Characterising the effects of neuroinflammation and hypoxia on white matter axonal injury in an ovine TBI model

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Abstract

Traumatic brain injury (TBI) is a life-threatening injury that results from mechanical forces to the brain. Such force damages axons within the white matter of the brain, driving death and disability associated with TBI, for which there are no effective treatments. However, the lack of clinically-translatable models means that understanding of white matter pathology is limited. To address this, our laboratory has developed an impact-acceleration ovine model of TBI. Briefly, 14 male merino wethers (18-24 months; 55-65kg) were randomised to receive TBI (n=8) or sham (n=6) surgeries. All animals were further randomised to receive either normoxia (TBI n=4; sham n=3) or transient hypoxia (65% pO_2 for 15 minutes; TBI n=4; sham n=3) after TBI or sham surgery. Following a 4-hour post-injury monitoring period, animals were euthanised, and brain tissue extracted then analysed for axonal injury and neuroinflammation within white matter tracts. Following TBI, significant calcium-mediated axonal injury was observed via spectrin N-terminal fragments in the three white matter regions of interest: corpus callosum ($p<0.05$), right internal capsule ($p<0.01$), and the left internal capsule ($p<0.05$). However, no marked neuroinflammation was observed post-TBI as assessed via levels of pro-inflammatory cytokines: TNF-α, IL-1β, and IL-6 ($p>0.05$). Calcium-mediated axonal injury was not associated with these pro-inflammatory cytokines ($p>0.05$). Furthermore, post-traumatic hypoxia had no effect on neuroinflammation, nor calcium-mediated axonal injury. Taken together, this study found neuroinflammation does not appear to drive the axonal injury seen in the initial stages of TBI. However, later time-points post-TBI are essential to characterise a temporal profile of these changes.

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Introduction

Traumatic brain injury (TBI) is the leading cause of death and disability for individuals under 45 years of age[1]. Physical (40%), cognitive (62%), behavioural (55%), and social (49%) deficits are common following TBI with widespread damage to axons, known as diffuse axonal injury (DAI), believed to be a leading cause[2-4]. DAI is present in 40-50% of all TBI hospital admissions and occurs in 80% of all motor vehicle-associated TBI cases[5].

In DAI, proximal segments of axons swell and disconnect from their respective distal axon terminals, resulting in reduced cortical connectivity and functional deficits[5]. The primary physical injury rarely disconnects axons but disrupts their cytoskeletons and ionic homeostasis. This damage sets forth progressive secondary pathophysiological mechanisms which evolve over weeks to years post-injury, and hence are potentially amenable to treatment[5-7].

The axonal cytoskeleton consists of microtubules, neurofilaments and actin, that not only maintain the structure of the axon, but also facilitate movement of products to and from the synapse[7]. Fast anterograde transport utilises microtubules to move products to the synapse, with a key mediator being the amyloid precursor protein (APP)[8]. *Ex-vivo* models have revealed that axonal stretching displaces microtubules, potentially impairing fast anterograde transport and leading to APP accumulation[9]. This is seen clinically, with APP extensively used as a marker of axonal disruption and injury[10]. However, APP accumulation alone may not reveal the full extent of axonal injury, with clinical studies detecting different sub-populations of injured axons independent to APP accumulation[11, 12]. For example, axonal injury can also be mediated through increases of intracellular Ca^{2+}, resultant from disruptions of ionic channels and stimulated releases of intracellular stores. This excess calcium ultimately activates calpain and caspases-3 and -9[5]. The proteolytic enzyme calpain is responsible for the breakdown of the axolemma anchoring protein spectrin, generating spectrin N-terminal fragments (SNTF), which can be used as a marker for calcium-mediated axonal damage[11].

Caspase and calpain work together to impair mitochondrial function and breakdown the axonal cytoskeleton, resulting in eventual bioenergetic failure and axonal disconnection[5, 13].

White matter consists of tracts of myelinated axons and represents a significant proportion of axons in humans[6]. Research by *Johnson et al (2013)* indicated that human corpus callosum tissue showed evidence of continued axonal injury for years after a single TBI[6]. In addition, diffusion tensor imaging (DTI) studies have revealed white matter undergoing vast degrees of atrophy, structural changes, and accelerated aging following injury[3, 14]. As such, modelling white matter injury is pertinent to understanding the pathology ultimately driving disability and death following TBI. Unfortunately, rodent models fail to reproduce comparable pathologies as their brains only contain 10% white matter, as opposed to the 60% white matter seen in humans[15]. Interestingly, unmyelinated axons are more susceptible to axonal injury compared with myelinated axons, and hence TBI pathologies in rodent models may be exaggerated[16].

In addition to low white matter proportions, the rodent brain is lissencephalic, meaning injury force is distributed evenly across the surface of their smooth brains[17]. This strongly contrasts the human gyrencephalic brain, whereby injury force is concentrated deep in the base of the sulci[17]. As such, the rodent model is not optimal to study post-traumatic white matter pathology with large animals, such as pigs and sheep, favoured as models for their rich white matter and gyrencephaly. Hence our laboratory developed an impact-acceleration ovine model of TBI. Sheep are ideal models due to their less developed neck musculature (compared to pigs) allowing for increased free head motion, akin to clinical injury[17]. Equivalent young adult male sheep were used in this study as they represent the demographic with the highest incidence of clinical TBI[1]. This model was established in our laboratory 20 years previously, showing evidence of APP related axonal injury 4 hours following severe TBI[18-20]. As such this study opted to replicate this early 4-hour time point with a modified injury device, to reduce the risk of skull fracture as a pre-requisite by the ethics committee for working towards a survival model of TBI. To assist with developing a clinically relevant model of injury, a subset of

injured animals underwent a brief period of hypoxia following injury. Hypoxia occurs in 45% of severe TBI patients and is associated with a trebled length of intensive care unit stay and worsened long term outcomes[21]. TBI with hypoxia occurs in situations where there are disruptions to brainstem respiratory centres or concurrent chest injuries, ultimately resulting in impaired breathing[21]. Rodent studies modelling post-traumatic hypoxia have shown exacerbated neuroinflammation and axonal injury[22-24].

