The Role of Substance P in Chronic Traumatic Encephalopathy

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Kelly McAteer

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Publications and Presentations

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Publications

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Presentations

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Abbreviations

ANOVA	analysis of variance
AT180	tau phosphorylated at threonine 231, immunohistochemistry specific antibody
BBB	blood brain barrier
BM	Barnes Maze
CNS	central nervous system
DAB	diaminobenzidene
ELISA	enzyme linked immunosorbent assay
FST	forced swim test
IHC	immunohistochemistry
mTBI	mild traumatic brain injury
NAT	N-acetyl L-tryptophan
NFT	neurofibrillary tangle
NK_1	neurokinin receptor-1
NMDA	N-methyl D-aspartate
NHS	normal horse serum
OF	Open Field
pTau	phosphorylated tau
rmTBI	repetitive mild traumatic brain injury
SEM	standard error of the mean

smTBI	single mild traumatic brain injury
SP	Substance P
SPC	streptadavin peroxidase conjugate
T231	Tau phosphorylated at threonine 231
TBI	traumatic brain injury
$TRPV_1$	transient receptor potential vanilloid receptor-1
WB	Western Blot
YM	Y Maze
001	EU-C-001, NK_1 antagonist
4PL	four parameter logistic curve

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Abstract

Chronic traumatic encephalopathy (CTE) is believed to be a neurodegenerative disease associated with contact sports and exposure to repetitive mild traumatic brain injury (rmTBI). CTE has attracted significant attraction over the past few years due to the high incidence of sports-related head injuries and the emergence of dementia-like symptoms many years following active play. In addition, patients with suspected CTE display a very unique pattern of hyperphosphorylated tau deposition post-mortem, with accumulation located at the base of the cortical sulci. To date, the link between exposure to rmTBI and the development of both dementia-like symptoms and post-mortem pathology associated with CTE has not been ascertained, highlighting the need for the study of potential mechanisms driving these processes.

Substance P (SP) is a neuropeptide involved in the process of neurogenic inflammation, which has been shown previously to be involved in many processes within the brain following traumatic brain injury (TBI) including blood brain barrier disruption and development of oedema. Significantly increased levels of SP have also been frequently observed following TBI, as well as other studies showing that SP release can increase with the application of more frequent and intense stimuli, such as that observed in rmTBI. It could therefore be that SP may contribute to the long term behavioural and pathological changes observed in CTE. Therefore the overall aims of this thesis were to fully ascertain the role of SP release following rmTBI and to determine whether the changes observed following rmTBI can be attenuated with a therapeutic intervention.

Significant increases in SP concentrations were observed following both rmTBI and severe TBI in the cortical regions in the acute phases of injury, although this did not appear to have a pronounced effect on tau as analysed by Western Blot (WB). However when analysed using immunohistochemistry changes in tau deposition were observed at the same timepoints. To further analyse the role that SP may play in these injury processes, blockade of the $TRPV_1$ receptor, where SP is believed to be released from when mechanically stimulated, was performed prior to injury.

Administration of a $TRPV_1$ antagonist both prior to and following injury prevented SP release in severely injured animals. However in rmTBI animals, SP release was only attenuated in those pre-treated with a $TRPV_1$ antagonist.

Therefore to further assess the effects of SP release following rmTBI, administration of an NK_1 antagonist, which is known to block the effects of SP release, was employed following injury. It was found that the antagonists may have had an effect on the acute release of SP following rmTBI, however this did not translate to changes in long term outcomes. Overall the effects on tau phosphorylation were believed to be negligible in this study and the SP/NK_1 system did not appear to be involved.

To summarise, it is believed that SP release is indeed affected following rmTBI. However, it does not appear to be involved in the phosphorylation of tau in the acute or chronic phases of injury. However, there is still much to be discovered about the role of the SP/NK1 system and its potential involvement following rmTBI and these studies provide the groundwork for future developments in this area.

Chapter 1: Introduction and Literature Review

1.1 INTRODUCTION & EPIDEMIOLOGY

Concussion is defined as a subset of traumatic brain injury (TBI) that is induced by biomechanical forces and results in a complex series of pathophysiological processes affecting the brain (McCrory et al. 2013). It is typically caused by a direct blow to the head, face, neck or other part of the body with an impulsive force transmitted to the head, usually resulting in the rapid onset of acute impairment of neurological function that resolves spontaneously (Marshall 2012). These clinical symptoms may or may not involve loss of consciousness and can also include headache, changes in behaviour, amnesia and insomnia (Khurana & Kaye 2012). Although most cases do resolve over time, a small percentage of patients with concussion, between 5-43%, can exhibit symptoms for weeks to months following the initial injury, termed post-concussion syndrome (Hiploylee et al. 2017).

Recent studies have highlighted a steady increase in sport related concussion hospitalizations, with an average annual increase of 5.4% in hospitalization rates in Victoria over a 9 year period (Finch, Clapperton & McCrory 2013). Football codes, including rugby, Australian football (AFL) and soccer accounted for 36% of concussion related hospitalizations between 2002 and 2011 (Finch, Clapperton & McCrory 2013). An estimated 1.6-3.8 million sport related concussions occur in the United States each year, however this number is believed to be severely under reported, with up to 50% of concussions going unreported (Harmon et al. 2013). Hospitalisation rates for sports related concussion have also increased, with a 60.5% rise seen in admissions for concussion between 2002 & 2011 in Victoria, with the associated costs estimated to be nearly $18,000,000 (Finch, Clapperton & McCrory 2013).

High levels of public concern regarding concussion, especially within professional sporting circles, have sparked an increased research presence within the past few years. This is due to the recent link

associating participation in contact sports, exposure to repeated events of concussion and the later development of dementia like symptoms in the years following the initial event (McKee et al. 2016).

1.2 DEFINITION OF CONCUSSION AND MILD TBI

Traumatic brain injury (TBI) is specifically defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force (Menon et al. 2010). Alteration in brain function can manifest as any period of loss of consciousness, memory loss of events immediately before or after the injury, neurological deficits including loss of balance, sensory loss, paralysis, or aphasia, or any alteration in mental state at the time of injury, such as confusion, disorientation or slowed thinking (Brenner, Vanderploeg & Terrio 2009; Menon et al. 2010). Due to advances in modern medicine including imaging techniques such as diffusion tensor MRI, changes in brain pathology can be detected in some cases, and can assist in the diagnosis of TBI if clinical symptoms are more subtle or delayed in their presentation (Krishna et al. 2012; Menon et al. 2010). External forces that can result in a TBI can include events such as the head either striking an object or being struck by an object, the brain undergoing rapid acceleration/deceleration movement without direct external trauma to the head, a penetration of a foreign body to the brain, or forces generated from blast or explosion events (Brenner, Vanderploeg & Terrio 2009; Menon et al. 2010).

Historically, severity of TBI was classified by using the Glasgow Coma Scale (GCS), a system used to assess impaired consciousness (Teasdale & Jennett 1974). The GCS is divided into three components; eye opening, verbal responses and motor responses summed to give a total score, with a GCS score of 13-15 defined as mild, 9-12 as moderate and 3-8 as severe (Teasdale & Jennett 1974). A concussion is thought to be a subset of mTBI resulting in the rapid onset of short-lived impairment of neurological function that reflect a functional disturbance rather than a structural injury and may or may not involve a period of loss of consciousness (McCrory et al. 2017).

1.3 MECHANISMS OF MILD TRAUMATIC BRAIN INJURY

TBI, regardless of severity level, involves a complex series of biochemical changes within the brain involving neurotoxicity, metabolic imbalances and general disruption of ionic and cellular homeostasis following the initial physical insult, described as secondary injury processes (Signoretti et al. 2011). Indeed, an animal study by Sandhir, Onyszchuk and Berman (2008) found that in adult mice injured using a controlled cortical impact model of TBI that basal levels of mRNA expression of CD11b and IBA_1, two markers of microglial activation, were most increased three days post-injury in the hippocampus. Additionally, this study looked at mRNA expression of GFAP and S100β, markers of astrocyte activation, and found that these were increased following TBI seven days post-injury (Sandhir, Onyszchuk & Berman 2008). This study also looked at the effects of TBI on neuroinflammation in aged mice and found that across the board, these markers were increased at all timepoints and return of these markers to control levels was delayed compared to the adult mice, which resolved rapidly following the peak in injury (Sandhir, Onyszchuk & Berman 2008). These secondary injury processes have also been observed in clinical cases, with a study by Bellander et al. (2011) that assessed the cerebrospinal fluid (CSF) of twenty patients suffering severe TBI to look for markers of secondary injury, namely a “membrane attack complex”, C5b9, as well as S100B and neuron-specific enolase (NSE). In the first 48 hours following the injury, initial peaks of all of these markers were observed. Further peaks of C5b9 were observed in CSF in patients exhibiting at least one secondary insult (e.g. insufficient respiration, unstable circulation, seizures) at least 24 hours after the primary injury (Bellander et al. 2011). CSF levels of S100B and NSE were also increased in these patients exhibiting secondary insults, however this was not statistically significant (Bellander et al. 2011).

The initial acceleration-deceleration forces involved with the injury almost immediately set into motion these start of the biochemical imbalances, with a sudden flux in ions through now disregulated ion channels and transient membrane defects caused by the stretch injury (Barkhoudarian, Hovda & Giza 2011; Farkas, Lifshitz & Povlishock 2006). Following this, there is a sudden release of neurotransmitters, of most importance is the release of glutamate, an excitatory amino acid, which plays a pivotal role by binding to the N-methyl-d-aspartate (NMDA) ionic channels (Faden et al. 1989). The activation of NMDA leads to further depolarisation which in turn leads to an influx of calcium ions into the cells leading to mitochondrial overloading (Xiong et al. 1997). This calcium influx into the mitochondria is then believed to be responsible for changing the permeability of the inner membranes of the cell, leading to further dysfunction of general cell function, disruptions in oxidative phosphorylation and swelling of organelles (Schinder et al. 1996). These dysfunctional mitochondria are then believed to become the main producer of reactive oxygen species (ROS), thereby inducing oxidative stress and lipid peroxidation (Vagnozzi et al. 1999). These events are believed to occur quite rapidly following the initial insult, starting one minute after trauma and persisting for 24-48 hrs following injury (Signoretti et al. 2011).

In addition to these metabolic changes, neuroinflammatory cascades are also activated, with microglia and astrocytes becoming activated shortly following the injury (Lucke-Wold et al. 2014). This has been observed both pre-clinically in animal models of rmTBI (Broussard et al. 2018; McAteer et al. 2016) and clinically in cases of brain injury (Smith et al. 2013; Thelin et al. 2017). Interestingly, in cases of CTE, NFT aggregation has a predisposition to occur around perivascular and subcortical areas that are near reactive microglia and astrocytes (Geddes et al. 1999). This lends further support to the role that neuroinflammation may play in CTE development.

To try and restore ionic homeostasis, adenosine triphosphate (ATP) dependant pumps work at maximal capacity, requiring a high level of glucose oxidation to facilitate the increased energy demand, however, due to the damage caused by calcium overloading and the production of ROS,

these reactions are impaired and the mitochondria simply cannot keep up with the required phosphorylation demands necessary to produce ATP (Signoretti et al. 2011). This results in a rapid decrease levels of ATP leading neurons to enter glycolysis (Figure 1.1) (Signoretti et al. 2011). These changes are are thought to be fully reversible in most mTBI sufferers due to the resolution of the correlated clinical symptoms of concussion in all but a small cohort of patients (Tavazzi et al. 2005).

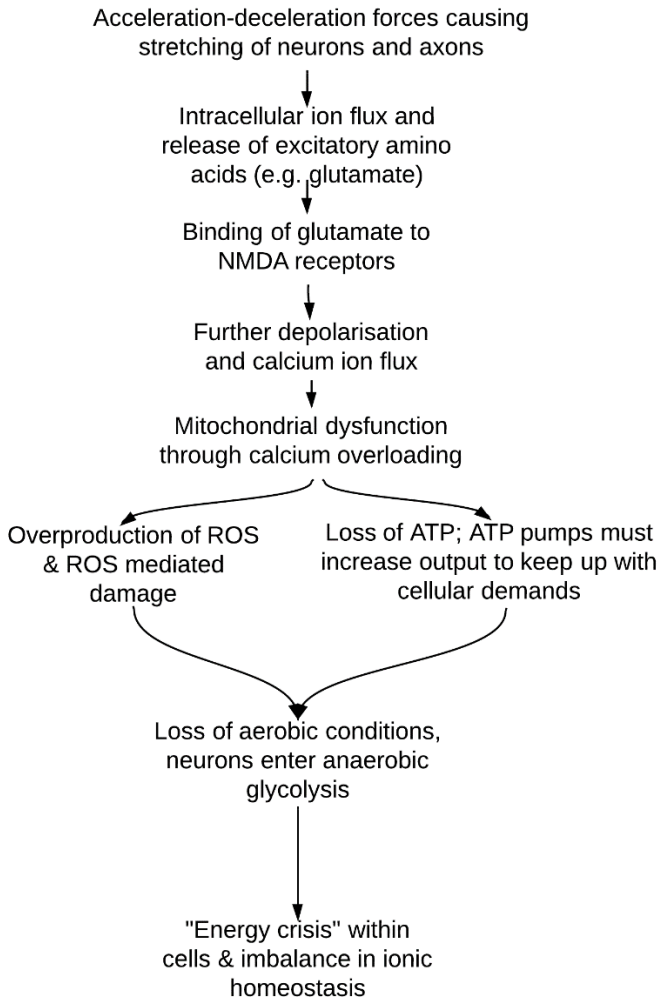


Figure 1.1: Overview of biochemical events taking place following concussion/mTBI, adapted from Signoretti et al. 2011 (Signoretti et al. 2011).

Although these changes are said to be reversible, there is a period following injury where if another concussion is sustained it can cause an exacerbation of injury and can sometimes be irreversible

(Giza & Hovda 2014; Hovda et al. 1993; Vagnozzi et al. 2007). Studies by Vagnozzi et al. (2007) (Vagnozzi et al. 2008) observing patients with sports related mTBI, found that although clinical symptoms resolved 3 days post injury, changes in brain metabolism did not resolve until 30 days post-injury, reflecting a window to increased brain vulnerability if a second impact were to occur (Vagnozzi et al. 2007). Similarly within pre-clinical studies, receiving a second mTBI either 3 or 5 days following the initial injury led to worsening of ionic homeostasis, increases in axonal injury and exacerbation of cognitive and motor deficits (Longhi et al. 2005). By increasing the time to the second injury to 7 days this exacerbation of symptoms did not occur, indicating that with a sufficient period of rest, acute exacerbation of symptoms of a mTBI with a subsequent injury can be prevented (Longhi et al. 2005).

1.4 LINK BETWEEN CONCUSSION AND LATER NEURODEGENERATION

Apart from multiple concussive impacts being linked to an acute exacerbation of symptoms, contact sports have also long been linked to the later emergence of disturbances in cognitive function (Guskiewicz et al. 2007), with the first such instance noted in boxers in a study from 1928 describing athletes that appeared “punch-drunk” in nature following repeated blows to the head (Martland 1928). The condition was termed *dementia pugilistica* in 1937 (Millspaugh 1937), and was considered neuropathologically distinct from other neurodegenerative diseases in a study from 1973 (Corsellis, Bruton & Freeman-Browne 1973). The link between repeated concussion and later neurodegeneration then returned to the spotlight with reports of distinct neuropathology within former professional American football players (NFL), as well as others exposed to repetitive concussion including wrestlers, soccer players, rugby players and those in the military (McKee et al. 2013; Omalu et al. 2005; Omalu et al. 2010). A key paper by Omalu *et al* (2005) reported the presence of diffuse Aβ plaques, neurofibrillary tangles (NFTs) and tau-positive neuritic threads in neocortical areas in a former NFL athlete who had a history of cognitive impairment, mood disorder

and parkinsonian symptoms before death (Omalu et al. 2005). The pattern of tau deposition is distinct from other neurodegenerative diseases, with NFTs, thorned astrocytes and dystrophic neurites aggregating in the superficial cortical layers of the brain, particularly at the base of the sulci and surrounding blood vessels (Stern et al. 2011), with this presentation now known as chronic traumatic encephalopathy (CTE). Additional neuropathological features of CTE include deposits of phosphorylated TAR-DNA binding protein 43 (TDP-43) as reactive neuronal cytoplasmic inclusions, persistent neuroinflammation, evidence of axonal injury particularly within the deep cortex and subcortical white matter, as well as loss of white matter, most evident in the corpus callosum (McKee et al. 2009). This is accompanied by gross atrophy, most pronounced in the frontal, temporal and medial lobes (McKee et al. 2016). CTE has been classified into four distinct disease stages that result in an increase in both the severity of clinical symptoms of patients and the associated neuropathology (Table 1). It should be noted that the diagnosis of CTE as its own distinct neuropathology is still under scrutiny and the incidence of what is believed to be pure CTE diagnoses is still unknown.

Although diagnosed post-mortem, CTE has been linked to two types of clinical presentations, with manifestation of symptoms years, sometimes decades after the repetitive concussions were sustained (Stern et al. 2013). The first type of presentation manifests earlier in life at approximately 40 years of age and involves changes in mood, such patients are usually more aggressive, impulsive, physically and verbally violent and depressed (Stern et al. 2013). The second type of presentation manifests at a much older age than the first, at approximately 60 years of age and involves changes in cognition, showing impairments in episodic memory with patients in this category more likely to develop dementia than in the first (Stern et al. 2013). Regardless of the type of initial presentation patients will progressively develop symptoms from both groups.

Stage	Clinical Features	Gross pathological changes	Pattern of tau deposition	TDP-43 immunoreactivity	Axonal injury
I	Loss of attention & concentration, increased aggression	None	Focal epicenters of perivascular pTau in the sulcal depths limited to the superior & dorsolateral frontal cortices	None	Minimal
II	Depression, mood swings, short-term memory loss, loss of attention & concentration, aggression	No cerebral atrophy, mild enlargement of ventricles	pTau pathology in multiple discrete foci of the cortex. Some small NFTs present in hypothalamus, hippocampus, thalamus, substantia nigra (SN).	Some TDP-43 immunoreactivity	Minimal
III	Memory loss, executive dysfunction, explosive behaviours, loss of attention & concentration, depression, mood swings, aggression	Mild cerebral atrophy with dilation of ventricles, septal abnormalities, atrophy of the mammillary bodies & thalamus, thinning of the corpus callosum	NFTs widespread throughout the cortex, hippocampus and amygdala. NFTs also observed in olfactory bulbs, hypothalamus, mammillary bodies and SN.	TDP-43 reactive neurites observed in cerebral cortex, medial temporal lobe & brainstem	Axonal loss & distorted axonal profiles observed in subcortical white matter (frontal & temporal cortices)
IV	Executive dysfunction, memory loss, severe memory loss & dementia, profound loss of attention & concentration, aphasia, explosive behaviours, aggression, paranoia, depression, visuospatial difficulties, suicidal tendencies	Atrophy of the cerebral cortex & white matter, medial temporal lobe, thalamus, hypothalamus & mammillary bodies. Ventricular enlargement, cavum septum pellucidum	Severe pTau abnormalities widespread throughout cerebellum, diencephalon, basal ganglia, brainstem & spinal cord.	Severe TDP-43 immunoreactivity in cerebral cortex, medial temporal lobe, diencephalon, basal ganglia & brainstem	Marked axonal loss in subcortical white matter tracts with distorted axonal profiles

Table 1.1: Proposed progression of CTE stages (adapted from McKee et al. (2013))

1.5 STRUCTURE AND FUNCTION OF TAU PROTEIN

The key feature of neurodegeneration following TBI is accumulation of abnormal forms of tau. Tau is a microtubule associated protein with strong binding affinity for microtubules; its primary function is to provide structural support to the microtubules within axons, therefore enabling axonal transport (Ballatore, Lee & Trojanowski 2007). It is expressed in six different isoforms determined by both their splicing of pre mRNA and by the number of binding domains, with three isoforms expressing three binding domains and three isoforms expressing four binding domains (Goedert et al. 1989; Wang, Xia, et al. 2013). The longest isoform of tau contains 441 amino acids and 80 potential phosphorylation sites, however phosphorylation synonymous with other tau pathologies of neurodegenerative diseases such as Alzheimer's Disease (AD) has been linked to only 40 different phosphorylation sites (Wang, Xia, et al. 2013).

Tau is a phosphoprotein and requires lower levels of phosphorylation to maintain normal function, especially in developmental stages where cytoskeletal plasticity is important (Cohen et al. 2011; Noble et al. 2013; Schwalbe et al. 2015). It is especially important in microtubule formation and stability, and is also associated with promoting neurite outgrowth (Kadavath et al. 2015; Weingarten et al. 1975). The ability of tau to bind to microtubules is maintained by its level of phosphorylation at its proline-rich region and at the C-terminal tail regions (Lindwall & Cole 1984; Mondragón-Rodríguez et al. 2013). Normal phosphorylation levels of pTau are maintained via the action of tau-protein kinases and phosphatases and the addition and removal of phosphate groups from the tau molecule are important in allowing movement of cargo down the microtubules (Mietelska-Porowska et al. 2014; Vershinin et al. 2007). However abnormal phosphorylation of tau at the P-region and C-terminus leads to its detachment from the microtubules which then causes instability and disassembly of microtubule complexes, inhibiting axonal transport (Hong et al. 1998; Mondragón-Rodríguez et al. 2013). When detached from the microtubules, hyperphosphorylated tau also has

the propensity to form insoluble aggregates via its hexapeptide motifs in the repeat domain (von Bergen et al. 2000), developing into the neurofibrillary tangles seen in neurodegenerative diseases such as AD and CTE (Kadavath et al. 2015; McKee et al. 2013). The development of soluble and insoluble tau oligomers is also believed to be involved with disease state tauopathy, with oligomeric tau noted in samples of AD and frontotemporal lobe dementia (FTLD) (Berger et al. 2007; Noble et al. 2013). Oligomeric tau aggregates are formed in the early stages of tau aggregation, display altered confirmation and have been associated with neurodegenerative phenotypes (Berger et al. 2007; Lasagna-Reeves et al. 2011; Lasagna-Reeves et al. 2012). Phosphorylated oligomeric tau species have also been discovered in cortical synapses extracted from AD samples (Henkins et al. 2012), adding weight to the possible role for hyperphosphorylated tau multimers in tau associated neuropathies. In addition to hyperphosphorylation, tau in disease states can become modified in a number of different ways including N- and C-terminal cleavage which can increase its propensity for aggregation (Binder et al. 2005). Indeed, altered confirmation has been shown to be a major determinant in inducing development of tauopathy *in vivo* (Terwel et al. 2005).

The phosphorylation state of tau is highly dependent on the proper regulation of the actions of protein kinases and phosphatases. Many kinases are known to influence tau phosphorylation, with protein kinase A (PKA), protein kinase C (PKC), glycogen synthetase kinase 3-beta (GSK-3β), extracellular signal-regulated kinase (ERK1/2) and cyclin-dependent kinase 5 (CDK5) receiving particular attention (Wang, Xia, et al. 2013). Whilst several serine/threonine protein phosphatases have been shown involvement in dephosphorylation of tau, protein phosphatase 2 (PP2A) appears to be the principal tau phosphatase *in vivo*, accounting for approximately 70% of all human brain phosphatase activity (Liu et al. 2005).

1.6 EVIDENCE THAT MILD TRAUMATIC BRAIN INJURY ALTERS TAU DYNAMICS

As stated earlier, tau hyperphosphorylation and aggregation has long been associated with a history of repeated concussion and is the key diagnostic feature of CTE (McKee et al. 2014; McKee et al. 2013). Indeed, previous work by McKee et al. (McKee et al. 2014) found focal accumulations of pTau at the base of the sulci following rmTBI within 6 months of a reported concussion in post-mortem human tissue. However it is exposure to multiple traumas over many years of participation in contact sports such as boxing, NFL, hockey, rugby and soccer that appears to be determinant of the severity of tau deposition observed in CTE (McKee et al. 2013). Following a rapid acceleration-deceleration brain injury, stretching of the axons occurs resulting in disruption of microtubule assembly and axonal swelling, referred to as diffuse axonal injury (Blennow, Hardy & Zetterberg 2012). This results in impaired axonal transport and disruption of tau binding to tubulin, beginning the process of tau phosphorylation and the development of tau-positive NFTs (Figure 1.2) (Ling, Hardy & Zetterberg 2015).

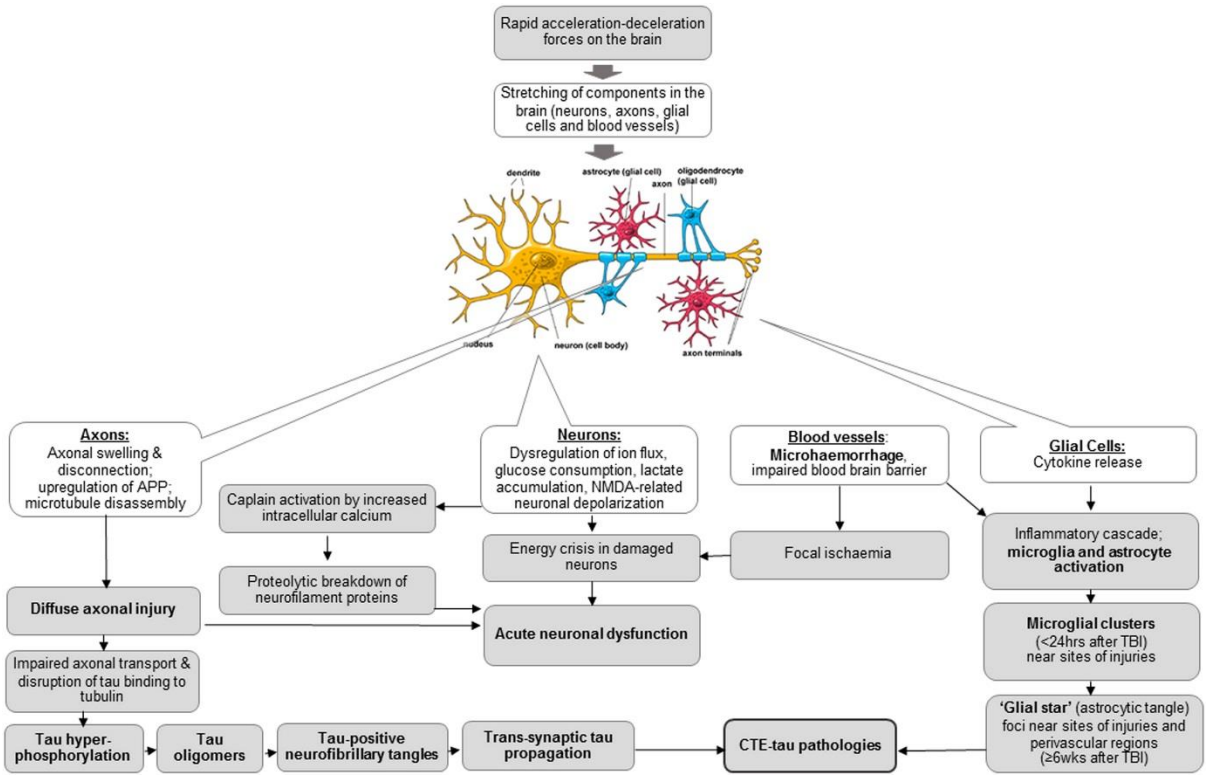


Figure 1.2: Diagram showing changes in tau phosphorylation following rapid acceleration-deceleration injury on the brain, adapted from (Ling, Hardy & Zetterberg 2015).

Many pre-clinical studies on the effects of rmTBI on changes in tau phosphorylation have demonstrated an increase in pTau levels in the chronic phases of injury (Kane et al. 2012; Luo et al. 2014; McAteer et al. 2016). A study by Kane et al. (Kane et al. 2012) found that after exposure to 5 mild closed head impacts spaced 24 hrs apart, levels of pTau were significantly increased in rmTBI animals compared to control animals 30 days following the final injury. pTau increases have also been observed in other closed head models, with increases observed following three mTBIs at a three month timepoint in closed head injury models, in both CCI, 24 hrs apart (Luo et al. 2014) and weight drop, 5 days apart (McAteer et al. 2016). A study by Kondo et al. (2015) also observed changes in tau following both repeated weight drop and single blast injury, specifically changes in cis-tau, which is the pathological conformation of tau observed in AD (Albayram et al. 2017). Cis-tau was found to be increased in rmTBI animals that were injured 7 times across 9 days within the cortex 24 hrs post-injury and had spread to other brain regions including the hippocampus 6 months post-injury. Additionally this study found robust cis-tau pathology at diffuse axons in the frontal cortex in neuropathologically verified human cases of CTE (Kondo et al. 2015). However, these increases in pTau have not been observed in similar models of rmTBI, with a study by Winston et al. (Winston et al. 2016) finding no pTau alterations in transgenic mice expressing human tau, following 30 total impacts over 6 weeks at 24 hr and 30 day timepoints following the final injury. Similarly studies by Mouzon et al. (Mouzon et al. 2012) and Mannix et al. (Mannix et al. 2013) also did not demonstrate changes in pTau following rmTBI. These studies all differ in the animal used, the spacing of the injuries, the number of injuries sustained and the final timepoint which although they cover a wide spectrum of different disease states, it may explain the differences observed.

