated depression levels when compared to a matched non-TBI cohort, even up to 15 years after injury [9].

Indeed, following a focal injury, lesion volume was found to increase nearly 5 fold over one-year post-injury [10], whereas, following a mixed focal/diffuse injury induced by lateral fluid percussion, cortical and hippocampal tissue loss increased significantly from one week to one year post-injury [11]. This is supported by clinical imaging studies, which have shown progressive white matter damage, particularly within the frontal and temporal regions, as well as loss of cortical grey matter, up to a year post-injury [12], in line with reports of progressive reductions in brain volume as assessed up to 14 months post-TBI [13].

The exact mechanisms that drive this ongoing neuronal injury are yet to be fully elucidated, with the development of an aberrant persistent chronic neuroinflammatory response thought to be one key mechanism [14]. Indeed, multiple studies have demonstrated that a neuroinflammatory response may persist following resolution of the acute effects of a TBI, with inflammatory markers present in the brain parenchyma, serum and cerebrospinal fluid of TBI patients at chronic time points (months to years later) [15–18]. In rodents, microglial activation has been demonstrated up to one-year following a focal TBI, with associated progressive lesion expansion, hippocampal degeneration, myelin loss and oxidative stress [10].

Although a number of studies have shown progressive neuronal loss

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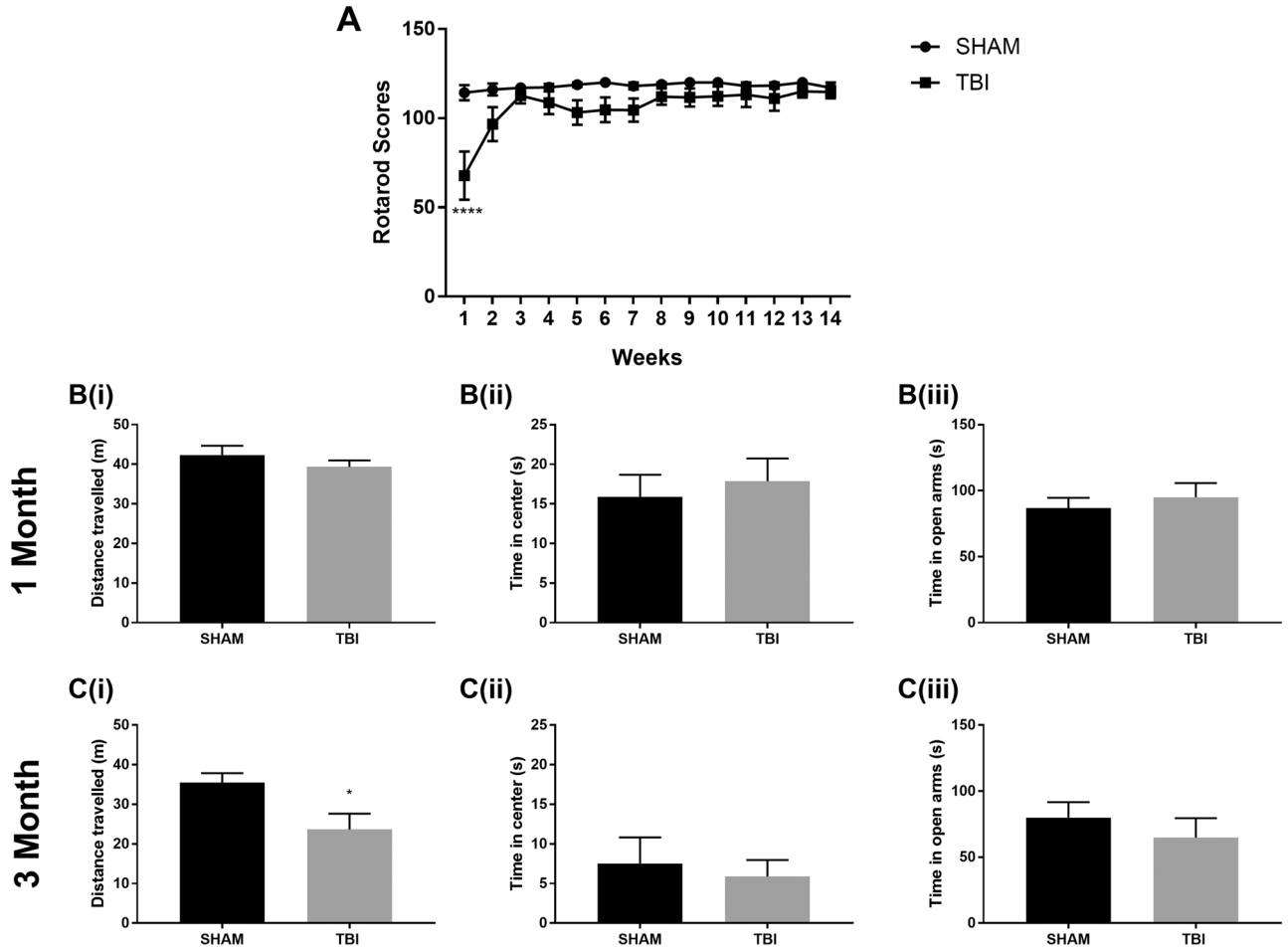

Fig. 1. Functional outcomes measured post injury. A) Motor outcome as measured on the rotarod, weekly for 3 months. Locomotor activity as measured on the open field at B(i)) 1 month and C(i)) 3 months. Anxiety-like behaviour as measured in the open field at B(ii)) 1 month and C(ii)) 3 months and on the elevated plus maze at B(iii)) 1 month and C(iii)) 3 months. Graphs represent the mean ± SEM, (n = 13–19 per group; ****$p < 0.0001$,*$p < 0.05$ compared to shams).

up to one year post-injury, a more detailed examination of the events that occur in the sub-acute and early chronic stages post-TBI that may promote this ongoing neuronal injury have received less attention. Furthermore, these studies have been predominantly conducted utilising focal [10] or mixed focal models [11], rather than a purely diffuse injury. A diffuse injury model would be clinically more relevant as it mimics the hallmarks seen in majority of the human TBI cases (motor vehicle accidents) such as unconsciousness post injury and widespread diffuse axonal injury [19,20]. As such, this study sought to investigate the effects of a moderate-severe diffuse TBI at 1 and 3 months post-injury on synaptic and axonal integrity and neuroinflammation, as well as on functional outcome.

2. Results

2.1. Motor outcome

Motor outcome was assessed weekly up to 3 months (Fig. 1A) on the rotarod. Sham and TBI animals showed no significant differences in their pre-training rotarod scores but a significant injury effect on the scores was seen in the weeks following the injury ($F_{1,19} = 5.146$, $p = 0.035$). TBI animals showed a significantly impaired rotarod scores when compared to shams (67.8 ± 13.47 s vs 114.3 ± 4.23 s in sham animals, $p < 0.0001$) at 24 h post injury (indicated by week 1 on Fig. 1A). However, by the third week (day 15) post-injury, TBI animals had returned to sham levels, (112.7 ± 4.46 s vs 117 ± 2.09 s, $p > 0.9999$) and maintained this for the rest of the testing period.

2.2. Locomotor activity

General locomotor activity was assessed as the distance travelled in the open field test (OFT). At 1 month post-injury (Fig. 1B(i)), TBI animals showed no difference in locomotor activity compared to shams (39.36 ± 1.6 m vs 42.35 ± 2.3 m in shams; $t(32) = 1.089$, $p = 0.2843$), but at 3 months post-injury (Fig. 1C(i)), there was a significant decrease in locomotor activity in the TBI animals when compared to shams (23.6 ± 4.0 m vs 35.5 ± 2.4 m; $t(23) = 2.479$, $p = 0.0209$).

2.3. Anxiety-like behaviour

Anxiety-like behaviour was measured as time spent in centre of OFT and time spent in the open arms of the elevated plus maze (EPM). No significant differences in time spent in centre of OFT and open arms of EPM were seen between the TBI animals and sham animals at 1 month (17.9 ± 2.9 s vs 15.9 ± 2.8 s in shams; $t(32) = 0.485$, $p = 0.6314$) and (95.0 ± 10.8 s vs 86.7 ± 7.9 s in shams; $t(32) = 0.591$, $p = 0.5587$) respectively (Fig. 1B (ii) and (iii)). Similarly, at 3 months post-injury, no significant differences were seen between groups in time spent in centre of OFT (5.9 ± 2.1 s vs 7.5 ± 3.3 s in shams; $t(23) = 0.427$, $p = 0.6735$) (Fig. 1C(ii)) as well as time spent in open arms of EPM (64.8 ± 14.7 s vs 80.0 ± 11.7 s in shams; $t(23) = 0.798$, $p = 0.4329$) (Fig. 1C(iii)).