What remains unclear is the capability of an ovine model of a mild-moderate TBI with or without post-injury hypoxia in replicating clinically-relevant features of white matter damage. Our preliminary investigations found minimal effects of the modified sheep TBI model on accumulation of APP as a marker of axonal injury, irrespective of whether animals were exposed to hypoxia following injury (Jessica Sharkey, personal communication). However, as outlined above, axonal injury may not just be represented by accumulation of APP. Hence this study sought to characterise different populations of vulnerable white matter axons exhibiting altered calcium homeostasis, using SNTF in this novel ovine model. Additionally, the study evaluated how clinically relevant factors such as post-traumatic hypoxia and neuroinflammation influenced this axonal injury.

Neuroinflammation is the inflammatory process within the brain and is mediated by resident immune cells, including microglia[25]. Neuroinflammation plays both a beneficial and detrimental role following TBI, stimulating pro- and anti-inflammatory properties respectively[26]. TBI stimulates microglial activation, resulting in the production and release of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, which have destructive actions on the brain parenchyma[27]. Both TNF-α and IL-1β peak in brain tissue within hours post-injury, with high concentrations associated with poor patient outcomes[28]. These cytokines are synthesised from the nuclear factor-kappa B (NF-kB) signalling pathway and can potentially initiate neuronal apoptosis and the destruction of myelin[29]. IL-6 utilises the Janus kinase-signal transducer an activator of

transcription pathway and as such its upregulation can be delayed compared with TNF-α and IL-1β, peaking in brain tissue concentration 6 hours post-injury[29].

Recent research has examined how neuroinflammation modulates axonal injury, with microglial processes converging APP-positive injured axons within white matter tracts in large animal TBI models[30]. Although, how neuroinflammation modulates calcium-related axonal pathology in white matter tracts remains unknown, inhibiting calpain in rodent TBI models reduces NF-kB-related inflammation 6 hours post-injury, suggesting that this form of axonal injury modulates the neuroinflammatory process[31]. However, this is yet to be replicated within white matter tracts of large animal TBI models.

This study opted to further characterise white matter pathology by detecting different populations of disrupted axons using SNTF and characterising neuroinflammation in white matter tracts of the novel ovine TBI model. Three white matter regions of interest were selected for this study: the corpus callosum and the bilateral internal capsules. The corpus callosum is a highly vulnerable white matter tract following TBI, and thus it was anticipated to show the highest degree of evidence of axonal injury[6]. The internal capsule houses axons critical for motor function and sensation, hence disrupting these axons is likely to produce post-TBI disabilities[3]. A brief period of post-injury hypoxia was also induced to determine how this modulates axonal injury and neuroinflammation in this model.

Aims and Hypothesis

Hypothesis: Neuroinflammation will influence the axonal injury found in the ovine TBI model.

Aim 1: Characterise changes to axonal integrity using SNTF following ovine TBI.

Aim 2: Characterise the expression of pro-inflammatory cytokines in the white matter in the ovine TBI model using ELISA.

Aim 3: Determine any relationship between neuroinflammation and axonal injury through regression analysis.

Ethical Considerations

Generation of TBI tissue (SAM 396.19) was approved by the SAHMRI (South Australian Health and Medical Research Institute) animal ethics committee. All animals were anaesthetised for surgical and perfusion procedures with extensive clinical and monitoring records kept.

Methods

Tissue Generation

This study utilised archival fresh and fixed brain tissue, which was generated using the ovine TBI model. Male merino wethers (n=14; 55-65 kg; 18-24 months) were randomly allocated into 4 groups: sham normoxia (n=3), sham hypoxia (n=3), TBI normoxia (n=4) and TBI hypoxia (n=4) groups (*Fig. 1*). Sham animals underwent all procedures, except for induction of injury.

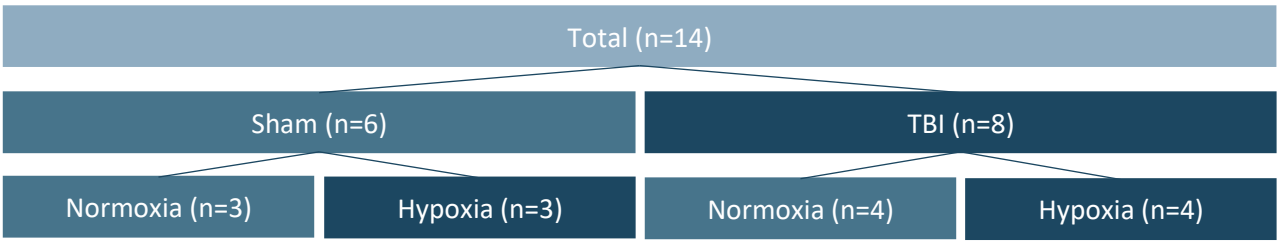

Figure 1: A schematic overview of the experimental groups within the study

Anaesthetisation

Animals were induced with an intravenous mix of ketamine (0.05mg/kg; Troy Laboratories Australia) and diazepam (0.04ml/kg; Ceva Australia), intubated and maintained with 3% isoflurane in a 70:30 nitrogen:oxygen mixture. Animals were then maintained under anaesthesia with a mix of 1.5-2% isoflurane and intravenous ketamine (2ml/hr) during surgery.