Although not yet fully studied, the cumulative effects of subconcussive impacts may yet play a role in later neurodegenerative disease development. Many studies have looked at the acute effect of subconcussive trauma in athletes following games or training sessions. A study by Johnson et al. (2014) assessed twenty-four college level rugby players and assessed brain scans of the brain-resting state 24 hrs both pre and post-game. In players with a history of previous concussion, there was a decrease in functional connectivity from the retrosplenial cortex, responsible for spatial awareness and memory and the dorsal posterior cingulate cortex, involved in working memory and attention (Johnson et al. 2014). A long term study by Merchant-Borna et al. (2016) also assessed the effects of multiple subconcussive impacts on white matter integrity at the end of a season of college football. Total head impacts for the season ranged from 431 (running back) to 1850 (center) dependent on playing position, with no clinically evident concussions detected within the study period. The study found correlations between the time between hits combined with the time interval between the hit and post-season DTI assessments and white matter changes observed at the end of the football season (Merchant-Borna et al. 2016). Additionally, a study by Abbas et al. (2015) assessed twenty-two clinically asymptomatic high school American football athletes that showed significantly different changes in functional connectivity measures when compared to non-contact sport controls, even before the start of the season. This suggests that neurological changes as a result of cumulative subconcussive hits may have accumulated over many years of playing the sport, resulting in long term brain changes when compared to their healthy peers (Abbas et al. 2015).

Nonetheless, given the evidence that tau is abnormally phosphorylated following TBI and that this may contribute to later neurodegeneration, it is important to understand how this occurs. Of interest are alterations in the activity of various kinases and phosphatases that control the phosphorylation state of tau following rmTBI.

1.7 PROTEIN KINASES AND PHOSPHATASES INVOLVED IN TAU HYPERPHOSPHORYLATION

1.7.1 GSK-3β

GSK-3β belongs to a family of protein-serine kinases that were first discovered as regulators of glycogen metabolism (Stambolic & Woodgett 1994). GSK-3β is widely expressed throughout the CNS where it localises to neurons and is involved in a number of cellular processes including gene transcription, apoptosis and microtubule stability (Anderton et al. 2001; Hooper, Killick & Lovestone 2008; Woodgett 1990). GSK-3β activity is modulated by its phosphorylation state; with phosphorylation at Ser9 inhibiting its activity and phosphorylation at Tyr216 increasing its activity (Dajani et al. 2001; Hughes et al. 1993). GSK-3β is a serine/threonine kinase that phosphorylates unprimed sites at Ser/Thr motifs, however it has the potential to phosphorylate at sites previously primed by another kinase such as p25 or CDK5 (Mandelkow et al. 1992; Sengupta et al. 1997). Previous work has indicated that with regard to tau protein, GSK-3β primarily phosphorylates at the unprimed Ser396/404 motif and the previously primed Thr231 site (Cho & Johnson 2003)

A study by Shapira et al. (Shapira et al. 2007) found that following mTBI, activity of GSK-3β may actually be inhibited acutely, with phosphorylation at its Ser9 site increased in the hippocampus at 3, 6 and 24 hrs post injury. The inhibition of GSK-3β was confirmed within this study by looking at the downstream protein β-catenin, which is typically degraded with enhanced GSK-3β activity and increased levels of this protein noted acutely (Shapira et al. 2007). A study by Dash et al. (Dash et al. 2011)also found that following moderate CCI injury in rodents, GSK-3β phosphorylation at Ser9 was significantly increased at a 3 day timepoint post-injury. Similarly, a study Zhao et al. (Zhao, Fu, et al. 2012) also observed increases in pGSK-3β at Ser9 72 hrs following moderate CCI injury in a rodent model. It is thought that the acute inhibition of GSK-3β activity via its phosphorylation at Ser9 is protective following TBI, as it has been previously shown to activate pro-apoptotic pathways (Shapira et al. 2007). However further work is needed to determine whether GSK-3β activity is

further altered chronically following TBI and whether this has downstream effects on tau phosphorylation. The protein kinase Akt is also believed to act on the phosphorylation of GSK-3β at Ser9, thus modulating the downstream effects of GSK-3B activation (Zhao, Fu, et al. 2012).

1.7.2 Akt

Akt is a serine/threonine-specific kinase that is involved in multiple key cellular processes including glucose metabolism, apoptosis, cell proliferation, transcription and cell migration (Altomare & Testa 2005; Dudek et al. 1997; Freeman-Cook et al. 2010). Akt is widely expressed throughout the CNS and is activated by its phosphorylation at Thr308 and Ser473 (Fukunaga & Kawano 2003; Ksiezak-Reding et al. 2003). This is mediated by the binding of Akt to lipids generated by phosphatidylinositol 3-kinase (PI3K), which generates signalling lipids that allow recruitment of Akt to the plasma membrane and allows for phosphorylation of Akt by intracellular kinases (Freyberg, Ferrando & Javitch 2010). Akt has been shown to phosphorylate the AD specific sites Thr212 & Ser214 on tau *in vitro*, with prior phosphorylation of Ser214 by Akt blocking PKA, but not GSK-3β activity (Ksiezak-Reding et al. 2003).

Decreases in pAkt have been observed post-TBI previously, with a study by Farook et al. (Farook et al. 2013) finding decreases at 12 and 24 hr timepoints in the pericontusional cortex following a moderate CCI, which also correlated with an increase in TUNEL stained cells at the same timepoints, indicating increased cell death in the acute phase of injury. A study by Zhang et al. (Zhang et al. 2005) observed increases in Akt, both the phosphorylated and total forms, following human traumatic brain injury compared to control cases. This study also assessed changes in a pre-clinical rodent CCI model of TBI, and found increases in pAkt phosphorylated at Ser473 in the ipsilateral cortex and hippocampus 6 hrs post-injury (Zhang et al. 2005). Furthermore, a study by Noshita et al. found decreases in pAkt levels within the CA1 region of the hippocampus as early as 1 hr post-injury in a rodent model of moderate CCI (Noshita et al. 2002). However this effect of injury appeared to rebound, with increases in pAkt levels observed 4 hrs post-injury (Noshita et al. 2002). Interestingly,

pAkt also appeared to co-localise with its downstream signalling elements including pGSK-3β at the 4 hr timepoint (Noshita et al. 2002).

Akt also interacts with the GSK-3β signalling pathway, with Akt phosphorylating GSK3β at Ser9 and therefore inhibiting its activity (Cross et al. 1995; Dajani et al. 2001). Indeed the decrease in in GSK-3β activity seen following TBI may be in part due to activation of Akt, as seen following both mTBI (Shapira et al. 2007). The study by Shapira et al. (Shapira et al. 2007) reported increases in pAkt from 3-24 hrs post-injury within the hippocampus. This appears to be an acute response with a decrease in pAkt levels then seen at 72 hrs post-injury (Shapira et al. 2007).

1.7.3 CDK5

CDK5 belongs to the family of cyclin-dependent kinases that have been shown to play a role in critical neuronal development when combined with its regulatory subunit, p35 (Patrick et al. 1999). The activity of CDK5 is determined by the availability of proteins p35 or p39, which are expressed within neurons and have a short half-life, 30 minutes and 120 minutes respectively (Kimura, Ishiguro & Hisanaga 2014). CDK5 has been reported to phosphorylate tau at between 9-13 sites, including Ser202/Thr205, Ser235 and Ser404 (Illenberger et al. 1998; Kimura, Ishiguro & Hisanaga 2014).

It has been shown that following moderate CCI TBI, CDK5 activity is increased in the injured cortex at 6 hrs post injury as observed in increases in cyclin A & D1 expression, facilitating an increase in cell cycle activation and microglial activation, with treatment via a CDK5 inhibitor reducing these effects (Kabadi et al. 2012). Similar results were observed in another study conducted by Kabadi et al. (Kabadi et al. 2014) which found increases in cyclin G1 and other markers of cell cycle activation following moderate LFP injury, which again were reduced by administration of a CDK5 inhibitor. In AD it has been shown that another regulatory subunit, p25, accumulates in the brains of patients and that when it combines with CDK5 this can have an adverse effect on cytoskeletal structure and

tau hyperphosphorylation, leading to morphological degeneration and apoptosis (Patrick et al. 1999). A study by Yousuf et al. (Yousuf et al. 2016) observed increases in p25 within the ipsilateral hippocampus in a mouse model of moderate CCI 24 and 48 hrs post-injury, with no changes in levels of CDK5 observed at the same timepoints. The same study also reported increased phosphorylation of tau at Ser202 at 2, 24 and 48 hr timepoints post-injury (Yousuf et al. 2016). However further elucidation into how the p25/CDK5 complex responds to rmTBI and tau phosphorylation is still warranted.

1.7.4 ERK1/2

ERK1/2 is a widely expressed protein kinase that is involved in the regulation of cell cycles including mitosis, meiosis and post-mitotic functions in differentiated cells and is also involved in anti-apoptotic cascades (Chang et al. 2003). ERK1/2 is part of a large family of mitogen activated protein kinases (MAPKs) that are believed to phosphorylate tau at a large number of sites, including Ser199/Ser202, Ser235, Ser396/Ser404, Ser424, Thr181, Thr212 and Thr231 (Billingsley & Kincaid 1997). Activation of ERK1/2 occurs via phosphorylation of not only its Tyr202/187 sites, but also phosphorylation of the Thr202/185 sites (Roskoski 2012).

A study by Kuo et al. (2013) (Kuo et al. 2013) showed that following moderate FPI, pERK1/2 levels dramatically increased in the hippocampus at 72 hrs post injury, although pERK1/2 levels were unaffected in the amygdala at the same timepoint (Kuo et al. 2013). In a study by Zhao et al. (2012) (Zhao, Luo, et al. 2012), pERK1/2 levels increased following moderate-severe weight drop TBI, with activation observed 30 minutes post injury, lasting up to 6 hrs post injury before reducing to control levels in the ipsilateral cortex. These results also appeared to correlate with a further study conducted on an *in vitro* scratch model, finding increases in pERK1/2 30 minutes post injury and lasting up to 6 hrs post injury before returning to control levels (Zhao, Luo, et al. 2012). Increases in

pERK immunoreactivity were also observed in a study by Otani et al. (Otani et al. 2002) at 5 minutes post moderate CCI, with gradual decreases observed to sham levels by a 6 hr timepoint. Additionally, a study by Raghupanthi et al. (Raghupathi et al. 2003) found increases in ERK1/2 activation following moderate lateral FPI in the ipsilateral cortex at 2 hrs post-injury, with activation still present 72 hrs post-injury. Furthermore a study by Enomoto et al. (Enomoto et al. 2005) found increases in pERK1/2 in the ipsilateral cortex of rodents following lateral FPI at 10 minutes post-injury, sustained up to 120 minutes post-injury; however in the contralateral hippocampus pERK1/2 levels were transiently increased 10 minutes post-injury, returning to basal levels by 30 minutes post-injury. However changes in ERK1/2 phosphorylation do not appear to change in the chronic phase of injury, with a study by Atkins et al. (Atkins et al. 2009) finding that following moderate FPI in rodents, no significant changes in levels of pERK were observed at 2, 8 or 12 weeks post-injury. Increases in pERK by way of administration of pro-apoptotic drugs have also been linked to an increase in tau hyperphosphorylation at Ser199/Ser202 and that inhibition of ERK1/2 activity reduced this phosphorylation and apoptotic cascades (Guise et al. 2001).

1.7.5 JNK

JNK belongs to the same family of MAPKs as ERK1/2, responding to stressful stimuli such as cytokines as well as playing a role in cellular apoptosis (Ip & Davis 1998). JNK activation occurs through phosphorylation on its Thr183 and Tyr185 motifs by MAP kinase modules (Lin 2003). JNK is believed to phosphorylate tau at the Ser202/Thr205 and Ser422 sites, which are both associated with AD pathogenesis (Ploia et al. 2011; Reynolds et al. 1997; Yoshida et al. 2004).

pJNK levels were also been found to be increased within the hippocampus following moderate FPI TBI 5 minutes post injury, however levels of both phosphorylated kinases declined steadily over a 30 minute period, returning to sham levels (Otani et al. 2002). Additionally JNK activation was found to

be significantly increased in the white matter below the injury site following moderate FPI 2 hrs post-injury, maintaining activation as far as 72 hrs post-injury (Raghupathi et al. 2003). Following moderate CCI TBI, at 24 hrs post injury phosphorylation of JNK was not only found to be increased in injured axons within the ipsilateral cortex and the thalamus, it was also been found to co-localise with pTau phosphorylated at Ser199 (Tran, Sanchez & Brody 2012). In the same study, conducted by Tran et al. (Tran, Sanchez & Brody 2012), treatment with a JNK inhibitor reduced TBI associated tau phosphorylation, however it did not reduce axonal injury. It appears from the literature that activity of MAPKs are indeed increased following TBI, however more research into how MAPK interactions differ in rmTBI and how this may affect abhorrent tau phosphorylation is warranted.

1.7.6 PP2A

PP2A is a serine/threonine phosphatase that regulates the function of many cellular molecules including Akt, p53 and β-catenin via their dephosphorylation (Seshacharyulu et al. 2013). It plays a critical role in numerous cellular processes including cell proliferation, signal transduction and apoptosis (Seshacharyulu et al. 2013). PP2A is composed of a structural A subunit, a catalytic C subunit and a multitude of regulatory B subunits, with a study by Yu, Yoo and Ahn (2014) finding that the B subunit PPP2R2A mediated tau dephosphorylation at Ser199, Ser202/Thr205, Thr231, Ser262 & Ser422. Within the adult brain, PP2A is a primary regulator of tau phosphorylation, both by directly acting on the tau protein and removing phosphate groups at sites Thr181, Ser199/Ser202, Thr205, Ser396 and Ser404, and indirectly by regulation of several tau kinases including PKA, ERK1/2 and GSK-3B (Billingsley & Kincaid 1997; Goedert et al. 1995; Iqbal et al. 2009; Qian et al. 2010). Following a moderate FPI TBI, a study by Shultz et al. (Shultz et al. 2015) found not only significant increases in pTau at Ser198 and Ser262 at 72 hrs post injury, but also significant decreases in PP2A activity at 24 hrs and 72 hrs post injury. Significant increases in pTau and decreases in PP2A activity were also observed at 12 weeks in this study in injured animals, which were attenuated with administration of a PP2A activator, sodium selenate (Shultz et al. 2015). Indeed, following 2 rmTBIs

spaced 5 days apart increases in pTau were observed at 24 hr, 7 days and 12 weeks post injury, with decreases in PP2A activity also observed at the same timepoints and again these changes reverting to sham levels with administration of the PP2A activator (Tan et al. 2016).

Although there is a probable link between TBI, kinase activation and tau phosphorylation, how this results in the neuropathology observed in cases of CTE is currently unknown. In addition to this there is no current explanation for the unique deposition seen in CTE, namely the perivascular accumulation of NFTs or their accumulation at the base of the cortical sulci. However previous studies in TBI have looked at the role of neurogenic inflammation and the neuropeptide Substance P (SP) within the injury process, and it is thought that this may play a role in the development of CTE pathology.

1.8 SUBSTANCE P AND NEUROGENIC INFLAMMATION

SP is a member of the tachykinin family of kinins, which also include calcitonin gene-related peptide (CGRP) and neurokinin A (Vink & van den Heuvel 2010). SP is released from both the central and peripheral endings of sensory neurons and is the most abundant tachykinin in the CNS (Kramer et al. 1998; Otsuka & Yoshioka 1993). SP is a mediator of neurogenic inflammation, defined as local inflammation that arises from the release of inflammatory mediators from sensory neurons; and has a high affinity for the tachykinin receptor neurokinin-1 (NK_1) (Geppetti et al. 2008; Harrison & Geppetti 2001). Blood vessels within the CNS are surrounded by sensory neurons that contain SP and any release of the neuropeptide can potentiate neurogenic inflammation (Vink & van den Heuvel 2010). Release of SP has been shown to promote plasma protein extravasation, vasodilation, oedema and cell migration (Geppetti et al. 1995), as well as having influence on mediators of classical inflammation including the activation of microglia and astrocytes (Corrigan et al. 2016). Studies have shown that following exposure to stressors, SP content is altered in the hippocampus,

septum, periaqueductal grey matter and the ventral tegmental area (Brodin et al. 1994; Lisoprawski, Blanc & Glowinski 1981; Rosen et al. 1992).

1.9 SUBSTANCE P AND THE $TRPV_1$ RECEPTOR

SP is known to be released from sensory neurons, primarily C fibres, and is released perivascularly via the activation of the transient receptor potential vanilloid receptor-1 ($TRPV_1$) (Gazzieri et al. 2007; Ralevic et al. 1990; Vink & van den Heuvel 2010; Woie et al. 1993). The $TRPV_1$ receptor is widespread throughout most tissues including the central nervous system (CNS) and is involved in detection of scalding heat and pain as it is normally activated by noxious heat and chemical stimuli such as capsaicin, the main ingredient in hot chillies (Caterina et al. 1997; Veronesi & Oortgiesen 2006). However, recent data suggests that $TRPV_1$ can become activated via mechanical stimulation such as stretch injury to neurons (Jones, Xu & Gebhart 2005). Indeed, Brederson et al. (2012) (Brederson et al. 2012) demonstrated that administration of a $TRPV_1$ antagonist following mechanical stimulation of peripheral sensory axons reduced mechanical allodynia. Similarly McGaraughty et al. (McGaraughty et al. 2008) found that a TRPV1 antagonist also reduced mechanical allodynia when inflamed peripheral neurons in rats had a mechanical stimulus applied. The physical acceleration and rotational forces involved in TBI could therefore cause the release of SP through the mechanical activation of the TRPV1 receptor.

1.10 THE NK_1 RECEPTOR

The NK_1 receptor is a G protein coupled receptor found throughout the central nervous system and has high specificity for all members of the tachykinin factory, but has highest affinity for SP (Harrison & Geppetti 2001). Expression of NK_1 receptors within the CNS is highest in the caudate putamen and superior colliculus, with expression also noted in the inferior colliculus, hypothalamus, hippocampus, substantia nigra and cerebral cortex (Dam & Quirion 1986; Quirion & Dam 1986). The NK_1 receptor is

also highly expressed in brain regions that are involved in regulation of stress and affective behaviour (Kramer et al. 1998). The binding of SP to the NK_1 receptor is known to elicit the transmission of pain signals and neuroinflammation (Seto et al. 2005; Thornton & Vink 2012). However it is also believed that the NK_1 receptor can act as an auto-receptor and has the potential to modulate SP release through the initial binding of SP, presumably released from the $TRPV_1$ receptor (Malcangio & Bowery 1999; Patacchini, Maggi & Holzer).

1.11 SUBSTANCE P RELEASE IN TRAUMATIC BRAIN INJURY

Many studies have shown that there are significant increases in SP reactivity following TBI. A study by Donkin et al. (Donkin et al. 2009) found significant increases in perivascular SP reactivity following severe TBI at 5 and 24 hr timepoints post injury, and increases in SP in plasma concentrations at 30 minutes post injury. In human TBI, SP immunoreactivity has also been observed, with a study by Zacest et al. (Zacest et al. 2010) finding increases in perivascular SP release as well as increases in cortical neurons and astrocytes. SP immunoreactivity also appears to increase relative to the intensity of injury, with a study by Corrigan et al. (Corrigan, Vink & Turner 2016) finding increases in immunoreactivity as the severity of the injury increased. Following severe TBI, SP is believed to play a major role in the secondary injury processes prevalent post-injury, especially with regards to neuroinflammation, increased BBB permeability and oedema formation (Vink, Gabrielian & Thornton 2017). This may differ dependent on the severity of TBI, as BBB breakdown is less pronounced following mTBI compared to severe TBI (Sahyouni et al. 2017) and oedema not typically observed apart from in exceptional circumstances (McCrory, Davis & Makdissi 2012). Inhibition of neurogenic inflammation by way of depletion of sensory neuropeptides prior to TBI has also resulted in reduction of oedema and BBB permeability, with a study by Nimmo et al. (Nimmo et al. 2004) finding that following chronic treatment with capsaicin, a $TRPV_1$ receptor agonist, TBI related oedema and BBB permeability was indeed reduced. SP may also play a role in determining levels of axonal injury,

as following administration of an NK_1 antagonist in a model of diffuse severe TBI in female rodents, SP immunoreactivity was decreased as well as axonal injury compared to vehicle treated animals (Corrigan et al. 2012). However, how SP release responds to rmTBI has not yet been fully investigated.

1.12 SUBSTANCE P RELEASE IN REPETITIVE INJURY

An important point to emphasise, especially in the context of rmTBI, is that SP release is proportional to the intensity and frequency of stressful stimuli, and that previous exposure to stress, such as neuroinflammation, can amplify the interactions between SP and the NK_1 receptor (Mantyh 2002; Stucky, Galeazza & Seybold 1993). If the intensity or the frequency of these stressful stimuli is further increased, this can have a two-fold effect, through the increased activation of NK_1 receptors adjacent to the site of release and through diffusion of SP further away from the site of release, allowing for the stimulation of more distant neurons (Mantyh 2002). Allen et al (1997) demonstrated that in response to more intense or frequent stimuli, approximately three to five times more neurons are activated compared to lesser forms of injury. Given the neuroinflammatory cascade that is observed following mTBI, it is plausible that repetitive injuries can exacerbate SP and NK_1 interactions, causing an increase in protein kinase activity and subsequent tau hyperphosphorylation.

1.13 NK_1 RECEPTOR AND KINASE INTERACTIONS

Of interest, NK_1 activation has also been linked to the activation of protein kinases that are also involved in tau phosphorylation, most notably ERK1/2. As discussed previously, ERK1/2 activation as well as NK1 activation are known to both be increased following TBI and therefore present an interesting pathway for tau phosphorylation following rmTBI. A study by Monastyrskaya et al.

(Monastyrskaya et al. 2005) found that *in vitro*, activation of NK_1 receptors via application of SP increased phosphorylation of ERK1/2 in human aortic smooth muscle cells. A study by Ji et al. (Ji et al. 2002) also found that ERK1/2 activation can also have effects on NK_1 expression, with sustained activation of ERK1/2 producing upregulation of NK_1 in dorsal horn neurons. The effects of NK_1 signalling on Akt have also been examined, with a study by Akazawa et al. (Akazawa et al. 2009) finding that stimulation of NK_1 by SP in glioblastomas increases Akt phosphorylation, and that blockade of NK_1 by an antagonist lowers basal phosphorylation of Akt. Given that Akt has the potential to both phosphorylate tau at Thr212 & Ser214 (Ksiezak-Reding et al. 2003), as well as interacting with GSK-3B via its phosphorylation at Ser9 thereby inhibiting GSK-3B activity (Zhao, Fu, et al. 2012), this provides an interesting avenue to discover how NK_1 interactions with Akt influence tau phosphorylation. The potential activation of other protein kinases including GSK-3β, CDK-5 and JNK by NK_1 has not yet been investigated but provides an interesting pathway for aberrant tau phosphorylation following SP release in TBI. Given the importance of the tau protein in maintaining microtubule stability, its abnormal phosphorylation via the proposed cascade involving mechanostimulation of $TRPV_1$, subsequent SP release, NK_1 activation and protein kinase activation may explain the neuropathology observed in CTE (Figure 1.2).

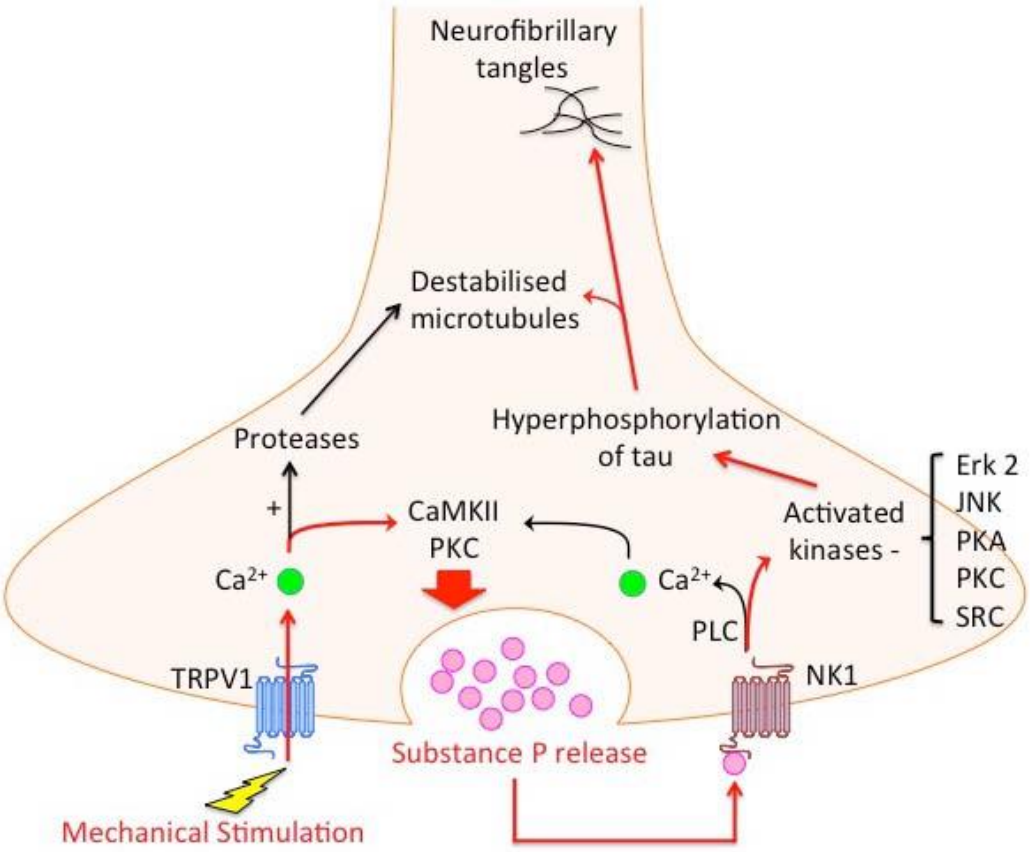


Figure 1.3: Working model of the biochemical events occurring after mTBI. Stretch injury caused by mTBI activates the $TRPV_1$ receptor, triggering the release of SP. SP then binds to the NK_1 receptor, activating a range of kinases known to act on the tau protein. Tau then becomes hyperphosphorylated, causing destabilisation of microtubules and formation of NFTs.

1.14 CONCLUSIONS AND AIMS

The central question to be answered by this project is that of the involvement of SP release and NK_1 activation in rmTBI and whether SP release is amplified in response to multiple traumatic insults. This project also aims to determine the effects of TBI on the $TRPV_1$ receptor and whether blocking the initial release of SP will attenuate changes in acute tau phosphorylation. This project will also attempt to characterise the differences between severe and rmTBI in determining SP release and whether this has any downstream effects on kinase activation and tau phosphorylation. Following this, this project will aim to determine the effects of NK_1 blockade on SP release following rmTBI and whether this will have positive outcomes in the acute and chronic phases of injury. Therefore the overall hypothesis for this study is that SP release, facilitated by mechanostimulation and activation of the $TRPV_1$ receptor, results in tau hyperphosphorylation and functional differences as observed in rmTBI and CTE and that administration of a NK_1 antagonist will improve these outcomes following injury.

As such the broad aims of this thesis are:

- To determine the time course of SP release, $TRPV_1$ expression, NK_1 expression and tau phosphorylation following smTBI, rmTBI and severe injury.
- To determine whether blockade of the $TRPV_1$ receptor prior to injury will have any effects on SP release and the downstream effects following both mild and severe TBI.
- To determine whether administration of an NK_1 antagonist will improve outcomes in the acute and chronic phases of injury following rmTBI.

Chapter 2: Materials and Methods

2.1 ANIMALS

2.1.1 Ethics

All experimental procedures were conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th edition (2013), established by the National Health and Medical Research Council. All experimental procedures were approved by the University of Adelaide Animal Ethics Committee (M-2012-225B).

2.1.2 Animal Preparation

12 week-old male Sprague-Dawley rats (360-380g) were used for all experiments (University of Adelaide Waite Campus). 12 week male Sprague-Dawley rats were used in these experiments as they have been used with a model of impact acceleration TBI to produce a moderate-severe TBI within the laboratory previously (Corrigan et al. 2012) and to allow for direct comparisons between the severe and rmTBI components of the experiments. Animals were group housed in a conventional facility on a 12 hour day-night cycle with a standard diet of rodent pellets and water *ad libitum*. All animals were acclimatised to the housing facilities for several days following delivery before inclusion in any experiments.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Induction of Mild Traumatic Brain Injury

Animals were injured using a modified version of the Marmarou impact acceleration model of diffuse TBI to allow an average of 110g of linear acceleration force (Li et al. 2011; Marmarou et al. 1994) (Figure 2.1).

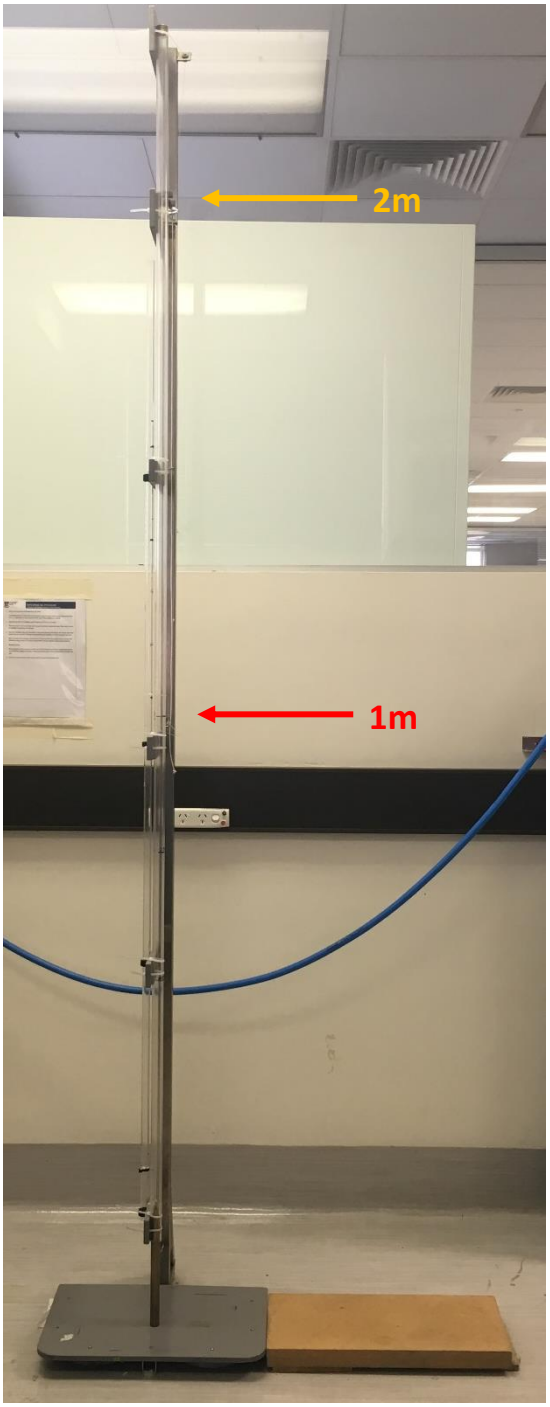


Figure 2.1: Image of the modified Marmarou impact acceleration device. mTBIs were induced via release of the 450g weight at a height of 1m (red). Severe TBI was induced via release of the weight at a height of 2m (yellow).