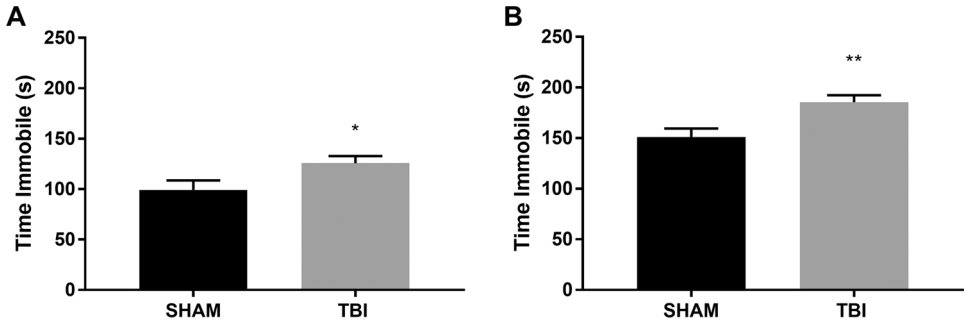

Fig. 2. Depressive-like behaviour as measured in forced swim test at A) 1 month and B) 3 months. Graphs represent the mean ± SEM, (n = 13–19 per group; **p < 0.01, *p < 0.05 compared to shams).

2.4. Depressive- like behaviour

Depressive-like phenotype was assessed based on the immobility time in the forced swim test (FST). TBI animals spent more time immobile than shams at 1 month post-injury (125.8 ± 7 s vs 99.2 ± 9.4 s in shams; $t(32) = 2.32$, p = 0.0269) (Fig. 2A), with this persisting at 3 months post-injury (185.6 ± 6.8 s vs 151.2 ± 8.4 ss in shams; $t(23) = 3.217$, p = 0.0038) (Fig. 2B).

2.5. Cognition

Cognitive outcome was assessed using the Y-Maze for spatial memory and Barnes maze for learning, memory and cognitive flexibility (ability to reprogram previously learned task) (Fig. 3). Y-Maze was performed at 1 month and 3 months post-injury, while the Barnes maze was only performed on the 3 month animals. Spatial working memory in the Y-Maze showed no significant changes in novel preference between the TBI group and the sham control group at any of the time points post-injury; 1 month (0.37 ± 0.03 vs 0.42 ± 0.03 in shams, $t(32) = 1.009$, p = 0.3205), 3 month (0.37 ± 0.04 vs 0.39 ± 0.03 in shams, $t(23) = 0.326$, p = 0.7475) (Fig. 3A–B). On the Barnes Maze, no significant differences were noted in time taken to locate the escape box on any of the training days during the acquisition phase ($F_{1,23} = 0.049$, p = 0.8276)(Fig. 3C). Nor was there any difference in ability to locate the old escape box on the probe day (shams 27.8 ± 12.6 vs TBI 14.0 ± 4.2 s; $t(23) = 1.076$, p = 0.293) (Fig. 3D). In terms of learning the location of the new escape box on probe day, there was a trend of injury effect ($F_{1,23} = 3.979$, p = 0.0581). The sham animals showed greater cognitive flexibility taking a significantly shorter time on Trial 1 compared to TBI animals (63.0 ± 16.3 s vs 24.0 ± 4.6 s in shams, p = 0.014), although both groups had similar times on trial 2 (24.0 ± 7.7 s vs 15.24 ± 3.1 s in shams, p = 0.528) (Fig. 3E).

2.6. Early chronic neuroinflammatory changes in prefrontal cortex (PFC) post-TBI

Levels of inflammation were assessed by counting the number of cells that were immunopositive for GFAP (glial fibrillary acidic protein) (Fig. 4), a structural protein in astrocytes and IBA1 (ionized calcium binding adaptor molecule 1) (Fig. 5), a calcium binding protein seen in microglia within the PFC and hippocampus. At 1 month post injury, GFAP immunopositive staining (GFAP + ve) was decreased within the PFC in TBI animals (135.8 ± 14.39 cells/mm^2) compared to shams (193.4 ± 13.48 cells/mm^2) ($t(7) = 2.92$, p = 0.019) (Fig. 4C). However, the number of GFAP + ve cells in the hippocampus of TBI animals (193.0 ± 22.9 cells/mm^2) and shams (207.5 ± 5.91 cells/mm^2) did not differ significantly between groups ($t(7) = 0.55$, p = 0.601) (Fig. 4D). At 3 months post injury, the number of GFAP + ve cells did

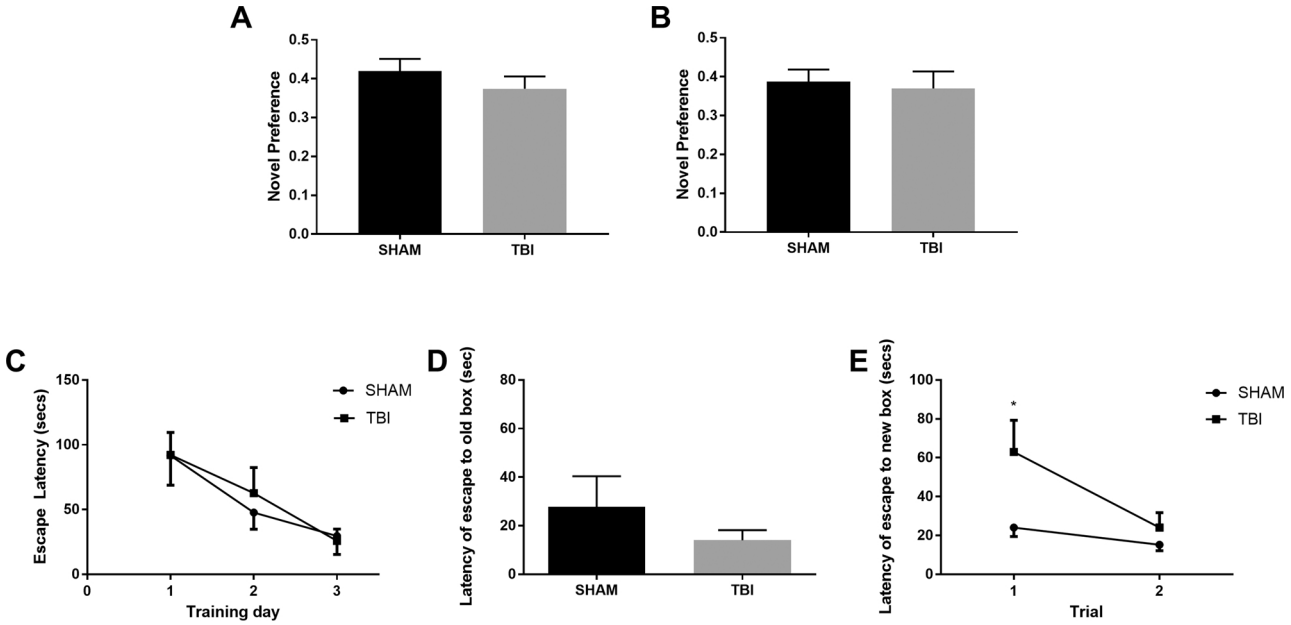

Fig. 3. Cognition assessed through Y-maze for spatial working memory at A) 1 month and B) 3 months post-injury and the Barnes maze at 3 month post-injury (C–E). For the Barnes Maze, C) learning ability in the acquisition phase, D) recollection memory during the probe trial and E) cognitive flexibility on probe day are shown. All graphs show mean ± SEM, (n = 13–19 per group; *p < 0.05 compared to shams).