TBI

Animals were placed in the prone position with the head suspended by a cervical sling, allowing free head motion in the sagittal plane. A mounted modified captive bolt animal stunner gun (Karl-Schermer; Ettlingen, Germany; Cat No. MKL) with a concave silicon tip, was set perpendicular to the head between the right supraorbital process and the right external auditory meatus[32]. A 21-calibre

round was fired, propelling the 385g captive bolt to impact the head. Following delivery of injury, any localised superficial haemorrhaging was treated with pressure and sutured closed if necessary.

Post-traumatic Hypoxia

Immediately post-injury, hypoxic animals had ventilation parameters altered to induce hypoxia. Specifically, the ventilated gas mixture was changed to 90:10 nitrogen:oxygen, with femoral arterial blood gas analysis performed every 5 minutes. Oxygen saturation levels below 65% were maintained for 15 minutes before normal ventilation was restored, allowing oxygen saturation to return to baseline conditions. The normoxic animals had no alteration of ventilation parameters and thus no change in oxygen saturation.

Perfusion

Animals were subject to physiological monitoring under anaesthesia for 4 hours post-injury ± hypoxia, before being humanely killed under anaesthesia. Heparinised saline was administered 10 minutes before the perfusion and the animal placed in a supine position. The bilateral common carotid arteries were exposed, catheters were inserted proximally, and 5 litres of cold tris-buffered saline was pumped through these arteries at a pressure of approximately 120mmHg to perfuse the brain. The jugular veins were incised to drain the perfusate.

Post-mortem Processing

Following perfusion, anaesthesia was discontinued, the head decapitated, and the brain extracted. The brain was then placed in a custom matrix on ice to mitigate degradation of small proteins and had alternating 10mm coronal sections cut. Sections were either post-fixed in 10% formalin, processed and embedded in paraffin wax blocks or had the corpus callosum and bilateral internal capsules (*Fig. 2*) dissected 4cm anteroposterior and snap-frozen in liquid nitrogen before being stored at -80℃.

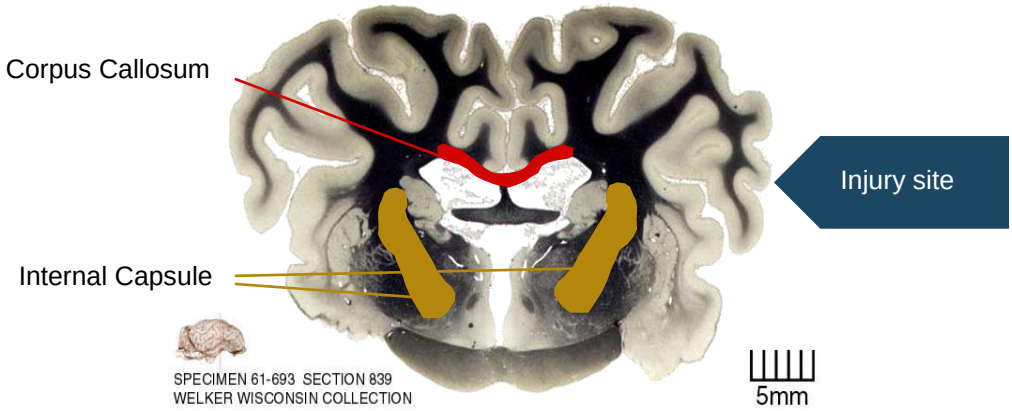

Figure 2: Representation (4cm anteroposterior) of the white matter regions of interest with respect to injury location[32]*. Corpus callosum (red); Internal capsules (yellow).*

Immunohistochemistry for Axonal Injury

Embedded brain tissue sections containing the corpus callosum and internal capsule were cut into 5µm sections using a microtome (Leica) and mounted onto glass slides. Tissue was dewaxed and immersed in methanol and 1.5% hydrogen peroxide to block endogenous peroxidase activity. Heat-induced epitope retrieval was done with an EDTA buffer to recover antigen reactivity. Blocking was done using normal horse serum (NHS; Vector laboratories; Cat No. S-2000-20) for 30 minutes, and the primary antibody for SNTF (1:4000; Merek; Cat No. ABN-2264) was applied and incubated overnight at room temperature. Negative control tissue received NHS only. Biotinylated horse anti-rabbit IgG (1:250; Vector laboratories; Cat No. BA-1100) was applied for 30 minutes following a tertiary of horseradish peroxidase streptavidin (1:1000; Vector laboratories; Cat No. SA-5004) for 1hr. Antigen visualisation was done through the application of 3,3′-diaminobenzidine (DAB; Vector Laboratories; Cat No. SK-4100), before counterstaining in haematoxylin. Slides were coverslipped and scanned using a Hamamatsu NanoZoomer 2.0RS (Hamamatsu, Japan) to obtain high-resolution images.

The sheep brain atlas was then used to confirm the specific size and location of regions of interest for each animal, maintaining consistency in regions across the dataset regardless of anteroposterior location[33]. Regions of interest were then outlined with area recorded using Hamamatsu NDP.view 2 (v2.7.52). A blinded investigator then manually counted SNTF-positive axons at 40x magnification.