Animals were anaesthetised via inhalation of 5% isofluorane (Henry-Schein) using a normoxic mixture of air (70% nitrogen, 30% oxygen; BOC), at a rate of two litres per minute. Once absence of reflexes was apparent, animals were maintained on 2% inhalational isofluorane via a nose cone for the duration of the procedure. The head of the animal was then cleaned with 70% ethanol and then shaved using hair clippers and a midline incision made using a No. 22 scalpel to expose the skull and the connective tissue was removed. A polyacrylamide adhesive (Bostik) was then used to adhere a stainless steel disc (10mm diameter, 3mm depth) directly to the skull of the animal centrally between the lambda and bregma sutures. Animals were then removed from isofluorane and secured to a foam bed (Type E bed foam, Foam to Size). Once secured, the animal was placed under the injury device and TBI delivered via the release of a 450g brass weight from a height of one meter directly onto the steel disc. Following the injury, the animal was rapidly removed from the injury device to prevent a rebound hit, removed from the foam bed and placed back onto 2% inhalational isofluorane and checked for skull fractures. The steel disc was then removed, the incision closed using wound clips (AUTOCLIP Wound Clips, 9mm, Benton Dickinson) and 0.2mL of lignocaine (Ilium) applied subcutaneously for local anaesthesia. The animal was then removed from anaesthesia and allowed to recover in a warmed box breathing room air. Animals not scheduled to receive an injury on that day underwent the same surgical procedures without being exposed to the injury, as outlined in Table 2.1. Animals in the repetitive injury group received three injuries, spaced five days apart over a ten day period. This spacing was based on previous work, (Shultz et al. 2012) with the spacing allowing for resolution of the inflammatory responses occurring between injuries that would be seen two to four weeks following a concussion in a human (McAteer et al. 2016). Single injury animals underwent two surgical procedures without injury, then received an injury on the final surgical day, all spaced five days apart over a 10 day period. Sham animals received a total of three surgical procedures but no injuries, spaced five days apart over a 10-day period.

Experimental Group	Experiment Day 0	Experiment Day 5	Experiment Day 10
SHAM	No injury	No injury	No injury
Single mTBI (smTBI)	No injury	No injury	**mTBI**
Repetitive mTBI (rmTBI)	**mTBI**	**mTBI**	**mTBI**

Table 2.1: Injury schedule for repetitive mild TBI animal groups

2.2.2 Induction of Severe Traumatic Brain Injury

Animals were also injured using the Marmarou impact acceleration model of diffuse TBI (Marmarou et al. 1994) (Figure 2.1), but the height from which the weight was dropped was increased to two metres, providing a linear acceleration force of approximately 250 g (Li et al. 2011). Animals were anaesthetised via inhalation of 5% isofluorane using a normoxic mixture of air (70% nitrogen, 30% oxygen, BOC) at a rate of two litres per minute. Once an absence of reflexes were apparent, the animal was then transferred to a vertical support, suspended by its upper incisors and a laryngoscope used to visualise the trachea. An endotracheal tube was then inserted into the trachea and the animal transferred to a ventilator (Harvard Apparatus). Animals were mechanically ventilated at a stroke rate of 85 strokes per minute on 2% inhalational isofluorane using a normoxic mixture of air. Once the animal was stable on the ventilator, helmet placement occurred as described above. Animals were then removed from isofluorane with the endotracheal tube still inserted and secured to a foam bed. Once secured, the animal was moved into the injury device and the weight released directly onto the metal steel disc. Following the injury, the animal was removed from the foam bed, placed back onto the ventilator receiving 2% inhalational isofluorane and checked for skull fractures. Animals then underwent a hypoxic period where they received a 90% nitrogen, 10% oxygen mix of air (1.8L N_2; 0.2L O_2) for 10 minutes to simulate loss of oxygen to the brain following a severe TBI due to cessation of breathing. During this period, the incision was closed using wound clips and 0.2mL of lignocaine (Ilium) applied subcutaneously for local anaesthesia. Animals also received 5mL of sterile 0.9% saline (Baxter Healthcare, AHB1324) subcutaneously to

prevent dehydration following injury. Once the hypoxic period had finished, the air supplied was turned back to a normoxic (1.4L N_2, 0.6L O_2) mix and anaesthesia was turned off to allow the animal to recover. Once the animal had regained normal breathing patterns the endotracheal tube was removed and the animal was allowed to recover in a warmed box breathing room air. Sham operated animals underwent all surgical procedures without receiving injury or hypoxia.

2.2.3 Post Surgery Recovery & Animal Monitoring

Animals were closely monitored in a heated recovery box immediately following injury. Once the animal had regained its righting reflex and appeared stable it was allowed to return to its home cage. Animals were continuously monitored in the days following injury, with wet food and 5mL of 0.9% saline given subcutaneously if the animal lost more than 5% of its initial starting weight in a 24 hour period.

2.3 DRUG PREPARATION AND ADMINISTRATION

2.3.1 Capsazepine ($TRPV_1$ antagonist)

Capsazepine (Sigma-Aldrich, C191) was stored at 4°C and prepared on the day of use. A 10^{-1} molar stock solution was prepared by adding 663µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, D2650) to 25mg of solid capsazepine. To make up the drug for administration, a solution of 10% polyethylene glycol sorbitan monooleate (Tween 80, Sigma-Aldrich, P6224) 10% ethanol (Chem Supply) and 80% 0.9% sterile saline (Baxter Healthcare, AHB1324) was prepared and 3.063mL of this solution was then added to 464µL of the 10^{-1} stock capsazepine to produce a final concentration of 5mg/mL. The vehicle for any experiment using capsazepine as the experimental drug consisted of a 10% ethanol, 10% Tween 80, 10% DMSO, 70% saline mixture that was prepared at the same time.

2.3.2 N-acetyl L-tryptophan (NK_1 antagonist)

N-acetyl L-tryptophan (NAT, Sigma-Aldrich, A6376) was stored at 4°C and prepared on the day of use. 1.25mg of NAT was dissolved in 5mL of 0.9% sterile saline (Baxter Healthcare, AHB1324) and then neutralised to pH 7.0-7.4 using hydrochloric acid (HCl) or sodium hydroxide (NaOH) to produce a final concentration of 2.5mg/kg. This drug and final concentration was used as it had been used within the laboratory previously in a model of severe TBI, with no adverse effects observed (Donkin et al. 2011).

2.3.3 EU-C-001 (NK_1 antagonist)

EU-C-001 was stored at 4°C and prepared on the day of use. 10mg of EU-C-001 was dissolved in 10mL of warmed 0.9% sterile saline (37°C, Baxter Healthcare, AHB1324) and was kept agitating and at 37°C until used, producing a concentration of 1mg/kg. This drug and final concentration was used as it had been used within the laboratory previously in a model of severe TBI, with no adverse effects observed (Donkin et al. 2011). This NK1 antagonist was chosen as it is lipid soluble and can more easily cross the BBB and allows for comparison against the non-lipid soluble NAT (Donkin et al. 2011)

2.3.4 Drug Administration

All animals received experimental drugs intravenously via the tail vein under anaesthesia. Animals were anaesthetised with 5% inhalational isofluorane for three minutes, then transferred to a nosecone where they received 1.5% inhalational isofluorane to maintain anaesthesia. The animal's tail was then cleaned and bathed in warm water to dilate the tail veins. Following this, the drug or vehicle injection was then delivered as a slow intravenous bolus using a 25G, 5/8th inch needle (Benton Dickinson) at a dosage of 1mL/kg. The animal was then removed from anaesthesia and allowed to recover in a warmed box before being returned to their home cage.

2.4 FUNCTIONAL OUTCOME TESTING

All functional outcome measures were undertaken at six and twelve weeks post injury. Experiments were performed at the end of the animal's dark cycle to ensure that performance would not be affected by the time of day. All measurements were tracked and measured using ANY-maze behavioural testing software (Stoleting Co., USA). Animals undertook each of the functional outcome tests in order from least stressful (Open Field) to most stressful (Elevated Plus Maze) over a nine day testing period (Figure 2.2)

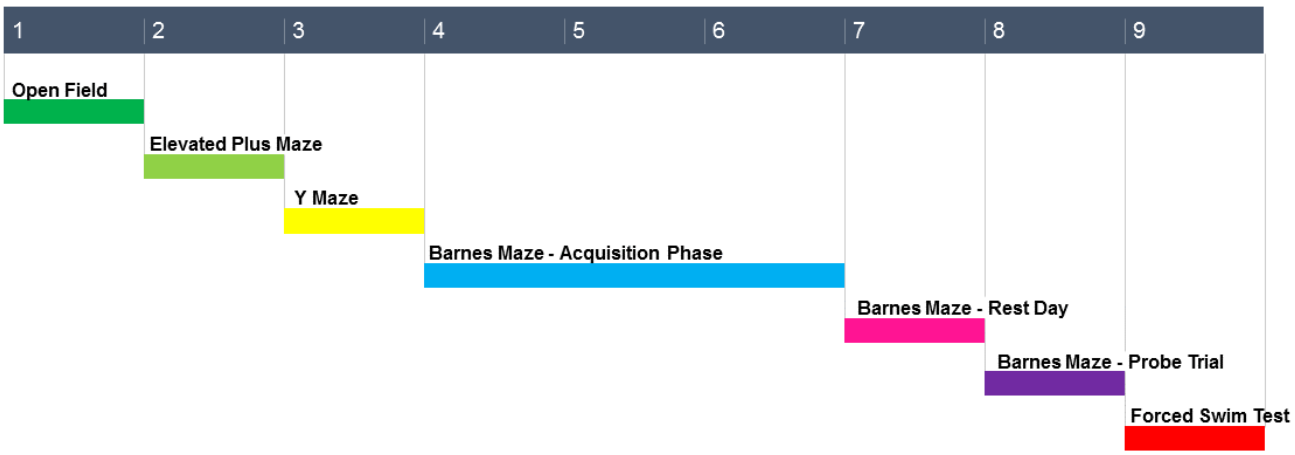


Figure 2.2: Functional Outcome testing schedule

2.4.1 Open Field

The open field is a common measure of exploration and anxiety in rodents (Gould, Dao & Kovacsics 2009). It is comprised of a 1m x 1m box divided into a 10 x 10 grid in which the animal is placed in the centre and allowed to explore freely for five minutes (Figure 2.3A). This test is based on the innate curiosity of the rat, as they are normally quite curious they will explore the entire area quite readily, whilst anxious animals will stick to the walls and corners of the box. Animals were placed in the centre of the arena and the experimenter left the room immediately afterwards. The distance travelled by the animal and time spent in outer and inner zones was recorded using ANY-maze

software. The number of and time spent grooming and rearing events was recorded manually by the experimenter using video output and ANY-maze software.

2.4.2 Elevated Plus Maze

The elevated plus maze is a common measure of anxiety in rodents (Walf & Frye 2007). The maze is comprised of two "closed" and two "open" arms (118cm length, 15cm width, 23cm height, Figure 2.3B), with rats exhibiting anxious behaviours preferring the closed arms over the open arms. The animal is placed in the centre of the maze, at the junction of the four arms facing towards the open arms and is allowed to explore the maze freely for five minutes. Animal movements, including distance travelled and time spent in each arm, as well as head time spent in each arm were recorded using ANY-maze software.

2.4.3 Y Maze

The Y Maze is designed to assess spatial and recognition memory in rodents, utilising the rodent's innate curiosity and its desire to explore new areas (Conrad et al. 1996). The three arms are arbitrarily assigned into start, novel and other arms, with these randomly alternated between animals (Figure 2.3C). The animal is first introduced into the maze with the novel arm blocked off and allowed to freely explore for three minutes. One hour after the initial exposure the rat is reintroduced into the maze with all three arms open and allowed to explore freely for three minutes (Figure 2.3D). Normal animals are curious to explore the newly introduced area, whereas animals that are otherwise compromised will tend to stay within the two familiar areas. Animal movements, including arm entries and amount of time spent in each arm were recorded using ANY-maze software.

2.4.4 Barnes Maze

The Barnes maze is a common cognitive task used to assess learning and memory in rodents and utilises the rodent's innate behaviour to seek small darkly lit spaces in preference to wide-open, brightly-lit spaces (Barnes 1979). It consists of a circular maze 1.2m in diameter, with eighteen

escape holes (5cm diameter) placed around the circumference, with an escape box located underneath one of these holes (Figure 2.3E). A floodlight is turned on when the animal is placed in the centre of the maze as an aversive stimulus to motivate the animal to find the escape box. Visual stimuli, such as bright pieces of paper are also placed around the maze to help the animal self-orientate. At the start of the trial, the animal is placed in the centre of the maze and allowed to freely explore for three minutes. If the animal discovers and enters the box within this time, the aversive stimulus is switched off and the animal removed from the maze and immediately allowed to return to its home cage. The time taken for the animal to located and enter the escape box is recorded as the Barnes maze latency. If the animal fails to find the escape box, or if the animal finds the escape box but does not enter within the allotted three minutes, it is gently guided to the box by the experimenter and detained for twenty seconds with all aversive stimuli switched off before being returned to its home cage. Animals undertook two trials, fifteen minutes apart over a three day period and then were given one day to rest, with a probe trial taking place on the fifth day. The probe trial involves moving the location of the escape box 90° from its original position to assess the animal's ability to learn the new location of the escape box. As with the normal trials, animals have three minutes to freely explore the maze and locate the escape box. Animals undertook two trials, fifteen minutes apart on the probe trial day. Animal movements, including distance travelled, time spent in the maze and in each quadrant of the maze, as well as time taken to locate and enter the escape box were recorded using ANY-maze software.

2.4.5 Forced Swim Test

The forced swim test (FST) is a common assessor of depression in rodent models (Slattery & Cryan 2012). A glass cylinder (25cm diameter x 60cm height) is filled with water (23-25°C) to a depth of 30cm (Figure 2.3F). Animals were placed in the water for a period of five minutes and their movements tracked using ANY-maze software. Normal animals will continuously move for the entire test period, whereas animals displaying depressive-like symptoms will have an increase in time spent immobile. Behaviours such as swimming (horizontal movements), climbing (vertical movements) and

immobility (no movement) were recorded manually by the experimenter using ANY-maze software. On completion of the trial, animals were immediately dried off using hand towels and placed in a pre-warmed cage for at least 20 minutes before being returned to their home cage.

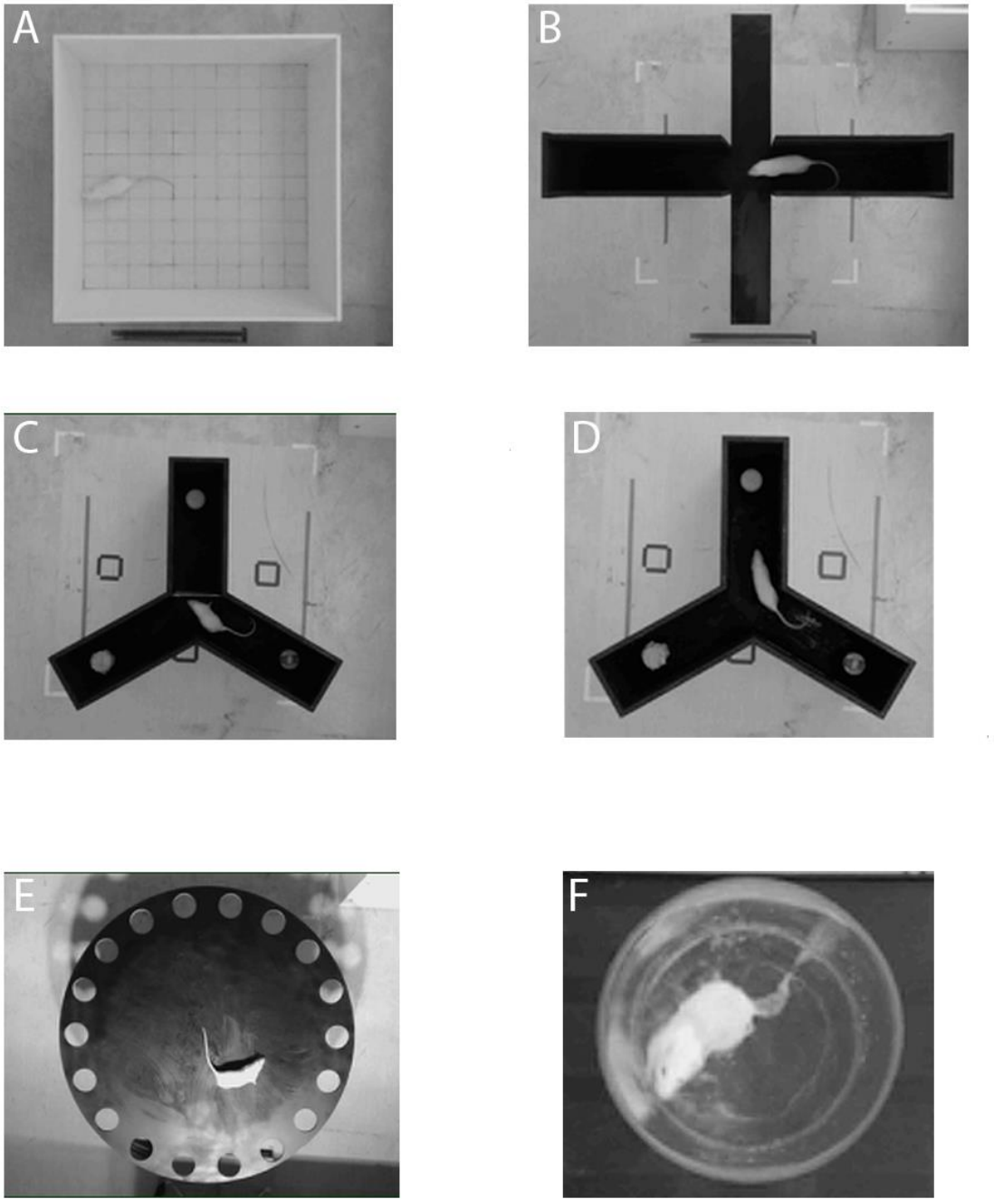


Figure 2.3: Overhead images of the functional outcome tests performed, including the Open Field (A), Elevated Plus Maze (B), Y Maze first trial with closed off novel arm (C), Y Maze second trial with open novel arm (D), Barnes Maze (E) and Forced Swim Test (F).

2.5 BIOLOGICAL SAMPLE COLLECTION

2.5.1 Collection of Fresh Tissue

At the specified time points of each study, animals were anaesthetised via 5% inhalational isofluorane until absence of reflexes was apparent. Animals were then transferred to a nose cone where anaesthesia was maintained for the duration of the procedure. A midline incision was made using surgical scissors across the abdomen of the animal to expose the liver and diaphragm. The ribcage was then opened and the diaphragm removed to visualise the heart. A blunt 19G needle (Benton Dickinson) connected to a peristaltic pump (Langer Instruments, BT100-2J) was then inserted into the apex of the heart and the tip of the needle advanced into the ascending aorta. The right atrium was then incised to allow vascular flushing. A solution of 0.9% saline was then perfused into the heart at a rate of 30rpm for approximately 7 minutes, until the perfusate ran clear. The animal was then disconnected from the pump, immediately decapitated and the brain removed. The cortex directly underneath the impact site and hippocampus were dissected out, separated into left and right hemispheres and immediately snap frozen in liquid nitrogen (BOC). Samples were stored at -80° until use.

2.5.2. Collection of Fixed Tissue

Animals were prepared as per Section 2.5.1, with the use of 10% buffered formalin (Australian Biostain Pty Ltd) instead of 0.9% saline to allow for fixation of brain tissue. Following the end of the procedure, animals were left intact for 2 hours prior to whole brain removal. Samples were stored in 10% buffered formalin for at least 48 hours before processing (as described in section 2.7.1).

2.6 FRESH TISSUE ANALYSIS

2.6.1 Tissue Homogenisation

Frozen cortical samples were removed from -80°C storage and allowed to defrost on ice. Once defrosted, samples were weighed and a proportional amount of the homogenisation buffer placed in a standard Eppendorf tube in 10 times brain region weight of standard radioimmunoprecipitaion

(RIPA) buffer (150mM sodium chloride, 50mM Tris pH 8.0, 0.5% sodium deoxycholate, 0.1% sodium deoxycholate, 1% NP-40) and 10µL/mL protease inhibitor cocktail (Sigma-Aldrich, P8340). Samples were homogenised on ice using a handheld pellet pestle (Sigma-Aldrich, Z359971) then centrifuged at 15000rpm for 30 minutes at 4°C. The supernatant was then aliquoted into standard Eppendorf tubes and stored at -80°C until use.

2.6.2 Protein Estimation Assay

Protein levels in each sample were assayed using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, 23225). 25µL of known bovine serum albumin (BSA) standards (0, 0.04, 0.2, 0.4, 1 µg/µL) were loaded in triplicate into a standard 96 well microplate (Corning Costar). Samples were diluted in the well (4µL sample, 21µL milliQ dH_2O, total 25µL) and loaded in triplicate, including a blank lysis buffer in place of the sample to allow for background readings to be subtracted from the final optical density. Kit reagents A & B were mixed at a 50:1 ratio to create the working reagent. 200µL of the working reagent was added to each well and the plate was placed on a plate shaker for 30 seconds to allow the well contents to mix thoroughly. The plate was then incubated at 37°C for 30 minutes and then immediately read on a microplate reader (BioTek) at 562nm. The protein content of each sample was determined by calculating the linear regression of the standard protein concentrations and extrapolating the values of the unknown samples to the equation produced. If the R^2 value of the linear regression of the BSA standards was below 0.95, the assay was repeated to ensure validity.

2.6.3 Substance P ELISA

Samples were assayed using a SP Parameter Assay Kit (R&D Systems, KGE007). Standards were prepared according to manufacturer's instructions to produce a 7 point curve (78.1, 156, 313, 625, 1250, 2500 pg/mL). 100µL of Calibrator Diluent RD5-45 (RD5-45, R&D Systems, 895905) was loaded in duplicate to allow for a non-specific binding (NSB) reading. 50µL of RD5-45 was loaded in duplicate to provide a zero point for the standard curve. 50µL of each standard was loaded in

duplicate. 200µg of each sample was loaded into each well as per protein estimations and RD5-45 added to bring the total well volume to 50µL and assayed in duplicate. 50µL of Primary Antibody Solution (R&D Systems, 893079) was added to each well, excluding the NSB wells. 50µL of Substance P Conjugate (R&D Systems, 893078) was then added to each well and the plate sealed using an adhesive strip. The plate was then inserted into a horizontal orbital microplate shaker (ThermoFisher Scientific) and incubated at room temperature for three hours at 500 ± 50 rpm.

On conclusion of the incubation period, the plate was washed using an automated plate washer (Bio-Plex Pro, Bio-Rad) primed with wash buffer (R&D Systems, 893003). The plate was aspirated and washed four times with 400µL of wash buffer per wash. During the wash procedure, kit components Colour Reagent A (R&D Systems, 895000) and Colour Reagent B (R&D Systems, 895001) were mixed in equal quantities to create the substrate solution and protected from light using aluminium foil. Following the final aspiration, 200µL of substrate solution was added to each well. The plate was protected from light using aluminium foil and incubated on the benchtop at room temperature for 30 minutes. Once 30 minutes had elapsed, 50µL of Stop Solution (R&D Systems, 895032) was added to each well and the plate immediately read on a microplate reader (BioTek). Readings were taken at 450nm and 570 nm to allow for wavelength correction. The measurements taken from 570nm were subtracted from the measurements taken at 450nm to compensate for optical imperfections in the plate. Duplicate readings for each standard, control and sample were averaged and the average NSB optical density was subtracted from each value. A four parameter logistic curve was generated using GraphPad Prism software (GraphPad Software) and unknown sample optical density measurements were interpolated onto the standard curve to determine SP concentrations.

2.6.4 Cell Signalling Assay

To analyse kinase activity, including the total and phosphorylated forms of Akt, MAPK/ERK1, JNK and GSK-3β, assays were performed using custom made Bio-Plex Pro Cell Signalling Assay kits (Bio-Rad). Positive and negative controls provided by the manufacturer were prepared by reconstituting with 250µL of milliQ dH_2O, vortexed and incubated at room temperature for 20 minutes. Samples were

removed from -80°C storage and allowed to defrost on ice. Coupled magnetic beads as provided by the manufacturer were diluted to 1x using the provided wash buffer. Magnetic beads were vortexed for 30 seconds then 288µL was diluted into 5,472µL of wash buffer and vortexed again. 50µL of the diluted beads were then immediately added to each well of the assay plate. The plate was then washed twice with 200µL of wash buffer using an automated plate washer (Bio-Plex Pro, Bio-Rad). 50µL of blank (detection antibody diluent), samples and controls were then loaded into the plate and the plate sealed with adhesive tape. The plate was then covered with aluminium foil and incubated on a horizontal orbital plate shaker (ThermoFisher Scientific). To fully resuspend the beads, the plate shaker was set to 900-1,100 rpm for 30 seconds, then for incubation turned down to 500 ± 50 rpm for 15-18 hours at room temperature.

Following the incubation, the adhesive tape was removed and the plate washed three times with 200µL of wash buffer in an automated plate washer. During this the detection antibodies were prepared by vortexing the provided 20x stock solution for 30 seconds, then adding 150µL to 2,850µL of detection antibody diluent for a total volume of 3mL. 25µL of diluted detection antibody was added to each well, then the plate sealed with adhesive tape and covered with aluminium foil. The plate was then incubated on a plate shaker and beads resuspended at 900-1,100 rpm for 30 seconds. The plate shaker was then set to incubate at 500 ± 50 rpm for 30 minutes at room temperature. Following this incubation the adhesive tape was removed and the plate washed three times with 200µL of wash buffer in an automated plate washer. Streptadavin-PE (SA-PE) was then prepared from stock by taking 60µL of 100x stock and diluting in 5,940µL of detection antibody diluent for a total volume of 6mL. 50µL of the 1x SA-PE solution was then added to each well of the washed plate, the plate sealed with adhesive tape and wrapped in aluminium foil. The plate was placed back on the plate shaker and beads resuspended at 900-1,100 rpm for 30 seconds. The plate was then incubated at 500 ± 50 rpm for 10 minutes at room temperature. Following this the sealing tape was removed and the plate washed three times with 200µL of wash buffer in an automated plate washer. 125µL of resuspension buffer was then added to each well and the beads resuspended

by sealing the plate and placing on a plate shaker at 900-1,100 rpm for 30 seconds. The plate was then loaded into a Bio-Plex MAGPIX reader (Bio-Rad) and the output read by Bio-Plex Manager Software (Bio-Rad). Readings were then collated and analysed using GraphPad Prism statistical software (GraphPad Software).

2.6.5 Western Blot

2.6.5.1 Gel Electrophoresis and Transfer

Samples for gel electrophoresis were prepared by adding 10µL of 4x Bolt LDS Sample Buffer (Life Technologies, B0007), 4µL of 10x Bolt Sample Reducing Agent (Life Technologies, B0009) and 30µg of sample as determined by protein estimation, made up to a total volume of 40µL using milliQ dH_2O. Samples were then heated in a dry bath to 70°C for 10 minutes before gel loading. Pre-cast Bolt 4-12% Bis-Tris Plus 12 well gels (Invitrogen, NW04122) were prepared and placed into a standard electrophoresis chamber filled with 400mL of 1x Bolt MOPS SDS Running Buffer (Life Technologies, B0001). The empty wells were filled with running buffer then 40µL of sample was loaded into each well. 4µL of protein ladder (Bio-Rad Precision Plus Protein Dual Colour Standards, 1610374) was added to each well in the left most lane to allow for visualisation of molecular weights. Once loaded, the electrophoresis chamber was closed and connected to power. Gels ran at 100W, 500mA for 2.5 hours.

Once electrophoresis was completed, gels were removed from their plastic casing and placed into an iBlot 2 Dry Blotting System (Life Technologies). Gels were transferred onto polyvinylidene difluoride (PVDF) membranes using pre made transfer stacks (Life Technologies iBlot 2 Transfer Stacks, PVDF, mini, IB24002), using a pre-set program consisting of 20V for 1 minute, 23V for 4 minutes then 25V for 2 minutes. Once the transfer had completed, membranes were washed in tris-buffered saline with 0.1% Tween 20 (TBS-T) 3x for 5 minutes each. Protein transfer to the membrane was then visualised using commercial Ponceau S Solution (Sigma-Aldrich, P7170). Once the membrane had been visualised, the Ponceau S solution was washed off using TBS-T and primary antibody

application was performed with one of two methods, dependant on the antibody and which are detailed below in Table 2.2. The two differing incubation methods were optimised to produce the best blots for each antibody and to avoid minimising wastage of materials.

