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Research Article

Temporal Changes in Tau Phosphorylation and Related Kinase and Phosphatases Following Two Models of Traumatic Brain Injury

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Abstract

A history of traumatic brain injury (TBI) is linked to later neurodegeneration, with a key feature accumulation of hyperphosphorylated tau. Tau is a microtubule stability protein that undergoes frequent cycles of phosphorylation and dephosphorylation due to kinases and phosphatase activity. Hyperphosphorylation of tau destabilizes microtubules interrupting axonal transport, as well as promotes aggregation disturbing synaptic dysfunction. Aberrant phosphorylation of tau post-injury is thought to be a key player in later neurodegeneration. However, it is not known whether type of TBI- a single severe injury compared to repeated mild injuries- affects the time course of tau accumulation or the pattern of changes in kinases and phosphatases that facilitate this phosphorylation. To investigate, male Sprague Dawley rats were subjected to either a single moderate/severe or 3 mild TBIs spaced 5 days apart (rmTBI) utlising the Marmarou impact-acceleration model. Levels of cortical ptau (AT180, pSer422, oligomeric tau), pGSK3β, pCDK5, pERK1/2, pAkt and PP2Ac were evaluated at 24h, 7 days, 1 month and 3 months post-injury, with changes in tau phosphorylation confirmed via immunohistochemistry. A similar time course of AT180 tau phosphorylation was seen irrespective of the nature of the initiating insult, with a spike at 24h post-injury return to baseline and then increasing chronically at 3 months post-injury. In line with this, levels of PP2Ac were decreased at 24h and 3 months post-injury, indicating a potential loss of phosphatase activity. Interestingly, minimal changes were seen in the kinases examined, with a spike in phosphorylation of GSK3β, at the inhibitory Ser site, at 24h and 3 months following rmTBI, but not single moderate severe TBI, suggesting a possible protective effect only post-rmTBI. This study highlights that changes in levels of phosphorylated tau are similar, regardless of the initiating injury, and highlights the need to further understand the driving mechanisms behind this phenomenon.

Keywords: traumatic brain injury, tau, GSK3β, PP2A

Introduction

Traumatic brain injury (TBI) is a critical health and socioeconomic problem worldwide [1]. Increasing evidence suggests that a traumatic brain injury (TBI) is not just an acute event but sets in motion a number of

ongoing pathological cascades that increase the risk of later developing dementia [2]. In support of this, a recent retrospective cohort study by Barnes et al., utilised the Veterans Health Administration health care database to demonstrate that the adjusted hazards ratio for a dementia diagnosis were 3.77 for those with a moderate to severe TBI and approximately 2.5 for those who had a mild TBI (mTBI) [3]. This aligns with a number of previous studies that have found ~ two-fold increased risk for the later development of dementia post-TBI [4,5]. Furthermore, this is supported by pathological studies showing that TBI increases the deposition of pathological proteins associated with neurodegenerative disease, particularly hyperphosphorylated tau [6,7].

However, it is not known whether the type of dementia differs depending on the nature of the initiating insult. A single moderate-severe TBI has been linked with an increased risk of Alzheimer's disease (AD) [8,9], whereas a history of repeated concussive/sub-concussive impacts are thought to drive the development of chronic traumatic encephalopathy (CTE) [6]. Alternatively, TBI associated dementia may be its own distinct entity, with features that may overlap with other neurodegenerative diseases. Indeed, the deposition of neurofibrillary tangles (NFTs) seen following trauma is similar following a single moderate-severe TBI and following repeated injury, with NFTs typically seen at the depths of the sulci, particularly in perivascular regions, first appearing in the superficial cortical layers [6,10,11].