Counting selection criteria (*Fig. 3*) was kept consistent with pre-existing calcium-mediated axonal injury papers[5]. Briefly, distinct segments of SNTF counted as a single injured axon (*Fig. 3B*), smaller segments travelling in the same plane were also counted as one axon (*Fig. 3C*). Small dots alluded to a cross-section of an injured axon and hence were counted (*Fig. 3A*). These counts were then standardised against region of interest area and was reported as SNTF-positive axons per mm^2.

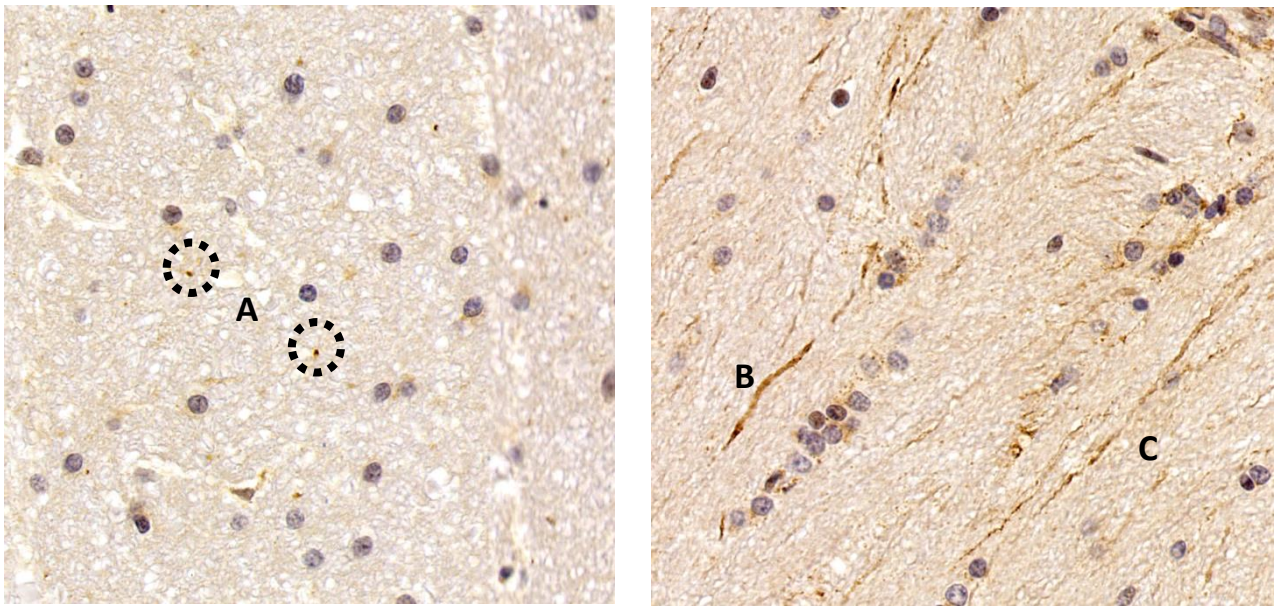

Figure 3: Representative 40x micrographs of SNTF-positive tissue. *This represents the three types of injured axon counted:* ***A)*** *A cross section of an injured axon (circled);* ***B)*** *Segments of SNTF-positive tissue indicating continuous damage along the axon;* ***C)*** *Two segments of SNTF-positive tissue in the same plane, likely indicating discontinuous injury from the same axon.*

ELISA Analysis for Neuroinflammation

Frozen corpus callosum and bilateral internal capsule samples were partially defrosted and cut into 50mg (~$2mm^2$) sections. Radioimmunoprecipitation assay buffer, phosphatase inhibitor (Sigma Aldrich; Cat No. 04906845001) and a Roche mini EDTA-free protease inhibitor tablet (Sigma Aldrich; Cat No. 04693159001) were added to the tissue and homogenised with a pellet pestle. Samples were sonicated and centrifuged at 14,000rpm at 4℃ for 15 minutes, with supernatant aliquoted in 3x100µL samples to avoid excess freeze-thaw cycles and stored at -80℃. Protein concentrations were then estimated using a Pierce BCA protein assay kit (Thermo-scientific; Cat No.

23227) as per the manufacturer's instructions. One ELISA kit from MyBiosource (San Diego, CA) was used for each cytokine of interest: TNF-α (#MBS778330), IL-1β (#MBS778369), and IL-6 (#MBS778334). Neat supernatant was loaded in duplicate and the assay was completed as per the manufacturer's instructions with plates read using a Synergy HTX Multi-Mode Microplate Reader (Biotek Instruments). Absorbance readings (450nm) were calibrated as standard curves and cytokine concentrations were interpolated. These concentrations were standardised against the total protein in each sample and were expressed as pg/ml.

Statistical Analysis

All statistical analysis was done in GraphPad prism (v8.4.3) and presented as mean ± SEM. All data was tested for normality using the Shapiro-Wilk test and returned as parametric. SNTF counts and ELISA data was analysed using a two-way analysis of variance (ANOVA), testing for the effects of TBI and hypoxia as factors. Where relevant, Sidak's multiple comparisons were used as a post-hoc analysis. A simple regression analysis was used to determine any correlation between cytokine concentrations and SNTF-positive axons. Statistical significance was determined as $p<0.05$.

Results

All animals underwent their respective TBI/sham and hypoxia/normoxia procedures without any adverse complications. 1x normoxic TBI and 1x hypoxic TBI corpus callosum sample was not included due to limited availability of ELISA kit supplies.