Target	Manufacturer/Cat No.	Host/Clonality	Dilution	Primary Antibody Incubation Method
Total Tau (Tau-5)	Millipore; 577801	Mouse monoclonal	1:500	iBind
pTau (T231)	Abcam; ab151559	Rabbit monoclonal	1:500	iBind
NK_1	Abcam; ab183713	Rabbit monoclonal	1:1000	Skim milk
$TRPV_1$	Novus Biologicals; NB100-98897	Rabbit polyclonal	1:1000	Skim milk

Table 2.2: Details of primary antibodies used in western blotting.

2.6.5.2 iBind Antibody Application

1x iBind Fluorescent Detection (FD) solution (Life Technologies, SLF1019) was prepared according to manufacturer's instructions. Briefly, 6mL of iBind 5x FD Buffer and 75µL of iBind 100x Additive was added to 23.9mL of milliQ dH_2O and was used for antibody dilution, washing and blocking of non-specific binding within the iBind device. The iBind device was loaded with an iBind card (Life Technologies, SLF1010) and soaked in 6mL of 1x iBind FD solution. The membrane was then placed onto the iBind card in the specified area and rolled out gently to remove any air bubbles. The iBind device was then shut to allow for loading of reagents. 2mL of primary antibody including housekeeper (GAPDH, Abcam ab14247, 1:1000) at the required concentration (as per Table 2.2) was added to the first chamber followed by 2mL of iBind 1x FD solution in the second chamber. 2mL of fluorescent secondary antibodies (LI-COR 800CW donkey anti-rabbit/anti mouse, 1:3000; LI-COR 680LT donkey anti-chicken, 1:2000) were loaded into the third chamber and 6mL of 1x iBind FD Solution was loaded into the last chamber. The iBind unit was then incubated overnight at 4°C under dark room conditions. Following the incubation, the membrane was removed from the iBind device and washed 3x TBS-T for 5 minutes each before being visualised on an Odyssey Fluorescent Imaging

System using Image Studio Lite Software (LI-COR). Images were then converted to black and white and analysed in ImageJ 1.48 (National Institute of Health). Output readings were analysed using GraphPad Prism statistical software (GraphPad Software).

2.6.5.2 Skim Milk Antibody Application

Following the wash steps, membranes were placed in a 5% skim milk powder/TBS-T (SM-TBST) solution to block non-specific binding for 2hrs at room temperature. Following this, membranes were removed from the blocking buffer and placed into a sealed plastic bag containing 4mL of primary antibody, including housekeeping protein (GAPDH, Abcam ab14247, 1:10,000) diluted in 2% SM-TBST. Antibodies were incubated at 4°C overnight on a rotator to allow adequate distribution of antibody solution. Following this incubation, the membrane was removed from the bag and washed 3x with TBS-T for 5 minutes each. The membrane was then sealed in a plastic bag with 4mL of secondary antibody (LI-COR 800CW donkey anti-rabbit/anti-mouse, 1:20,000; LI-COR 680LT donkey anti-chicken, 1:10,000), diluted in 2% SM-TBST for 2hrs at room temperature on a rotator. Following this incubation, membranes were removed from the bag and washed three times with TBS-T for 5mins each. Membranes were then visualised on an Odyssey Fluorescent Imaging System using Image Studio Lite Software (LI-COR). Images were then converted to black and white and analysed in ImageJ 1.48 (National Institute of Health) and output readings analysed using GraphPad Prism statistical software (GraphPad Software).

2.7 HISTOLOGICAL ANALYSIS

2.7.1 Sample Preparation and Sectioning

Whole fixed brains, prepared as described in Section 2.5.2 were placed in a rodent brain blocker (Kopf, PA002), and sectioned so that a 3mm section from Bregma -1.3mm to Bregma -4.3mm would be contained in the same block to allow for serial sectioning. Sections were then processed overnight in a Tissue-Tek VIP through a series of ethanol baths for 20mins each (50%, 70%, 80%, 95%, 2x100%), then 2x 1.5hr xylene baths followed by four paraffin baths with increasing times (1x

30min, 2x 60min, 1x 90min). Sections were then individually embedded in paraffin wax and the aforementioned 3mm block was sectioned serially using a microtome (Leica Microsystems). 5µm sections were taken at Bregma -1.8mm, -3.0mm and -4.1mm and mounted onto SuperFrost Plus microscope slides (Menzel-Gläser).

2.7.2 Immunohistochemistry

Slides were dewaxed and washed in xylene and ethanol as described in 2.7.2. Following the final ethanol wash, slides were immediately transferred to a methanol/1.5% hydrogen peroxide (H_2O_2) bath for 30mins to block endogenous peroxidases. Sections were then washed 2x in phosphate buffered saline and Tween 20 (0.01% PBS-T) for 5mins each. Antigen retrieval was then performed by placing sections into citrate buffer (pH 6.0) and microwaving at boiling point for 10mins. Sections were then placed on the bench top still in citrate buffer and allowed to cool to room temperature before washing in PBS-T 2x for 5mins each. Slides were then placed into slide incubation trays and sections outlined using a wax marker (Dako, S2002). Slides were then incubated with 3% normal horse serum/PBS-T (NHS, Sigma-Aldrich, H0146) for 1hr at room temperature to prevent non-specific binding of the primary antibody. Following this incubation, NHS was removed and the primary antibody was applied overnight at 4°C at the appropriate dilution as per Table 2.3.

Target	Manufacturer/Cat No.	Host/Clonality	Dilution
pTau (T231; AT180)	Thermo Scientific; MN1040	Mouse monoclonal	1:1000

Table 2.3: Details of primary antibodies used in immunohistochemistry.

Sections were then washed in PBS-T 3x for 5mins each before application of the appropriate secondary antibody against the host species (Vector Laboratories) at a 1:250 dilution in 3% NHS/PBS-T for 30mins at room temperature. Sections were washed once again in PBS-T three times for five minutes each before application of streptadavin peroxidase conjugate (SPC, Pierce, 21124) at a 1:1000 dilution in 3% NHS/PBS-T for 1hr at room temperature. Following this slides were washed in

PBS-T three times for 5mins each, then antibody binding was visualised using 3.3'diaminobenzidine (DAB, Sigma-Aldrich, D8001) applied to sections until they turned brown. Sections were then immediately washed thoroughly with running distilled water for 10mins, then counterstained with haematoxylin for 1min and washed again. Slides were then differentiated with acid alcohol and lithium carbonate (as per section 2.7.2), then placed into 2x 100% ethanol incubations for 2mins each, followed by 2x histolene incubations for 2mins each. Slides were cleaned and mounted with DPX mounting medium (Sigma-Aldrich, 06522) and allowed to dry for 48hrs before being digitised via the Nanozoomer Digital Pathology System (Hamamatsu). A $1mm^2$ area directly below the cortex was selected in each sample and cell counts were performed by two independent blinded assessors. Results from each assessor were averaged for the final count and these results were analysed using GraphPad Prism (GraphPad Software).

2.8 STATISTICAL ANALYSIS

All statistical analysis was performed using GraphPad Prism software (GraphPad Software, v7.02). Most data were analysed using a one-way analysis of variance (ANOVA) with Tukey post-hoc analysis except for Barnes maze data, which were analysed using a two-way ANOVA with Tukey post-hoc analysis. SP ELISA data was analysed by first creating a standard curve with a four parameter logistic fit. The unknown values were then interpolated to the standard curve. Data were then sorted into experimental groups and analysed using a one-way ANOVA with Tukey post-hoc analysis.

Chapter 3: Characterisation of the time course of SP release following varying severity levels of TBI

3.1 INTRODUCTION

SP release following severe TBI has been widely characterised, with multiple studies showing increases in SP immunoreactivity in pre-clinical models (Corrigan et al. 2012; Donkin et al. 2011; Donkin et al. 2009), as well as in post-mortem human tissue (Zacest et al. 2010). Although such SP release is widely documented following severe TBI, to date there have been no studies assessing the time course of SP release in smTBI or rmTBI and how this differs to what is already known about severe TBI. Indeed, it has been shown previously that SP release is proportional to the intensity and frequency of stressful stimuli (Allen et al. 1997; Mantyh 2002), but this has not been fully assessed in an experimental TBI model. The profile of SP release following repetitive mTBI and whether this response is amplified versus the single increase already observed in severe TBI therefore requires further investigation.

Of specific interest are how these changes in SP relate to alterations in expression of the NK_1 and $TRPV_1$ receptors following TBI. Activation of $TRPV_1$ by a number of stimuli including heat, protons, inflammatory mediators like bradykinin and prostaglandin, as well as potentially via mechanical activation leads to the release of SP. Whether expression of $TRPV_1$ changes in response to repeated concussive impacts has yet to be determined. Similarly the expression of the receptor to which SP preferentially binds, the NK_1 receptor, has not been examined following repeated injury. This is of particular interest given the speculation that activation of NK1 may further potentiate the release of SP when activated under stress conditions (Singewald et al. 2008).

Although SP has been shown to play a role in a number of secondary injury processes including axonal injury (Donkin et al. 2011) and BBB breakdown (Nimmo et al. 2004), whether it is involved in the hyperphosphorylation of tau in later stage disease processes remains unknown. Classical

inflammation has been implicated in the underlying mechanisms of other chronic neurodegenerative diseases, including Alzheimer's disease (Metcalfe & Figueiredo-Pereira 2010). Indeed, acute activation of the immune system through induced inflammation can exacerbate tau phosphorylation (Lee et al. 2010; Sy et al. 2011). The role that neurogenic inflammation and SP may play in these late stage disease processes, especially with regards to excess tau phosphorylation, has not yet been investigated and provides an attractive opportunity to explore the relationship between neurogenic inflammation and tau phosphorylation.

Therefore, this study sought to characterise the release of SP and its relationship to changes in tau phosphorylation acutely following TBI, encompassing moderate-severe TBI, a single mild TBI andrmTBI. This study chose to focus on the phosphorylated tau isoform Thr231 as it has been shown previously to be upregulated following injury in a mouse model of severe TBI (Hawkins et al. 2013). Additionally, Thr231 has been implicated in tau-mediated neurodegeneration via conversion to its pathological cis conformation from its physiological trans conformation (Lu et al. 2016) and therefore presents an interesting avenue of study following rmTBI.

3.2 STUDY DESIGN

This study was divided into 2 smaller studies. In the first study the time-course of SP release and tau phosphorylation was examined at 5, 24 and 72 hrs following either sham surgery, smTBI, rmTBI or a single severe TBI. Animals were randomly allocated with a total of 48 animals used (n=4/gp/timepoint). In the second study, animals (n=32, n=4/gp/timepoint) were randomly assigned into sham, smTBI, rmTBI and severe TBI groups for IHC analysis at the 5 hr and 24 hr timepoints, which represented the peak of SP release following severe and rmTBI respectively. Animals assigned into the smTBI and rmTBI groups received mTBI at the prescribed time-points (as described in Chapter 2.2.1) and animals assigned into the severe TBI group received a single moderate-severe TBI

(as described in Chapter 2.2.2). Animals were sacrificed at the pre-determined time-points and fresh or fixed tissue collected (as per Chapter 2.5.1-2.5.2).

3.2.1 Fresh Tissue Analyses

Fresh tissue was homogenised and protein levels estimated (as per Chapter 2.6.1 – 2.6.2) and levels of normal and phosphorylated tau, as well as NK_1 and $TRPV_1$ expression were analysed using WB (as described in Chapter 2.6.5). Levels of cortical SP release were assessed using a commercial SP ELISA kit (as described in Chapter 2.6.3).

3.2.2 Fixed Tissue Analyses

Animal brains were sectioned (as per Section 2.7.1) and immunohistochemically stained for pTau (AT180) as described in (Chapter 2.7.2). Sections were digitised and analysed by blinded assessors (as per Chapter 2.7.2).

3.2.2 Statistical Analysis

SP ELISA data was analysed by first creating a standard curve with a four parameter logistic fit. The unknown values were then interpolated to the standard curve. Data were then sorted into experimental groups and analysed using a one-way ANOVA with Tukey post-hoc analysis. Immunohistochemistry cell count data were analysed using a one-way ANOVA with Tukey post-hoc analysis. All western blot data were analysed for differences between groups at each of the prescribed timepoints using a two-way ANOVA with Tukey post-hoc analysis. All statistical analyses were performed in GraphPad Prism (GraphPad Software, v7.0.2.). All data are displayed as mean ± SEM.

3.3 RESULTS

3.3.1 SP ELISA

At 5 hrs post-injury, a significant increase in SP concentration was observed in the severe TBI cohort (1652±206.1pg/mL; p=0.011), when compared to shams (1060±453.2pg/mL). SP concentration levels in severe TBI animals were also significantly increased when compared to the smTBI group (755.9±115.8pg/mL) at the 5 hr timepoint (p=0.0005). At 24 hrs post-injury, the rmTBI group showed the most profound increase in SP concentration, when compared to all other groups at all time-points (2221±179.5pg/mL) and was significantly increased when compared only to the sham group (p<0.0001), but also the smTBI group (p<0.0001), severe TBI group at 24 hrs post-injury (p<0.0001) and severe TBI group at 5 hrs post-injury (p=0.0237). At 72 hrs post-injury, SP concentrations were significantly lower than the sham group in all experimental groups (p=0.038 smTBI = 491.5±35.48pg/mL; p= 0.015 rmTBI = 520.7±69.63pg/mL; p=0.008 SEVERE = 449.4±19pg/mL). However, there were no significant differences between the different injury groups at this time-point (Figure 3.1).

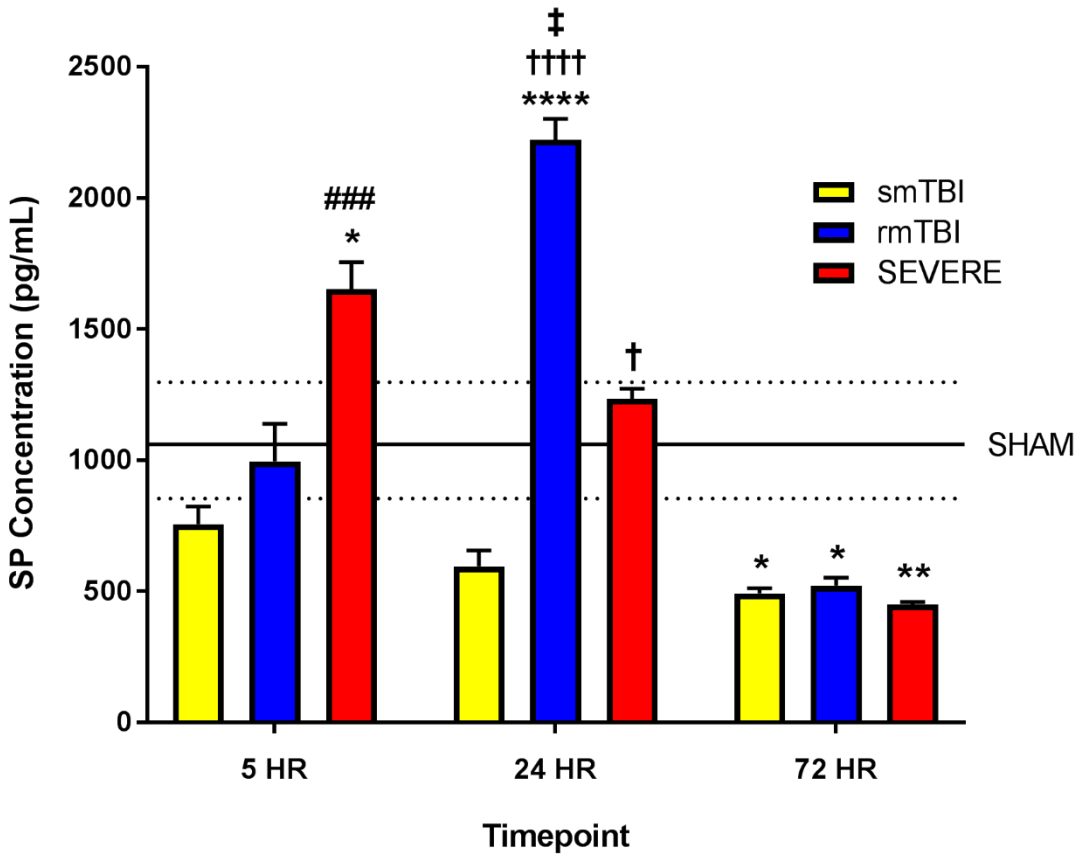


Figure 3.1: Time course of cortical SP concentrations following smTBI, rmTBI and severe TBI. Significant increases in SP concentration were noted in the SEVERE 5HR and rmTBI 24HR groups when compared to shams. SP concentrations in the SEVERE 5HR group were also significantly increased when compared to the smTBI group at the 5HR timepoint. The largest SP increase was observed in the rmTBI 24HR group which was significantly increased when compared to not only the sham group, but also the smTBI 24HR group, the severe 24HR group and the severe 5HR group. Significant decreases in SP concentration were observed in all experimental groups when compared to shams at the 72 hr time-point (SHAM n=6, all other groups n=4; *p<0.05, **p<0.01, ****p<0.0001 compared to sham; ###p<0.001 compared to smTBI 5HR; †p<0.05, ††††p<0.0001 compared to smTBI 24HR; ‡p<0.05 compared to SEVERE 5HR).

3.3.2 NK_1 & $TRPV_1$ Western Blot

Analysis of changes in TRPV1 relative density found a significant effect of both time (p=0.002) and injury type (p=0.011). At 5 hrs post-injury no significant effect of injury compared to shams was seen in TRPV1 levels (smTBI p=0.136; rmTBI p=0.390 and severe TBI p=0.117). At 24hrs post-injury, again no significant differences were noted relative to shams, although a significant increase in $TRPV_1$ band density was observed in the severe TBI group (0.973±0.195) when compared to the smTBI group (0.448±0.215; p=0.008). By 72 hrs this difference between the severe TBI and smTBI group was no longer evident, with the rmTBI group showing a significant decrease in $TRPV_1$ blot density when compared to shams (0.738±0.226; p=0.002). When analysing changes across the time course within each group, no difference in TRPV1 blot density was observed in sham or smTBI animals from 5-72 hrs post-injury, whereas in rmTBI animals a significant decrease was seen from 24 to 72 hrs post-injury (p=0.0004). Furthermore in severe injured animals a significant increase was seen from 5 to 24 hrs post-injury (p=0.002), with a significant decrease from 24 hrs to 72hrs post-injury (p=0.03), with no difference between 5 and 72 hr levels of TRPV1 (p=0.981).

Evaluation of NK_1 blot density similarly found a significant effect of both timepoint (p<0.0001) and injury type (p=0.01). No significant changes relative to sham animals were observed at the 5HR (smTBI p=0.993; rmTBI p>0.999; SEVERE p=0.731) or 72HR timepoint (smTBI p=0.9998; rmTBI p=0.868; SEVERE p=0.647). However there was an increase in NK_1 blot density at 24 hrs following severe injury (1.68±0.31) when compared to the shams (1.11±0.123; p=0.004), smTBI (1.11±0.076; p=0.005) and rmTBI (1.013±0.141; p=0.001) groups (Figure 3.2A). Indeed no change across the time course was seen in sham, smTBI or rmTBI animals with only the severe injury group showing a significant increase in NK1 blot density when comparing the 5 and 72 hr to the 24 hr timepoint (p<0.001).

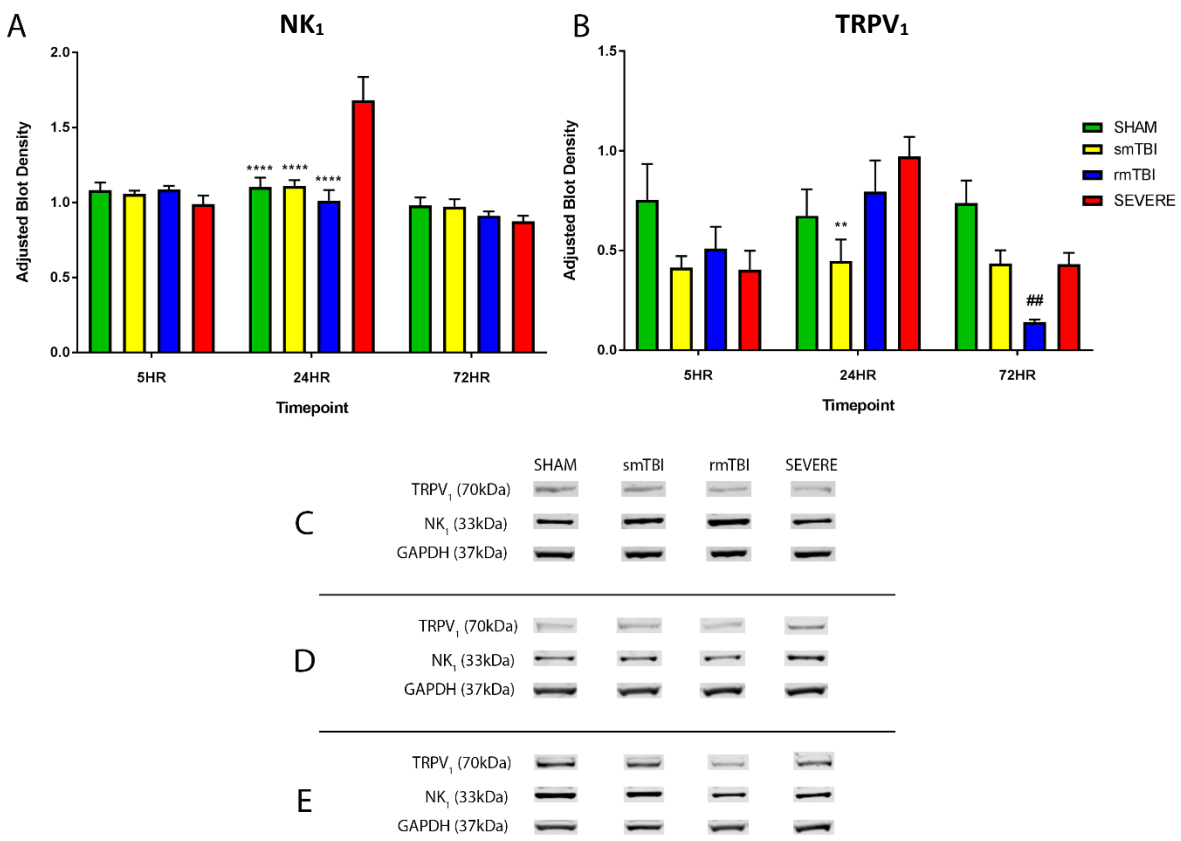


Figure 3.2: Time course of NK_1 (A) and $TRPV_1$ (B) expression as determined via western blot. Representative NK_1, $TRPV_1$ and housekeeper (GAPDH) bands from 5 hr (C), 24 hr (D) and 72 hr (E) animals. (n=4/gp, **p<0.01, ***p<0.001, ****p<0.0001 compared to SEVERE 24HR; ##p<0.01 compared to SHAM 72HR).

3.3.3 Tau Western Blot Data

Analysis of changes in T231 band density found that the timepoint had a significant effect (p<0.0001), however injury type did not (p=0.386). At 5 hrs post-injury, a significant decrease (p=0.033) in T231 band density was observed in the severe group (0.439±0.171) when compared to the rmTBI (0.997±0.119) group (Figure 3.3A). There were no other significant differences observed between the other experimental groups in T231 band density when compared to shams at this time-point (smTBI p=0.999, rmTBI p=0.887, SEVERE p=0.159).The 24 hr timepoint showed a significant increase in T231 density in the severe group (2.456±0.079), when compared to the sham (1.624±0.476; p=0.0007), smTBI (1.372±0.457; p<0.0001) and rmTBI (1.413±0.42; p<0.0001) groups.

However by the 72 hr timepoint, the significant increase in T231 density in the severe group was no longer evident, with no significant differences observed between any groups when compared to sham at this timepoint (smTBI p=0.999, rmTBI p=0.993, SEVERE p=0.971).

When comparing groups across the time course, a significant increase in T231 density was noted from 5 to 24hrs (p=0.001) and a significant decrease observed from 24hrs to 72 hrs (p=0.002) in the sham animals. A significant increase in density was also observed in the smTBI group from 5hrs to 24 hrs (p=0.037). Although not significant, a trend toward a decrease was observed between 24 and 72 hrs (p=0.054). In the rmTBI group, no significant difference was observed between 5 and 24 hrs (p=0.096), however a significant decrease in T231 density was noted from 24 to 72 hrs (p=0.016). A significant increase in density was observed in the severe group from 5 to 24 hrs (p<0.0001) however between the 24 and 72 hr timepoints this had significantly decreased back to sham levels (p<0.0001). In all groups, no significant changes were observed between the 5hr and the 72hr timepoint (SHAM p=0.977, smTBI p=0.985, rmTBI p=0.713, SEVERE p=0.151).

With regards to Tau-5 band density, level of injury appeared to have a significant effect (p=0.029) however the timepoint did not (p=0.108) (Figure 3.3B). At 5 hrs post-injury, there were no significant differences observed between groups when compared to sham (smTBI p=0.997, rmTBI p=0.672, SEVERE p=0.997). Groups were also not significantly different from shams at the 24 hr timepoint (smTBI p=0.267, rmTBI p=0.632, SEVERE p=0.711), however a significant increase in band density was observed in the severe group (1.618±0.222) when compared to smTBI (0.993±0.125) at this timepoint (p=0.029). At the 72 hr timepoint, a significant increase in Tau-5 band density was observed in the rmTBI group (1.535±0.471) when compared to sham (0.932±0.146, p=0.038). The severe group appeared to show a trend towards a decrease when compared to sham at this timepoint (1.499±0.471, p=0.055). The smTBI group was not different from shams at this timepoint (1.106±0.335, p=0.849). Time did not appear to have a significant effect across sham, smTBI or rmTBI groups on Tau-5 band density. In the severe group, a significant increase was observed from 5 to 24

hrs (p=0.016) and a trend towards an increase also observed from 5 to 72 hrs (p=0.059). No significant difference was observed between 24 and 72 hrs in the severe group (p=0.844).

The T231/Tau-5 ratio was also analysed to compare the relevant amounts of normal tau to phosphorylated tau. The T231/Tau-5 ratio was not significantly affected by the severity of injury (p=0.333) but did appear to be significantly affected by the timepoint (p<0.0001) (Figure 3.3C). No significant differences were observed between any groups when compared to sham within the 5hr (smTBI p=0.994, rmTBI p=0.995, SEVERE p=0.133), 24hr (smTBI p=0.862, rmTBI p=0.999, SEVERE p=0.448) and 72hr (smTBI p=0.983, rmTBI p=0.341, SEVERE p=0.306) timepoints. Sham animals were also not affected by the timepoint, with no differences observed between 5-72 hrs. The T231/Tau-5 ratio in the smTBI group significantly increased from 5hrs (0.779±0.357) to 24 hrs (1.398±0.479, p=0.011) and then significantly decreased from 24 to 72 hrs (0.859±0.201, p=0.027). No change was observed between 5 and 72 hrs (p=0.917). In the rmTBI group, a trend towards an increase was observed from 5hrs (0.781±0.102) to 24 hrs (1.265±0.449, p=0.051) and a significant decrease in the ratio was observed from 24 to 72 hrs (0.594±0.195, p=0.005). However no changes were observed between 5 and 72 hrs (p=0.619). This was echoed somewhat in the severe group, with a significant increase in the T231/Tau-5 ratio observed from 5 hrs (0.386±0.134) to 24 hrs (1.541±0.225, p<0.0001) and a significant decrease observed from 24 to 72 hrs (0.581±0.122, p<0.0001) and again no differences observed between 5 and 72 hrs (p=0.595).