Given that TBI is known to promote hyperphosphorylation of tau, it is likely that there is an imbalance between kinase and phosphatase activity following TBI, although it is not known whether this differs following a single moderate-severe injury versus more mild repeated impacts. Phosphorylation of tau is essential for its normal function [12]. Tau stabilises microtubules, but frequent cycles of binding and detachment are required to permit axonal transport, with non-phosphorylated tau stably binding to the microtubules and phosphorylated tau (ptau) becoming detached [13]. This is regulated by the actions of kinases and phosphatases, with key tau kinases including glycogen synthetase kinase-3β (GSK-3β), mitogen-activated protein kinase (ERK1/2) and cyclin-dependent kinase 5 (CDK5) and Akt [13], while the principal phosphatase PP2A accounts for approximately 70% of human brain phosphatase activity [14]. An imbalance between the action of kinases and phosphatases can lead to the hyperphosphorylation of tau [15], which disrupts microtubules [16] and can lead to its aggregation [17]. This could drive neurodegeneration following TBI by disrupting axonal transport, interfering with synaptic transmission and promoting inflammation (see [18] for review). However, how tau phosphorylation and concomitant kinase and phosphatase activity evolve over time after injury, and how this may relate to the initial nature of the insult, have not yet been investigated.

As such, this study will investigate the time-course of tau phosphorylation and alterations in kinase (GSK-3β, ERK 1/2, CDK5 & Akt) and phosphatase levels (PP2A) following either a single moderate-severe TBI repeated mild TBI (rmTBI; 3 impacts over 10 days) up to 3 months post-injury.

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 12h light/dark cycle with uninterrupted access to food and water. Rats were randomly allocated to receive either sham surgery, a single moderate-severe TBI or rmTBI, with 3 injuries spaced 5 days apart. This time interval between injuries and number of injuries has been described as optimal to produce cumulative long-term functional deficits after rmTBI [19].

Rodent model of TBI

Male, Sprague-Dawley rats (350-400g) were injured using the diffuse impact-acceleration model of brain injury to create either a single moderate severe TBI (msTBI) or repeated mTBI (rmTBI), as has been described in detail previously [19,20]. In brief, for the severe injury, the weight is dropped from 2m onto a steel disc, whilst to deliver rmTBI, the weight is dropped onto the steel disc from 1m on days 0, 5 and 10. A 10cm thick foam cushion decelerates the head after impact, thus producing an acceleration/deceleration injury that is typical of concussion [21]. 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. Animals were then euthanized at either 24h, 7 days, 1 month or 3 months post-injury, with sham animals divided into two groups of n=6 with acute animals euthanized at either 24h (n=3) or 7 days post-injury (n=3) and chronic animals at either 1 month (n=3) or 3 months post-injury (n=3).

Immunohistochemistry

Rats were terminally anaesthetized with isoflurane and transcardially perfused with 10% formalin. Two sections per brain, 5 μm thick, were collected at 250μm intervals, representing the region from Bregma -2.5, -3mm. Slides were then stained for levels of tau phosphorylation (AT180, 1:1000, MN1040 Thermo Fisher). 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 1h, 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 [22,23]. For quantitation, a box (0.4mm^2) was placed in four locations within the cortex of each section and all cell bodies greater than 7.5μm in size, to exclude most non-neuronal cells were counted as either AT180+ve or –ve, with a % of AT180+ve cells then calculated Counts were performed twice and standard deviation between counts was typically <10%.

Biochemical analysis

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

Western Blot

Gel electrophoresis was performed using Bolt 4-12% Bis-Tris Plus gels (Life Technologies) with 30ug of protein loaded per well. Gels were run at 150V for 30-45 minutes, 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 minutes), stained with Ponceau S red solution (Fluka Analytical) (5 minutes) for protein visualization, and washed with distilled water until removal of Ponceau had been achieved. Membranes were incubated for 2.5 hours with primary and secondary antibodies (1:3000, Li-Cor) in 1X iBind solution using the iBind Western System (Life Technologies) (AT180, tau-5, GSK3β & PP2Ac) or alternatively in 5% milk overnight with just the primary antibody. Those incubated in milk were washed 3 times in TBS-T the next day and then the secondary antibody applied for 30 mins. Primary antibodies were used at individually optimised concentrations (Table 1) and the primary housekeeping antibody was chicken anti-GAPDH (1:4000, ab83956, Abcam). Western blots were imaged using an Odyssey Infrared Imaging System (model 9120;

software version 3.0.21) (LI-COR, Inc.) at a resolution of 169µm. 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 both to the housekeeper and to this loading control.

