Brainstem Neurodegeneration Following Traumatic Brain Injury in a Rodent Model

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ABSTRACT

Traumatic brain injury (TBI) is a risk factor for the later development of Alzheimer's disease (AD), a form of dementia, although the exact mechanisms of its involvement are yet to be determined. Research suggests that the first indication of post-traumatic AD may occur in key brainstem nuclei; the locus coeruleus (LC) and raphe nuclei (RN), which are responsible for the production of neurotransmitters which contribute to normal cognition. Fatal TBI leads to neuronal loss within the LC and RN within one-month post-injury, but whether non-fatal TBI similarly effects these structures at chronic time-points remains unknown. Accordingly, this project investigated brainstem neurodegeneration by examining archival rodent model tissue at 6 months post-TBI. Male Sprague Dawley rats (10-12 weeks) underwent Marmarou's weight drop model for diffuse TBI (n=8), or sham surgery (n=6). Brain tissue was collected at 6 months post-injury and the pons extracted for examination of neurotransmitter precursor enzymes dopamine beta hydroxylase (DβH), tryptophan hydroxylase (TPH) and neuronal marker NeuN, as markers for degeneration using western blot. At 6 months post-injury, TBI animals displayed significant decreases in DβH levels compared to shams ($p<0.05$), however no significant alterations in TPH levels were observed ($p=0.43$). A significant decrease in NeuN levels was seen in TBI animals compared to shams ($p<0.05$) at 6 months post-injury, suggesting that neuronal death had occurred. This study provides further support of the interaction between TBI and brainstem neurodegeneration, by demonstrating that both DβH levels and NeuN levels decrease following TBI at 6 months post-injury.

INTRODUCTION

Traumatic brain injury and neurodegenerative disease

Traumatic brain injury (TBI) occurs when the brain impacts with an object, or experiences acceleration or deceleration forces, or a combination[3]. It is estimated that globally over 50 million people suffer from a TBI annually, and that approximately half the global population will sustain at least one TBI within their lifespan[4]. Immediate primary complications following TBI can include diffuse or focal vascular changes, axonal and glial cell injury, all of which drive ongoing secondary-injury cascades including neuroinflammation, oxidative stress and excitotoxicity[3, 5]. Ongoing neuronal death may explain the link between TBI and increased risk of later neurodegeneration, including Alzheimer's disease (AD)[6, 7], chronic traumatic encephalopathy (CTE)[8] and Parkinson's disease[9]. A recent study of military veterans diagnosed with mild TBI between 2001 and 2014 (n=178,779) found a more than 2-fold increase in risk of dementia compared to controls (n=178,779) after adjusting for demographic factors and comorbidities[10]. *Wang et al.* displayed in a Taiwanese nation-wide study that patients had a 1.68 times greater risk of developing dementia within 5-years post-TBI[7]. Recently, The Lancet added TBI as a modifiable risk factor for dementia to their 2017 commission model[11], acknowledging the key involvement of TBI as a contributor to dementia development. Considering the documented link between TBI and neurodegenerative disease development, it is therefore critical to develop a comprehensive understanding of pathologies following TBI occurrence.

Neuropathology in AD

AD, a form of dementia, is associated with the accumulation of pathological proteins including amyloid beta (Aβ) and tau[12]. Aβ is a protein which can aggregate and form extracellular senile plaques, and in AD there is an increase in the presence of the Aβ42 peptide isoform, which is

more predisposed to aggregation[13]. Tau is a microtubule associated protein which acts to stabilise microtubules and facilitate axonal transport, its activity modulated through phosphorylation and de-phosphorylation with kinases and phosphatases respectively[14]. However, when tau becomes hyperphosphorylated, it can lead to misfolding, the disruption of normal axonal function and the formation of neurotoxic neurofibrillary tangles (NFTs), which are seen in both neurodegenerative diseases and cognitively normal ageing/elderly patients[15]. Indeed, AD has been associated with aggregations of both these proteins, and in 1991, Braak and Braak categorised the advancement of AD pathology into Braak Stages I-VI, as marked by the presence and corresponding severity of hyperphosphorylated tau and NFTs[12], (see *Figure 1*)[1], later revising this categorisation in 2011 to include brainstem pre-cortical stage involvement.

A 40-year average delay occurs between stages I-II and stages III-IV[16], with the gradual onset of cognitive symptoms reflecting neurodegeneration[16]. Given the chronic nature of these changes, it is possible that early pathological processes triggered by earlier events such as TBI may cause degeneration later in life. Indeed, TBI has been shown to drive the accumulation of both Aβ and NFTs. *Johnson et al.* found that long term TBI survivors (assessed 1-47 years post-TBI) of 60 years and younger had a significant difference in NFT expression compared to controls ($p=0.02$)[17]. Through immunohistochemistry (IHC), *Tran et al.* found intra-axonal Aβ pathology present in young transgenic mice (5-7 months) following controlled cortical impact, with no Aβ present in the age-matched controls[18]. A recent transgenic mouse study (expressing mutant human tau) documented the accelerated development of tau pathology in a rodent TBI model compared to shams, with tauopathy also occurring in regions contralateral to injury site, mainly in the amygdala[19]. After 6 months post-injury, both groups displayed advancing tau