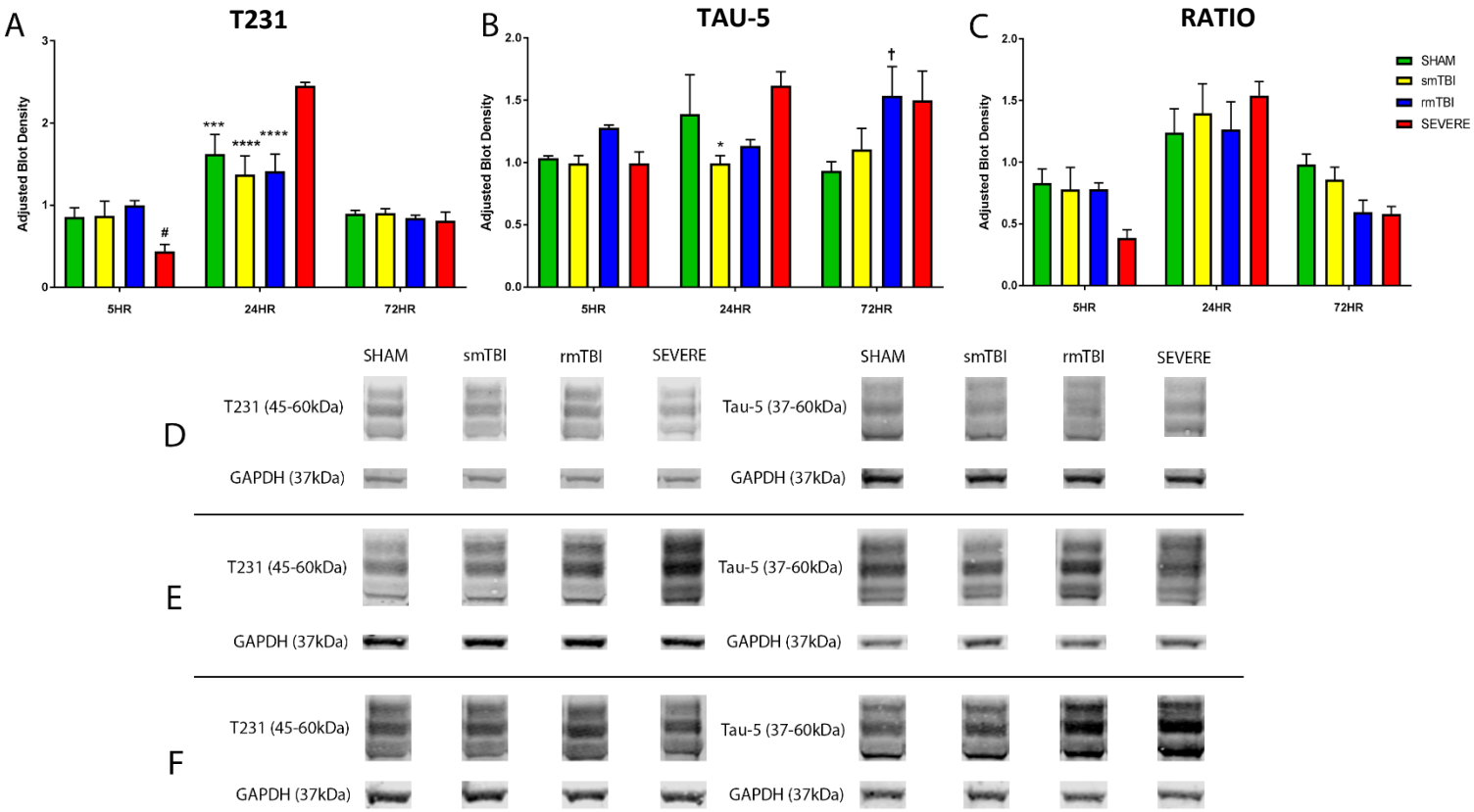
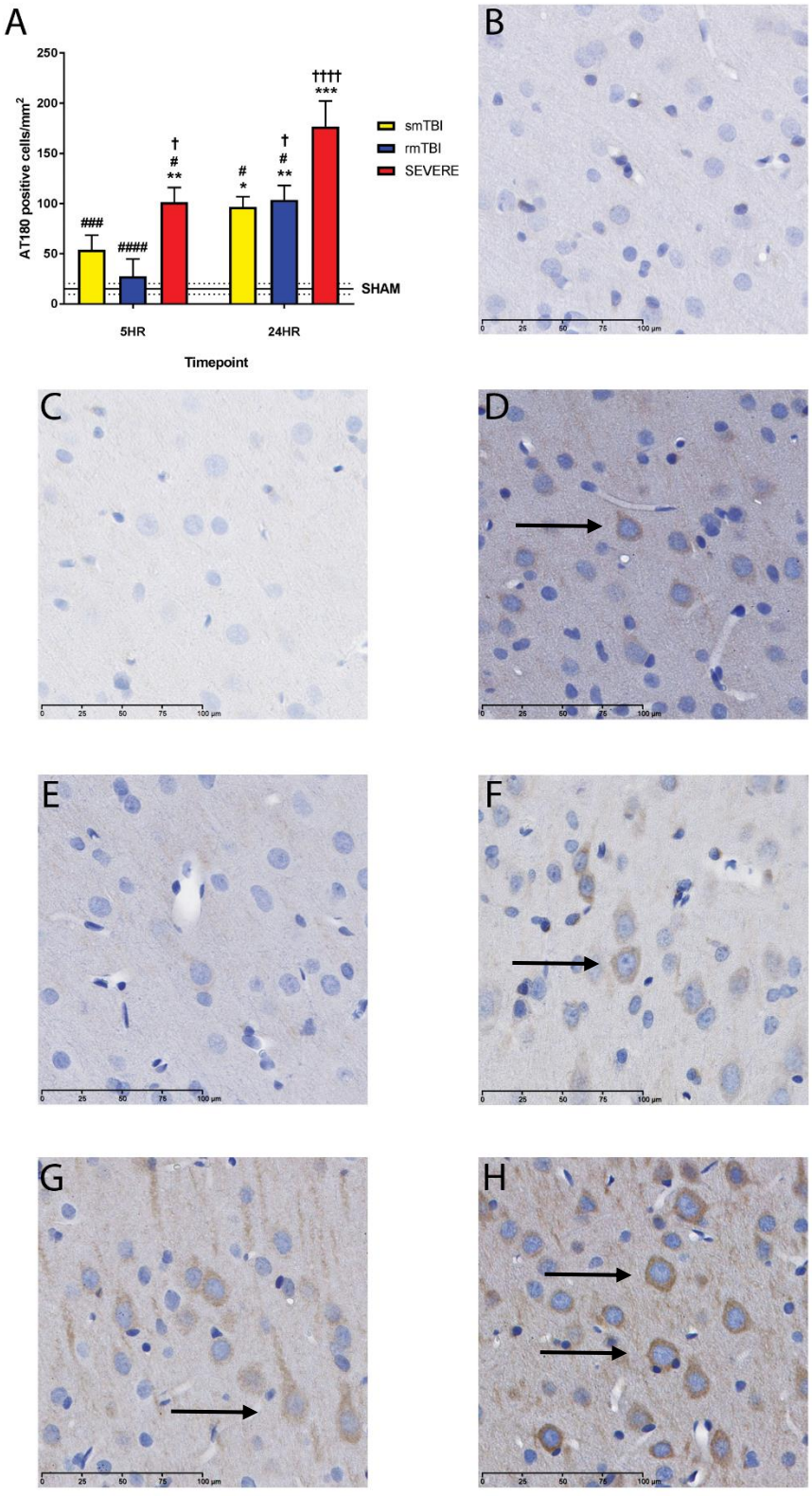


Figure 3.3: Time course of T231 (A), Tau-5 (B) and the T231/Tau-5 ratio (C) acutely following TBI. (D): Representative T231, Tau-5 and housekeeper (GAPDH) bands from 5 hr (D), 24 hr (E) and 72 hr (F) animals (n=4/gp, *p<0.05, ***p<0.001, ****p<0.0001 compared to SEVERE 24HR; #p<0.01 compared to rmTBI 5HR; †p<0.05 compared to SHAM 72HR).

3.3.4 Tau Immunohistochemistry

Given the spikes in SP release observed in severe TBI animals at the 5 hr time-point and in the rmTBI animals at the 24 hr timepoint, more targeted immunohistochemical analysis for phosphorylated tau within the cortex under the impact site was undertaken to further elucidate the potential interactions between increases in SP release and subsequent tau phosphorylation.

A significant increase in the number of AT180±ve cells was observed in the severe group at the 5 hr time-point (101.2±32.4; Figure 3.4G) when compared to sham (15±12.1, p=0.009, Figure 3.4B). When compared to the sham group, significant increases in AT180±ve cells were also observed in smTBI (96.6±23.07, p=0.015, Figure 3.4D), rmTBI (103.8±31.73, p=0.007, Figure 3.4F) and severe TBI (176.8±57.18, p<0.0001, Figure 3.4H) groups. AT180±ve cells were significantly decreased in the rmTBI group at 5 hrs (27.8±38), with significance observed between this group and the rmTBI group at 24 hrs (p = 0.036), and the severe group at both 5 hrs (p=0.027) and 24 hrs (p<0.0001). The severe 24 hr group also had a significantly increased number of AT180±ve cells when compared to the smTBI group at both 5 hrs (p=0.0001) and 24 hrs (p=0.01), the rmTBI group at 24 hrs (p=0.037) and the severe group at 5 hrs (p=0.028) (Figure 3.4A).


A
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smTBI
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0 25 50 75 100 µm

Figure 3.4: (A) Cell count data of AT180±ve cells in the cortex of animals at 5 hr and 24 hr timepoints (B-H) Representative IHC staining of AT180±ve cells in the cortex of animals at 5 hr and 24 hr timepoints. B: SHAM, C: smTBI 5HR, D: smTBI 24HR, E: rmTBI 5HR, F: rmTBI 24HR, G: SEVERE 5HR, H: SEVERE 24HR. All images taken at 40x, scale bar 0-100µm. (n=5/gp; *p<0.05, **p<0.01 compared to sham; #p<0.05, ###p<0.001, ####p<0.0001 compared to SEVERE 24HR; †p<0.05, ††††p<0.0001 compared to rmTBI 5HR).

3.4 DISCUSSION

The aim of this study was to characterise the pattern of acute SP release and the expression of both NK_1 and $TRPV_1$ following smTBI, rmTBI and severe TBI, and to determine whether the pattern of such SP release was related to increases in the levels of tau phosphorylation seen acutely. The peak of SP release following severe TBI was seen at 5 hrs, compared to 24 hrs in the rmTBI animals, with no increase in SP observed following a single mTBI. Levels of the NK_1 receptor, to which SP binds with the highest affinity, were only increased following severe TBI at the 24 hr time-point, whereas the $TRPV_1$ receptor, whose activation leads to SP release, was decreased in rmTBI animals at 72 hrs post-injury. A change across time was also observed in NK_1 expression, with the increase in NK_1 expression observed at the 24 hr timepoint in severe TBI not observed at the 5 or 72 hr timepoints. No direct relationship between SP-NK_1 system and tau phosphorylation could be seen acutely. Severely injured animals who have a peak of SP release at 5 hrs post-injury, showed increases in the number of AT180+ve cells as observed via IHC at both 5 and 24 hrs post-injury. In contrast both smTBI and rmTBI groups showed similar numbers of AT180+ve cells at 24 hrs post-injury, despite only the rmTBI group having an increase in SP levels at this time-point. Despite changes in the number of AT180+ve cells being observed via targeted IHC analysis, no differences were observed via WB analysis.

In regards to the release of SP following injury, although a rise in SP levels was seen in severe TBI animals at the 5 hr time-point, a much larger increase was observed in rmTBI animals at the 24 hr time-point. This matches previous work showing minimal increases of SP following smTBI, with increases in SP immunoreactivity observed as injury level increases (Corrigan et al. 2016). Given that SP release has previously been shown to be proportional to the frequency and intensity of stressful stimuli (Mantyh 2002) our findings are not surprising. Previous exposure to stressors such as neuroinflammation can amplify the interactions between SP and the NK_1 receptor and if the frequency of these stressful stimuli are further increased this can have a two-fold effect, both

through the increased activation of NK_1 receptors adjacent to the site of release and through the further diffusion of SP away from the site of release, allowing for the stimulation of more distant neurons (Mantyh 2002; Stucky, Galeazza & Seybold 1993). More frequent and intense stimuli on neurons can also lead to higher levels of neuronal activation, with three to five times the amount of neurons becoming activated compared to less stressful stimuli (Allen et al. 1997). Release of SP and activation of NK_1 receptors in response to insult or injury in the CNS has been shown to be both beneficial and detrimental. Studies by Chauhan et al. (2008; 2011) have found that in NK_1 deficient mice and mice treated with a NK_1 receptor antagonist they both showed decreases in the inflammatory response as well as decreased astrogliosis and demyelination following bacterial infection and meningitis. However in contrast, SP/NK_1 interactions are critical for resistance to Salmonella infection, as shown with reduced survival rates in wild-type mice following administration of a NK1 receptor antagonist in a study by Kincy-Cain and Bost (1996). This could potentially explain the shift in SP release between the 5hr & 24 hr marks, although SP was released in response to the initial injury and may have been beneficial, repeated insults would have driven further release of SP from the already primed system and therefore would now be detrimental (Johnson, Young & Marriott 2016).$TRPV_1$ expression also appeared to be affected in this study, with decreases in rmTBI animals observed at the 72 hr timepoint. However, previous literature indicates that when subjected to repeated stress over a 10 day period, $TRPV_1$ receptors appear to increase their expression both *in situ* & *in vitro* (Hong et al. 2011). Further investigation into what precipitates $TRPV_1$ activation and subsequent SP release in severe versus rmTBI is warranted.

Of interest is the apparent increase in NK_1 receptor expression in the severe group at the 24 hr time-point, consistent with previous studies reporting that increases in SP release levels induce expression of the NK_1 receptor on inflammatory response cells, such as astrocytes and microglia (Lin 1995; Rasley et al. 2002). Although expression was increased in the severe group, it was not increased in the rmTBI group, which had the largest increase in SP across all experimental groups and time-points. This could potentially be due to the behaviour of the NK_1 receptor following SP

binding as when SP initially binds to the NK_1 receptor this results in rapid internalisation into the cytoplasm via endosomes (Mantyh 2002). Such receptor internalisation is reversible (Mantyh 2002), and what might be occurring is a rebound effect following the severe injury. Specifically, as SP levels decline following severe TBI, the NK_1 receptors may be reversing their internalisation and returning to the cell surface. In contrast, the higher levels of SP release following rmTBI may be influencing receptor internalisation and therefore keeping these receptors internalised. However, the method used to assess NK1 expression in this study, WB, may not be appropriate in assessment of receptor internalisation and a different method such as immunocytochemistry or IHC should be employed in future studies to determine this. The behaviour of SP-NK_1 system interactions following rmTBI still needs further elucidation before any firm conclusions can be drawn as to the contribution of this ligand-receptor pair.

Increases in pTau acutely following severe TBI, such as that observed in this study at the 24 hr time-point have been observed previously in pre-clinical models (Rubenstein et al. 2017). However, although SP concentrations were higher in the rmTBI animals, levels of tau phosphorylation were not higher than that observed in the severe TBI animals. Furthermore, tau phosphorylation was also observed in the smTBI group at the 24 hr time-point, indicating that there may be other processes influencing tau phosphorylation. Indeed, previous studies report that tau phosphorylation may be exacerbated by classical inflammation (Collins-Praino & Corrigan 2017; Kitazawa et al. 2005; Sy et al. 2011). Given the observed delay between peak SP release and tau phosphorylation in this study, assessment of pTau changes at later timepoints via IHC is warranted to fully determine this time course.

However, how neurogenic inflammation might interact with these cascades has not yet been investigated. It could be that the initial disassociation of tau from the microtubules could be caused

by the initial primary axonal injury, then phosphorylation becomes exacerbated via the secondary injury processes, potentially driven by neurogenic cascades facilitated by SP/NK_1 feedback loops. Such processes may play a role at a later time-point in rmTBI animals, as previous studies assessing pTau following rmTBI have found increases at later time-points, up to three weeks post-injury (Petraglia et al. 2014). Axonal injury has also been seen in greater amounts following severe TBI compared to mTBI (Browne et al. 2011; Smith et al. 2000), and as such this may present a larger amount of dissociated tau available for phosphorylation compared to rmTBI. Additional analysis of changes in pTau deposition at further timepoints following both rmTBI and severe TBI, as well as additional characterisation of changes in axonal injury between the two injury levels can further elucidate the disease processes following injury Furthermore, in order to directly determine the effects of neurogenic inflammation on tau phosphorylation, further studies involving blockade of the SP pathway post-injury are required. Nevertheless, this presents an attractive opportunity to investigate these injury processes further and potentially identify targets to attenuate these processes.

It is important to recognise and reflect on the differences observed in the WB and IHC pTau results. This may be due to the larger area analysed with the WB homogenates versus the more targeted IHC analysis. Whilst great care was taken to extract the relevant areas for WB analysis, it is certainly possible that too much cortical tissue was removed in the dissection stages. Normal distribution of tau does indeed vary throughout the brain, with a study using human tissue finding that the frontal grey matter contained 30-45% more normal tau than corresponding white matter (Khatoon, Grundke-Iqbal & Iqbal 1994). In the model of injury used in this study, the injury occurs between the lambda and bregma sutures, which is approximately 9mm wide (Paxinos, Watson & Emson 1980). When examined via IHC, tau phosphorylation also appeared to occur in the more superior areas of the cortex, directly underneath the impact site where the most stress occurs during injury. A study

by Ho & Kleiven (Ho & Kleiven 2009) showed that in a 3D model of stress, comparing lissencephalic and gyrencephalic brains, that in lissencephalic brains stress appeared to run parallel to the surface of the tissue, whereas in gyrencephalic brains the presence of gyri reduced strain on the tissue and localised the stress to the base of the sulci. Given the small margin for error in assessing and removing the correct tissue, it is perhaps warranted that in further studies more targeted dissection is required to both remove the required tissue and ensure that native tau levels in other sections do not have an effect on the final result, as well as conducting parallel IHC studies assessing the distribution of tau.

3.5 CONCLUSIONS

This study demonstrated that SP release is indeed present in the cortex following both rmTBI and severe TBI and that repeated insults appear to potentiate the release of SP via mechanisms that are yet to be determined. Further investigation is needed to determine whether SP is linked to tau phosphorylation acutely following injury.

Chapter 4: Does blockade of the $TRPV_1$ receptor prior to injury prevent negative outcomes following TBI?

4.1. INTRODUCTION

In the previous chapter, it was demonstrated that following severe TBI, an increase in SP concentration was observed 5 hrs post-injury, which is in keeping with previous studies (Donkin et al. 2009). Interestingly following rmTBI, there was a sharp increase in SP release observed at 24 hrs post-injury that surpassed the levels of SP release observed in severe injury at 5 hrs. Thus the release of SP appears to be a feature of both rmTBI and severe injury, however, the mechanism behind what is triggering the release of SP in these settings requires further investigation.

Release of SP results from the activation of members of the transient receptor potential (TRP) family, which allow the influx of cations into neurons (Parenti et al. 2016). Of particular interest is the $TRPV_1$ receptor which has been proposed to be a putative mechanoreceptor (Brederson et al. 2012; McGaraughty et al. 2008). Indeed blockade or knockout of the $TRPV_1$ receptor reduces the sensitisation of afferent neurons in the colon caused by stretch activation (Jones, Xu & Gebhart 2005) and attenuates mechanical hyperalgesia in models of osteoarthritic pain (Chu et al. 2011). However, to date there has been no investigation into whether the mechanical forces following TBI similarly stimulate the $TRPV_1$ receptor leading to SP release. It is postulated that as a result of the primary insult in rmTBI, $TRPV_1$ may become sensitised and therefore may become more prone to the release of SP following subsequent injuries. Indeed, $TRPV_1$ has previously been shown to mediate mechanical hypersensitivity following tissue insult and injury (Jones, Xu & Gebhart 2005). As such, blockade of the $TRPV_1$ receptor could potentially reduce downstream effects and therefore tissue injury. Therefore the use of a competitive $TRPV_1$ antagonist, such as capsazepine, could therefore be employed to prevent the release of SP and therefore improve outcomes following TBI. Capsazepine itself has a long half-life, estimated to be approximately 96 hrs and can readily cross the blood-brain barrier so it would have no problems interacting with the $TRPV_1$ receptor (Bevan et al. 1992; Hu, Easton & Fraser 2005). Therefore it seems like an ideal candidate to allow for a mechanistic study of the SP/NK1 system following both rmTBI and severe TBI.

In addition in the previous chapter we were unable to demonstrate whether there was a relationship between SP release and tau phosphorylation following TBI. In order to investigate this further, preventing SP release through blockade of the $TRPV_1$ receptor and analysis on the effects of tau phosphorylation will allow a more directed examination of the relationship between these two events.

Accordingly, we sought to determine the role of mechanical activation of $TRPV_1$ by administering treatment with the $TRPV_1$ antagonist, capsazepine either before or after injury and examining the effect on SP levels following both severe and rmTBI. If mechanical input is indeed the primary stimulus of $TRPV_1$ activation and SP release then application prior to the mechanical injury should be more effective than treatment after the TBI. Furthermore we sought to determine whether capsazepine treatment following severe TBI altered levels of tau phosphorylation with associated changes in the activity of kinases involved in tau phosphorylation. This study aimed to focus solely on the effect of severe TBI on tau phosphorylation and kinase activity as we know that kinase dynamics of interest to this study are altered following severe TBI (Kuo et al. 2013; Tran, Sanchez & Brody 2012; Zhao, Luo, et al. 2012) and would allow for the establishment of baselines for the rmTBI studies. Alterations in kinase activity and tau phosphorylation following rmTBI were further assessed in the studies conducted as a part of Chapter 5.

4.2 STUDY DESIGN

A total of 60 animals were used in this study, which was divided into 2 parts. For the first study to investigate the role of mechanical stimulation of $TRPV_1$ in the facilitation of SP release following trauma, animals (n=40) were randomly assigned into the rmTBI or severe TBI cohorts and then subsequently sub-divided into sham, capsazepine pre-treatment, capsazepine post-treatment or

vehicle-treated groups for each injury condition. Animals received either 3x rmTBI (as described in Chapter 2.2.1) or a single moderate-severe TBI (as described in Chapter 2.2.2) and were administered either capsazepine (5mg/mL) or equal volume of vehicle via tail vein injection at either 30 mins pre-injury or 30 mins post-injury. Specifically, for animals in the rmTBI cohort this drug or vehicle administration was at 30 mins pre-injury or post-injury on the day of the final rmTBI injury. Capsazepine and vehicle treatments were prepared fresh on the day (as described in Chapter 2.3.1) and administered under anaesthesia at the prescribed time-points (as per Chapter 2.3.4). The terminal time-point for assessment of SP release differed between the rmTBI and severe TBI cohorts, as the results from Chapter 3 indicated that the peak of SP release differed between these injury conditions. As such, rmTBI animals were sacrificed at 24 hrs following the final injury, whereas moderate-severe TBI animals were sacrificed at 5 hrs following injury for fresh tissue collection.

A further subset of animals (n=20) were used for the second study, investigating whether prevention of SP release via blockade of the $TRPV_1$ receptor in severe TBI animals had any effects on kinase activation or tau phosphorylation. Animals were randomly assigned into sham, vehicle, capsazepine pre-treatment or capsazepine post-treatment groups. Animals were sacrificed at 5 hrs post-injury for collection of fixed tissue (as described in Chapter 2.5.1).

4.2.1 Fresh Tissue Analysis

Fresh tissue was homogenised, protein levels estimated (as per Chapter 2.6.1 – 2.6.2) and the levels of normal and phosphorylated tau, as well as NK_1 and $TRPV_1$ expression, analysed using WB (as described in Chapter 2.6.5). Levels of cortical SP release were assessed using a commercial SP ELISA kit (as described in Chapter 2.6.3). Cell signalling interactions were assessed using a custom made Bio-Plex Pro Cell Signalling Assay kit (Multiplex, Bio-Rad; Chapter 2.6.4).

4.2.2. Fixed Tissue Analyses

To further address the cortical distribution of pTau, immunohistochemistry was performed (as per Chapter 2.7.). Data were collected from two independent blinded assessors (as described in Chapter 2.7.2).

4.2.3. Statistical Analysis

SP ELISA data was analysed by first creating a standard curve with a four parameter logistic fit. The unknown values were then interpolated to the standard curve. Data were then sorted into experimental groups and analysed using a one-way ANOVA with Tukey post-hoc analysis. Western Blot density data was analysed using a one-way ANOVA with Tukey post-hoc analysis. Multiplex readout data was analysed using a one-way ANOVA with Tukey post-hoc analysis. pTau immunohistochemistry cell count data was analysed using a one-way ANOVA with Tukey post-hoc analysis. All statistical analyses were performed in GraphPad Prism (GraphPad Software, v7.0.2.)

4.3 RESULTS

4.3.1 The role of mechanical stimulation of the $TRPV_1$ receptor on SP release

4.3.1.1 SP ELISA

Following severe injury both capsazepine pre-treatment (952±71.49pg/mL; p=0.002) and post-treatment (1101±74.82pg/mL; p=0.012) led to a significant reduction in SP levels when compared to vehicle treated animals (1700±501.7pg/mL; Figure 4.4A). Indeed levels of SP in both capsazepine pre-treatment (p=0.934) and capsazepine post-treatment (p=0.477) animals were not significantly different to sham animals (853.6±145.2pg/m, p=0.934 & p =0.477 respectively).

In contrast, in rmTBI animals only the animals pre-treated with capsazepine showed a significant reduction in SP levels compared to vehicle-treated animals (1177±224.3pg/mL vs 1973±188.9pg/mL; p=0.005), with capsazepine post-treated animals having a similar level of cortical SP to vehicle controls (1697±501pg/mL; p=0.467). A trend towards an increase in SP concentration was also detected in the capsazepine post-treatment group when compared to the capsazepine pre-treatment group (p=0.075; Figure 4.1B).

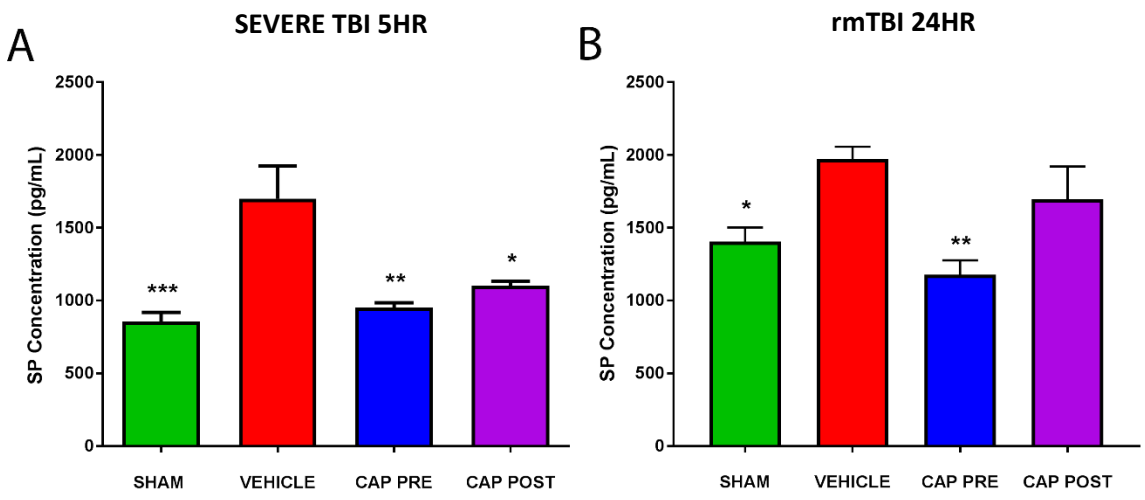

Figure 4.1: SP ELISA data showing changes in cortical SP expression across treatment groups in severe TBI at 5 hrs post-injury (A) and in rmTBI at 24 hrs post-injury (B). (A): Vehicle treated animals had a significantly higher SP expression compared to sham, capsazepine pre-treatment and capsazepine post-treatment following severe TBI 5 hrs post-injury. No significant differences were observed between capsazepine pre-treatment and capsazepine post-treatment (n=5 all groups; ***p<0.001, **p<0.01, *p<0.05 compared to vehicle). (B) SP concentrations were significantly elevated in the vehicle treated group when compared to both sham and capsazepine pre-treatment groups following rmTBI 24 hrs post-injury (n=5/gp; *p<0.05, **p<0.01 compared to vehicle).

4.3.1.2 NK_1 & $TRPV_1$ Western Data

No changes in the levels of NK_1 or $TRPV_1$ were observed following either rmTBI or severe injury or capsazepine treatment ($p = 0.39$ for NK_1 SEVERE; $p= 0.561$ for NK_1 rmTBI; $p = 0.20$ for $TRPV_1$ SEVERE; $p = 0.318$ for $TRPV_1$ rmTBI; Figure 4.2).

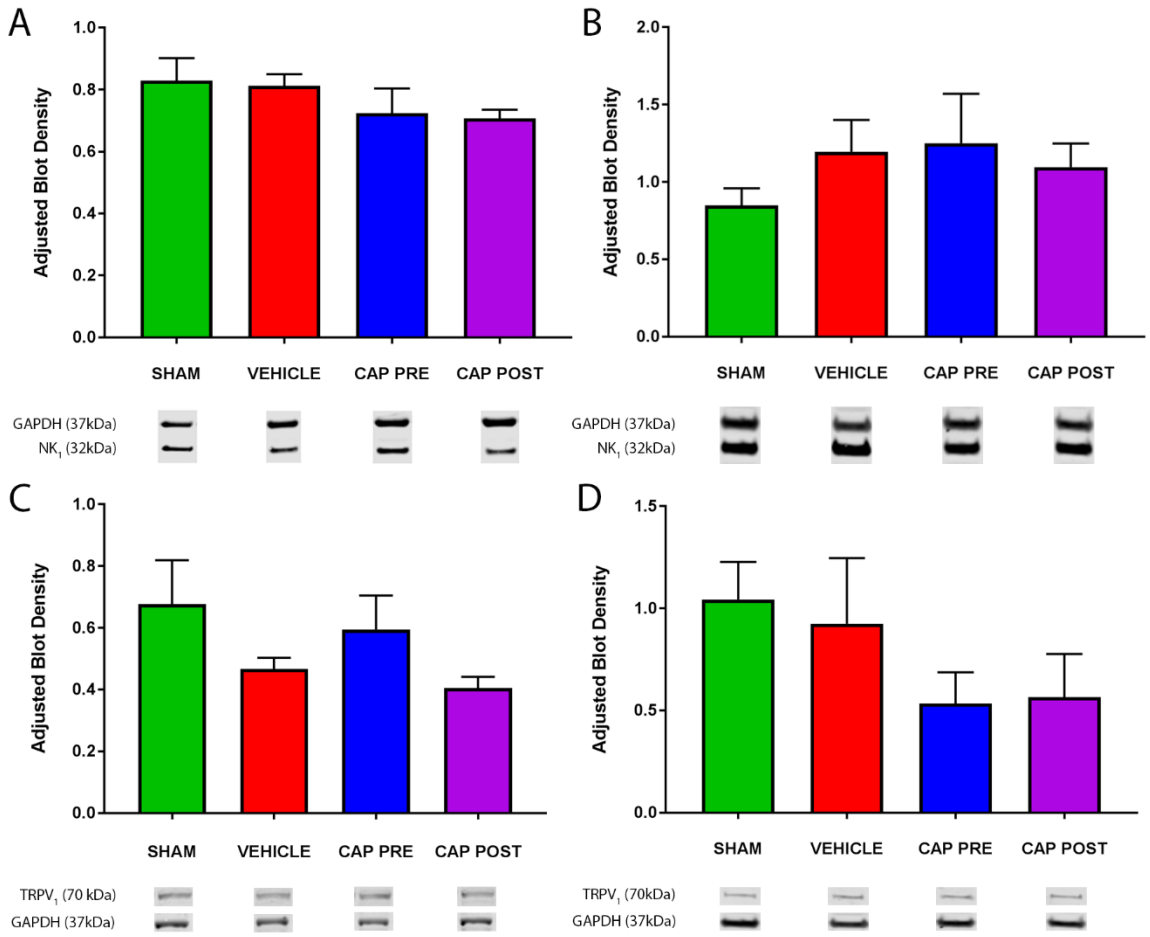

Figure 4.2: Western Blot data showing changes in NK_1 (A) and $TRPV_1$ (B) expression in capsazepine treated animals 5 hrs post severe TBI, and changes in NK_1 (C) and $TRPV_1$ (D) expression in capsazepine treated animals 24 hrs post rmTBI. No significant changes were observed across all groups (n=5/gp, $p>0.05$).