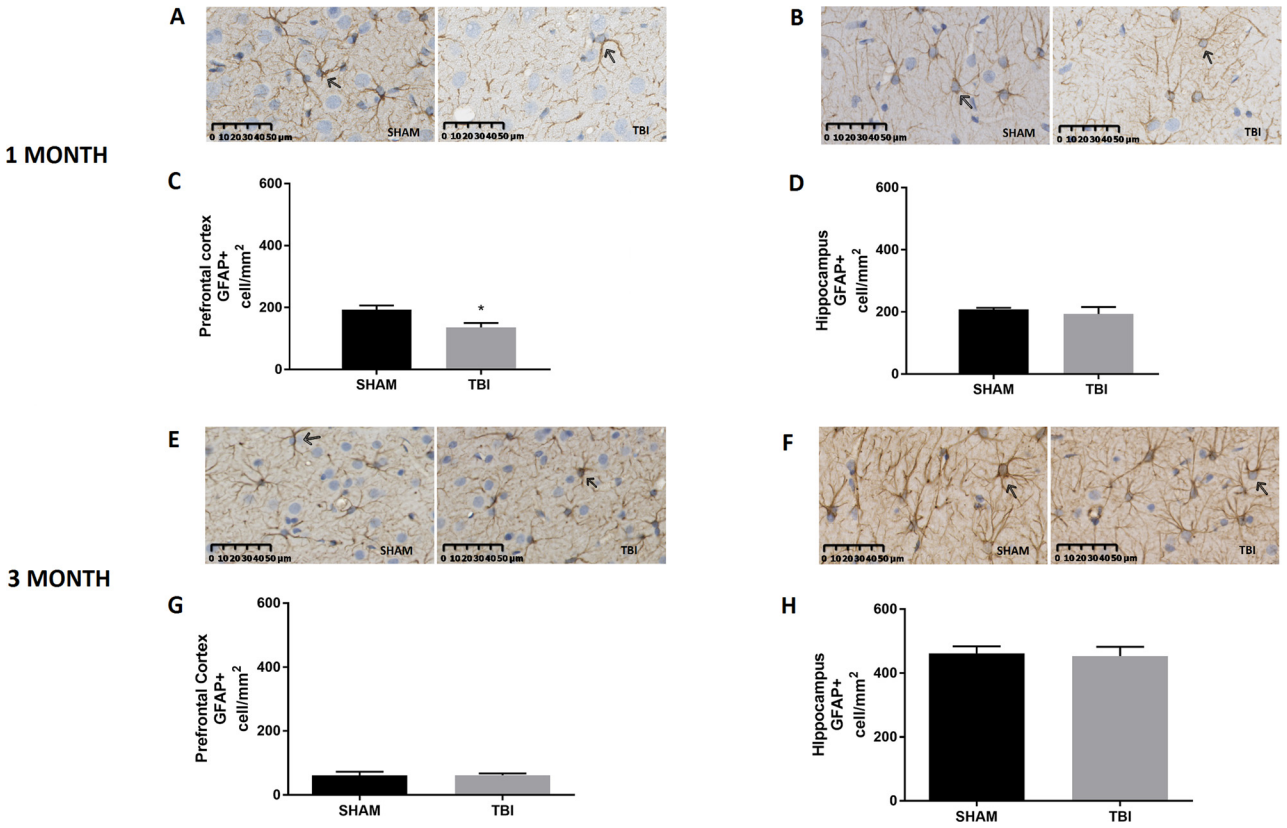

Fig. 4. Representative images of GFAP staining within the A,E) PFC and B,F) hippocampus at A–B) 1 month and E–F) 3 months post-injury, as well as their respective cell counts at C–D) 1 month and G–H) 3 months. Graphs represent the mean ± SEM, (n = 4–5 per group; *p < 0.05 compared to shams).

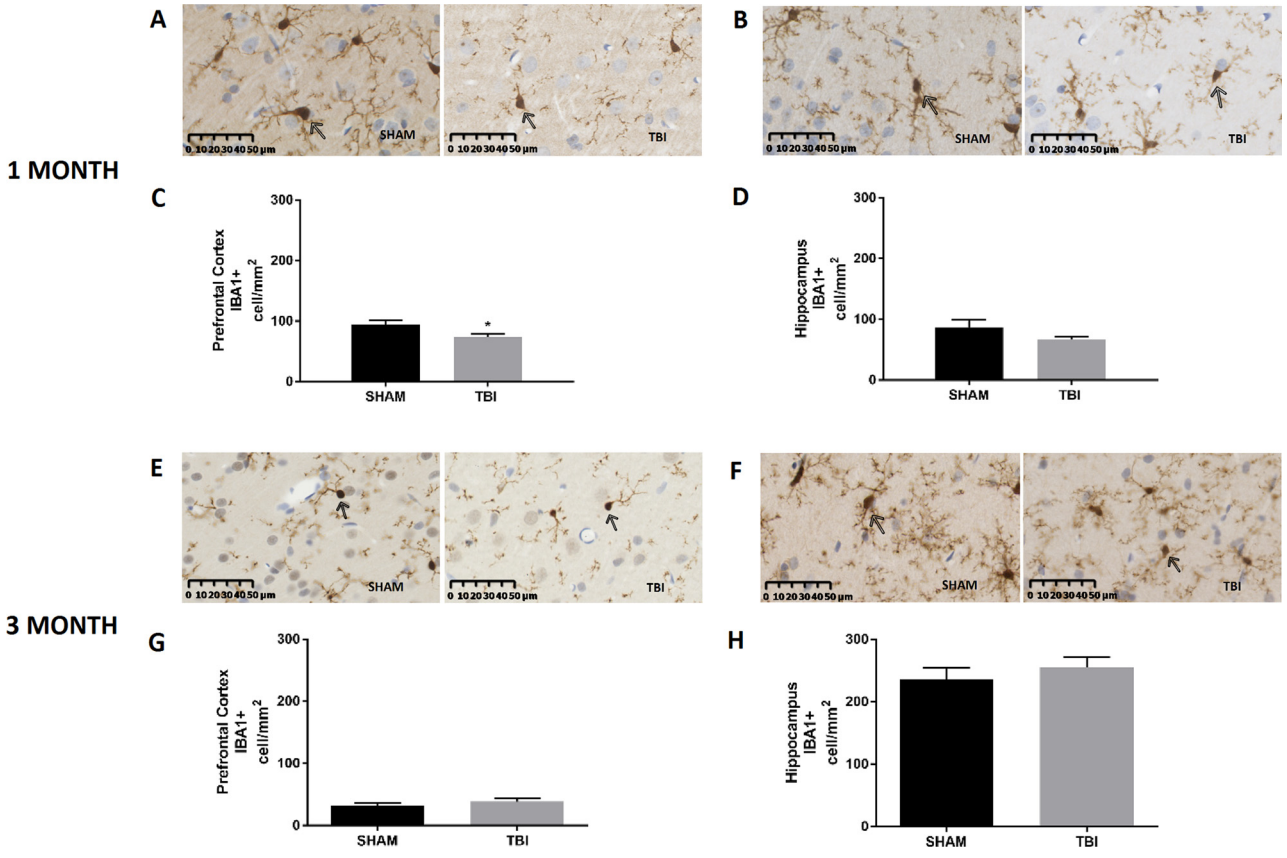

Fig. 5. Representative images of IBA1 staining in the A,E) PFC and B,F) hippocampus at A–B) 1 month and E–F) 3 months, as well as their respective cell counts at C–D) 1 month and G–H) 3 months. Graphs represent the mean ± SEM, (n = 4–5 per group; *p < 0.05 compared to shams).

not significantly differ between the shams and TBI in either the PFC (62.19 ± 5.67 cells/mm^2 vs 61.61 ± 11.58 cells/mm^2 in shams, $t(6) = 0.052$, $p = 0.96$) or the hippocampus (453.5 ± 29.01 cells/mm^2 vs 461.6 ± 22.11 cells/mm^2 in shams, $t(7) = 0.214$, $p = 0.84$) (Fig. 4G–H).

Similarly, the number of IBA1 + ve cells in the PFC of TBI animals (73.84 ± 5.48 cells/mm^2) was significantly decreased compared to shams (94.45 ± 6.70 cells/mm^2) ($t(7) = 2.33$, $p = 0.049$) at 1 month post-injury (Fig. 5C). In contrast, the hippocampus showed no significant differences in IBA1 + ve staining in the TBI animals (66.78 ± 5.03 cells/mm^2) compared to shams (86.99 ± 12.2 cells/mm^2) ($t(7) = 1.54$, $p = 0.176$). By 3 months post-injury, there was no significance difference in IBA1 + ve staining in the PFC (38.54 ± 4.72 cells/mm^2 vs 32.39 ± 3.96 cells/mm^2 in shams, $t(6) = 0.89$, $p = 0.409$) or the hippocampus (254.9 ± 17.28 cells/mm^2 vs 235.9 ± 18.5 cells/mm^2 in shams, $t(7) = 0.75$, $p = 0.478$) between the groups (Fig. 5G–H).