Table 1: Antibodies used within the study

Antibody	Supplier	Catalogue number	Concentration
Akt	Cell signalling	9272	1/250
pAKT (Ser 473)	Cell signalling	9271	1/250
GSk3beta	Cell signalling	27C10	1/250
pGsk3beta	Cell signalling	9336	1/250
Cdk5	Cell signalling	2506	1/500
pCdk5 (C-7)	Santa Cruz	377558	1/250
ERK1/2	Cell signalling	9102A	1/500
pERK1/2	Cell signalling	9102	1/250
PP2Ac	Millipore	05-421	1/250
AT180	Thermofisher	MN1020	1/1000
Tau-5	Thermofisher	AHB0042	1/1000
T22	ABN454	Merck Millipore	1/200

Statistics

All data was evaluated using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests for multiple comparisons. A p value of less than 0.05 was considered significant. All graphical data are presented as mean ± SEM.

Results

The effect of injury and LPS administration on tau phosphorylation was examined by examining the expression of AT180 and Ser422 relative to total tau via both western blot (Figure 1) and immunohistochemistry of AT180 (Figure 2).

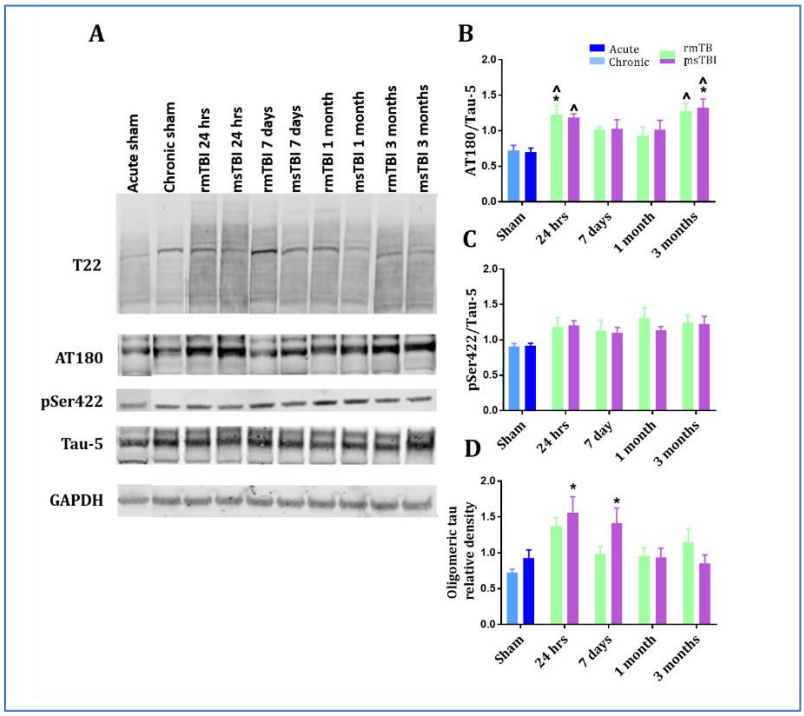

Figure 1: Evaluation of the temporal profile of tau phosphorylation and aggregation following rmTBI and msTBI. **(A)** Representative western blots and **(B)** analysis of the AT180:tau-5 ratio, **(C)** pSer422:tau-5 ratio and **(D)** oligomeric tau via T22. ^p<0.05 compared to chronic shams, *p<0.05 compared to acute shams, n=5-6 per group.