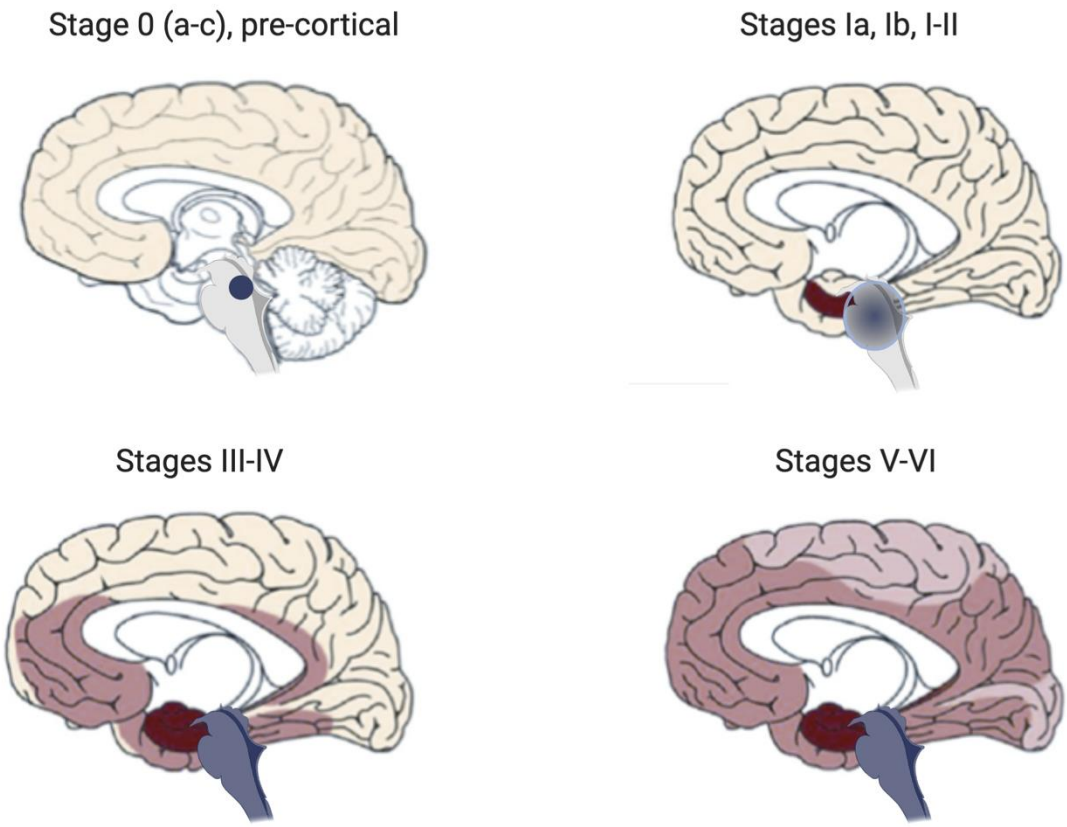

Figure 1. *Visual representation of Braak stages of AD-associated tauopathy and cortical spread, note LC involvement in Stage 0, adapted*[1].

pathology, with the TBI group demonstrating significant impairment in long-term memory whilst completing Barnes maze tests compared with controls ($p<0.01$)[19]. Such findings highlight that post-TBI tau pathology is observed at chronic time-points post-TBI, at sites distal to the injury site, and results in long-term memory deficits, suggesting that chronic degeneration is occurring.

However to date, whether the pattern of degeneration following TBI matches that seen in AD is yet to be examined. Brainstem nuclei are particularly susceptible to degeneration in the early stages of AD. The brainstem consists of the midbrain, pons and medulla oblongata, and is responsible for the regulation of many homeostatic functions within the body, with certain

nuclei responsible for the production of key neurotransmitters which facilitate this[20]. Early AD degeneration has been marked by the presence of tauopathy in both ageing and AD brains, with neurodegeneration particularly noted in the locus coeruleus (LC) and raphe nuclei (RN)[21] (see *Figure 2*) in early AD stages I & II, and even pre-cortical stage 0[22].