2.7. Evaluation of neuronal and synaptic integrity

Neuronal and synaptic structural damage post injury was assessed using a variety of markers; PSD-95 (postsynaptic density protein 95) and synaptophysin for assessing synaptic integrity, NF-L (neurofilament light chain) and NF-H (neurofilament heavy chain) for assessing neurofilament structure and axonal stability and MBP (myelin basic protein) for assessing neuronal myelination stability. In the PFC, at 1 month post injury, the relative density of PSD-95 and synaptophysin were significantly reduced in the TBI animals compared to shams (0.964 ± 0.214 vs 1.721 ± 0.041, $t(6) = 2.64$, $p = 0.039$ and 2.103 ± 0.469 vs 3.623 ± 0.229, $t(5) = 2.59$, $p = 0.049$, respectively) (Fig. 6). This had resolved by 3 months post-injury, with similar values reported in TBI and sham animals; PSD-95 (1.717 ± 0.322 vs 1.738 ± 0.031 in shams, $t(6) = 0.049$, $p = 0.962$), synaptophysin (1.77 ± 0.268 vs 1.72 ± 0.192 in shams, $t(8) = 0.144$, $p = 0.889$). In comparison, in the hippocampus, there were no significant differences in the relative density of PSD-95 and synaptophysin at either 1 month; PSD-95 (0.474 ± 0.056 vs 0.369 ± 0.037 in shams, $t(8) = 1.57$, $p = 0.154$), synaptophysin (0.786 ± 0.085 vs 0.973 ± 0.107 in shams, $t(6) = 1.363$, $p = 0.222$) or 3 months; PSD-95 (0.239 ± 0.028 vs 0.272 ± 0.024 in shams, $t(7) = 0.877$, $p = 0.41$), synaptophysin (0.519 ± 0.085 vs 0.515 ± 0.123 in shams, $t(8) = 0.022$, $p = 0.983$) post-injury (Fig. 6E–H).

Assessment of axonal integrity with NF-L found no significant differences within the PFC (1.393 ± 0.082 vs 1.405 ± 0.059 in shams, $t(8) = 0.123$, $p = 0.905$) or the hippocampus (1.009 ± 0.076 vs 1.113 ± 0.069 in shams, $t(8) = 1.019$, $p = 0.338$) at 1 month-post-injury; however, a trend towards a decrease in the hippocampus at 3 months post-injury was observed (0.88 ± 0.107 vs 1.184 ± 0.071 in shams; $t(6) = 2.38$, $p = 0.06$) (Fig. 7D). In contrast, a significant increase in levels of NF-H was seen at 1 month post-injury within the PFC (1.398 ± 0.11 vs 0.922 ± 0.138 in shams; $t(8) = 2.70$, $p = 0.027$), which had resolved by 3 months post-injury (0.838 ± 0.148 vs 1.216 ± 0.234 in shams, $t(8) = 1.365$, $p = 0.209$). No changes in NF-H were noted within the hippocampus at 1 month (1.459 ± 0.213 vs 1.5 ± 0.101 in shams, $t(8) = 0.177$, $p = 0.864$) or at 3 months (2.352 ± 0.427 vs 2.642 ± 0.204 in shams, $t(7) = 0.561$, $p = 0.593$) post injury. Integrity of myelin was evaluated with MBP, with a trend towards an increase in the PFC at 1 month post-injury (0.635 ± 0.068 vs 0.404 ± 0.074; $t(7) = 2.304$, $p = 0.055$) which had resolved by 3 months post-injury (1.006 ± 0.018 vs 0.966 ± 0.07 in shams, $t(8) = 0.54$, $p = 0.604$) (Fig. 7I & K). No differences in MBP were seen at 1 month (1.121 ± 0.151 vs 0.915 ± 0.109 in shams, $t(6) = 1.102$, $p = 0.313$) or 3 months (0.494 ± 0.062 vs 0.571 ± 0.103 in shams, $t(7) = 0.595$, $p = 0.571$) post-injury in the hippocampus.

2.8. Oxidative stress

Oxidative stress was assessed by evaluating levels of the antioxidant, SOD-1 (superoxide dismutase 1) (Fig. 8). In the PFC, there was a significant increase in the relative density of SOD-1 at 1 month post-injury (1.082 ± 0.033 vs 0.85 ± 0.032 in shams, $t(6) = 5.074$, $p = 0.002$), which had resolved by 3 months (0.67 ± 0.102 vs 0.602 ± 0.049 in shams, $t(7) = 0.547$, $p = 0.602$) post-injury. In the hippocampus, no changes in SOD-1 were noted at either time-point; 1 month (0.934 ± 0.043 vs 0.881 ± 0.052 in shams, $t(8) = 0.787$, $p = 0.454$), 3 months (0.679 ± 0.056 vs 1.09 ± 0.203 in shams, $t(7) = 1.764$, $p = 0.121$).

3. Discussion

The current study investigated the effect of moderate-severe TBI on chronic changes in axonal and synaptic integrity, neuroinflammation and persistent functional deficits at 1 and 3 months post-injury. It was found that, following TBI, animals showed persistent depressive-like behaviour with increased time spent immobile in the FST at 1 and 3 months post-injury. A decrease in cognitive flexibility on the Barnes Maze was seen at 3 months post-injury, but no impairment was noted in learning and memory during the acquisition phase of the task nor in recognition memory on the Y-Maze (Table 1). Within the PFC, synaptic loss was noted at 1 month post-injury, as indicated by decreased levels of synaptophysin and PSD-95, which corresponded to a concomitant decrease in the number of astrocytes and microglia. Furthermore, other neuronal changes such as increases in NF-H and MBP, were also observed at this early timepoint in the PFC. These changes were resolved by 3 months post-injury. In contrast, within the hippocampus, no changes in the number of inflammatory cells was noted at either time-point nor any effect on synaptic integrity, with the main finding a decrease in relative expression of NF-L at 3 months post-injury.

The most notable functional finding was that TBI led to the development of persistent depressive-like behaviour that had not resolved by 3 months post-injury (Table 1). Although no ongoing motor impairment was noted on the rotarod, with performance at sham level at 3 weeks post-injury, there was a decrease in locomotor activity at 3 months post-injury on the open field. This may relate to lack of motivation to explore the open field [21], but further studies will be needed to confirm this theory. Nonetheless, it appears that the increase in immobility time in the FST reflects a behavioural response, rather than gross motor impairment. This increase in immobility time is thought to be indicative of behavioural despair and, given that it decreases with administration of antidepressants [22], is thought to provide an indicator of depressive-like behaviour. The observations in this study are in line with clinical studies, which have reported the prevalence of depression in TBI patients to be as high as 77% [23], with 30–40% of individuals suffering from major depressive disorder within a year post-injury [24]. In contrast, pre-clinical studies have had mixed results, with reports of no difference in behaviour on the FST at 1 month post moderate controlled cortical impact [25–27] or 6 months post-lateral fluid percussion injury [28]. Conversely, Milman et al and Taylor et al found increased immobility at 2–3 months post-injury utilising a diffuse weight drop model and a more severe CCI model, respectively [29,30]. This suggests that, in order for depressive-like behaviour to be present at sub-acute-chronic time-points post-injury, a wider spread injury may be required, like the diffuse model of injury employed here.

Indeed, within this study, the profile of deficits, in depressive-like behaviour and reduced cognitive flexibility, align with structural changes that were mostly noted within the PFC and not the hippocampus (Table 2). The PFC plays a central role in emotional regulation, with reductions in PFC volume following TBI associated with the development of depressive symptoms post-TBI [24,31,32]. In regards to cognitive flexibility, lesions within the PFC lead to an impairment in the ability to modify a response in relation to new information of a learned