Assessment of Axonal Injury using SNTF

Assessment of calcium-mediated axonal injury revealed a significant group effect for TBI in all three regions of interest following TBI (*Fig. 4*). Specifically, the corpus callosum showed a significant main group effect for TBI ($p<0.05$), however post-hoc analysis revealed no significant differences for both normoxic sham vs TBI ($p=0.14$) and hypoxic sham vs TBI ($p=0.18$). The right (injured hemisphere) internal capsule showed a main effect for TBI ($p<0.01$) with post-hoc showing no significance for normoxic sham vs TBI groups ($p=0.30$) but revealed a significant increase ($p<0.01$)

from the hypoxic sham group (3.73 ± 0.23/mm^2; n=3) to the hypoxic TBI group (30.06 ± 6.44/mm^2; n=4). Finally, despite a significant main effect of TBI in the left (uninjured hemisphere) internal capsule ($p<0.05$), post-hoc analysis revealed no significant differences between normoxic ($p=0.13$) or hypoxic ($p=0.18$) sham vs. TBI.

No main effect of hypoxia on SNTF axonal injury was observed in this cohort for any of the white matter regions examined (corpus callosum: $p=0.68$; right internal capsule: $p=0.09$; left internal capsule: $p=0.97$).

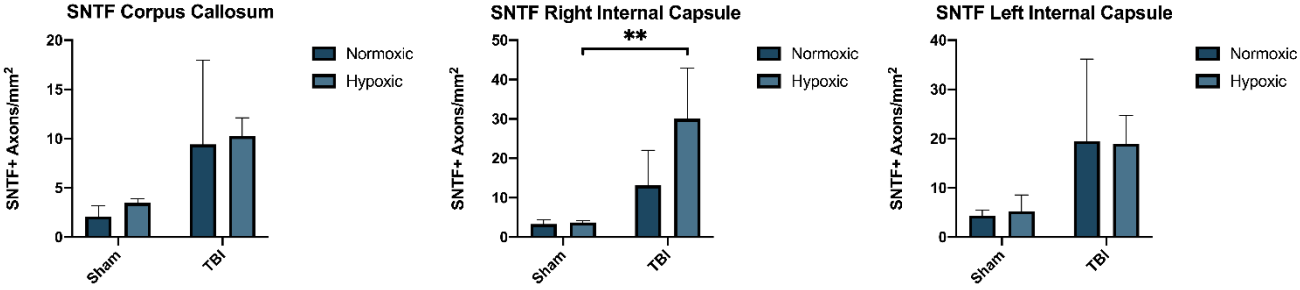

Figure 4: Number of SNTF-positive axons within white matter regions following TBI. *All three regions showed a significant main effect of TBI on SNTF+ve axons (n=8) compared to shams (n=6): corpus callosum ($p<0.05$), right ($p<0.01$) and left ($p<0.05$) internal capsule. Specifically, post-hoc analysis found significant increases in the hypoxic TBI group compared to the hypoxic sham group in the right internal capsule ($p<0.01$). Data mean ± SEM; 2-way ANOVA and Sidak's multiple comparisons; ** $p<0.01$.*

Assessment of Pro-inflammatory Cytokines in White Matter Homogenate

Analysis of white matter cytokine concentrations revealed a significant main effect for hypoxia in the left internal capsule for TNF-α ($p<0.01$) and IL-1β ($p<0.01$), and a trend in IL-6 ($p=0.07$). Post-hoc analysis showed a significant reduction of TNF-α between sham normoxic and hypoxic animals (1193 ± 19 vs 838 ± 75 pg/ml; $p<0.05$) groups, with a trend between TBI normoxic and TBI hypoxic groups ($p=0.09$).

Trends were observed in both sham (p=0.08) and TBI (p=0.05) animals for decreased IL-1β concentrations following hypoxia in the left internal capsule (*Fig. 5*). In contrast, there was no main effect of hypoxia observed in the corpus callosum (TNF-α: p=0.09, IL-1β: p=0.56 and IL-6: p=0.46) or the right internal capsule (TNF-α: p=0.40, IL-1β: p=0.43 and IL-6: p=0.76). Furthermore, no main effect of TBI was noted on the levels of inflammatory cytokines within the right internal capsule (TNF-α: p=0.14; IL-1β: p=0.93; IL-6: p=0.63), left internal capsule (TNF-α: p=0.41; IL-1β: p=0.40; IL-6: p=0.36), or corpus callosum (TNF-α: p=0.16; IL-1β: p=0.110; IL-6: p=0.51)