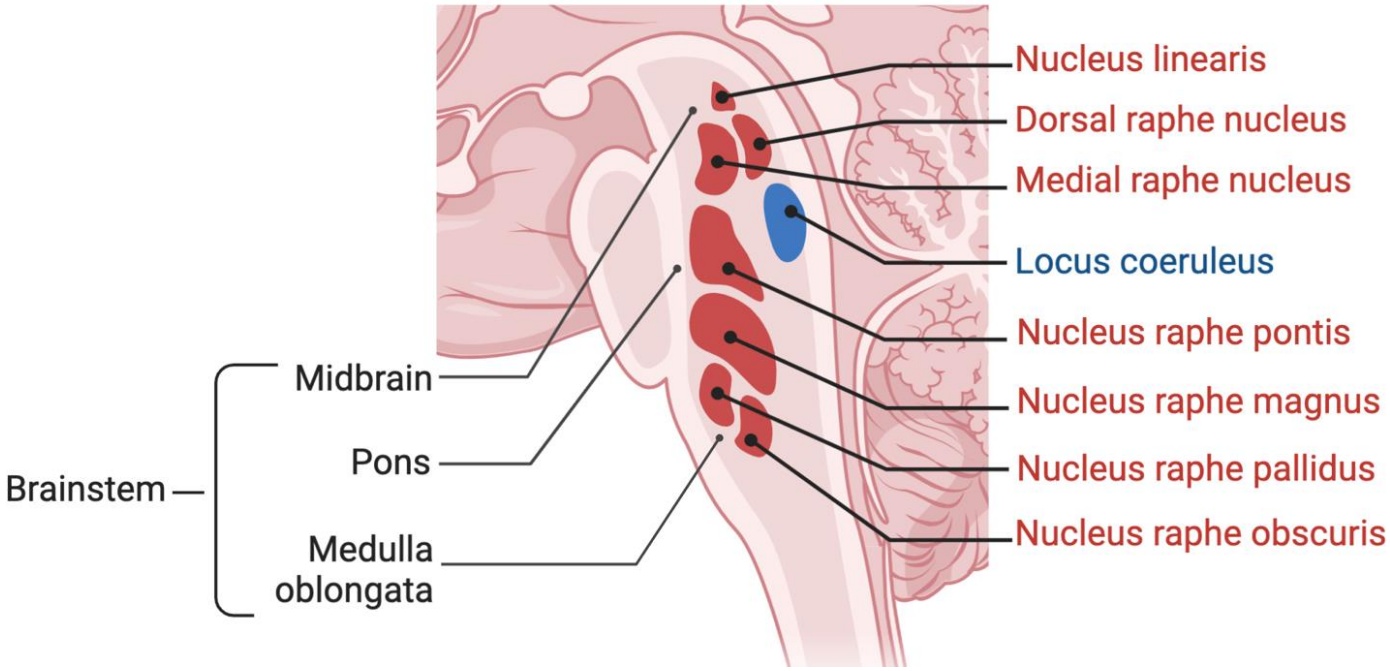

Figure 2. *Anatomy of brainstem nuclei of interest, adapted[2], (blue=LC, red=RN), created with BioRender.com*

The LC is located within the pons, adjacent to the fourth ventricle, and consists of a number of different neuronal types, including those neurons responsible for producing the catecholamine neurotransmitter noradrenaline (NA)[23]. This neurotransmitter plays a key role in modulating episodic and working memory, behavioural arousal, and also plays a role in attention and salience[24]. Many studies since the 1980s have documented the increased loss of LC neurons in AD[25-27]. More recently, *Zarow et al*. found that AD brains had an average LC cell loss of 67.9% ($p<0.05$), compared to healthy controls, as detected using IHC[28]. One very recent IHC study even went on to establish a temporal profile of LC degeneration in clinical AD, and surmised

that as the Braak stage increases by one unit, LC volume decreases by 8.4%[29]. However, this study was limited in its very small sample sizes for stages III (n=4), IV (n=1) and V (n=2), compared to other stage groups, and so their findings have low statistical power. However, *Ehrenberg et al*. also found that the number of neurons displaying hyperphosphorylated tau inclusions in the LC increases with the increments of Braak stage[22]. This study found tauopathy present in even in the initial Braak Stage I of AD[22], and taken with previous studies, suggests that this nucleus may be one of the first sites at which AD-related degeneration occurs. In AD patients, lowered NA concentrations measured at time of death are associated with increased cognitive impairment in the months preceding death[30], further demonstrating that LC integrity is key to maintaining cognitive function, especially in the case of neurodegenerative diseases.

The RN are a series of nuclei located along the midline of the brainstem throughout the midbrain, pons and medulla, and consists of rostral and caudal groups[31]. The rostral groups are responsible for producing the majority of the brain's serotonin (5-HT), which plays a role in regulating appetite, mood and sleep, as well as in cognitive memory and learning[31, 32]. Early studies from the 1980s found that AD patients experienced a 28% decrease in large neuron (diameter>25μm) density and 23% decrease in large neuron number within the RN when compared to controls[33]. A later study sought to assess a similar outcome, however this time, through Nissl staining and inclusion of all neuron sizes, found a significant 39.4% decrease in RN neuronal number in AD brains versus controls[34]. Furthermore, a more recent study specifically analysing 5-HT neurons via IHC within the dorsal and median RN in clinical AD found a 41% reduction in serotonergic neurons in the dorsal RN, and a 29% reduction in the median RN, compared to controls[35]. *Ehrenberg et al.* also found that the RN displayed neurons containing hyperphosphorylated tau in Braak Stage I,[22] indicating the involvement of RN degeneration early in the disease progression. Furthermore, *Gringberg et al.* suggest that the

dorsal RN may be the first areas affected by neurofibrillary changes in AD, reporting that in 100% of patients at Braak stage 1 and higher (n=118 out of 180), there were AD-related phosphorylated tau neurofibrillary changes in the dorsal RN[36].