4.3.2: Effect of SP blockade on kinase activation and tau phosphorylation in severe TBI

4.3.2.1 Kinase phosphorylation

Levels of phosphorylated Akt (pAkt) were consistent between all experimental groups with no significant differences observed (p=0.265; Figure 4.3A). Phosphorylated ERK (pERK) readings revealed similar results with no significant differences observed between all experimental groups (p=0.193; Figure 4.3B). Readings for phosphorylated GSK-3β (pGSK-3β) also revealed no significant differences across experimental groups (p=0.935; Figure 4.3C). Levels of phosphorylated JNK (pJNK) showed a significant decrease in capsazepine pre-treated (p=0.007), capsazepine post-treated (p=0.002) and vehicle-treated (p<0.0001) groups, when compared to shams. A significant difference was also noted between the capsazepine pre-treated and vehicle groups (p=0.032; Figure 4.3D. Differences between the capsazepine post-treated and vehicle groups were not significant (p=0.1378). Total levels of Akt, ERK1/2, GSK-3β and JNK were also assessed and no differences were observed between any of these groups in levels of total kinase detected (Akt p=0.543, ERK1/2 p=0.262, GSK-3β p=0.249, JNK p=0.864; Figure 4.4)

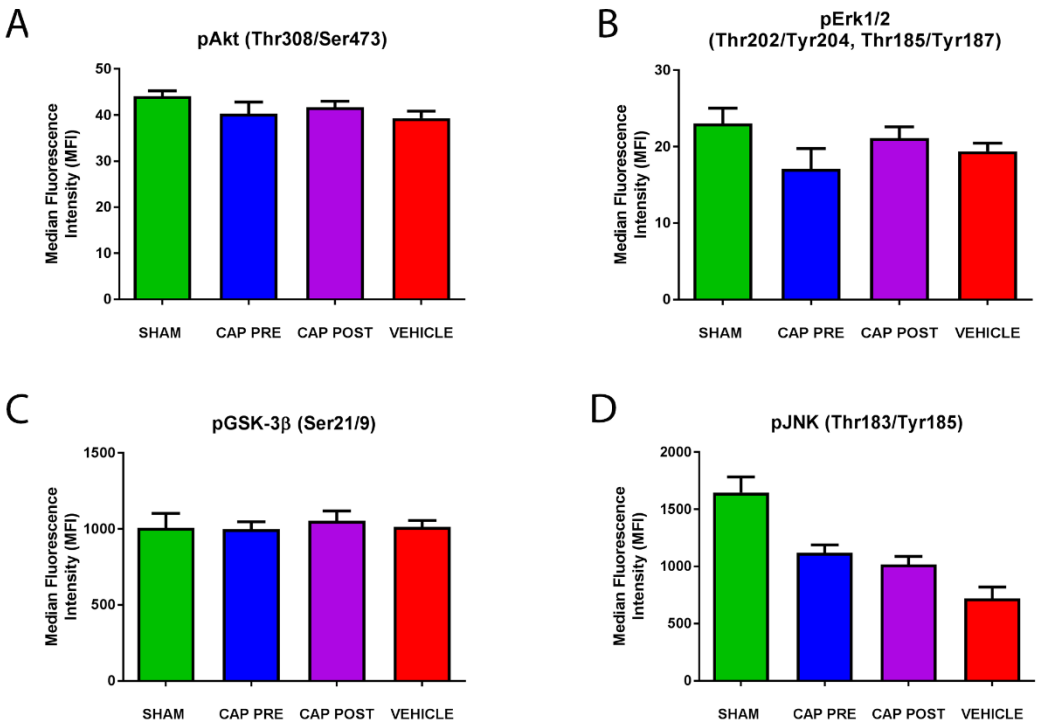

Figure 4.3: Multiplex data showing changes between groups in expression of pAkt (A), pERK1/2 (B), pGSK-3β (C) and pJNK (D). Differences in the levels of pAkt, pERK1/2, pGSK-3β were not significant between any of the groups (n=5, $p>0.05$). A significant decrease in pJNK was observed in vehicle (red, $p<0.0001$), capsazepine post-treated (purple, $p<0.01$) and capsazepine pre-treated (red, $p<0.01$) animals when compared to sham (green). A significant difference was also observed in the vehicle group when compared to the capsazepine pre-treatment group ($p<0.05$). (n=5/gp; **$p<0.01$, ****$p<0.0001$ compared to sham, #$p<0.05$ compared to capsazepine pre-treated).

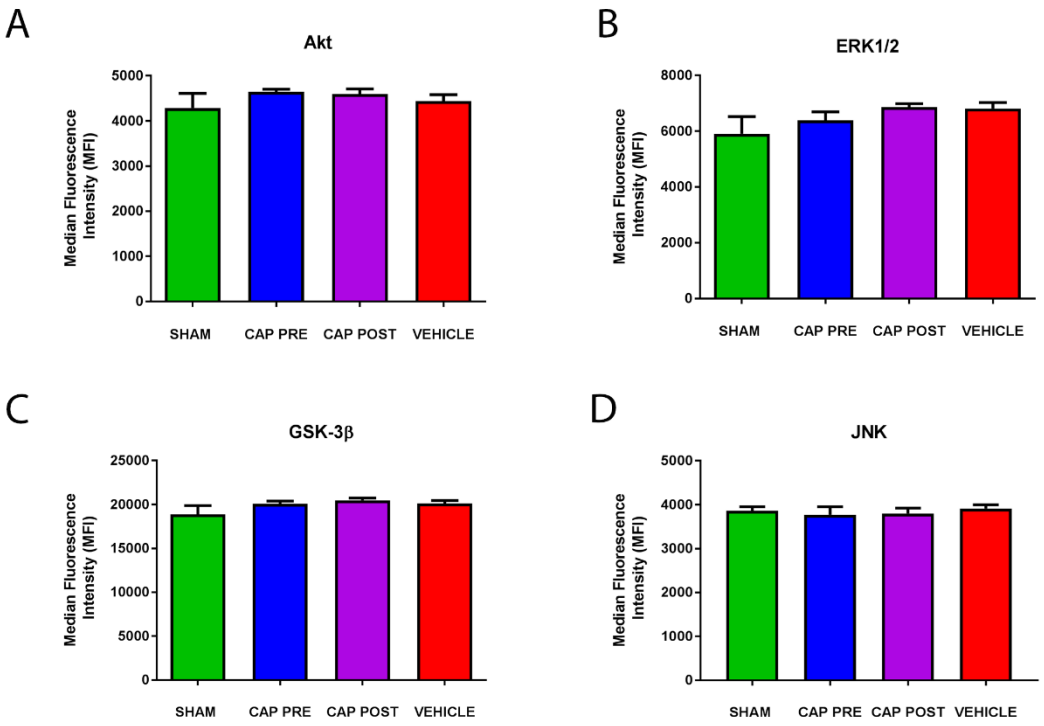

Figure 4.4: Multiplex data showing changes between groups in expression of total Akt (A), ERK1/2 (B), GSK-3β (C) and JNK (D). No significant differences were observed in total levels of Akt, ERK1/2, GSK-3β or JNK levels at 5 hrs post-injury in capsazepine treated animals (n=5/gp, $p>0.05$).

4.3.2.2 Effect of blockade of SP release on tau phosphorylation following severe TBI.

The effect of pre- and post-treatment with capsazepine on tau phosphorylation within the cortex were assessed within the cortex via both WB and immunohistochemistry. No effect of injury nor treatment allocation was seen on the levels of T231 ($p=0.56$), tau-5 ($p=0.15$) or the ratio of T231/tau-5 ($p=0.39$) via western blot (Figure 4.5). In contrast, the more specific evaluation via immunohistochemistry found that cell counts of reactive pTau cells directly underneath the impact site showed significant increases in pTau immunoreactivity in capsazepine post-treated (82.8±28.6, $p<0.0001$; Figure 4.6D) and vehicle-treated (63.4±19.55, $p=0.004$; Figure 4.6B) groups when compared to shams (1.2±1.3; Figure 4.6A). Capsazepine post-treated animals also showed a significant increase in pTau immunoreactivity when compared to capsazepine pre-treated animals (29.8±14.7; $p=0.002$; Figure 4.6C)

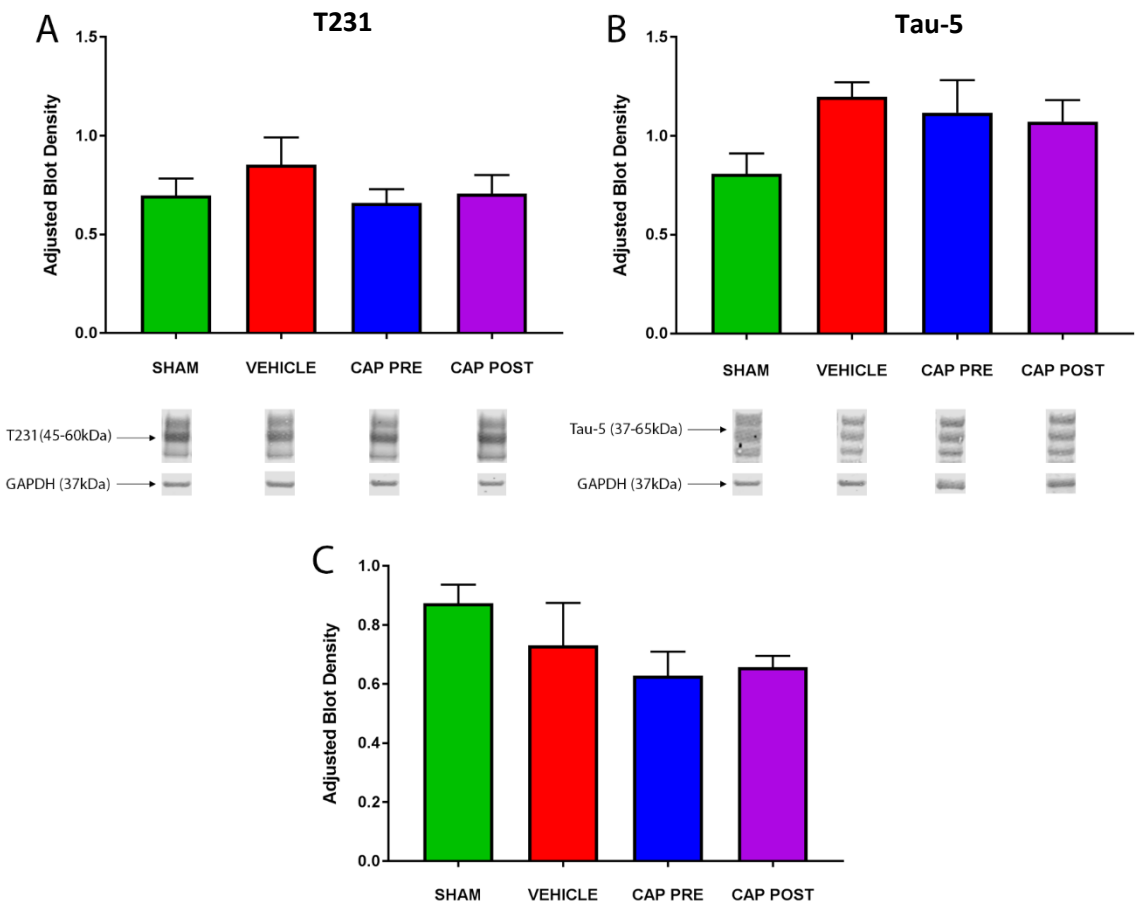

Figure 4.5: Western Blot data showing changes in pTau (T231) deposition (A), Tau-5 expression (B) and T231/Tau-5 ratio (C) in capsazepine treated animals 5 hrs post injury. No significant changes were observed across all groups, regardless of injury type or treatment allocation (n=5/gp, $p < 0.05$).

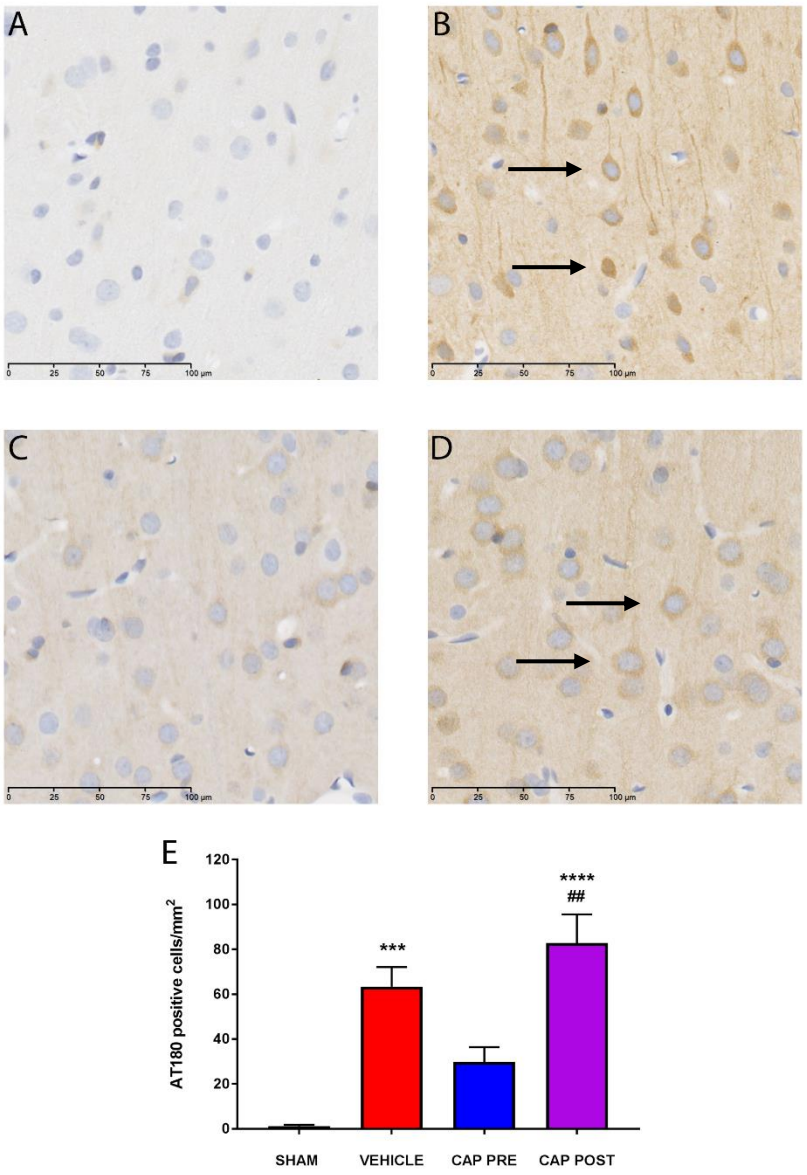

Figure 4.6: pTau immunoreactivity within the cortex directly below the impact site in Capsazepine treated animals 5 hrs post severe TBI. Increased pTau immunoreactivity was observed in capsazepine post-treated (D) and vehicle (B) animals at five hrs post-injury. Less immunoreactivity was noted in capsazepine pre-treated (C) animals and virtually no immunoreactivity was noted in sham (A) animals. Images are displayed at 40x magnification, scale bar 0-100µm. This was confirmed via cell count of pTau immunoreactive cells directly underneath the impact site in capsazepine treated animals 5 hrs post-injury (E)**.** (n=5 all groups; ***p<0.001, ****p<0.0001 compared to sham, ##p<0.01 compared to capsazepine pre-treated).

4.4 DISCUSSION

These studies showed that treatment with the $TRPV_1$ receptor antagonist capsazepine prevented the release of SP following rmTBI only when given prior to injury, whereas in severe TBI both pre and post-injury treatment successfully prevented SP release. Administration of capsazepine was not associated with alterations in the levels of the NK_1 or $TRPV_1$ receptor following either rmTBI or severe TBI. Given that we had previously shown increases in tau phosphorylation acutely following severe TBI, we also sought to investigate whether preventing the release of SP with capsazepine would prevent this. Of note although both pre and post-treatment with capsazepine led to a reduction in SP levels at 5 hrs post-injury, only the pre-treatment group had significantly increased expression of pJNK and decreased levels of AT180 positive cells when assessed via IHC. It is interesting to observe the differences in SP levels in sham animals when comparing the severe and rmTBI groups. This could be due to a multitude of factors including animal supply, surgical techniques, amount of anaesthesia, increased trauma due to intubation in severe sham animals, however these differences were controlled for as closely as possible within the scope of the experiments.

Given the effectiveness of capsazepine treatment in reducing SP levels regardless of timing of administration following severe TBI compared to in rmTBI when only pre-treatment was successful, there may be different mechanisms driving SP release in these two models of TBI. Reductions in SP when given prior to injury, suggest that mechanical activation of the $TRPV_1$ receptor is required, whereas effectiveness after injury is suggestive of activation of $TRPV_1$ by other mediators, such as inflammatory mediators including bradykinins and prostaglandins. Neuroinflammation is widely shown to be increased following severe TBI in the intermediate phase of injury, with numerous potential triggers including peripheral blood products, tissue and cellular debris and reactive oxygen species potentially starting 1 hr following the initial mechanical insult (Algattas & Huang 2014). Indeed, a study by Tompkins et al. (2013) found that in a model of blast wave induced severe TBI,

increases in interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNFα) were elevated 1 hr post-injury. Given that neuroinflammation is not limited to severe TBI with microglial activation also seen at 6 hrs following diffuse mTBI (Lafrenaye et al. 2015), further studies would be needed to determine whether the time-course of inflammation and its severity differs between severe and rmTBI and may explain the difference in effectiveness of capsazepine post-treatment in preventing SP release.

In line with the results from the previous chapter the peak of SP release was not associated with changes in the expression of the NK_1 or $TRPV_1$ receptor, with this unaltered by capsazepine treatment. Indeed previous studies have suggested that delayed time-points are required to observe changes in these receptors, with increased expression of $TRPV_1$ in response to cold stressors seen at 28 days-post-injury (Liu et al. 2017). Similarly increased NK_1 receptor expression following penetrative brain-injury was observed from 1-4 weeks post-injury (Lin 1995). Extension of the final timepoints may therefore elucidate changes in NK_1 and $TRPV_1$ expression following TBI in future studies.

As SP release was attenuated by capsazepine treatment both pre and post severe injury, we further investigated whether this altered kinase activation and tau phosphorylation post-injury. Capsazepine pre-treatment did indeed attenuate tau phosphorylation following injury as observed in IHC, whereas post treatment did not. Furthermore capsazepine pre-treatment significantly increased levels of pJNK compared to vehicle treated animals, although levels were still significantly decreased compared to shams. Given that both capsazepine pre and post-treatment decreased the expression of SP at 5 hr post-injury the mechanism driving the decrease in tau phosphorylation in only the pre-treated animals is difficult to explain. It is possible that pre-treatment is more effective at preventing other aspects of the secondary injury cascade, such as axonal injury- either due to the longer time-frame available or due to the prevention of initial SP release. Examination of SP levels

prior to the 5 hr time-point would allow elucidation of whether SP levels differ in the pre versus post-treated animals at the very early stage post-injury. However these changes in tau phosphorylation do not appear to be directly linked to changes in the activity of the kinases examined in this study.

It is also interesting to observe the decreases in pJNK following severe injury as a study by Otani et al. (Otani et al. 2002) indicated that following moderate TBI, pJNK levels decreased following a sharp increase 5 mins post-injury, reducing to non-significance from the sham group within 30 mins. This could potentially be a neuroprotective rebound effect, as in a study by Okami et al. (Okami et al. 2013) showed that through blockade of JNK, therefore preventing upregulation of the JNK signalling pathway and therefore inducing JNK dephosphorylation, has been shown to reduce neuronal cell death in an *in vitro* model of ischaemia after reperfusion at 3 and 24 hr timepoints.

4.5 CONCLUSIONS

Overall it appears that mechanical activation of the $TRPV_1$ receptor is key to facilitating SP release following rmTBI, and that secondary injury processes in conjunction with mechanical activation facilitate SP release following severe injury. It also appears that blockade of $TRPV_1$ can have an effect on tau phosphorylation, however how this happens still requires investigation.

Chapter 5: Does administration of an NK_1 tachykinin receptor antagonist improve outcomes following rmTBI?

5.1 INTRODUCTION

In the previous chapters (Chapters 3-4), it has been demonstrated that there is indeed SP release following severe traumatic brain injury, and that in rmTBI this response may be amplified (Chapter 3). As such, SP may therefore play a significant role in the secondary injury cascade following rmTBI. As outlined previously (Chapter 4) SP release from mechanically stimulated TRPV1 receptors may be a mediator of tau hyperphosphorylation, which is important as accumulation of abnormal tau in the form of neurofibrillary tangles is the hallmark diagnostic feature of CTE (McKee et al. 2016).

In addition, SP release may exacerbate a number of other secondary injury factors, such as axonal injury. Concussive insults are known to be associated with increases in intracellular calcium, due to the ionic fluxes induced by the biomechanical impact, as well as the indiscriminate release of neurotransmitters, including glutamate (Giza & Hovda 2014; Prins et al. 2013). Such increased levels of intracellular calcium promote over-activation of a number of deleterious enzymes, which in turn lead to cellular injury and axonal damage through disruption of key cytoskeletal components such as neurofilaments and microtubules (Giza & Hovda 2014). Indeed, SP can facilitate this process, with studies demonstrating that SP administration leads to a sustained potentiation of NMDA receptors (Castillo et al. 2011), as it facilitates the release of the excitatory neurotransmitter glutamate (Guiard et al. 2007) which binds to the NMDA receptor to promote calcium influx (Strong et al. 2014).

Axonal injury is a key mediator of cognitive deficits following concussion (McKee et al. 2014), with recent reports that reduced axon integrity in CTE cases is related to the degree of tau pathology in the associated grey matter (Holleran et al. 2017). The level of axonal injury has been shown to be markedly reduced following administration of an NK_1 tachykinin receptor antagonist following severe injury in females (Corrigan et al. 2012; Donkin et al. 2011), which is the principal receptor to which SP binds (Alves 2014). This reduction in axonal injury was associated with an improvement in acute behavioural outcomes encompassing both motor and cognitive domains in females (Corrigan et al. 2012; Donkin et al. 2011). However, the efficacy of NK_1 tachykinin receptor antagonist

treatment following rmTBI is yet to be determined. It should be noted that in the previous severe TBI studies two different forms of the NK_1 antagonist have been utilised encompassing NAT, which cannot cross an intact BBB due to its lipid solubility (Banks 2009) and EU-C-001 (001), a blood-brain permeable variant (Donkin et al. 2011).

Therefore, this chapter sought to assess the efficacy of both NK_1 tachykinin receptor antagonists, NAT and EU-C-001 following rmTBI, both in the acute (24 hrs post-injury) and more chronic phase of injury (12 weeks post-injury), through evaluation of their effects on kinase activity, axonal integrity and tau phosphorylation, as well as behavioural outcomes. It is hypothesised that EU-C-001 will be more effective due to its lipid solubility and enhanced ability to cross the BBB, in contrast to NAT which is not lipid soluble and requires the BBB to be open to penetrate the brain (Donkin et al. 2011). A smTBI group was also included in this study to assess whether there are any long term effects following injury, although there were no pathological differences observed in earlier studies (Chapter 3), there may be long term effects of a single injury that may not present acutely.

5.2 STUDY DESIGN

A total of 100 animals were used in the study: 1) acute study: n=50 animals, 24 hr survival time-point; 2) long-term study: n=50; 12 week survival time-point. For both survival time-points, animals were randomly assigned into experimental groups: sham, smTBI, rmTBI with vehicle treatment (rmTBI VEH), rmTBI with NAT treatment (rmTBI NAT) or rmTBI with EU-C-001 treatment (rmTBI 001).

Animals received mild TBI (as described in Chapter 2.2.1) and at thirty minutes following each injury, animals were re-anaesthetised and administered either NAT (2.5mg/kg), EU-C-001 (1mg/kg) or vehicle (0.9% saline) via tail vein injection, prepared as described in Chapter 2.3.2 - 2.3.3. At the pre-determined end time-points, n=5 animals/gp were allocated to fresh tissue collection and n=5 animals were allocated to fixed tissue collection. However due to technical difficulties and loss of corrupted data from the functional testing, group sizes have varied over the course of this study.

5.2.1 Functional Outcome Testing

At six and twelve weeks post-injury, animals were tested for measures of anxiety, spatial and recognition and working memory as well as depressive-like symptoms using the Open Field (OF), Elevated Plus Maze, Y Maze (EPM), Barnes Maze (BM) and Forced Swim Test (FST), as described in Chapter 2.4.

5.2.2 Fresh Tissue Analysis

Fresh tissue was collected at 24 hrs and 12 weeks post-injury, homogenised (as per Chapter 2.6.1) and protein levels estimated (as per Chapter 2.6.2). Levels of normal and phosphorylated tau were assessed at both time-points using WB (as described in Chapter 2.6.5). Markers of axonal integrity including myelin basic protein (MBP) and neurofilament light chain (NFL), as well as the synapse markers, in post-synaptic density protein 95 (PSD-95), and synaptophysin were analysed at the 12 week timepoint using WB (as described in Chapter 2.6.5). Levels of cortical SP release at 24 hrs post injury were assessed using a commercial SP ELISA (as described in Chapter 2.6.3). Cell signalling interactions were assessed at 24 hrs post injury using a custom made Bio-Plex Pro Cell Signalling Assay kit (Multiplex, Bio-Rad; as described in Chapter 2.6.4).

5.2.3 Fixed Tissue Analysis

To further examine the cortical distribution of pTau, immunohistochemistry was performed (as per Chapter 2.7.2). Data were collected by two independent and blinded assessors (as described in Chapter 2.7.2).

5.2.4. Statistical Analysis

All functional outcome measures, excluding the Barnes Maze, were analysed using a one-way ANOVA with Tukey post-hoc analysis. Barnes Maze data from days 1-3 of the protocol and the probe trial were analysed using a two-way ANOVA with Tukey post-hoc analysis for multiple comparisons.

SP ELISA data was analysed by first creating a standard curve with a four parameter logistic fit. The unknown values were then interpolated to the standard curve. Data were then sorted into experimental groups and analysed using a one-way ANOVA with Tukey post-hoc analysis. WB density

data, the multiplex kinase assays and pTau immunohistochemistry cell count data were analysed using a one-way ANOVA with Tukey post-hoc analysis. All statistical analyses were performed in GraphPad Prism (GraphPad Software, v7.0.2.). All data are displayed as mean ± SEM.

5.3 RESULTS

5.3.1 SP ELISA

A significant increase in cortical SP concentration was observed in rmTBI VEH tissue (1388±491.9 pg/mL) when compared to sham (567.5±187.1 pg/mL, p = 0.002), smTBI (613.9±338.4 pg/mL, p = 0.003), rmTBI NAT (589±127.2 pg/mL, p = 0.002) and rmTBI 001 (391±121.2 pg/mL, p = 0.0002) animals at 24 hrs post-injury (Figure 5.1). No other significant differences in cortical SP levels amongst groups were observed (p>0.05).

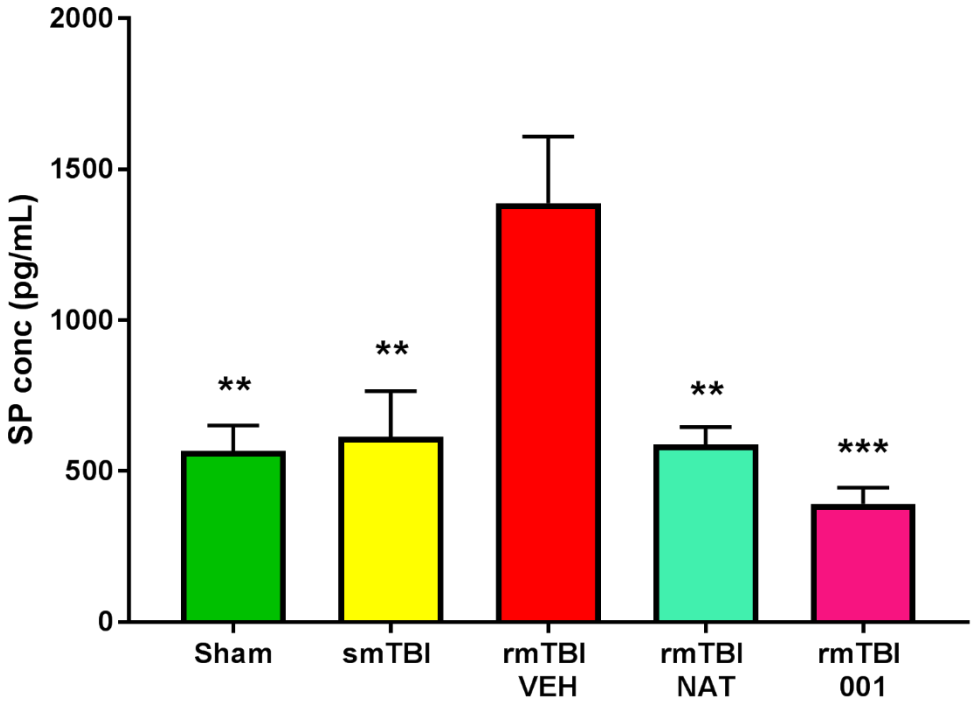

Figure 5.1: SP ELISA data showing changes in SP expression across treatment groups at 24 hrs post injury**.** A significant increase in SP concentration was observed in the rmTBI VEH group, which was prevented with administration of both NK_1 tachykinin receptor antagonists NAT and 001. (n=5/gp; **p<0.01, ***p<0.001 compared to rmTBI VEH).