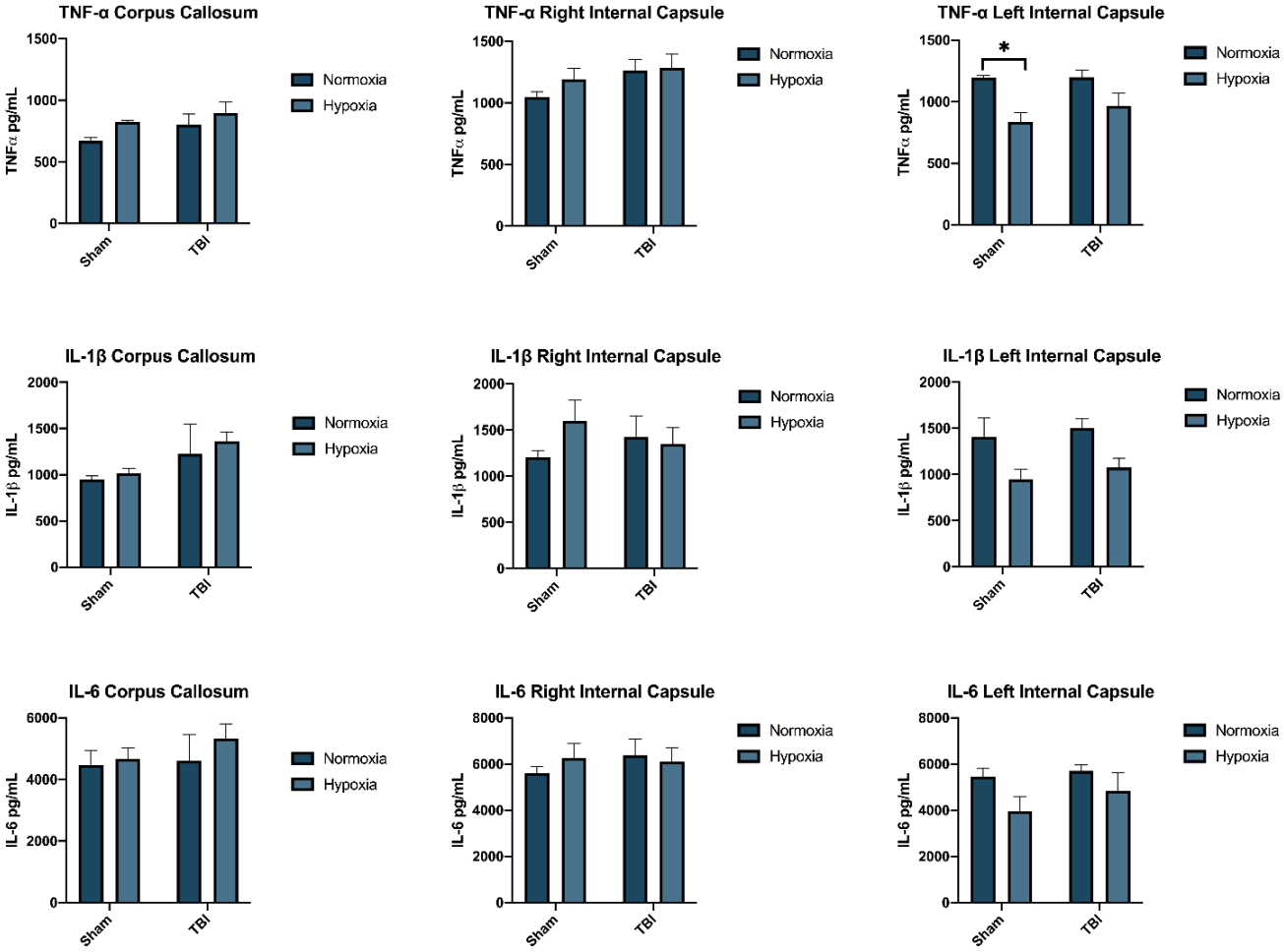

Figure 5: Cytokine concentrations for TNF-α IL-1β and IL-6 in the internal capsule and corpus callosum. *A main effect of hypoxia was noted for TNF-α (p<0.05) and IL-1β (p<0.05). Sidak's multiple comparisons revealed a significant decrease in TNF-α concentrations following hypoxia in sham animals (p<0.05). Data mean ± SEM; 2-way ANOVA and Sidak's multiple comparisons; * p<0.05; Corpus callosum: n=12, Bilateral internal capsules: n=14.*