TBI and brainstem neurodegeneration

Whilst TBI is an acknowledged risk for brain degeneration, few studies examine the effects of TBI on these particularly susceptible nuclei. Previous work suggests that the brainstem is vulnerable to TBI, such as *Fearing et al.'s in vivo* morphometric MRI findings, which demonstrated a decrease in midbrain brainstem volume in children (mean age=12.9 at time of scanning) following moderate-severe TBI (mean post-injury interval=3.1 years)[37]. Pre-clinical evidence by *Bolton et al.* revealed the presence of brainstem axonal injury in mice following repeated mild TBI at 24-hour intervals, compared to sham animals[38]. Whilst these studies confirm brainstem involvement post-TBI, the specificity of these studies did not extend beyond generalised brainstem injury and did not examine specific nuclei vulnerability. Another more recent study investigated LC and dorsal RN neuronal volume loss following TBI in humans, reporting that post-TBI, patients had a 29% loss in LC noradrenergic neurons and 17% loss in dorsal RN serotonergic neurons[39]. However, the small group size of n=8 patients, which also consisted of only severe, fatal TBI (20 days±10 days) and heterogenous injuries, meaning that the study findings are not representative of all TBI patients[39]. Taken together, existing studies are somewhat limited as they either do not examine specific brainstem nuclei or tend to evaluate brainstem degeneration only at an acute time period post-injury (see *Figure 3* for study rationale). As such, this study sought to establish whether TBI is associated with alterations in expression of neuronal markers associated with NA and 5-HT within the brainstem, suggestive of neurodegeneration within the LC and RN respectively. This was done using a rodent model to ensure injury and living condition consistency, whilst reducing downfalls found using post-

mortem human tissue including heterogenous injury, co-morbidities, environmental and demographic influence.

Aims and hypothesis

Hypothesis: Following rodent TBI, there will be a decrease in number of noradrenergic neurons in the LC and serotonergic neurons in the RN compared to sham rats at six months post-injury.

Aim 1: To determine whether a previous TBI affects relative expression of proteins associated with noradrenergic and serotonergic neurotransmission at six months post-injury using western blot analysis.

Aim 2: To determine whether a previous TBI results in neuronal death at six months post-injury using western blot analysis.