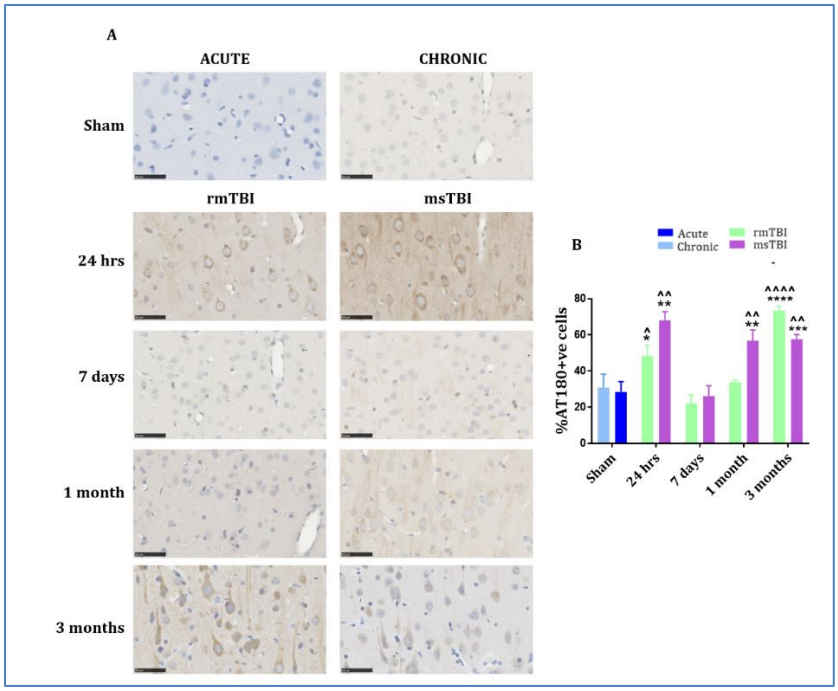

Figure 2: (A) Representative images of AT180 immunohistochemistry, **(B)** with counts of the percentage of AT180+ve cells within the cortex. ^p<0.05, ^^p<0.01, ^^^^p<0.0001 compared to chronic shams, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to acute shams; n=5-6 per group, scale bar=50μm).

No significant differences were noted in level of pSer422 at any timepoint following either type of injury ($F_{9,42}$=1.78 p=0.10). However, a significant change in the AT180: total tau ratio was noted ($F_{9,42}$=4.401 p<0.001), with both msTBI and rmTBI animals demonstrating significantly higher AT180: tau:5 levels compared to chronic shams (1.32 ± 0.28 & 1.27 ± 0.24 *vs*. 0.72 ± 0.14, respectively) at 3 months post-injury. At 24h post-injury, a significant increase in AT180: tau-5 was seen in rmTBI animals compared to acute shams (1.22 ± 0.31 *vs*. 0.72 ± 0.17, p<0.05), with a trend towards increase in the msTBI animals (1.19 ± 0.12, p=0.07). No significant differences compared to either sham group were seen at either 7 days or 1-month post-injury in either injury type. Levels of oligomeric tau were also examined, with a significant overall effect noted ($F_{9,42}$=3.53, p<0.01), with significantly higher levels in msTBI than acute shams at both 24h and 7 days post injury (1.56 ± 0.44, 0.4 ± 0.47 *vs*. 0.72 ± 0.122, p<0.05), before a return to sham levels at 1 and 3 months post-injury (Figure 1D). For rmTBI animals, there was a trend towards significance in the 24hr animals (1.36 ± 0.27, p=0.06), with no other alterations noted.

To confirm alterations in expression of AT180 seen via western blot, immunohistochemistry was undertaken to examine the percentage of cortical AT180+ve cells. There was a significant change in the %AT180+ve cells ($F_{9,42}$=8.0, p<0.0001), with both msTBI (68.01 ± 10.93) and rmTBI animals (48.28 ± 13.32) showing a significant increase at 24h post-injury compared to both acute (25.69 ± 15.0, p<0.001 and p<0.05 respectively) and chronic shams (27.13 ± 3.13, p<0.001 and p<0.05 respectively. No differences were seen in 7-day animals, with an increase then seen in msTBI animals at 1-month post-injury (56.74 ± 13.41, p<0.01 compared to both acute & chronic sham). At 3 months post-injury, both msTBI and rmTBI animals had significantly higher numbers of %AT180 +ve cells compared to both sham groups (msTBI 57.64 ± 5.82, rmTBI 73.42 ± 5.56, p<0.001).

Kinases

Kinase involvement was investigated by determining the levels of phosphorylated versus total ERK1/2, AKT, CDk5 and GSK3β. An overall significant effect was seen for pERK1/2/ERK1/2 ($F_{9,42}$=5.02, p<0.001), pGSK3β/GSK3β ($F_{9,42}$=9.96, p<0.0001), pCDK5/CDK5 ($F_{9,42}$=3.0, p<0.01) and pAKT/AKT ($F_{9,42}$=2.19, p<0.05). However, post-hoc

analysis did not show any significant differences between groups for pAKT/AKT. In contrast, post-hoc analysis of pGSK3β/GSK3β showed a significant elevation at 24h post-injury in rmTBI animals compared to the acute shams (1.18 ± 0.30 *vs.* 0.60 ± 0.11, p<0.05) and in 3-month animals relative to chronic shams (1.87 ± 0.1 *vs.* 0.78 ± 0.16; p<0.001). Levels at 3 months were significantly higher than the rmTBI 24h (1.18 ± 0.30, p<0.05), 7 day (0.84 ± 0.34, p<0.001) and 1 month (0.91 ± 0.10, p<0.001) post-injury animals. In contrast, no significant increases were seen following msTBI relative to shams at any-time point, although there was a small decrease in relative expression seen at 7 days post-injury, such that these animals were significantly different to msTBI 3-month animals (0.55 ± 0.24 *vs.* 1.18 ± 0.33, p<0.05).