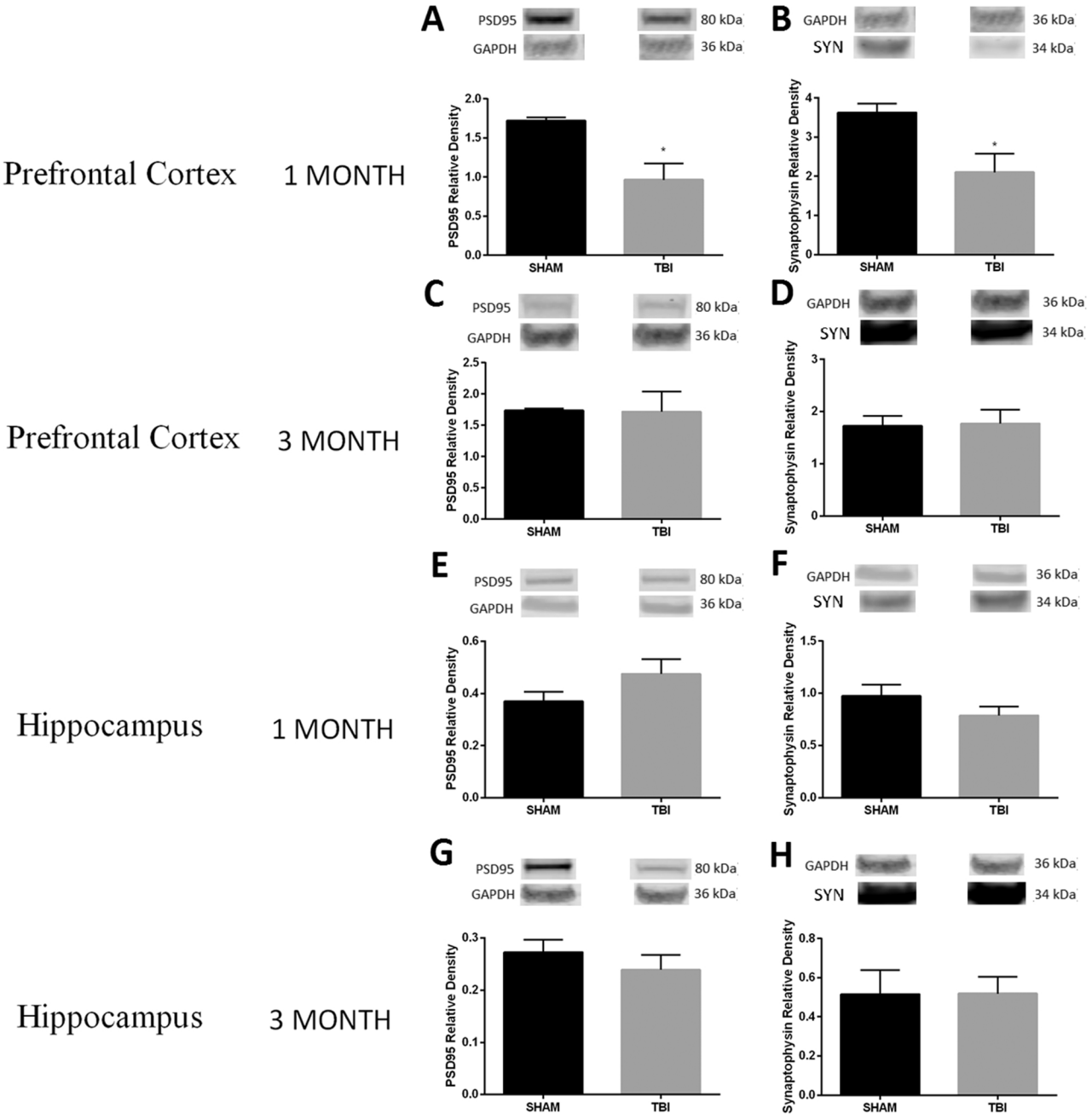

Fig. 6. Synaptic structural damage was assessed by post-synaptic density 95 (PSD-95) and synaptophysin markers. Western blot images of PSD-95 and synaptophysin markers as well as GAPDH (housekeeper protein) at the A–D) PFC and E–H) hippocampus for each of the time point. The graphs illustrate the relative density of A,C,E,G) PSD-95 and B,D,F,H) synaptophysin in TBI animals when compared to sham in the PFC at A–B) 1 month and C–D) 3 months post-injury, and in the hippocampus at E–F) 1 month and G–H) 3 months post-injury. Graph represent the mean ± SEM, (n = 5 per group; *p < 0.05 compared to shams).

task [33,34], similar to the deficit seen here, with post-TBI animals taking longer to locate the escape box when it was moved during the probe trial. These deficits were associated with decreased levels of PSD-95 and synaptophysin within the PFC, suggesting synaptic dysfunction. Few studies have examined the effect of TBI on synaptic morphology in the PFC region post-TBI, with Hoskinson et al finding alterations in dendritic spine density at 4 months following a parietal CCI injury [35] and Zhao et al finding a significant reduction of dendritic spine density in layer II/III pyramidal neurons of the medial PFC at two weeks post-FPI [36]. This supports the idea that TBI can cause significant disruption to the PFC region. Notably, although PSD-95 and synaptophysin had returned to sham levels by 3 months post-injury, functional deficits persisted, suggesting that there may have been persistent alterations in the circuitry (ex: serotonin circuitry) or changes in the functionality of the neurons (ex: receptor expression) of the PFC post-TBI. Interestingly a study by Park and Friston suggest that some functional outcome such as task-orientated cognition may be resulted from a divergence in structural and functional networks in the brain [37]. This implies that although structural networks may be recovered from injury (as seen in our study at 3 months), impairment in the functional networks (not investigated) may drive the persistent impairment seen. Further studies on the dynamics of these two networks in relation to cognition may provide better insight to post-TBI studies as well as neurodegeneration studies. Besides that, specific examination of synaptic morphology within different layers and specific regions of the PFC may provide further insight into circuitry alterations. It might also be beneficial to

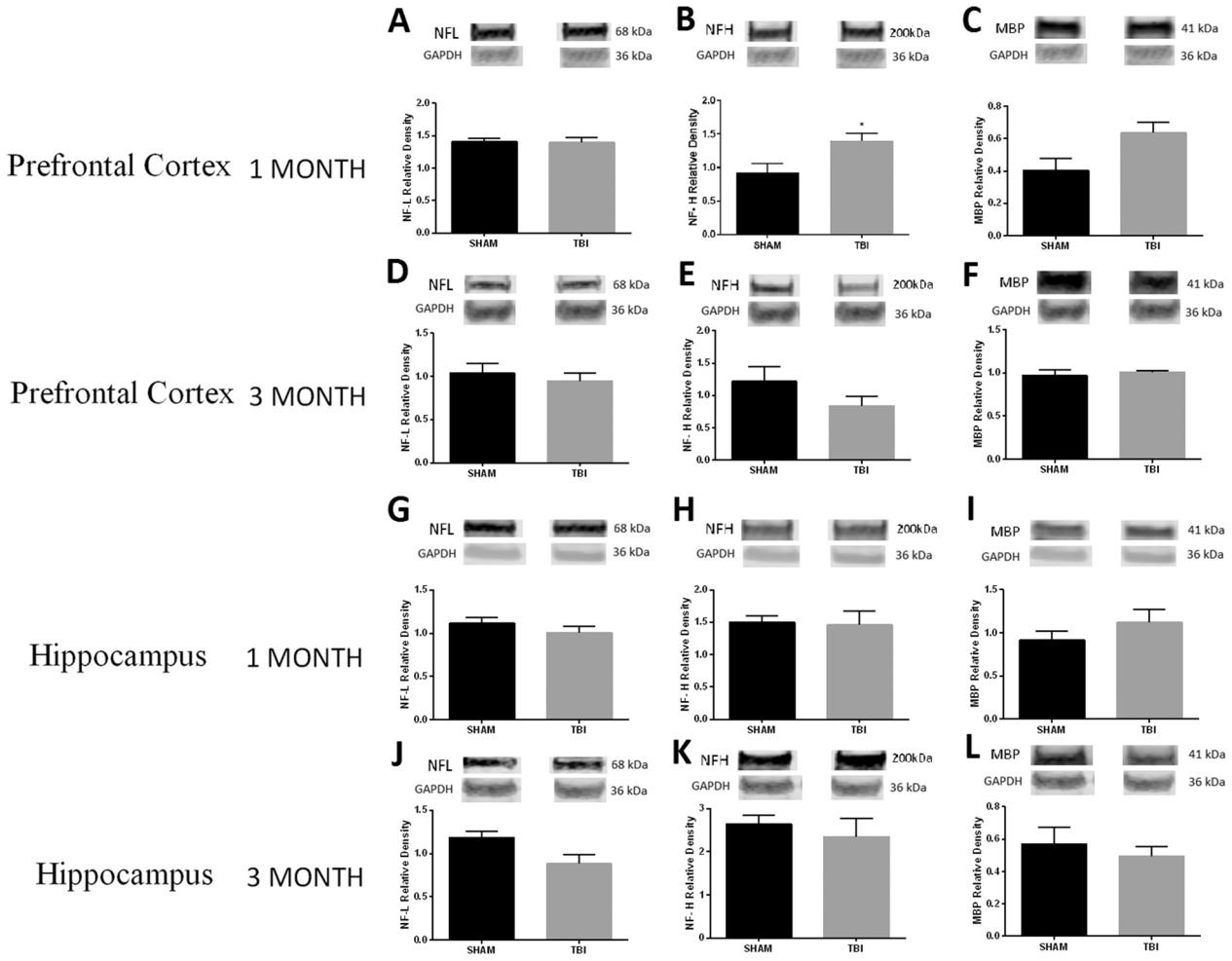

Fig. 7. Neuronal structural damage was assessed by neurofilament-light chain (NF-L), neurofilament-heavy chain (NF-H) and myelin basic protein (MBP) markers. Western blot images of NF-L, NF-H and MBP markers as well as GAPDH (housekeeper protein) in the A–F) PFC and G–L) hippocampus for each of the time point. The graphs illustrate the relative density of NF-L, NF-H and MBP in TBI animals when compared to sham in the PFC at A–C) 1 month and D–F) 3 months post-injury, and in the hippocampus at G–I) 1 month and J–L) 3 months post-injury. Graph represent the mean ± SEM, (n = 5; *p < 0.05 compared to shams).

investigate total neuron number in future studies, for a more precise measurement of synapse loss. Since persistent functional impairments were seen at 3 months, this could suggest there may be on-going neuronal network damage resulted from TBI (that may have branch from the structural damage seen at 1 month) which may drive early dementia or neurodegeneration.