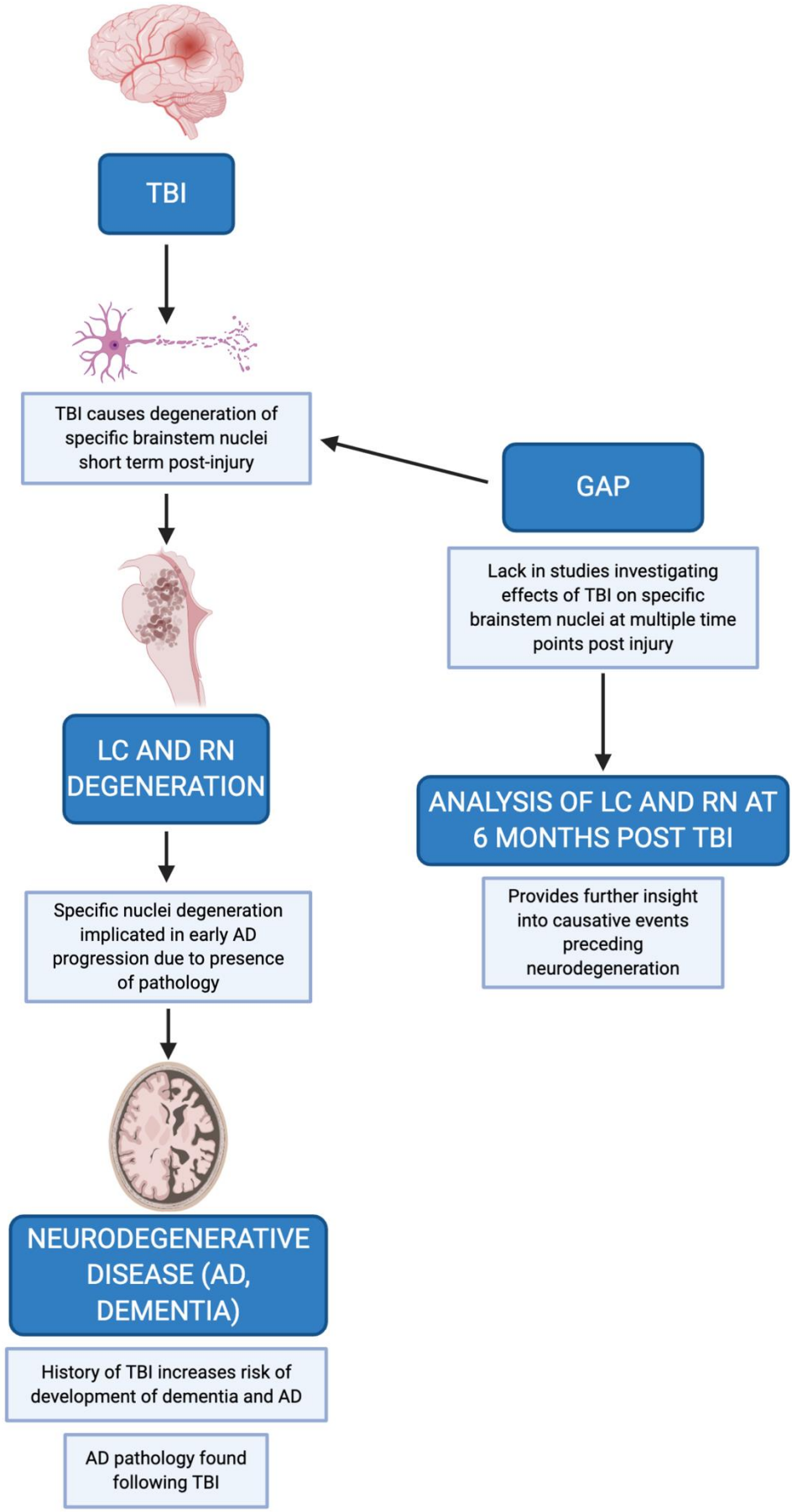

Figure 3. *Study rationale, created with BioRender.com*

MATERIALS AND METHODS

Archival Tissue

Archival brain tissue harvested from adult (10-12 weeks; 420-480g at time of injury) male Sprague Dawley rats was used in this study (University of Adelaide Animal Ethics Committee approval: M-2015-187)[40]. Animals were housed under standard laboratory conditions on a 12-hour light-dark cycle with access to food and water *ad libitum*.

Injury Model

Animals (n=14) were randomly allocated to sham or TBI groups (shams n=6; TBI n=8). Anaesthesia was induced prior to surgery via inhaled 5% isoflurane under normoxic conditions, animals were then maintained under anaesthesia with 2% isoflurane via nose cone, with TBI animals also undergoing intubation and mechanical ventilation. A midline scalp incision was made to allow for placement of a metal disk on the central skull, to ensure diffuse injury and prevent skull fracture. TBI (n=8) was then induced using Marmarou's weight drop model[41] where a 450g weight was dropped from a height of 2 metres onto the metal disk to achieve the desired moderate-severe diffuse TBI[40]. Following this, TBI animals were subjected to hypoxic conditions (2L/min nitrogen, 0.2L/min oxygen) for 10 minutes, as this more closely replicates clinical effects found following TBI[42, 43], and they also received subcutaneous saline (5mL of 0.9% (w/v) saline solution) to prevent dehydration. Sham animals (n=6) underwent only the incision to the scalp, without disk placement, weight drop or period of hypoxia. Following TBI hypoxia or sham surgery, animals were recovered and closely monitored with clinical record sheets until the end of the study.

Tissue Collection and Processing

At 6 months post-injury or sham surgery, anaesthetised animals underwent transcardial perfusion with 0.9% saline prior to brain removal, the brain stem dissected and brain tissue samples snap frozen in liquid nitrogen and stored at -80°C. Pons samples were then thawed, dissected and homogenised in RIPA lysis buffer with phosphatase inhibitor (Sigma Aldrich; Cat No. 04906845001) and a Roche mini EDTA-free protease inhibitor tablet (Sigma Aldrich; Cat No. 04693159001). Protein concentrations were estimated with Pierce BCA Protein Assay Kit (Thermo-Scientific; Cat No. 23227), with samples read at 562nm, with readings interpolated from a standard curve to generate protein concentrations for each sample. Samples were then stored at -80°C until further required, and it is noted that all steps prior to this point were conducted by people other than the author of this study[40].

Western Blot

Gel electrophoresis was performed on the archival tissue using Bolt 4-12% Bis Tris Plus gels (Life Technologies), with a total of 45µg of protein loaded per well and 5µg of ladder loaded in the first well of each gel. Gels were then run at 120V for 1 hour 20 minutes in 20X Bolt MES SDS Running Buffer, then 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) (3x 5 minute washes) before antibody incubation (*Table 1).* For precursor enzyme markers (see *Figure 4*), membranes were blocked with 5% skim milk TBST solution and then incubated with primary and housekeeper antibodies in a 2% skim milk TBST solution overnight at 4°C. Following overnight incubation, membranes were washed in TBST (3x 5-minute washes) before being incubated with corresponding secondary antibodies (LICOR IRDye 680RD Donkey anti-Chicken 926-68075, 1:1000; LICOR IRDye 800CW Donkey anti-Rabbit 926-32213, 1:1000) in darkness in 2% skim milk TBST solution at 4°C for 2 hours.

Membranes were then washed in TBST (3x 5 minute washes) in darkness. Neuronal marker NeuN membranes were incubated in the iBind Western System (Life Technologies) with both primary antibody and corresponding secondary antibody solution (LICOR IRDye 680RD Donkey anti-Chicken 926-68075, 1:2000; LICOR IRDye 800CW Donkey anti-Rabbit 926-32213, 1:3000) in 1X iBind solution for 2.5 hours at room temperature, in the dark. Following completed antibody incubation, membranes were washed in TBST solution (3x 5 minute washes) before being visualised using an Odyssey CLx Infrared Imaging System (model 9140).

Biological Marker	**Catalogue number**	**Dilution**	**Rationale**
Dopamine beta hydroxylase	ab209487 (Abcam)	1:500	Precursor enzyme for neurotransmitter produced in nuclei of interest
Tryptophan hydroxylase	ab217024 (Abcam)	1:1000	
NeuN	ab177487 (Abcam)	1:750	Marker of neuronal number
GAPDH	ab83957 (Abcam)	1:1000	Housekeeper protein

Table 1. *Antibody information*

Western Blot Analysis

Semi-quantitative analysis of band signals was conducted using Image Studio Lite (Version 5.2.5), adjusting for normalisation by using a single control sample in the first well of each gel for the same biomarker. As such, relative density was calculated based on adjusted density, factoring in the control sample across the gels, as demonstrated below.