Post-hoc analysis of pERK1/2/ERK1/2 showed a significantly higher level of relative expression at 24h compared to later-time points. Indeed, rmTBI 24h animals had significantly higher levels of pERK1/2/ERK1/2 compared to 1 and 3-month rmTBI animals (1.92 ± 0.55 *vs.* 1.04 ± 0.08 & 0.92 ± 0.5 respectively, p<0.05), showing a decline in activation of this kinase over-time following injury. A similar pattern was seen in msTBI animals, with significantly higher levels of pERK1/2/ERK1/2 at 24h post-injury compared to severe TBI animals at 3 months post-injury (1.42 ± 0.30 *vs.* 0.60 ± 0.19, p<0.05). No significant changes were noted between either msTBI or rmTBI animals and shams at any time-point post-injury, although a trend towards significance was noted in rmTBI 24h post-injury animals compared to acute shams (1.92 ± 0.55 *vs.* 1.22 ± 0.31, p=0.06).

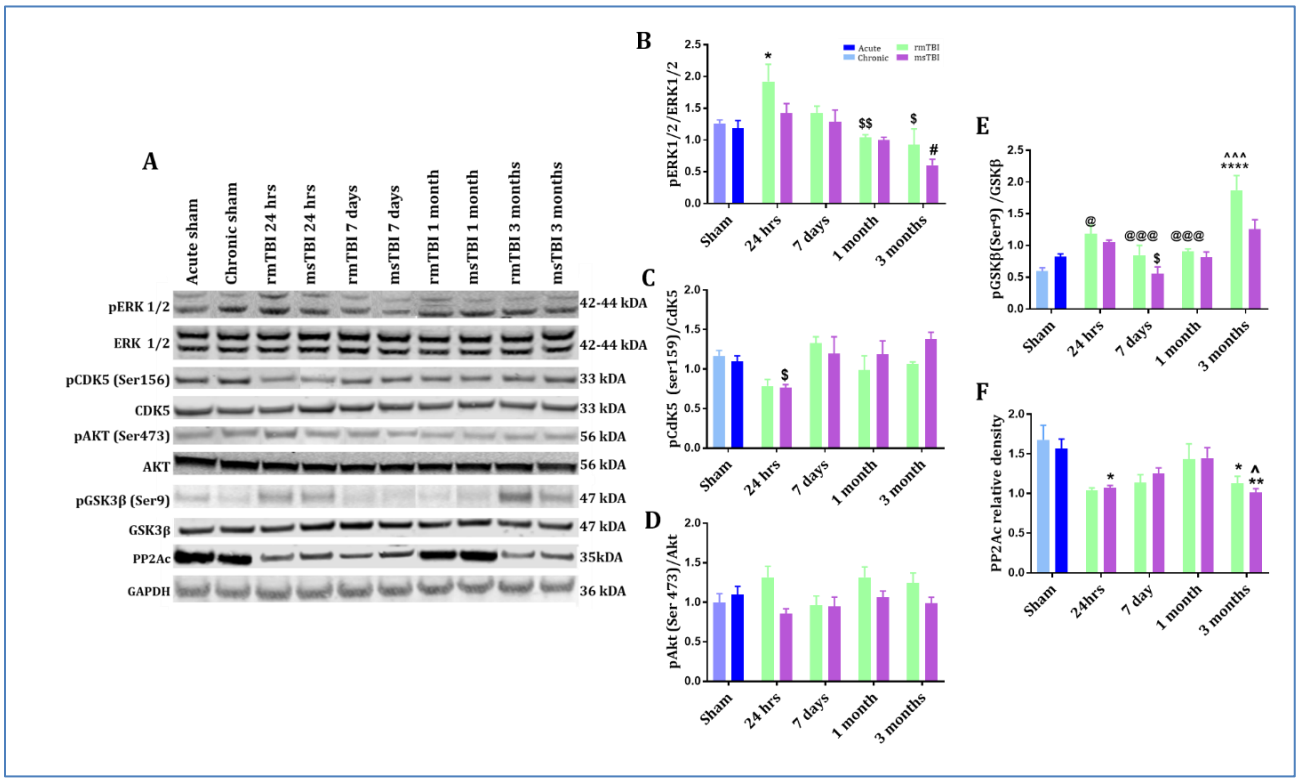

Figure 3: (A) Temporal profile of changes in the relative expression of key tau phosphorylating kinases in (B) pERK1/2, (C) pCDK5, (D) pAKT and (E) pGSK3β, as well as the major phosphatase, PP2Ac (F). ^p<0.05, ^^p<0.01, ^^^p<0.001 compared to chronic shams, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to acute shams, @p<0.05, @@@p,0.001 compared to rmTBI 3 month animals, $p<0.05 compared to msTBI 3 month animals, $p<0.05, $$p<0.01 compared to rmTBI 24h animals; n=5-6 per group

In contrast to the pERK1/2/ERK1/2 results, post-hoc analysis of pCDK5/CDK5 showed a decrease in relative expression at 24h post-injury in msTBI animals with an increase over-time such that they were significantly different to msTBI 3-month animals (0.76 ± 0.08 *vs.* 1.39 ± 0.17, p<0.05). Within the rmTBI animals, a trend towards

significance was seen from 24h to 7 days post-injury (0.79 ± 0.19 *vs*. 1.32 ± 0.18, p=0.07), but no difference at 3 months post-injury (1.06 ± 0.06, p=0.80). No differences were seen between injured animals and shams at any timepoint