As well as evidence of synaptic disruption, levels of NF-H were also significantly increased at 1 month post-injury within the PFC, before returning to baseline at 3 months, although no changes were noted in levels of NF-L. Neurofilaments are the dominant intermediate filament of axons [38,39] and are thought to be a key contributor to axon strength and resilience to mechanical stretch [40]. Immediately following diffuse impact acceleration and fluid percussion injuries, neurofilament compaction due to side-arm phosphorylation or proteolysis is known to be a key indicator of axonal integrity [41,42]. Activation of neuronal proteases is also associated with an acute reduction in levels of neurofilament as measured via western blot encompassing the light, medium and heavy subtypes [43,44]. The increase in NF-H at one month post-injury may therefore reflect a rebound reparative response following this acute injury phase involving disruption and loss of these proteins. Another potential explanation for the increase seen in NF-H in the current study is as a protective mechanism against toxic oxygen radical species. Wataya et al found that NF-H may act to sequester toxic lipid peroxidation byproducts in aldehydes, in order to protect critical active sites on proteins from oxidative attack [45]. NF-H is thought to preferentially perform this task as it is a lysine-rich protein, the component providing the buffering mechanism [46]. Unfortunately, within our study, we did not investigate oxidative stress markers directly, but instead used a measurement of superoxide dismutase 1 (SOD1), an antioxidant enzyme against superoxide radicals [47]. Although it can be argued that changes in SOD1 levels might be affected by the hypoxia insult during TBI induction, which was showed to be true in the hippocampus by Ramanathan et al., the study also showed that certain areas of the brain such as the cortex are resistant to hypoxia and therefore unaffecting the SOD1 levels [48]. Moreover, a recent study by Coimbra-Costa, showed that reoxygenation after acute hypoxia, as in our study, returned oxidative stress parameters and antioxidant enzymes to control or sham values suggesting the hypoxia treatment post-TBI may not affect the SOD1 levels in the brain [49]. Interestingly, our study found the levels of SOD1 were elevated, like those of NF-H, at 1 month post-injury within the PFC only, suggesting that this could be a similar protective mechanism against elevated levels of reactive oxygen species (ROS) resulted from the TBI. Indeed, overexpression of SOD1 is known to be neuroprotective in a number of models of brain injury [50,51]. Previous studies have shown ongoing oxidative stress within the injured parietal cortex at 1 month following FPI injury, as indicated by an increase in levels of oxidative damaged lipids and proteins [52,53]. Future studies should confirm whether there is evidence of ongoing oxidative stress within the PFC following a purely diffuse weight drop injury.

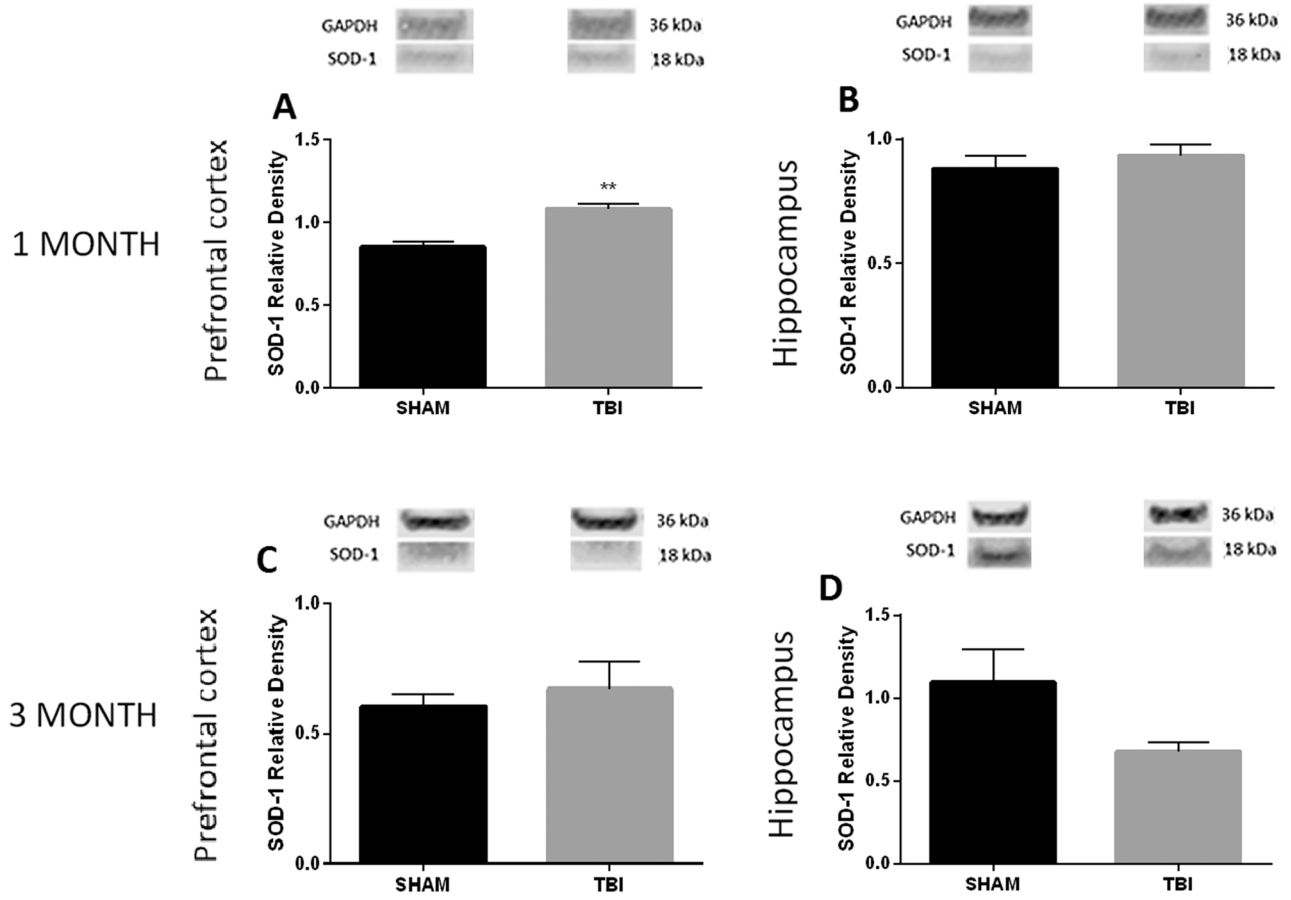

Fig. 8. Oxidative stress was assessed by superoxide-dismutase 1 (SOD-1) marker. Western blot images of SOD-1 and GAPDH (housekeeper protein) in the A,C) PFC and B,D) hippocampus for each of the time points. The graphs illustrate the relative density of SOD-1 in TBI animals when compared to sham in the PFC at A) 1 month and C) 3 months post-injury, and in the hippocampus at B) 1 month and D) 3 months post-injury. Graphs represent the mean ± SEM, (n = 5 per group; **p < 0.01 compared to shams).

Surprisingly, despite the pattern of behavioural deficits seen here and the evidence of synaptic dysfunction, increased neuroinflammation was not seen in the PFC at either 1 or 3 months post-injury. In fact, a reduction in the number of microglia and astrocytes was noted in this region at 1 month post-injury. Given that these cells have a number of beneficial functions, including release of neurotrophic factors, such as BDNF [54,55], modulation of neurotransmitter levels within the synapse [56] and supply of energy to neurons [57], this decrease may not be beneficial. Indeed, previous reports have found a decrease in levels of GFAP, a cytoskeletal protein expressed by many astrocytes, in the PFC of depressed patients [58–60]. It has been proposed that this alteration in astrocytes may influence glutamatergic signalling, thereby contributing to pathology [61,62]. The mechanism driving this decrease in resident immune cell numbers within the PFC at 1 month post-injury is not known, but it is possible that these cells may have migrated to other sites, such as the corpus callosum [63], with restoration of numbers by 3 months post-injury. Further studies are needed to confirm this result, as well as to assess if other anatomical regions are affected at these time points and later time points. Furthermore, as only total number of microglia were assessed, it is important to also confirm whether they are resting or reactive to provide a clearer picture of the neuroinflammatory reaction after injury. Neuroinflammation is significantly more complex than microglia or astrocytes alone. While this was beyond the scope of the current study, it is also important to assess

Table 1
Summary of behavioural results; changes in TBI when compared to shams at 1 month and 3 months post injury.