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

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

Figure 4. *Neurotransmitter synthesis, created with BioRender.com*

Statistical Analysis

All statistical analysis was done using GraphPad Prism (Version 8.4.3) and presented as mean ± SEM, with all data analysed using unpaired students t-tests and statistical significance deemed when $p < 0.05$. Grubbs tests were performed and any result more than 2 standard deviations from the mean was removed as an outlier.

RESULTS

History of TBI influences brainstem neurotransmitter enzyme expression

Neuronal neurotransmitter precursor enzyme markers were evaluated to determine if a history of TBI altered their expression at 6 months post-injury, as compared to sham animals. It was

found that previous moderate-severe TBI resulted in a significant decrease in DβH levels compared to shams (0.95±0.05 vs 1.59±0.24, $p<0.05$), indicating that a history of TBI does indeed cause neuronal dysfunction at the pontine level of the brainstem at 6 months post-injury, (*Figure 5A*). However, there were no significant changes observed in levels of TPH in TBI animals compared to sham animals (1.23±0.19 vs 1.08±0.14, $p=0.43$) at 6 months post-injury, which may suggest neuronal dysfunction of the raphe nuclei does not occur at the level of the pons in the brainstem, 6 months post-injury (*Figure 5B*).

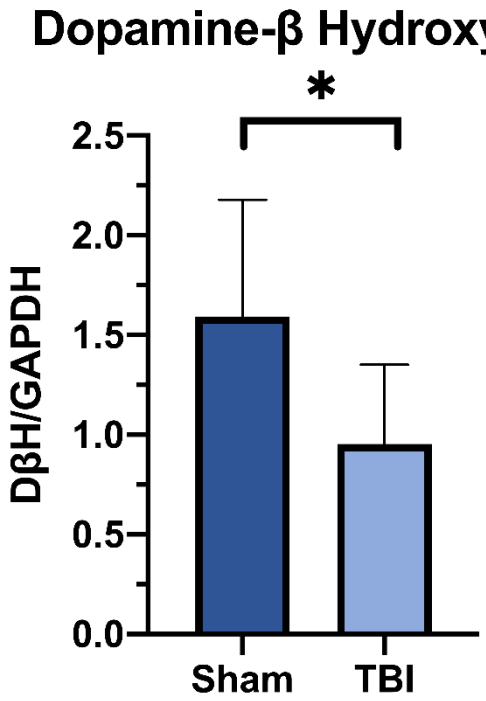

FIGURE 5A.

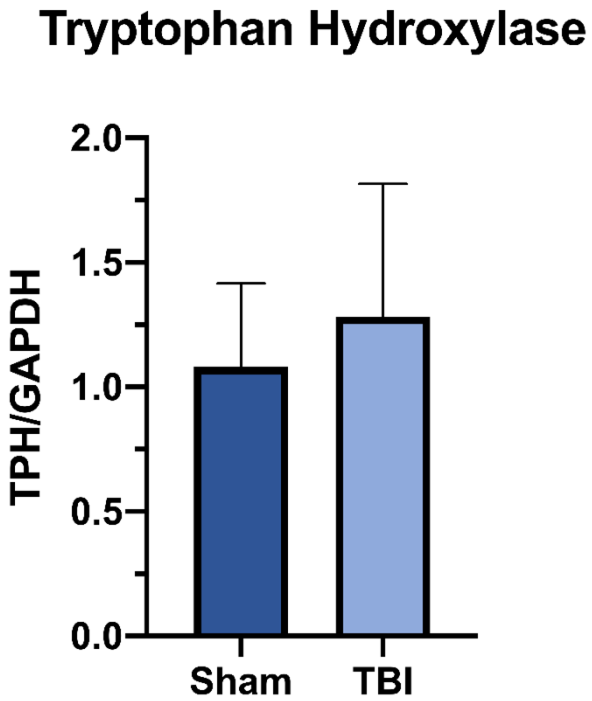

FIGURE 5B.

Figure 5. A) *Western blot analysis of DβH protein extraction following an unpaired t-test demonstrated a significant decrease in DβH in TBI rats compared to sham rats, *p<0.05.* ***B)*** *Unpaired t-test demonstrated no significant difference in TPH expression in TBI rats compared to sham rats (p=0.43)*

History of TBI influences NeuN expression within the pons

Levels of the neuronal marker NeuN were evaluated to determine if any significant changes in precursor enzyme levels were a result of loss in neuronal number or neuronal dysfunction. It

was found that past TBI did result in significant reduction in NeuN levels at 6 months post-injury compared to shams (1.17±0.14 vs 1.53±0.06, $p<0.05$), indicating that there may have been a loss in neuronal numbers following TBI when compared to sham animals (*Figure 6*). Data for one TBI animal was excluded from this dataset as an outlier, its value straying more than 2 standard deviations away from the mean (Grubbs test critical value=2.03, 95% confidence, outlier datapoint=2.20).