Analysis of the catalytic unit of the key phosphatase PP2A, showed a significant overall effect ($F_{9.42}$=4.30, p<0.001). At 24h post-injury, both rmTBI and msTBI animals had significantly lower relative expression than acute shams (1.04 ± 0.07 & 1.07 ± 0.07 *vs*. 1.68 ± 0.37, p<0.05). By 7 days post-injury, the msTBI animals still had significantly lower levels than acute shams (1.13 ± 0.20, p<0.05), with a trend towards significance in the rmTBI animals (1.25 ± 0.14, p=0.05). At 1 month, neither injury group were significantly different from the shams (acute+chronic), but at 3 months post-injury, the msTBI animals had significantly lower relative expression than chronic shams (1.01 ± 0.12 *vs*. 1.56 ± 0.23, p<0.05), but rmTBI animals did not (1.12 ± 0.20, p=0.2). No significant differences were seen between the rmTBI or msTBI groups overtime (Figure 3).

Discussion

We investigated dynamic changes in tau phosphorylation and tau-related protein kinases, in GSK3β, CDk5, ERK1/2 and AKT, as well as the chief phosphatase, PP2A, following two different severities of rodent models of TBI. Both repeated mTBI and a single msTBI increased levels of ptau as measured by AT180, with increases seen at 24h and 3 months post-injury. This may be due to reductions in the key phosphatase, PP2A, with levels of PP2Ac found to be significantly lower at both 24h (both rmTBI and msTBI) and 3 months (msTBI) post-injury. Interestingly, minimal effects on the kinases examined were seen, with an increase at 24h following rmTBI in levels of pERK1/2 and pGSK3β at this time point and at 3 months in pGSK3β. As the phosphorylation site investigated on pGSK3β inhibits its activity [24], this response may be protective and unrelated to the effects seen on tau phosphorylation.