Test Paradigm	Behaviour measurement	1 month	3 month
Open Field Test (OFT)	Distance Travelled (m)	(Δ = − 2.99)	↓ (Δ = − 11.83)* p = 0.021
	Time in Center (s)	(Δ = 1.97)	(Δ = − 1.64)
Elevated Plus Maze (EPM)	Time in open arms (s)	(Δ = 8.29)	(Δ = − 15.17)
Forced Swim Test (FST)	Time Immobile (s)	↑(Δ = 26.57)* p = 0.027	↑(Δ = 34.45)** p = 0.004
Y-Maze	Novel Preference	(Δ = − 0.045)	(Δ = − 0.018)
Barnes Maze	Escape Latency to box on Acquisition Training (s)	NA	Day 1: (Δ = − 0.523) Day 2: (Δ = − 15.80) Day 3: (Δ = 3.697)
	Escape Latency to Old box (s)	NA	(Δ = − 13.78)
	Escape Latency to New Box (s)	NA	Trial 1: (Δ = − 38.95)* p = 0.014 Trial 2: (Δ = − 8.781)

Note: *p < 0.05, **p < 0.01, ↓ = decrease in value when compared to shams, ↑ = increase in value when compared to shams, Δ = (mean of TBI – mean of sham).

Table 2
Summary of histopathological results; changes in TBI when compared to shams at 1 month and 3 months post injury, at the prefrontal cortex and hippocampus region.

Markers	Prefrontal Cortex		Hippocampus	
	1 month	3 month	1 month	3 month
GFAP (glial fibrillary acidic protein)	↓ (Δ = − 57.60)* p = 0.019	(Δ = 0.59)	(Δ = − 14.50)	(Δ = − 8.17)
IBA1 (ionized calcium binding adaptor molecule 1)	↓ (Δ = − 20.70)* p = 0.049	(Δ = 6.15)	(Δ = − 20.20)	(Δ = 19.06)
PSD-95 (postsynaptic density protein 95)	↓ (Δ = − 0.76)* p = 0.039	(Δ = − 0.02)	(Δ = 0.11)	(Δ = − 0.03)
Synaptophysin	↓ (Δ = − 1.52)* p = 0.049	(Δ = 0.05)	(Δ = − 0.19)	(Δ = 0.003)
NF-L (neurofilament light chain)	(Δ = − 0.01)	(Δ = − 0.09)	(Δ = − 0.10)	↓ (Δ = − 0.30) p = 0.06
NF-H (neurofilament heavy chain)	↑ (Δ = 0.48)* p = 0.027)	(Δ = − 0.38)	(Δ = − 0.04)	(Δ = − 0.29)
MBP (myelin basic protein)	↑ (Δ = 0.23) p = 0.055	(Δ = 0.04)	(Δ = 0.21)	(Δ = − 0.08)
SOD-1 (superoxide dismutase 1)	↑ (Δ = 0.23)** p = 0.002	(Δ = 0.07)	(Δ = 0.05)	(Δ = − 0.42)

Note: *p < 0.05, **p < 0.01, ↓ = decrease in value when compared to shams, ↑ = increase in value when compared to shams, Δ = (mean of TBI – mean of sham).

levels of markers such as chemokines and cytokines in order to fully evaluate the effect of TBI on neuroinflammation [64]. Additionally, TBI has the capacity to lead to long-term alterations in neurochemical signalling, which may be significant contributors to persistent behavioural deficits following injury, so future studies should also investigate levels of key neurotransmitters, including dopamine and serotonin.

In contrast to the evidence of structural changes within the PFC at subacute time points post-injury, this study found minimal pathology within the hippocampus. This lack of hippocampal pathology is supported by the lack of deficits in the learning phase of the Barnes Maze or in recognition memory as assessed by the Y-Maze. These tasks preferentially assess hippocampal dependent learning with Conrad et al demonstrating that bilateral damage to the CA3, CA4 or dentate gyrus led to a decrease in spatial memory on the Y Maze [65]. In regards to the lack of hippocampal mediated cognitive impairment seen in this study, previous studies, contrastingly, have shown persistent cognitive deficits post-TBI, with, for example, Pearce et al observing significant deficits in spatial learning ability in the MWM beginning at two months and lasting up to one year following lateral FP brain injury [66], with similar reports of cognitive deficits from one month to one year following CCI injury [67]. This most likely relates to the more significant hippocampal damage associated with these injury models, with CCI associated with a 60% loss of hippocampal synapses acutely, that had still not recovered to pre-injury levels by day 60 [68]. Similar levels of significant hippocampal cell death have been reported following FPI [69], unlike the lack of synaptic damage seen here at either one or three months post-injury. Previous studies utilising the diffuse impact-acceleration model have similarly reported a lack of hippocampal dependent cognitive deficits on the MWM or radial arm maze [70,71], with a corresponding lack of neuronal loss within this area [70].

In conclusion, this study found that the PFC is significantly affected at one month following a diffuse TBI. There was evidence suggestive of both synaptic and axonal disruption that were associated with a decrease in the number of astrocytes and microglia. These alterations within the PFC also coincide with the impairments on the FST and decreased cognitive flexibility seen after injury. In contrast, the hippocampus was relatively spared at 1 and 3 months post-injury, with future studies needing to examine later time-points to determine if hippocampal damage emerges. Nevertheless, our study provides evidence of early structural changes in the prefrontal cortex after moderate-severe diffuse TBI. While these changes are resolved at 3 months post-injury, future studies should investigate whether they re-emerge or progress to other areas, such as the hippocampus, at later time points, which may contribute to long-term deficits or even predispose individuals to the development of dementia and other neurodegenerative conditions known to be linked to TBI.

4. Experimental procedure

4.1. Animals

Adult male Sprague-Dawley rats (10–12 weeks) (were used under approval of the University of Adelaide Animal Ethics Committee (M-2015-027). Animals were housed under conventional laboratory conditions, with a 12-hour light-dark cycle and access to food and water ad libitum. Animals were randomly allocated to receive either sham surgery or moderate, diffuse TBI, with one subset subject to a functional assessment battery at 1 month post-injury (shams n = 14; TBI n = 19) and another at 3 months post-injury (shams n = 13, TBI n = 14). Following completion of functional assessment, animals were perfused and the brains collected for either histological or molecular analysis.

4.2. Injury model

The Marmarou impact-acceleration model [72] was utilized, as it has been extensively validated as a model of diffuse injury [19]. Animal weights ranged from 350 to 380 g at the time of TBI induction. Animals underwent anaesthetic induction via inhalation of 5% isoflurane under normoxic conditions. They were subsequently intubated, mechanically ventilated and maintained on 2% isoflurane throughout. A midline incision was made to facilitate the placement of a metal disc centrally between lambda and bregma. Animals assigned to undergo TBI were then transiently taken off ventilation, strapped onto a foam, with injury induced by releasing a 450 g weight from a height of 2 m down a clear tube onto the centre of the metal helmet. Contact was observed to ensure single, direct impact without a rebound hit. Animals were then subject to hypoxic conditions (2 L/min nitrogen; 0.2 L/min oxygen) as previously described [63]. This is because this model of TBI leads to a period of apnea in unventilated animals which can lead to high mortality rates [73]. In order to regulate this animals are ventilated and then subjected to a hypoxic period to allow standardisation across the cohort, whilst still replicating the natural history of this injury type. Wound closure was performed with surgical staples. Successful induction of moderate to severe TBI was assessed 24 h later by rotarod scores of below 100, weight reduction of 5–10% and clinical signs (paresis and hunched posture). Any animal not falling within these parameters at 24 h post-injury was excluded from further behavioural and histopathological assessment. Based upon clinical record sheets, four animals were excluded from the study cohort after the TBI induction, from the moderate to severe TBI group as they did not meet the criteria of successful TBI induction. No additional distinctions were made between severity of injury. Shams assessed at the same timepoint (24 h) exhibited none of the clinical signs and had rotarod scores of more than 100. A previous study from our group has assessed motor performance for the first 7 days post-injury in this model [63]. In comparison to sham animals, TBI animals exhibited significant deficits in rotarod performance on days 1–3 post-injury, but this performance no longer

significantly differed from shams by 4 days post-injury.

4.3. Functional studies

Functional tests assessing cognition, anxiety, depression and motor function were performed at 1 month and 3 months post-injury. All functional data was recorded using the ANY-maze Video Tracking System version 4.99 m (Stoelting Co.). The functional tests were done in order from least to most aversive (stress inducing) except the rotarod test which was done at specific timepoints throughout the experiment regardless of other tests. All behavioural tests were conducted with the observer blinded to injury/sham status.