5.3.2 Kinase Activation

The effect of NK_1 tachykinin receptor antagonist treatment on acutely altering the expression of kinases, pAKT, pJNK, pERK and pGSK3α/β, known to be involved in tau phosphorylation expression was evaluated. A significant decrease in levels of pAKT were seen in the rmTBI VEH ($p = 0.005$) and rmTBI NAT ($p = 0.011$) groups compared to shams. Furthermore the smTBI ($p = 0.005$), rmTBI VEH ($p = 0.0002$) and rmTBI NAT ($p = 0.0004$) had significantly lower levels than the rmTBI 001 group (Figure 5.2A).

Similar results were observed in pERK1/2 expression at 24 hrs post injury (Figure 5.2B), with significant decreases relative to the sham and rmTBI 001 animals seen following smTBI (SHAM $p = 0.031$; rmTBI 001 $p = 0.0002$), rmTBI VEH (SHAM $p = 0.004$; rmTBI 001 $p<0.0001$) and rmTBI NAT (SHAM $p = 0.014$; rmTBI 001$p<0.0001$) animals. The same pattern was observed in pJNK expression, with significant decreases relative to the sham and rmTBI 001 animals seen in the smTBI (SHAM $p = 0.026$; rmTBI 001 $p = 0.0009$), rmTBI VEH (SHAM $p = 0.002$; rmTBI 001 $p<0.0001$) and rmTBI NAT groups (SHAM $p = 0.008$; rmTBI 001 $p = 0.0003$) (Figure 5.2D). In contrast no effect of injury or NK_1 tachykinin receptor antagonist treatment was seen on pGSK-3α/β expression ($p=0.181$; Figure 5.2C). Overall no changes were observed when looking at total expression of Akt, ERK, GSK-3α/β or JNK across all injury and treatment groups (Figure 5.3)

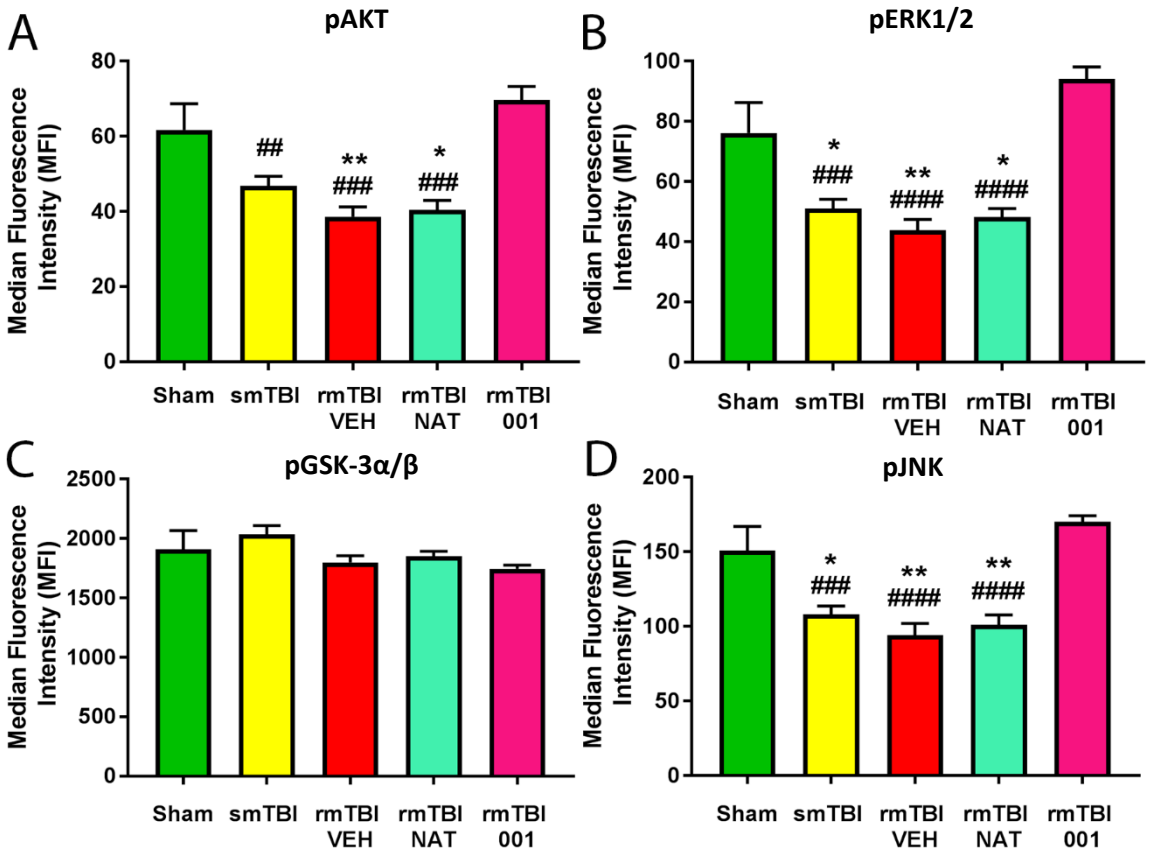

Figure 5.2: Multiplex data showing changes between groups in expression of pAKT (A), pERK1/2 (B), pGSK-3α/β (C) and pJNK (D) in NK_1 tachykinin receptor antagonist treated animals at 24 hrs post injury. Treatment with 001 following rmTBI preserved levels of pERK1/2, pJNK and pAkt at 24 hrs following injury. There was no effect of injury on the levels of pGSKα/β. (n=5 all groups; *p<0.05, **p<0.01 compared to sham, ##p<0.01, ###p<0.001, ####p<0.0001 compared to rmTBI 001).

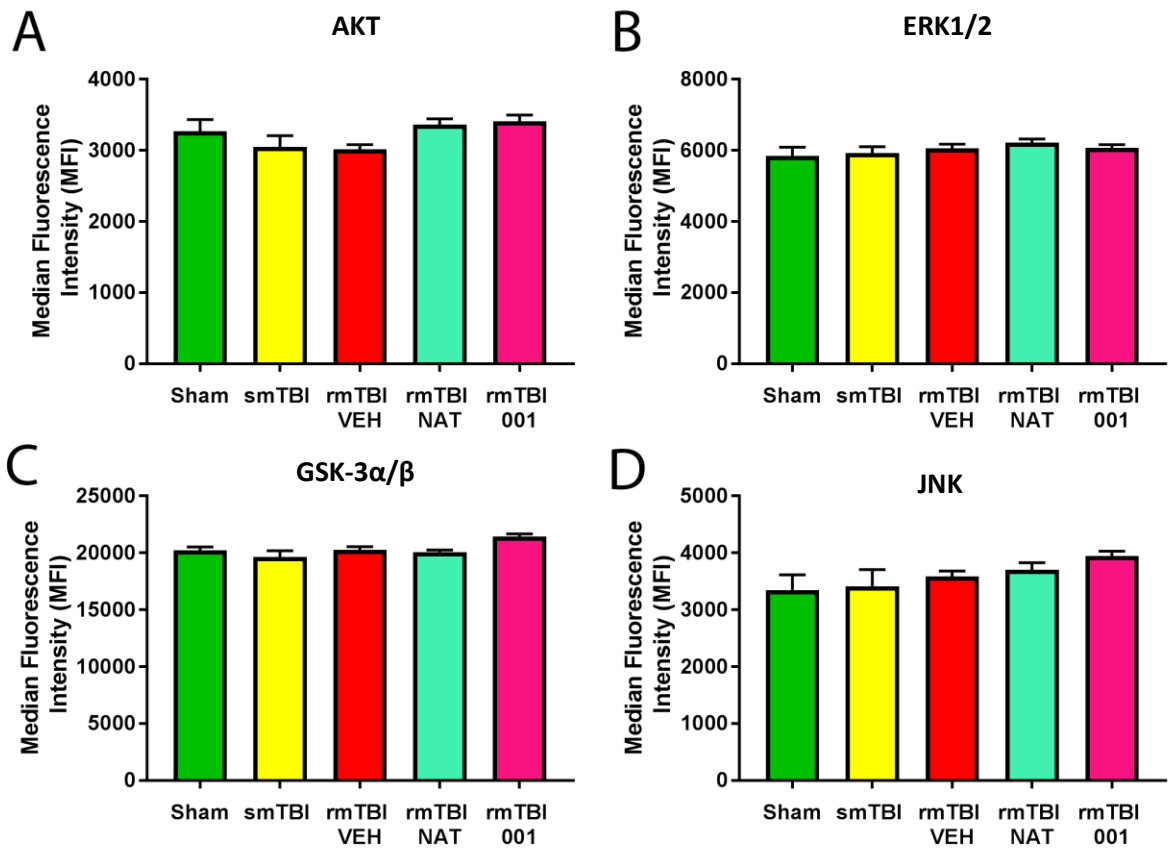

Figure 5.3: Multiplex data of total Akt (A), ERK1/2 (B), GSK-3α/β (C) and JNK (D) in any animals at 24 hrs post injury. No changes were observed across all experimental groups (n=5gp/, p>0.05).

5.3.3 Tau Phosphorylation

The effects of blockade of SP signalling on tau phosphorylation were examined via both WB, as well as IHC of the number of AT180+ve neurons directly under the impact site. At 24 hrs post-injury, no changes in pTau ($p = 0.81$), Tau-5 ($p = 0.84$) or the pTau/Tau-5 ratio ($p=0.76$) were observed between any of the experimental groups within the cortical impact site via WB (Figure 5.4A-C). However, with a more directed analysis via IHC cell counts of the cortex located directly underneath the impact site, a significant increase in the number of pTau immunoreactive cells was seen in the rmTBI VEH (67±13.91; $p = 0.019$) and rmTBI NAT groups (65.4±21.05; $p = 0.026$), with a trend towards an increase in the rmTBI 001 group (59.8±22.4; $p=0.069$) when compared to the smTBI group (27.6±12.18; Figure 5.4D). No significant differences in pTau immunoreactive cells were noted in the sham animals (45.5±17.86) compared to the smTBI group (Figure 5.5).

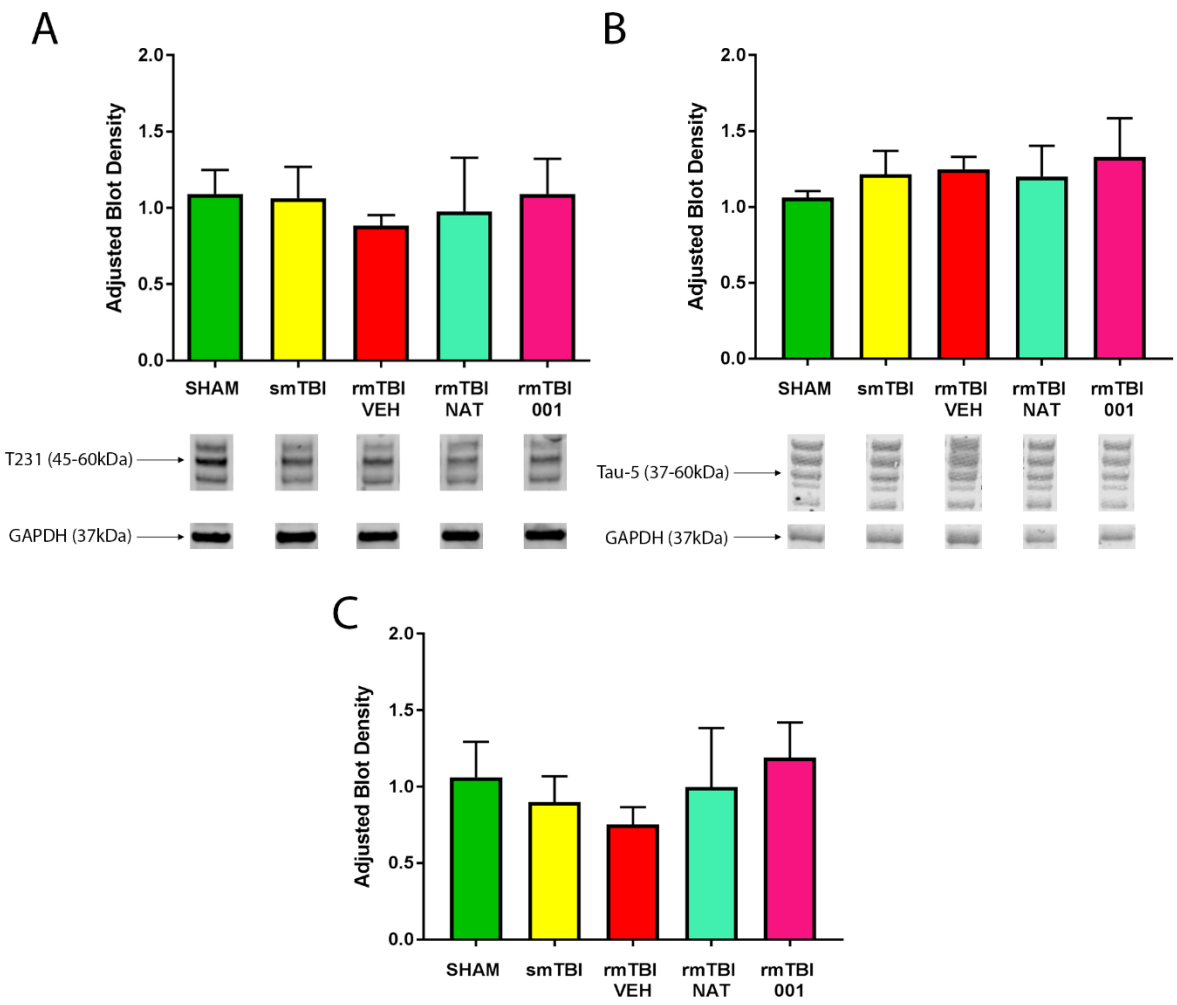

Figure 5.4: Analysis of pTau (T231) (A), Tau-5 expression (B) and the T231/Tau-5 ratio (C) in NK_1 tachykinin receptor antagonist treated animals at 24 hrs post injury via WB. There were no changes in levels of tau phosphorylation at the T231 site noted at 24 hrs following injury (n=4 per group, $p<0.05$).

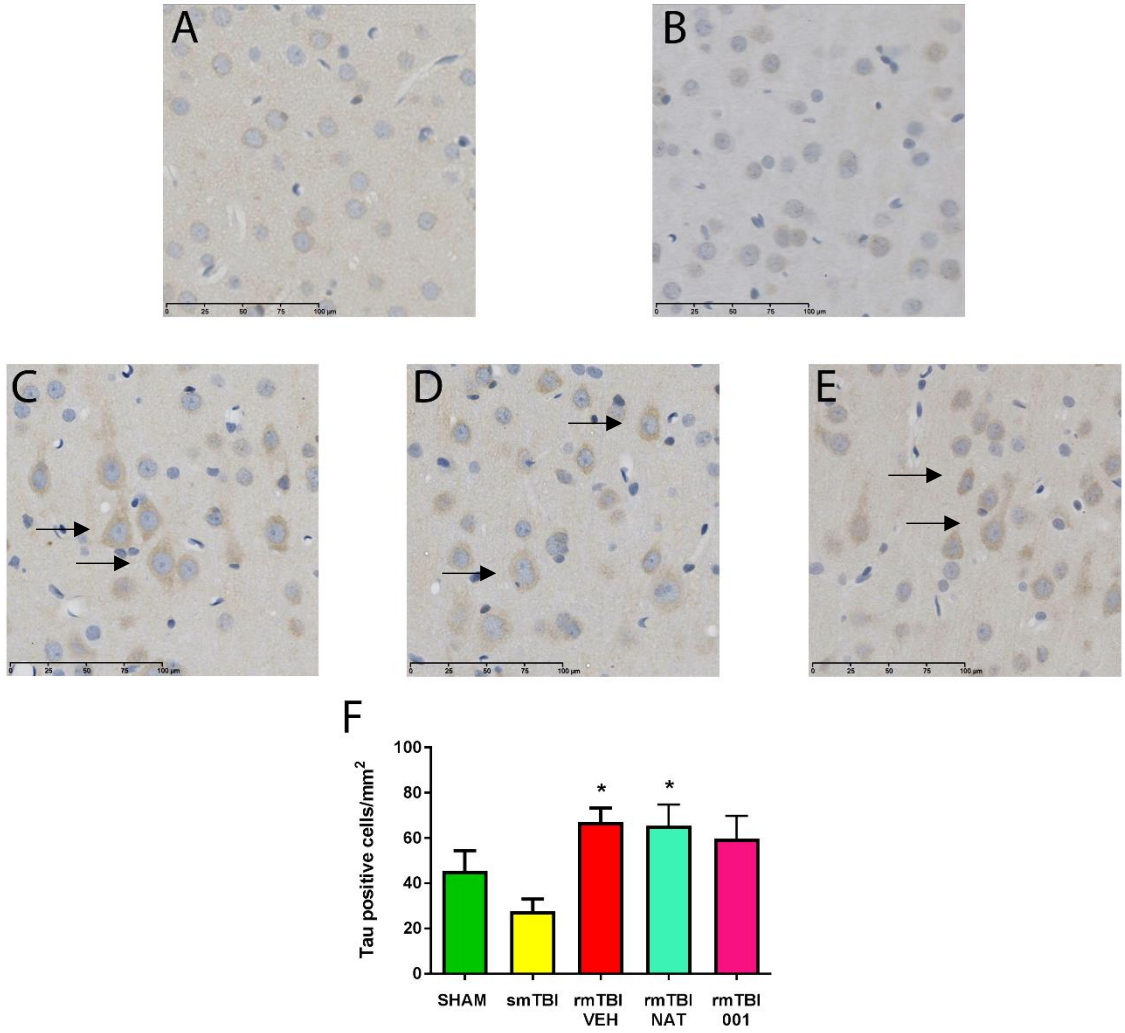

Figure 5.5: Representative IHC images of AT180 staining in cells directly underneath the impact site in sham (A), smTBI (B), rmTBI VEH (C), rmTBI NAT (D) and rmTBI 001 (E) animals 24 hrs post-injury. IHC staining showed a significant increase in AT180 positive cells in rmTBI VEH and rmTBI NAT groups at the 24 hr time-point (arrows), when compared to the smTBI group. Staining also appeared to be increased in the rmTBI 001 group, however this was not significant when compared to the other groups. Sham and smTBI groups generally appeared to have less staining overall when compared to all other groups. Images taken at 40x, scale bar 0-100µm. These changes were confirmed via cell count of the pTau immunoreactive cells (F) (n=5/gp; *p<0.05 compared to smTBI).

At 12 weeks post-injury, WB analysis showed significant differences in T231 levels (Figure 5.6) with smTBI ($p=0.001$), and all the rmTBI animals, irrespective of treatment (mTBI VEH $p=0.04$, rmTBI NAT $p<0.0001$ and rmTBI 001 $p=0.01$) having higher levels compared to shams. Amongst injury groups, a significant increase in T231 expression was observed in the rmTBI NAT group when compared to the rmTBI VEH group ($p = 0.02$), with no significant differences observed between the rmTBI VEH and rmTBI 001 groups ($p = 0.96$). However, when converted to a ratio of T231/Tau-5 expression no significant differences in tau phosphorylation were noted between any of the vehicle or NK_1 tachykinin receptor antagonist treated groups ($p=0.63$; Figure 5.6C). This may be potentially due to differences in expression of total-tau, with a significant increase in tau-5 expression in the rmTBI NAT group when compared to sham animals ($p = 0.02$; Figure 5.6B). Despite these observations, WB analysis did not match the IHC analysis, with no significant differences in AT180+ve cells observed across any of the groups ($p = 0.352$; Figure 5.6D).

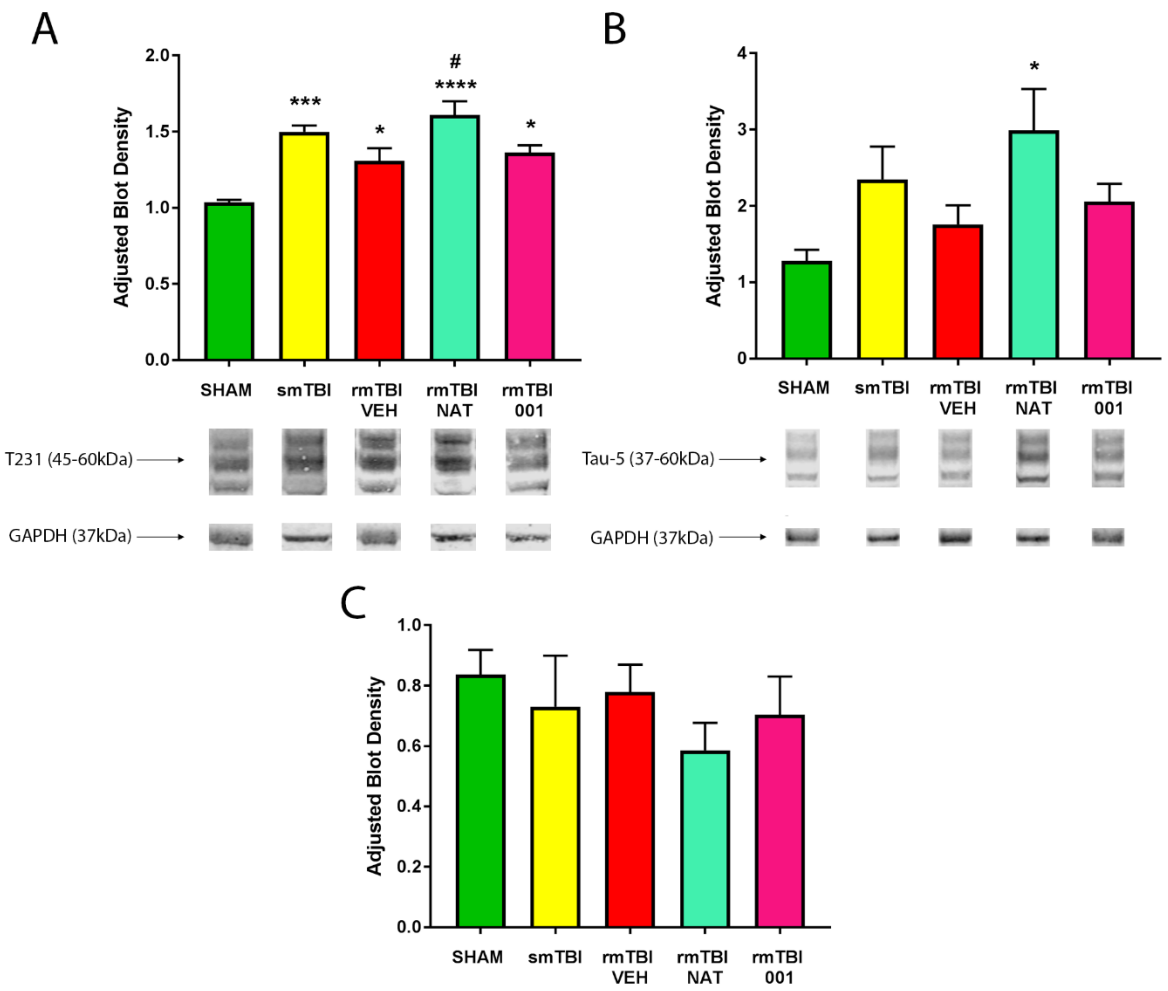

Figure 5.6: Analysis of changes in the levels pTau (T231) deposition (A), Tau-5 expression (B) and the T231/Tau-5 ratio (C) in NK_1 tachykinin receptor antagonist treated animals at 12 weeks post-injury. Significant increases in T231 expression were observed in all experimental groups when compared to sham animals, and in the rmTBI NAT group when compared to the rmTBI VEH group (A). Tau-5 expression was also significantly increased in rmTBI NAT animals when compared to sham (B). No effect of NK_1 antagonist treatment was seen on the Tau231/Tau-5 ratio (C) (n=4/gp; *p<0.05, ***p<0.001, ****p<0.0001 compared to sham, #p<0.05 compared to rmTBI VEH).

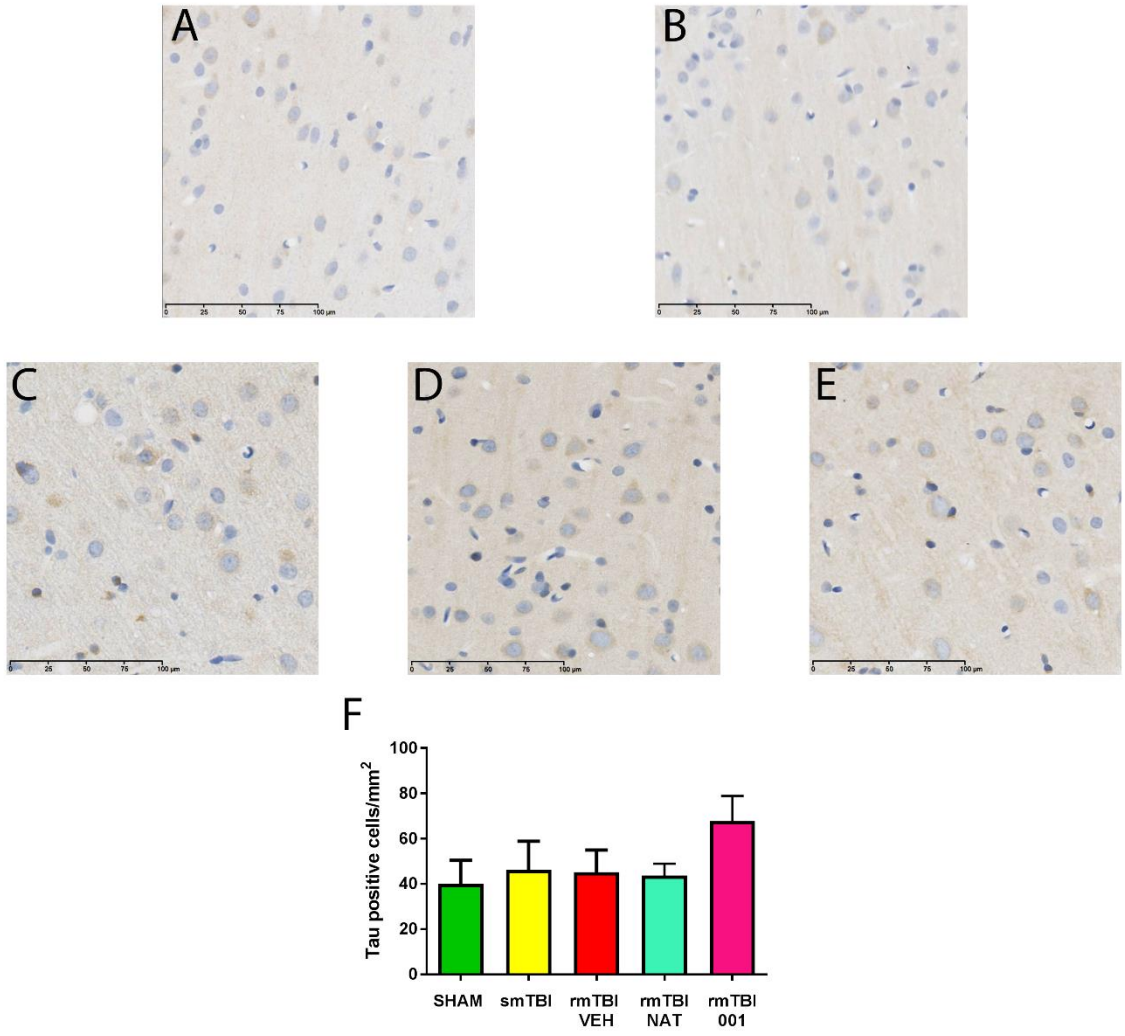

Figure 5.7: Representative IHC images of AT180 staining in cells directly underneath the impact site in sham (A), smTBI (B), rmTBI VEH (C), rmTBI NAT (D) and rmTBI 001 (E) animals 24 hrs post-injury. No significant differences in AT180 staining were observed across all experimental groups. Images taken at 40x, scale bar 0-100µm. These observations were confirmed via cell count of the pTau immunoreactive cells (F)**.** (n=5/gp; p>0.05).

5.3.4 Axonal and Synaptic Integrity

WB analysis was used to investigate the effects of NK_1 tachykinin receptor antagonist treatment following rmTBI on preservation of synaptic and axonal integrity at 12 weeks post-injury in the cortex under the impact site (Figure 5.8). Levels of PSD-95, a post-synaptic scaffolding protein, were significantly increased following both smTBI (4.09±0.93; p = 0.004) and rmTBI NAT (4.13±1.32; p = 0.004), when compared to shams (1.474±0.51) at 12 weeks post -injury. Significant differences were also observed in the smTBI and rmTBI NAT groups when compared to both the rmTBI VEH (1.67±0.7; p = 0.008; p = 0.007 respectively) and rmTBI 001 (1.18±0.45; p = 0.001; p = 0.001 respectively) groups (Figure 5.8A). In contrast, levels of synaptophysin, a presynaptic vesicular protein representing an index of synapse formation, were unaltered by injury (p = 0.767) (Figure 5.8D).