Regression Analysis Between Axonal Injury and Neuroinflammation

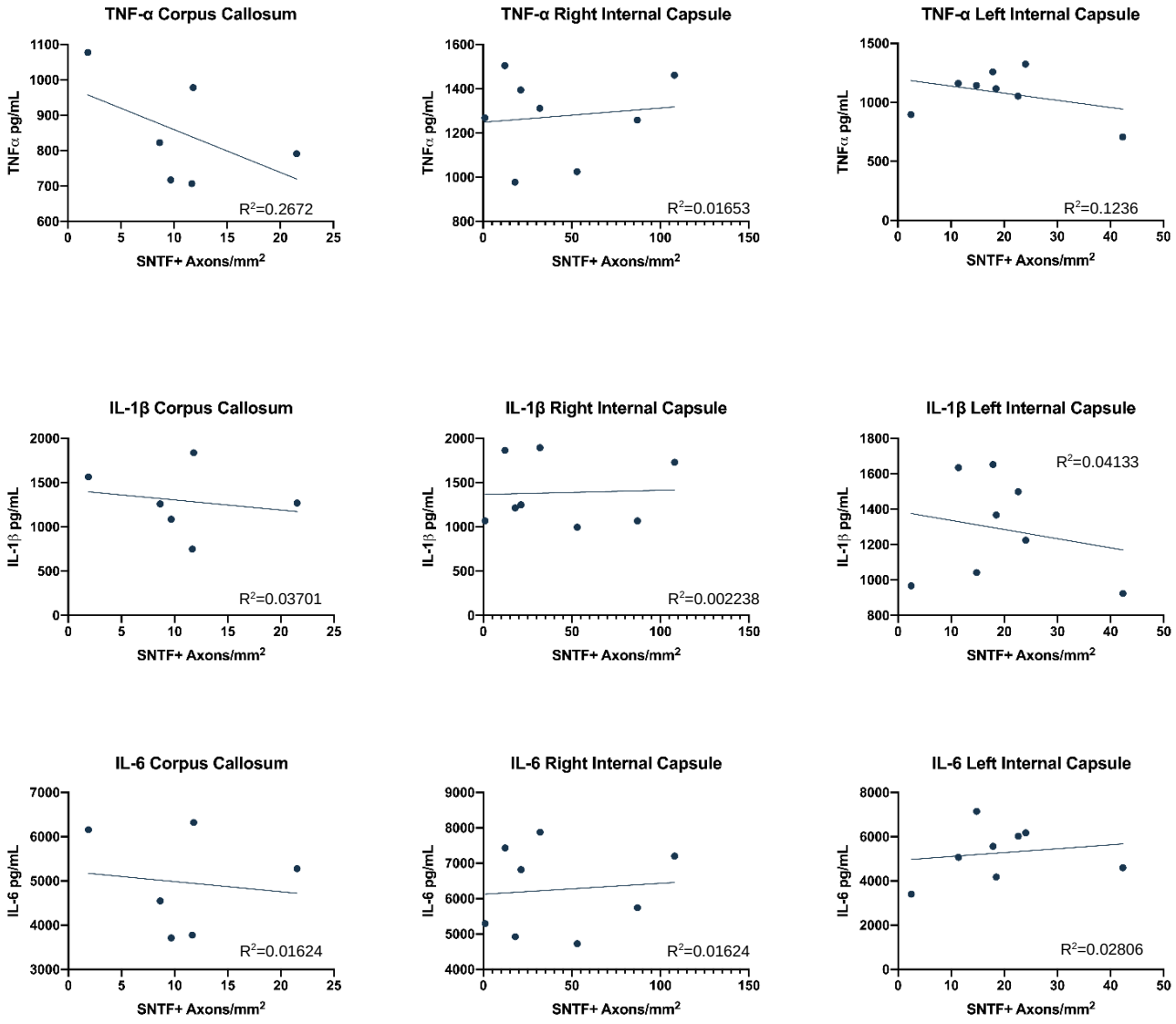

Figure 6: Simple linear regression analysis between pro-inflammatory cytokines and SNTF+ axons. *No correlations were observed between TNF-α, IL-1β and IL-6 levels and number of SNTF-positive axons/mm^2 in white matter tracts following TBI; Corpus callosum: n=6, Bilateral internal capsules: n=8.*

Following a simple linear regression analysis between SNTF-positive axons per mm^2 and pro-inflammatory cytokine concentrations in injured animals, there were no significant correlations observed (*Fig. 6*). Corpus callosum SNTF-positive axons per mm^2 showed no significant correlations to concentrations of TNF-α ($p=0.29$; $R^2=0.27$), IL-1β ($p=0.71$; $R^2=0.04$), or IL-6 ($p=0.81$; $R^2=0.02$).

Similarly, the right internal capsule showed no significant correlations between neuroinflammation and SNTF: TNF-α ($p=0.76$; $R^2=0.02$), IL-1β ($p=0.91$; $R^2<0.01$), or IL-6 ($p=0.82$; $R^2<0.01$). Finally, the left internal capsule showed no relationship either: TNF-α ($p=0.39$; $R^2=0.12$), IL-1β ($p=0.63$; $R^2=0.04$), or IL-6 ($p=0.69$; $R^2=0.03$).

Discussion

This study was one of the first large animal TBI studies to examine neuroinflammation and calcium-mediated axonal injury within white matter tracts post-TBI. Our ovine model demonstrated evidence of this SNTF-positive axonal injury within white matter tracts, a form of injury previously not classified in sheep post-trauma.

Calcium-mediated Axonal Injury

We have demonstrated that SNTF immunostaining detects a subpopulation of injured axons within the white matter following ovine TBI, which was not observed with APP staining. To our knowledge, this is the first time SNTF staining has been successfully used to assess axonal injury in an ovine TBI model. Not only does this confirm that SNTF-related axonal injury is a common feature of injury post-trauma across various species, but also reaffirms the translational merit of the ovine model in producing clinically relevant axonal injury. Indeed, SNTF is seen clinically across all TBI severities, with its abundance indicative of disruptions of axons via the activation of proteolytic enzymes[11]. Additionally, elevated serum concentrations of SNTF have been linked with a decline in cognitive performance and abnormal DTI scans following TBI, highlighting its clinical relevance in assessing disability post-TBI[34]. SNTF detected axonal injury in all regions of interest in our study, suggesting that all white matter tracts are susceptible to this form of injury. Following manual counting, we observed that the right internal capsule showed the highest concentration of SNTF-positive axons, likely due to it being closest to the impact site. However, the corpus callosum is thought to be the most susceptible white matter region to axonal injury and hence intuitively should have the highest

degree of axonal injury following TBI[6]. This was not observed in our study however differing axonal densities between these two white matter tracts may be key[35].

APP staining is dependent on a high degree of impaired axonal transport before positive staining is observed and hence is less sensitive to subtle injury compared to SNTF[11, 12]. To further this, calcium can impair mitochondrial functioning and is thought to impair fast anterograde transport before it accumulates at sites of impaired microtubules via bioenergetic failure, hence leading to a population of SNTF-positive, APP-negative axons[11]. This may indicate why SNTF pathology was observed where APP pathology was not and reinforces the use of multiple markers for axonal injury to get a complete picture post-TBI.

There was no significant effect of hypoxia on the number of SNTF-positive axons following TBI. To date, no studies have explored the relationship between calcium-mediated axonal injury and hypoxia. Research suggests that calcium homeostasis is altered by hypoxia, with intracellular increases expected following post-traumatic hypoxia[36]. Hypoxia reduces ATP production and impedes the cells ability to efflux calcium following TBI[36]. This may result in prolonged high intracellular calcium concentrations, sustaining the activation of destructive proteolytic enzymes, ultimately exacerbating axonal injury over an extended timeframe. Hence the survival time-point used in our study may have been too acute to see the effects of this potentially persistent heightened intracellular calcium and subsequent exacerbated damage.