4.3.1. Rotarod

The rotarod is used as a standard motor coordination evaluation test for rodents [74]. Animals were placed on an elevated horizontal rod that rotates along the longitudinal axis. Animals were first habituated on the stationary rod for 10 ss. Then, for every 10 s thereafter, the rotation of the rod was accelerated at a constant rate of 3 rpm until the 100 s mark (maximum acceleration speed of 30 rpm). Animals were kept on the rotarod at the maximum speed for a further 20 s before decelerating the speed and removing the animal from the test. The rotarod score was measured by the latency of the animal to fall off the rod. Animals were trained for 3 consecutive days or until a score of 120 (baseline) was achieved. Following injury, animals were tested on the rotarod at 24 h then every 7 days following, i.e., day 8, day 15 and so on till the endpoint of the study.

4.3.2. Open field test

The open field test (OFT) is a common tests of locomotor activity [75]. Animals were placed in the centre of a large square box (95 cm × 95 cm) with walls at height 44.5 cm and the total distance travelled over a 5 min period was recorded.

4.3.3. Elevated plus maze

The elevated plus maze (EPM) is widely used in anxiety research [75]. Animals were placed in the centre of an elevated (50 cm in height) cross-shaped maze consisting of two open and two closed (walls of height 40 cm) maze arms (each of length 50 cm), facing the open arms, for 5 min. Time spent in the closed arms versus open arms was recorded, with increased time spent in the closed arms thought to represent anxiety-like behaviour.

4.3.4. Y-maze

The Y-Maze is used to test cognition in terms of spatial recognition memory [76]. In the Y-Maze, animals are placed in an equal angled Y-shaped arena, with each arm of the maze identical in size and shape but visually distinct (due to cues on the wall) from the others. The test involves two 3-minute trials separated by 1 h. In the first trial, one arm was closed off with a clear wall (novel arm) to enable the animal to visually recognise its presence; in the second trial, this novel arm became accessible (wall removed). In cases of reduced spatial reference memory, the animal spends less time within the novel arm.

4.3.5. Barnes maze

The Barnes maze evaluates spatial learning and memory in rats [77]. The maze is an elevated, open circular black platform with 18 holes evenly distributed along its edges. One of the holes is pre-determined as the escape hole with a black escape box placed below the hole. The Barnes maze test was preformed over the course of five days; three days of acquisition trials, a rest day (no interaction with the animals) and a probe day. During the acquisition days, animals were subject to two trials spaced 15 min apart. They were placed in the centre of the Barnes maze in a brightly lit room with the time taken for the animal to find and enter the escape box recorded. On day 5, the escape box was relocated to a new hole and two trials conducted 1 h apart. In trial 1, the time taken for the animal to reach the old position of the escape hole was recorded. In both trials, the time taken to locate and enter the newly relocated escape box was recorded as a measure of cognitive flexibility.

4.3.6. Forced swim test

The forced swim test (FST) is widely utilised to assess depressive-like behaviour [46]. The animal was placed within an inescapable glass cylinder filled halfway with 25 °C water, adjusted for the animal's length so that the hind legs does not touch the bottom of the cylinder, for 5 min. The time spent immobile was recorded as a measure of behavioural despair.

4.4. Tissue collection and processing

Animals were randomly assigned for further processing, either by molecular analysis or immunohistochemistry, during euthanasia. Animals that were to be used for molecular analysis were transcardially perfused with 0.9% saline and the brain dissected with the prefrontal cortex (PFC) and hippocampus taken (n = 5 per group). Samples were snap-frozen in liquid nitrogen before being stored at −80 °C. The samples were then homogenised via sonication in freshly prepared buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 140 mM 2-mercaptoethanol) with protease inhibitor cocktail (Sigma), 10 u L/mL aprotinin, leupeptin, pepstatin A and 10 mM PMSF. Each sample underwent 3 bursts of 10 s duration under a sonicator probe. Homogenised samples were centrifuged for 30 min at 14,000 rpm and 4 °C, before supernatant was collected. Protein concentration was estimated with Pierce BCA Protein Assay (ThermoScientific) at 750 nm absorbance.

Animals that were to be used for immunohistochemical analysis were transcardially perfused with 0.2 m L heparin + 10% formalin. Brains were removed and post-fixed in 10% formalin for 24 h, then blocked into 2 m m coronal sections and embedded in paraffin-wax. To examine the PFC, three consecutive 5 µm coronal slices were taken beginning at + 4.20 mm from Bregma for each animal. For hippocampal sections, three serial 5 µm coronal slices per animal were taken starting at -1.60 mm representing anterior hippocampus, −2.80 mm representing mid hippocampus and at −3.80 mm representing posterior hippocampus. Tissue mounted slides were allowed to dry at 37 °C overnight.

4.5. 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 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 (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 visualisation, and washed with distilled water until removal of Ponceau had been achieved.

Membranes were incubated for 2.5 h with primary and secondary antibodies in 1X iBind solution using the iBind Western System (Life Technologies). Primary antibodies were used at individually optimised concentrations: mouse anti-post-synaptic density protein 95 (PSD-95) (1:1000, ab2723 or 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), rabbit anti-superoxide dismutase 1 (SOD1) (1:1000, ab13498, Abcam), and the primary housekeeping antibody chicken anti-GAPDH (1:4000, ab83956, Abcam). Secondary antibodies to the respective primary antibodies (donkey anti-rabbit, donkey anti-mouse and donkey anti-chicken, IRDye 800CW; LI-COR, Inc.) were used at 1:3000. Western blots were imaged using an Odyssey Infrared Imaging System

Table 3
Primary antibodies investigated using immunohistochemistry.

Primary Antibody	Analysis Target	Antigen Retrieval	Host animal and Dilution	Manufacturer
GFAP	Astrocyte reactivity	Citrate	Rabbit 1: 40,000	DAKO
Iba1	Microglial reactivity	Citrate	Rabbit 1: 20,000	Wako

[GFAP: Glial Fibrillary Acidic Protein, Iba1: Ionized calcium Binding Adaptor molecule 1].

(model 9120; software version 3.0.21) (LI-COR, Inc.) at a resolution of 169 µm. Semi-quantitative analysis of band signals was performed using ImageJ version 1.49 and Image Studio Lite version 5.2. Normalization of blot runs at 1 month and 3 month were performed using a single control sample of the respective time points. Thus, relative density of the samples were calculated based on the adjusted density for each blot, as below:

$$\text{Adjusted density} = \frac{band\ signal\ of\ sample\ protein/housekeeper}{band\ signal\ of\ control\ protein/housekeeper}$$

$$\text{Relative density} = \frac{adjusted\ density\ of\ protein}{adjusted\ density\ of\ housekeeper}$$

4.6. Immunohistochemistry

Immunohistochemistry (IHC) was performed as per standard procedure. In brief, slides were oven-dried, de-waxed in xylene, rehydrated in ethanol and then placed into methanol with 0.5% hydrogen peroxide to block endogenous peroxidases. Then the slides were washed twice in phosphate buffered saline (PBS) and were blocked in normal horse serum (NHS) (1:30) for 30 min before incubation overnight with primary antibody (Table 3). The following day, slides were washed twice in PBS before application of secondary antibody (DAKO, 1:250, 30 min). Slides were once again washed twice with PBS, and then incubated with streptavidin peroxidase conjugate (SPC) (1:1000, 60 min). Slides were given a final wash in PBS, then incubated with 3,3′-Diaminobenzidinetetrahydrochloride (DAB) (1:50, 7 min) for antigen retrieval. Lastly, slides were counterstained with haematoxylin, placed in ethanol and subsequently in xylene, before mounting on cover slips.

Following staining, sections were scanned with Nanozoomer slide-scanner (Hamamatsu, Japan) and images viewed on NDPview (version 2). GFAP and Iba1 immunoreactivity was assessed quantitatively by counting the reactive and immunopositive cells per mm^2 within the hippocampus (CA1 + CA3 + DG region) and PFC (prelimbic region). The experimenter was blinded to the experimental group during cell counting and counts were performed twice Numbers obtained for each of the two counts were correlated to assess inter-attempt variability. This resulted in an r-value of 0.771.

4.7. Statistics

Except where outlined below, all data was analysed via two-tailed unpaired t-test using GraphPad Prism software. A repeated two-way analysis of variance was performed on the rotarod scores and on the acquisition days of the Barnes maze test. P values < 0.05 were considered statistically significant.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contribution statement

AA, JT and HC were involved with generation and analysis of experimental data. FC and LCP oversaw the experimental design, experimental analysis and production of the manuscript. All authors have viewed and edited the submitted manuscript.

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