It was found that levels of ptau as measured by AT180, but not pSer422, showed two prominent peaks following both rmTBI and a single msTBI at 24h and then at 3 months post-injury. Some discrepancy was noted between results from immunohistochemistry and western blot for AT180, with an earlier increase at 1-month post-injury in the single msTBI animals persisting to 3 months seen in the IHC, versus an increase only at 3 months via western blot. This may relate to the more targeted analysis within the immunohistochemistry. Nonetheless, these findings are in line with previous reports that have found an increase in ptau following rmTBI both acutely [19,25] and chronically [19,26,27]. Similar findings have also been reported following a single moderate severe-TBI [28-31]. This suggests that tau pathology is a significant pathological feature following both repeated mild and single severe injury. Initial increases in tau phosphorylation may relate to early axonal disruption, which facilitates detachment of tau from microtubules and provides a pool of tau that can be readily phosphorylated [32]. Conversely, the later peak in ptau may relate to other ongoing secondary injury processes following injury, such as chronic inflammation and oxidative stress [2]. A persistent inflammatory response is a feature following both rmTBI [33] and single severe TBI [34,35], with inflammation known to promote phosphorylation of tau [18]. Such continued chronic elevations in ptau after injury may be an important link in the increasing the risk of neurodegenerative disease after TBI, although future studies are needed to assess whether these elevations continue at later timepoints following injury.

Interestingly, although we saw effects at the AT180 site, no significant changes were seen at pSer422. Tau phosphorylation is complex, as tau contains 80 potential serine/threonine and 5 potential tyrosine sites on its longest isoform, with phosphorylation occurring at least 30 of them [36]. As such, this may reflect differential processes that occur at each of the phosphorylation sites, which respond to the activity of different kinases. For example, the AT180 site is proposed to be phosphorylated by p38, LRK2, PKA, CDK5, GSK-3 and PSK1&2, whilst s422 is thought to be phosphorylated by GSK-3, PSK1&2, PKA, p38 and CAMK12 [37]. Furthermore, these sites also have differential pathological effects. For example, phosphorylation at Thr231 (detected by AT180) is associated with inhibition of

tau's ability to bind microtubules [38], as well as promoting aggregation [39]. In contrast, phosphorylation at Ser422 is thought to prevent cleavage of tau by caspase-3, with mixed reports as to whether this worsens [40] or impedes [41] disease progression. Aggregation at Thr231 is also known to promote neurodegeneration via caspase-3 activation [38,39], so the elevations seen here at 3 months post-injury are consistent with ongoing pathology following TBI. In regard to the failure to see an increase in pSer422, this is in line with other studies showing that TBI induced phosphorylation differs depending on site examined. For example, Zhao et al. (2017) showed that, following a single severe focal injury, elevations at 24h and 72h post-injury were seen at Ser404, but not Ser262 or Thr205 [42]. Further work is needed to look at other phosphorylation sites and map the pattern and evolution of changes post-TBI.

As well as specific phosphorylation sites, levels of oligomeric tau were also examined, with a spike seen at 24h post-injury following both injury types, which persisted to 7 days following msTBI, but returned to sham levels by 1 and 3 months. When tau detaches from the microtubules, the increase in soluble tau can make it prone to self-aggregation leading to formation of tau oligomers [17]. These tau oligomers are thought to drive dysfunction by disrupting synapses, impeding mitochondrial function and also seeding the spread of toxic forms of tau by acting as templates for misfolding [39]. This study suggests that oligomers form rapidly regardless of injury type, most likely due to the aforementioned release of tau from microtubules in response to axonal stretching. However, it appears that this pool of oligomeric tau is rapidly removed, potentially by mechanisms such as autophagy [43]. Further work could investigate whether this initial increase in oligomeric tau propagates to other regions within the brain and the mechanisms driving its removal.

Given the changes in tau phosphorylation seen post-injury, the mechanisms driving this alteration were of interest. Tau phosphorylation state is dependent on the balance of phosphatase and kinase activity. Here it was seen that expression of PP2Ac followed a similar pattern, regardless of injury type, with a decrease at 24h, followed by a return to baseline by 1 month and a decrease again at 3 months post-injury. PP2A is a heterotrimeric protein that regulates key signaling pathways in the brain [44], and is composed of a core A-subunit, a catalytic C-subunit (PP2Ac), and a regulatory B-subunit [45]. PP2Ac is thus physically responsible for the dephosphorylation process, with different B units influencing substrate specificity [46]. Down regulation of the PP2Ac subunit has previously been shown to be associated with a decrease in PP2A activity [47-49]. Although PP2A activity would need to be directly measured to confirm this, this result is suggestive of a deficit in phosphatase activity, permitting an increase in tau phosphorylation post-injury. Such an alteration could help to explain the alterations seen in AT180 at both 24h and 3 months post-injury.