One major limitation of these results is variations in the coronal section analysed within this tissue. The sheep brain atlas reveals coronal anatomical structures every 20mm[33]. Comparing anatomical structures seen within our tissue with the structures found in the atlas highlighted a potential variance of up to 40mm[33]. This discrepancy could result in variation in the amount of axonal injury detected due to varying proximity to the impact site.

Pro-Inflammatory Cytokines

Many large animal experimental models of TBI have found microglial activation, an indicator of neuroinflammation, to be observed as early as 15 minutes and up to 6 hours post-injury[11, 30, 37, 38]. Our study utilised pro-inflammatory cytokines as a measure of neuroinflammation, as they are a known product of activated microglia[39]. Hence, based on previous studies, our 4-hour time-point was within reasonable time frames to detect post-traumatic neuroinflammation, however, we did not observe marked neuroinflammation. The paucity of pro-inflammatory cytokine expression could be attributable to several factors, such as the severity of the injury or the use of ketamine during surgical procedures. Research suggests that the severity of injury plays a role in the development of neuroinflammation post-injury, with a TBI rodent model of increasing injury severity noting increased expression of NF-kB 24 hours post-injury via western blots[40]. Low APP-positive axons and the paucity of neuroinflammation may suggest that the level of injury achieved in this model may not be great enough to induce significant neuroinflammation. Ketamine has well documented neuroprotective properties, suppressing the release of pro-inflammatory cytokines within the rodent brain[41], such that a similar effect could potentially drive the effects seen in our study. Continuous ketamine infusion has not been used in previous ovine or other large animal models of TBI and hence may explain why those studies showed evidence of neuroinflammation[30].

Pro-inflammatory cytokine expression showed a significant decrease following hypoxia for both TNF-α and IL-1β, but this was limited to the left internal capsule. Ketamine has been shown to influence the NF-kB pathway and hence may explain why TNF-α and IL-1β were affected but IL-6 was not[42]. Interestingly, when ketamine is paired with transient hypoxia in foetal sheep tissue, there is an attenuation of the neuroinflammatory response with a reduction in pro-inflammatory cytokines and microglial activation[43]. Hence, the interaction of anaesthetics coupled with hypoxia may have led to the further downregulation of neuroinflammation observed in this region. However, why this effect

was only observed in the left internal capsule remains unclear. Variation between animals may have exacerbated group effects, a limitation of a low sample size in both sham groups (n=3/group).

Calcium-mediated Axonal Injury and Neuroinflammation

To date the relationship between calcium-mediated axonal injury and neuroinflammation has not been studied in large animal models. Regression analysis revealed no correlations between calcium-mediated axonal injury and pro-inflammatory cytokine levels in injured animals. Given that no increase in pro-inflammatory cytokine expression was noted following TBI, it suggests that this class of axonal injury does not drive neuroinflammation within white matter tracts at this early post-injury time-point. This contrasts research suggesting that calpain-mediated axonal injury which suggested that this form of injury exacerbated neuroinflammation 6 hours post-injury[31]. However, our study may have been too acute to fully see the effects of this relationship.

Study Limitations

One major limitation was the early 4-hour time-point following TBI, which may have been premature to observe significant neuroinflammation and APP-positive axonal injury development within the white matter. Hence extending the study to incorporate longer time-points (i.e., 24 hours) may give further insight into how neuroinflammation and axonal injury interact. However, in this study we were limited to the 4-hour time-point in the first instance to adhere to ethical requirements. Another limitation was the use of animals from a single age range and sex. It has been well documented that ageing exacerbates neuroinflammation following TBI[44]. The elderly represent the second-largest demographic for TBI patients, hence extending the study to include older sheep may improve the clinical relevance of this model[1]. In addition, sex also modulates neuroinflammation, hence including female sheep may also improve its relevance to the clinical TBI population[45].

The sample sizes used in the study were low, with only n=6 sham animals and n=8 TBI animals used, and these were further sub-divided into normoxia and hypoxia groups, further reducing the statistical power. Such low sample sizes make establishing clear relationships difficult due to variations

between animals and injury severities, hence a larger sample size may yield different results. In addition, the two corpus callosum samples not analysed in the cohort may have influenced the conclusions drawn on the neuroinflammation present within the cohort. White matter analysis was limited to 3 regions due to time and supply constraints.

However, analysing more regions, such as the corona radiata, may provide a more robust spatial profile of both axonal injury and neuroinflammation following ovine TBI. Also, analysis of microglial activation and levels of anti-inflammatory cytokines, such as IL-10 and IL-4, within these regions was not performed but is essential in understanding the nature of the neuroinflammatory process following TBI. Finally, analysing peripheral serum and cerebrospinal fluid levels of both inflammatory cytokines and SNTF, alongside brain tissue, may give further insight into how white matter pathologies translate into clinically-measurable biomarkers[34].

Conclusion

Our study sought to characterise white matter pathology in the novel ovine model of TBI. Our results found that SNTF pathology occurs independently of both neuroinflammation and hypoxia, suggesting that axonal injury drives neuroinflammation in white matter tracts[6, 30]. Hence ameliorating axonal injury with novel therapeutics could indeed assist in mitigating the neuroinflammatory response seen following TBI. However, an extension to later post-TBI time-points is required to maximise insight into these injury processes. Nevertheless, we have shown that our novel ovine model was successful in producing some clinically-relevant TBI pathology, providing further insight into axonal injury and therefore warrants use in future pre-clinical TBI studies.

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