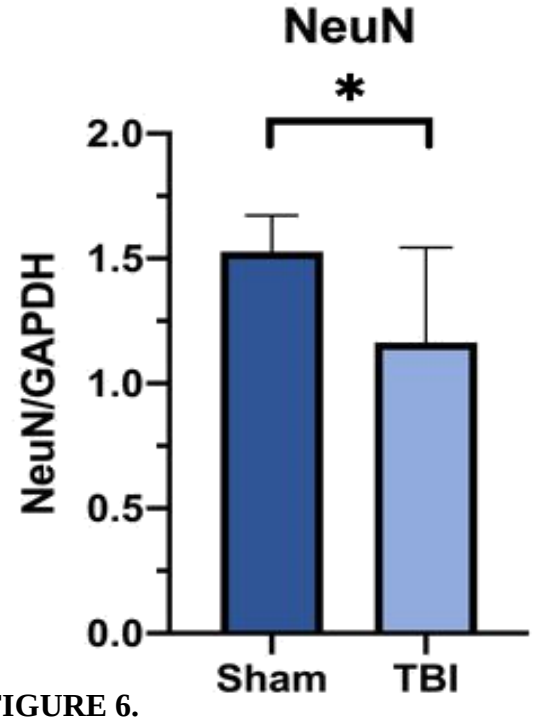

FIGURE 6.

Figure 6. *Western blot analysis of NeuN protein extraction following an unpaired t-test demonstrated a significant decrease in NeuN in TBI rats compared to sham rats, *p<0.05.*

DISCUSSION

The findings of this study provide a deeper understanding of particular brainstem nuclei dysfunction following TBI. The results are concordant with previous studies in that they support brainstem neurodegeneration post-TBI, but in the context of specific nuclei associated

with neurodegenerative disease, demonstrate that at a further time-point following-injury, neuronal dysfunction or death may be occurring.

Locus coeruleus degeneration following TBI

In this study, the presence of precursor neurotransmitter enzymes and neuronal markers were used to assess neuronal health of the LC and RN. We found that a history of a single, moderate-severe TBI did indeed reduce the levels of DβH at 6 months post-TBI, suggesting that at this time-point, noradrenergic neuronal dysfunction or death may be occurring in the LC, within the pons. This method of assessing neurotransmitter precursor enzyme presence, rather than levels of the key neurotransmitters of interest themselves provides better insight into neuronal health, due to the short half-lives of neurotransmitters. Direct NA level assessment may also not indicate neuronal dysfunction, as other compensatory mechanisms such as downregulation of NA transporters would not result in a reduction of NA[44]. The change in DβH levels found in this study support the findings of *Valko et al.* in extending their evaluation of degeneration occurring immediately after fatal TBI, by displaying that it can also be detected at a longer time-point following moderate-severe TBI[39]. Further IHC analyses may confirm LC degeneration as the origin of this dysfunction.

Neuronal death following TBI

Examining levels of NeuN allowed for further assessment of neuronal death versus neuronal dysfunction, and it was found that following TBI at this time-point, there was a significant reduction in NeuN protein. This suggests that the pons undergoes neurodegeneration, and not just neuronal dysfunction, at 6 months following TBI. This further supports previous DβH findings, suggesting that neuronal degeneration may be the cause of pre-cursor enzyme level changes. One limiting factor of NeuN is that it does not allow for the specificity of labelling

particular types of neurons, as it only identifies nuclei of nervous cells[45], and so whilst the reduction in neuronal number within this study was significant, it only indicates that neuronal death has occurred within the pons, and does not identify specific nuclei or the noradrenergic neurons which are of interest. Given the diffuse nature of the TBI model injury in this study, this could explain why neuronal death has occurred within the pons. The lack of specificity in protein extractions and nature of western blot analysis also mean that this data does not support neuronal loss within specific nuclei. Such that, using IHC to assess DβH levels would allow for a more quantitative method of analysis, and would allow for confirmation of this.

Raphe nuclei following TBI

No significant changes in TPH levels found between TBI and shams, indicating that at 6 months post-TBI, neurodegeneration or neuronal dysfunction was not observed within the RN, which contrasts what previous research suggests of RN degeneration. It is however likely that the reason for discrepancy in this finding compared to DβH results lies within the limitations of this study. In only using brainstem pons protein extractions, this greatly limited the total potential TPH levels being assessed, as the RN also occur at midbrain and medulla oblongata levels of the brainstem. As such, these results suggest that perhaps RN degeneration does not occur at the level of the pons at 6 months post-TBI, and that perhaps degeneration occurs first at other levels of the brainstem. Studies suggest that degeneration of the RN in diseases such as AD may occur more severely at a caudal level and less in the rostral[46]. With the rostral RN being the site of most 5-HT production, use of TPH as a marker may not reflect accurate RN degeneration at this time point following TBI. It would be prudent to ensure protein extractions from other levels of brainstem (medulla and midbrain) are also assessed in future studies to gain a more complete picture of long-term brainstem alterations following TBI.