This is supported by the overall lack of findings in relation to the kinases studied here. Of note, despite the similarity in tau phosphorylation pattern seen between the rmTBI and msTBI groups, one kinase showed differential expression dependent on injury type. GSK3β increased at 24h and 3 months following injury in rmTBI, but not msTBI animals. GSK3β activity is modulated by its phosphorylation state. As it is constitutively active, and phosphorylation at Ser9, as examined here, inhibits its activity [24]. Thus, the increase in expression noted here in the rmTBI animals would actually be protective against aberrant tau phosphorylation, through it inhibition of GSK-3β activity. Previous studies have found that activity of GSK3β is acutely decreased following a focal injury up to 3 days post-injury [50,51], with this thought to be a protective response by preventing activation of pro-apoptotic factors, such as Bcl-2-associated X protein (Bax) [52]. Indeed, treatment with the GSK-3 inhibitor lithium post-TBI significantly improved spatial learning and memory with a corresponding reduction in hippocampal cell loss [53]. This is particularly significant as GSK3β has also been shown to play a critical role in the phosphorylation of tau in AD [54]. In models of AD, reducing GSK3β activity via either pharmacological compounds [55] or even exercise [56] has been shown to lead

to reduced brain pathology. In the current study, it appears that inhibitory pGSK3β phosphorylation occurs in the acute and chronic phases following rmTBI, a phenomenon not seen in msTBI animals, suggesting that this protective mechanism may be lost with more severe injury. Thus, msTBI may set the stage for greater tau hyperphosphorylation and, subsequently, a potentially higher risk of later neurodegenerative disease development compared to rmTBI. However, while impaired cognitive flexibility was recently demonstrated at 3 months post-msTBI in a diffuse axonal injury model [20], mild effects on cognitive function have also been seen at 3 months post-rmTBI [19]. Thus, additional studies at later time points are needed to more accurately assess these potential injury differences.

In regard to the other kinases, minimal changes were noted. Phosphorylated CDK5 was decreased at 24h post-injury before returning to sham level at 7 days, with levels staying relatively stable over time in both rmTBI and msTBI animals, suggestive that this kinase was not a significant contributor to tau phosphorylation post-injury. In contrast, pERK1/2 was higher at 24h compared to 3 months in both rmTBI and msTBI animals. This is in line with previous reports that levels of pERK1/2 rise acutely following TBI, with elevations noted up to 72h post-injury following a single moderate fluid percussion injury [57,58] with return to sham levels at chronic time-points (2, 8 and 12 weeks post-injury) [59]. This acute activation is thought to be protective, as administration of inhibitors of the ERK cascade prior to trauma leads to improvement of motor and cognitive deficits and reduction in lesion volume (Mori, 2002). Similarly, a combined JNK and ERK1/2 inhibitor reduced cell death in stretch injured neurons [60]. This may relate to the many different downstream effects of ERK1/2 activation, including enhancing synaptic plasticity [61], stimulating the inflammatory response through activation of microglia and astrocytes [62] and inhibiting apoptotic cascades depending on the external environment [63]. Nonetheless, ERK1/2 is also believed to phosphorylate tau at a large number of sites, including Ser199/Ser202, Ser235, Ser396/Ser404, Ser424, Thr181, Thr212 and Thr231 [64], thus the acute increase in activation of this pathway could play a role in the early increase in levels of ptau post-injury. However, given that chronically levels of the kinase return to sham level, it does not appear to play a role in the later chronic increases in tau phosphorylation.

This study provides further support to the role of TBI in promoting hyperphosphorylation of tau, with both rmTBI and msTBI leading to increases in levels of AT180 at 3 months post-injury. Notably a biphasic response was seen, with increases seen acutely at 24h post-injury and then again at 3 months, suggesting that differential mechanisms may be driving the response at these time-points. None of the kinases examined, in GSK3β, Akt, CDK5 and ERK1/2, were activated at 3 months post-injury, suggesting either the involvement of other kinases, such as PKA or PKC, or that this relates to a lack of phosphatase activity, which corresponds to the decrease in levels of PP2Ac seen here. Nonetheless aberrant phosphorylation of tau appears to be an important chronic secondary injury factor post-TBI, following both rmTBI and msTBI and may be key contributor to later neurodegeneration.

Acknowledgments

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