Mechanisms of neuronal degeneration

Whilst the exact mechanisms that link TBI and development of pathologies and neurodegeneration later in life remain unclear, research suggests pathophysiological links between post-TBI nervous tissue biochemical changes and the eventual development of neuropathology[47]. These changes may be responsible for the results found in this study or may provide possible avenues for further exploration. Following TBI, secondary injury cascades can cause metabolic changes, mitochondrial dysfunction, blood-brain-barrier dysfunction, presence of oxidative free radicals, excitotoxicity, axonal injury and neuroinflammation, with these responses exacerbating the original injury[5, 48]. Diffuse axonal injury is found in 40-50% of all TBI hospital admissions[49], and it is suggested that the mechanical breaking of axons in injury may result in impaired axonal transport and the aggregation of proteins such as tau and amyloid precursor protein (APP), which is cleaved to produce Aβ[50]. TBI also triggers an acute pro-inflammatory response to the site of brain damage, which may become chronic due to the continued release of pro-inflammatory cytokines and other inflammatory mediators by resident glial cells[5, 51]. It has been suggested that a chronic neuroinflammatory response to TBI may in turn facilitate the build-up of neurotoxic protein aggregates commonly found as pathological hallmarks in neurodegenerative diseases like AD, including Aβ plaques and NFTs[51]. As such, it is possible that assessment for neuroinflammatory and axonal injury markers in the brainstem may provide further insight into the cause of degeneration which has been found to occur following TBI.

Why the brainstem and these particular nuclei demonstrate a vulnerability to TBI is yet to be fully determined. One explanation for specific brainstem vulnerability in humans could be that the brainstem is particularly susceptible to axonal damage following TBI due to orientation of neuronal axons[52, 53]. The brainstem also consists of neurons which have very long, overlapping dendritic processes[54], which may also predispose them to receiving damage in cases of TBI.

Recently, it has been suggested that LC neurons may already be predisposed for neurodegeneration due to their high metabolic activity and excitability, leading to heightened mitochondrial demand, potential dysfunction and oxidative stress[55]. Oxidative stress may occur in these LC neurons due to their autonomous pacemaker nature, and increased Ca^{2+} entry through L-type channels, triggering mitochondrial Ca^{2+}-augmented oxidant production[56].

Potential explanation for LC degeneration suggested by this study may also lie in the connection between TBI and tauopathy. As previously discussed, tau pathology is promoted following TBI,[17], and is also noted early in the progression of AD[1, 21, 22], even pre-clinically[1]. When tau becomes hyperphosphorylated, this promotes detachment of tau from microtubules, which destabilises them and inhibits their role in axonal transport, eventually resulting in axonal injury[57]. This in turn results in increased soluble tau present in neurons, which can aggregate to form granular tau oligomers and NFTs[58], with some studies suggesting that it may be these oligomers which are neurotoxic and induce mitochondrial dysfunction[59]. Neuronal death may also occur due to the accumulation of intracellular NFT formations, or perhaps destabilised microtubule dysfunction resulting in neuronal death[60]. Indeed, it is also still debated as to whether the hyperphosphorylated tau itself is toxic, or whether this increases sensitivity to secondary insults[61]. Regardless, this makes for a compelling argument for the involvement of tau in the gradual degeneration of the LC, and it could be that the altered physiological environment following TBI may accelerate this accumulation of tau and neuronal dysfunction.

While many pathologies provide potential mechanisms for neuronal death following TBI, and with TBI a confirmed risk factor for the development of neurodegenerative diseases such as dementia, it is important to evaluate extensively any potential origins of degeneration. As such,

future studies investigating brainstem involvement may be crucial to extending the knowledge on this link, and this in turn may allow for better elucidation of mechanisms underlying neurodegeneration.

Study Limitations

This study is limited in that it consists of tissue taken at only one time-point, 6 months post-TBI. A more comprehensive future model and inclusion of multiple time-points post-TBI would allow for the establishment of a temporal profile of brainstem degeneration. This is critical, as this project does not identify if the changes observed are ones that developed immediately following TBI and have not resolved, or are emerging in the chronic phase post-TBI. Inclusion of brain tissue collection at 1, 2, 6 and 12 months post-injury would elucidate if the degeneration occurs earlier or later post-TBI, and whether neurodegeneration is accelerated at more chronic post-injury time-points. The use of western blot for protein level analysis is also limited in that it provides only semi-quantitative analysis of protein levels. Mass spectrometry-based assays would allow for a more comprehensive analysis of present enzyme concentration[62]. In addition, including another quantitative method of analysis such as IHC would also allow for identification of targeted protein expression location, as well as neuronal number expressing target proteins, whilst maintaining tissue integrity. However, the findings of this study provide adequate preliminary support, enough to warrant the use of more quantitative methods of analysis in future to assess neuronal number in these locations. Given the evidence of tauopathy in these nuclei in both ageing and neurodegenerative disease, it would also be useful to use tau markers (tau-5) and phospho-tau markers (tau-5, AT180, Ser404, Ser396) to assess neurodegeneration in this rodent model. The use of a larger animal model (e.g. sheep) may also provide further insight into brainstem degeneration, as they possess better translatability to humans, with more synonymous pathophysiology.

Conclusion

In conclusion, a history of TBI is linked to a decrease in NeuN expression and DβH, suggesting a loss of NA neurons within the pons at six months post-injury. Whilst TPH levels did not change following TBI, this may be region of interest or time post-injury dependent. Nevertheless, these findings highlight the complexities of TBI and neurodegeneration, and demonstrate that brainstem neurodegeneration at 6 months post-TBI in a rodent model is present, supporting the hypothesis that TBI can continue to have implications long after the initial injury event. The implications of this, combined with the role of neurotransmitters produced by these nuclei and neurodegenerative disease development, may provide invaluable insight into more accurately understanding the pathophysiology of diseases such as AD and dementia.

Word count: 4498

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