In regards to axonal integrity, MBP, which is essential for normal myelination was found to significantly differ between groups (Figure 5.8B; p=0.02). Specifically, there was a significant increase in MBP levels in rmTBI VEH animals (1.65±0.52; p<0.05) and a trend towards an increase in rmTBI NAT animals (1.51±0.39; p=0.05), compared to the rmTBI 001 animals (0.69±0.25). No differences in MBP band density were seen in relation to sham (1.11±0.15) or smTBI (1.25±0.50) animals (Figure 5.8B). The axonal structural protein NFL was also assessed, however no significant differences were seen, irrespective of injury or treatment type (p=0.77; Figure 5.8C).

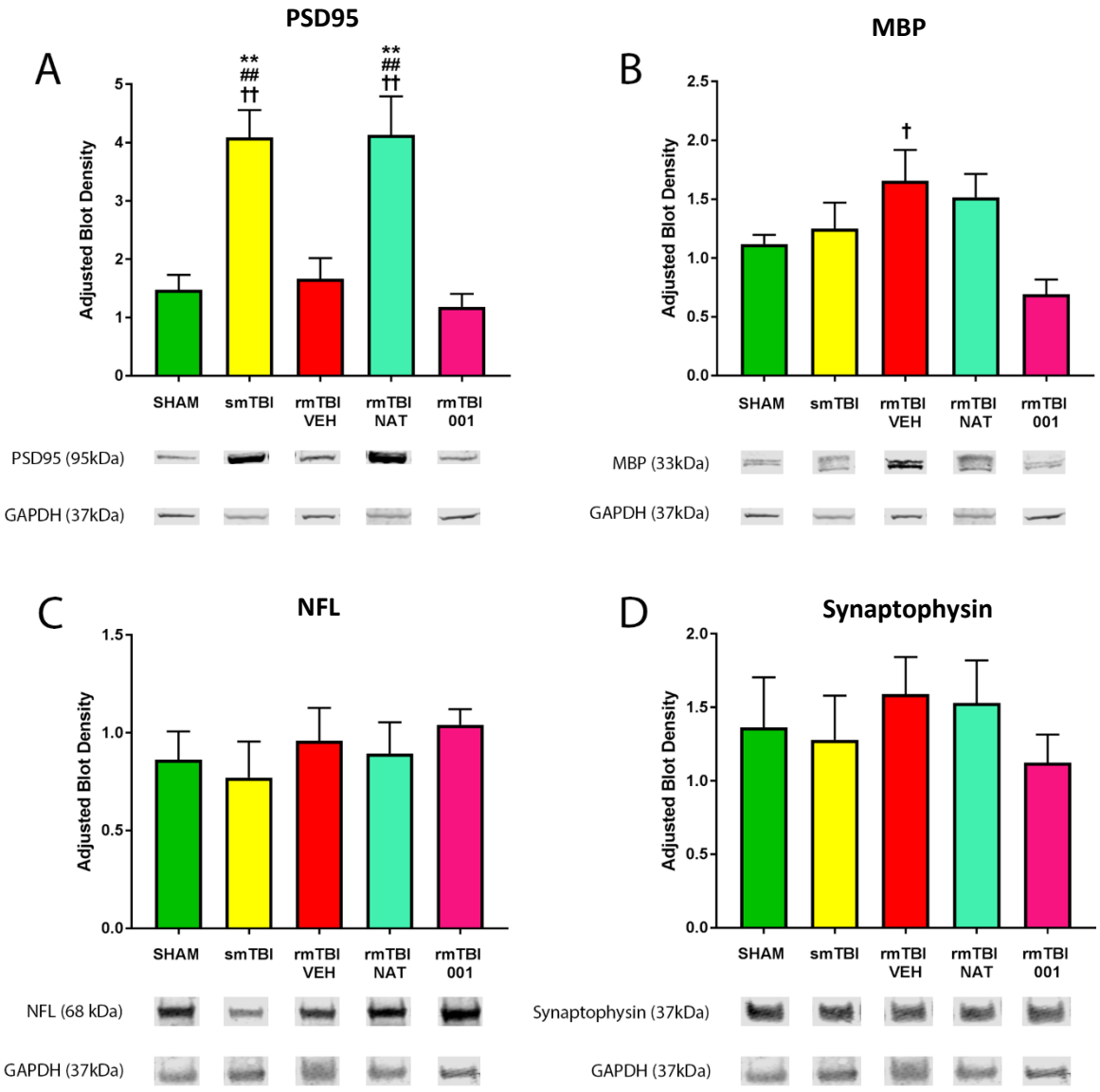

Figure 5.8: WB data showing changes in PSD95 (A), MBP (B), NFL (C) and synaptophysin (D) in NK_1 treated animals 12 weeks post injury. (n=4/ gp; **p<0.01 compared to sham, ##p<0.01 compared to rmTBI VEH, †p<0.05 compared to rmTBI 001, ††p<0.01 compared to rmTBI 001).

5.3.5 Functional Outcome Testing

The effect of NK_1 tachykinin receptor antagonist treatment on locomotion, anxiety-like behaviour, depressive-like behaviour and cognition was assessed at 6 and 12 weeks post-injury. Distance travelled in the OF showed no differences at 6 weeks post-injury between any of the groups ($p = 0.61$; Figure 5.9A). However, at 12 weeks post-injury, a significant increase in the total distance travelled was observed in the rmTBI 001 group compared to shams (38.59±13.27m vs. 24.99±11.54m, p=0.015), in addition to a trend towards increased distance travelled in the rmTBI VEH group (35.75±4.8; p=0.086). No significant differences were seen between the smTBI (33.79±5.38m; p=0.226) and rmTBI NAT groups (32.73±9.04m; p=0.346) when compared to shams (Figure 5.9B).

Within the EPM no difference in terms of time spent in the open arm was noted at either 6 (p=0.977; Figure 5.9C) or 12 weeks (p=0.188; Figure 5.9D) post injury. Similarly, the amount of time spent in the novel arm within the Y Maze was not significantly different between any group at either time-point (6 weeks: p=0.791; 12 weeks p=0.253; Figure 5.9E&F). Depressive-like behaviour was evaluated as time spent immobile on the FST, with no differences seen at 6 weeks (p=0.129; Figure 5.9G), but an overall effect seen at 12 weeks post-injury (p=0.013). This was driven by a significant reduction in time spent immobile in the smTBI animals (110.7±52.69) compared to the rmTBI NAT animals (180.4±52.65s; p=0.023), with a trend compared to the sham (167.7±54.78s; p=0.091) and rmTBI 001 groups (167.3±24.04s; p=0.095). No differences were observed between the smTBI and rmTBI VEH groups at any time post-injury (129.5±55.44s; p=0.91; Figure 5.9H).

Learning and memory was also examined on the Barnes Maze, with the time taken to enter the escape box averaged over two trials on three separate training days. All groups displayed a significant improvement in test duration time over the three day testing period at both 6 and 12

week time points ($p<0.0001$ 6 & 12 weeks), however such changes were not significantly different between groups (6 weeks: $p=0.643$; 12 weeks: $p=0.199$; Figure 5.10A&B). Probe trial data analysis of the time taken to find and enter the new escape box location was used as the primary outcome measure, and although the time taken to complete the trial did improve across all groups ($p=0.029$ 6 weeks, $p=0.01$ 12 weeks), no significant differences were observed between groups at both the 6 and 12 week time-points (6 weeks: $p=0.322$; 12 weeks: $p=0.715$; Figure 5.10C&D).

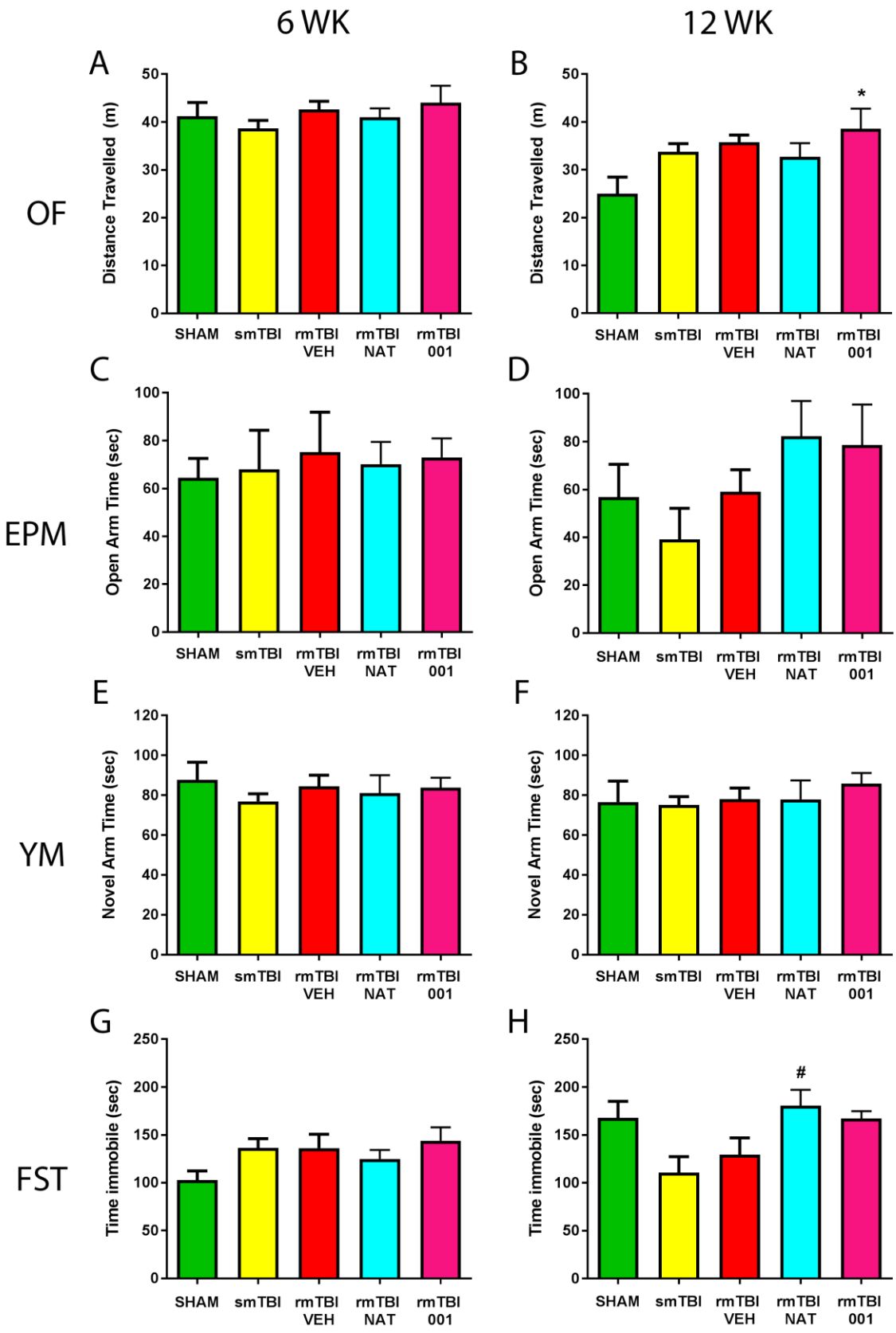
6 WK
12 WK
OF
EPM
YM
FST
A
B
C
D
E
F
G
H
Distance Travelled (m)
Open Arm Time (sec)
Novel Arm Time (sec)
Time immobile (sec)
SHAM
smTBI
rmTBI VEH
rmTBI NAT
rmTBI 001
*
#

Figure 5.9: Effect of NK_1 tachykinin receptor antagonist treatment on functional performance on the OF (A&B), EPM (C&D), Y Maze (E&F) and FST (G&H) performance at 6 (left) and 12 (right) weeks post-injury. No differences were noted between groups at the 6 week time-point for all groups in all tests (OF: n=10/gp, $p>0.05$; EPM: SHAM, smTBI, rmTBI NAT n=10, rmTBI VEH n=6, rmTBI 001 n=7, $p>0.05$; Y Maze: SHAM, rmTBI VEH n=9, smTBI n=10, rmTBI NAT n=7, rmTBI 001 n=6, $p>0.05$; FST n=10/gp, $p>0.05$). No differences were observed between groups in EPM and Y Maze performance at the 12 week time-point (EPM: n=10/gp, $p>0.05$; SHAM n=9, smTBI n=10, rmTBI VEH, rmTBI NAT n=7, rmTBI 001 n=8, $p>0.05$). A significant increase in distance travelled was observed in rmTBI 001 animals when compared to sham in the OF at 12 weeks post-injury (n=10/gp; *$p<0.05$ compared to sham). In the FST at 12 weeks post-injury, a significant increase in immobility time was also observed in the rmTBI NAT group when compared to the smTBI group (n=10/gp; #$p<0.05$ compared to smTBI).

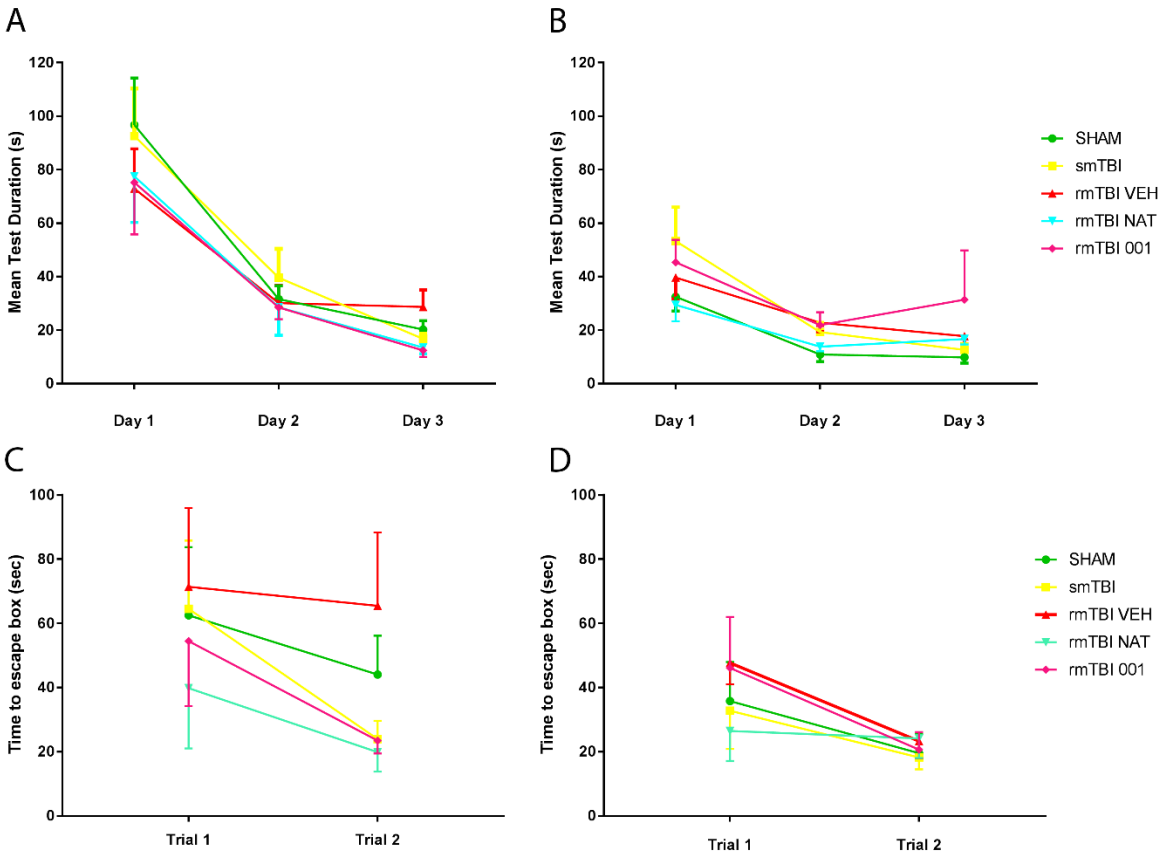

Figure 5.10: Barnes Maze performance over the acquisition phase (A&B) and the probe trial (C&D) at 6 (left panel) and 12 (right panel) weeks post-injury. Although performance did improve over time following injury, there were no significant differences observed between all groups in the acquisition phase at either 6 or 12 weeks post-injury. Similarly, although performance improved over time in the probe trial, no significant difference was observed between all groups at both the 6 and 12 week time-points (6 week: SHAM, smTBI n=8; rmTBI VEH, rmTBI NAT, rmTBI 001 n=7, $p>0.05$; SHAM n=9, smTBI, rmTBI 001 n=8, rmTBI VEH, rmTBI NAT n=7, $p>0.05$).

5.4 DISCUSSION

Although it has been demonstrated in these studies that administration of an NK_1 tachykinin receptor antagonist appears to have acute effects following injury through a reduction in SP release, alteration of the levels of pAkt, pERK and pJNK and a reduction in the levels of tau phosphorylation (when the specific region under the impact zone was assessed immunohistochemically), this did not translate to long-term benefits in improving tau phosphorylation, functional outcomes or changes in markers of axonal integrity. Such findings were unexpected, however there are several caveats. Specifically, the fact that in this study, in contrast to our previous work (McAteer et al. 2016), rmTBI animals did demonstrate any significant functional deficits at either 6 or 12 weeks post-injury, nor accumulation of hyperphosphorylated tau, as assessed via immunohistochemistry, which would be amenable to treatment. As such, these key differences in the pattern of injury observed may contribute to the discrepancy in outcomes observed between our studies. Intriguingly, there was implication that the NK_1 antagonist, NAT, may actually worsen outcome with these animals, showing an increase in levels of PSD-95, a postsynaptic membrane protein that is found adjacent to the presynaptic sites of neurotransmitter release, and an increase in time spent immobile in the FST, which was not present in rmTBI vehicle treated animals. In contrast some benefits of 001 were seen with preservation of sham levels of MBP at 12 weeks post-injury, which was increased in both rmTBI vehicle and NAT treated animals.

Attenuation of SP release via the administration of NK_1 tachykinin receptor antagonists has been observed previously in models of stress exposure. A study conducted by Singewald et al (Singewald et al. 2008) found that at resting, administration of a NK_1 tachykinin receptor antagonist increased SP release, but when subjected to swim stressors, SP release was diminished. It was therefore suggested that under normal conditions SP and the NK_1 receptor act in a self-regulatory capacity, regulating endogenous concentrations via receptor-mediated negative feedback loops. In contrast,

under stressful conditions SP release is further facilitated through the action of NK_1 activation, creating a positive feedback loop leading to higher levels of SP and internalisation of NK_1 receptors that have higher SP binding affinity (Lever et al. 2003; Malcangio & Bowery 1999; Singewald et al. 2008). Therefore administration of a NK1 antagonist can block this positive feedback loop and prevent further SP release via this mechanism. This could also explain the increases in SP observed in the rmTBI vehicle treated animals and the subsequent decrease in concentration in the NK_1 tachykinin receptor antagonist treated groups, due to the blockade of potential SP binding sites and therefore preventing activation of this self-regulation loop.

In addition to investigating acute changes in SP, the effect of rmTBI and subsequent NK1 tachykinin receptor antagonist treatment on levels of activated kinases (pAKT, pERK, pJNK and pGSK3α/β) were also investigated. rmTBI significantly decreased pAkt, pERK and pJNK expression at 24 hrs post-injury, which was prevented by treatment with 001, but not NAT. This may be because higher levels of 001 may have entered the central nervous system, as it is more BBB penetrable than NAT (Donkin et al. 2011), and therefore able to more easily access the cerebral parenchyma. In addition to this, 001 has an additional trifluoromethyl benzyl ester group which increases binding affinity to the NK1 receptor (Cascieri et al. 1994; MacLeod et al. 1994), which may have had an impact on these results.

The effect of these alterations in acute kinase levels require further investigation, given the wide range of functions of these kinases. Protein phosphorylation is the most widespread post-translational modification used in signal transduction and can affect every single cellular process, not limited to cell metabolism, growth, membrane transport and countless others (Ubersax & Ferrell Jr 2007). ERK activation requires phosphorylation at its regulatory Tyr and Thr residues, and has been shown to interact and phosphorylate a large number of substrates (Yoon & Seger 2006). A number of transcription factors are phosphorylated by active ERK including Elk1 (Gille, Sharrocks & Shaw 1992), p53 (Murphy et al. 2002), c-Fos (Milne et al. 1994), and c-Jun (Morton et al. 2003), which are

all important for the initiation and regulation of proliferation and oncogenic transformation (Shaul & Seger 2007). However in most cell types, ERK activation is transient, with peak activation and phosphorylation observed 5-15 minutes after stimulation (Yao & Seger 2004). Dephosphorylation of ERK is mediated by removal of the phosphates from the Tyr and Thr residues by Ser/Thr phosphatases such as PP2A (Alessi et al. 1995; Yao & Seger 2004). Decreases in pERK may be therefore linked to a negative feedback loop involving the actions of phosphatases in controlling excessive ERK activity following acute activation (Yao & Seger 2004). The decrease in pERK expression observed in this study echoes the results of other studies, with dephosphorylation of ERK1/2 in the hippocampus observed following a single mTBI in rodents at 4 and 18 days post-injury (Kuo et al. 2013). The dephosphorylation of ERK has also been linked to the induction of apoptosis via capsase-3 activation (Yufune et al. 2016; Zhao, Luo, et al. 2012) and thus may have detrimental effects acutely post-injury.

With regards to the other kinases assessed in this study, decreases in pAkt have been linked to phosphorylation of tau at Thr212/Ser214 *in vitro* (Ksiezak-Reding et al. 2003). Decreases in pAkt may also be detrimental, as administration of an Akt inhibitor prior to moderate controlled cortical impact TBI increases apoptotic cell death, with depletion of the Akt inhibitor leading to rescue of pAkt and attenuation of TBI-induced cell death (Farook et al. 2013). Furthermore, administration of a histone deacetylase inhibitor, believed to interact with the PTEN and Akt pathways, both prevented such decreased dephosphorylation and improved functional outcomes following TBI (Wang, Jiang, et al. 2013). Reduction of pJNK also improves outcomes following TBI, with a moderate reduction of activity via a known JNK inhibitor found to reduce JNK phosphorylation, which in turn reduced tau phosphorylation following TBI (Tran, Sanchez & Brody 2012).

However, in this study any effects of alterations in kinase activation appeared to be distinct from alterations in tau phosphorylation. Acutely, no increase was seen in T231/Tau 5 ratio via WB,

whereas more targeted immunohistochemical analysis saw an increase in all rmTBI groups regardless of treatment, although this did not reach significance in the 001 treated animals. Nevertheless, observing differences between WB and IHC analysis of tau is not uncommon, as due to the multitude of tau isoforms and phosphorylation sites it can be difficult to select appropriate targets. Indeed, Espinoza et al (Espinoza et al. 2008) assessed the differences in 3R and 4R tau labelling with IHC and WB techniques in a cohort of human AD samples, reporting that although IHC detected both 3R and 4R tau, when analysed with WB, not all tangles appeared to be labelled using the same 3R and 4R monoclonal antibodies. It should be highlighted that WB analysis encompasses a much larger area of tissue compared to IHC, and thus if changes in tau phosphorylation are highly localised these may not be seen via WB as they are "diluted" in the large sample. Furthermore, in lissencephalic brains, tau deposition may run parallel to the surface of the tissue given the shear stress profiles previously observed (Cloots et al. 2008), and given the distinctive accumulation of tau within certain cortical layers, this is difficult to differentiate with WB analysis.

With regards to changes in pTau in the chronic stage of injury in this study, at 12 weeks post-injury an increase in T231 was noted in all injury groups, relative to sham via WB. However it appears that rather than an increase in phosphorylation of tau, this seemed to be caused by an overall increase in levels of tau itself, as once expressed as a ratio (T231/tau-5) this effect was lost. Increases in total tau in blood plasma have been observed in former military personnel and are strongly associated with exposure to mTBI and the development of post-concussive syndrome (Olivera et al. 2015). This also has been observed in animal models, with Ojo et al (Ojo et al. 2016) reporting a two-fold increase in total tau at 6 months following mTBI that was delivered twice weekly for a period of either 3 or 4 weeks. In contrast to the acute time-point, such alterations were not reflected in the IHC analysis, with no differences observed compared to sham tissue. This may, in part, be explained by the way that tau phosphorylation was assessed, as number of AT180+ve neurons, as the increase

seen via WB may have been driven by an increase in intensity in the AT180+ve cells, rather than an increase in the overall number of positive cells. Furthermore, it may suggest that by 3 months post-injury alterations in tau are no longer confined directly to the impact site, and may have spread to encompass greater areas of tissue beyond the immediate injury impact site. This is most evident in CTE disease progression, with stage I of the disease localised to discrete pTau foci in the cerebral cortex and around small blood vessels, whereas in stage IV severe pTau pathology is observed throughout most regions of the cerebral cortex and medial temporal lobe (McKee et al. 2013).

Nonetheless, it is evident that NK_1 tachykinin receptor antagonist treatment had no effect on the levels of tau phosphorylation, at either the acute or long-term time-points examined. Future studies could also examine different phosphorylation sites, although T231 is implicated in NFT formation in other tauopathies such as Alzheimer's disease, there are upwards of 40 phosphorylation sites directly identified that are also involved (Hanger, Anderton & Noble 2009). Investigation of other phosphorylation sites that are important for maintenance of microtubule assembly, such as Ser262, (Biernat et al. 1993), or Ser214, which when phosphorylated, along with T231, can decrease polymerisation of microtubules by tau (Illenberger et al. 1998), may therefore be warranted to give more insight into the effects of rmTBI and potential therapeutic effect of NK_1 antagonist treatment on tau phosphorylation (Schwalbe et al. 2015). Hyperphosphorylation of tau in neurodegenerative disease states occurs at many different sites and is mediated through a multitude of pathways. A study by Cavallini et al. (Cavallini et al. 2013) assessing the phosphorylation of tau in human neuroblastoma cells and primary cortical neurons found that GSK3α/β and MAPK13 were the most active tau kinases at sites associated with AD disease states. Fyn kinase activation has also been linked to tau hyperphosphorylation, it has the ability to hyperphosphorylate tau in the dendritic spines leading to microtubule instability (Rubenstein et al. 2017). Indeed, in a study by Chin et al. (Chin et al. 2005), overexpression of Fyn in a mouse model of AD resulted in accelerated synaptic

loss and cognitive impairments. It could be that NK1 blockade is not having the intended effects on kinase activity, or not interacting with other kinases that could be interacting with tau. The hyperphosphorylation of tau following TBI is an incredibly complex and multifaceted and necessitates a need to highlight the important interactions between tau and kinases in order to understand disease progression in tauopathies.

Despite having no effect on tau phosphorylation, treatment with 001 did preserve MBP levels at sham levels, in contrast to rmTBI vehicle and NAT animals which showed a significant increase at 3 months post-injury. MBP is the second most abundant protein in CNS myelin and is essential for its formation (Boggs 2006). MBP plays an essential role in the compaction and formation of the myelin sheath within the CNS allowing for proper transmission of signals down neurons (Readhead et al. 1990). The mechanism driving an increase in MBP following rmTBI is unknown; it may reflect a rebound effect following an acute alteration of MBP, as increases have been observed in the acute phase of injury. Indeed, MBP has been proposed as a potential biomarker of mTBI, with increases in MBP serum levels reported at 24 hrs, 72 hrs and 14 day time-points following rodent mTBI (Rostami et al. 2012). In line with our findings, increases in MBP were observed in juvenile rats following a moderate CCI TBI at a 60 days post-injury, which were associated with changes in white matter morphology. Further histological examination is required to confirm whether alterations in MBP seen here relate to structural abnormalities in myelination.

In contrast to the changes in MBP which were observed in rmTBI VEH and NAT animals, altered PSD-95 expression was seen in the smTBI and rmTBI NAT animals only. PSD-95 is a scaffolding protein that is expressed within excitatory synapses and is important in glutamatergic neurotransmission (Wakade et al. 2010). PSD-95 expression has also been observed to change expression following neurological injury, with acute decreases following moderate CCI observed in the ipsilateral

hippocampus 7 days post-injury (Wakade et al. 2010). Loss of PSD-95 and post-synaptic scaffolding proteins has also been observed in immature rodents following TBI and in human cases of AD (Gobbel et al. 2007; Proctor, Coulson & Dodd 2011; Walker & Tesco 2013), which does not match the results in this study which report an increase in PSD-95 expression. Although much work has been done assessing the short-term effects of mTBI on axonal integrity, the results from this study warrant further investigation into axonal responses in the chronic phase of injury.

Despite these minor structural changes, limited alterations in functional outcome were observed following rmTBI at either 6 or 12 weeks post-injury. This is in contrast to our previous experience with this model where we observed decreases in locomotion at 6 and 12 weeks post-injury as observed on the OF, as well as mild changes in spatial learning and memory as seen in the Y Maze at 12 weeks post-injury and Barnes Maze at both 6 and 12 weeks have been observed in rmTBI animals and were not observed in the current study (McAteer et al. 2016). Potential explanations for such changes in functional outcome performance not being replicated in the present study are varied. Specifically, the animals used in this study, although sourced from the same supplier, may for some inexplicable reason experienced a phenotypic shift (Wong, Gottesman & Petronis 2005) and therefore did not respond to the rmTBI as they had previously. It could also be the case that the sham animals, although not injured, could have become influenced by outside factors. Although such factors are strictly controlled for, any change to the housing or diet of the animals within the holding facility could also have an effect on these results. Although functional deficits in this model have been observed previously, the longer inter-injury interval may explain the milder deficits observed in this study. Multiple studies have used a shorter, three day interval and in turn, observed greater functional deficits (Huang et al. 2013; Kane et al. 2012; Longhi et al. 2005)In addition to this, corruption of critical functional data due to technological faults reduced group sizes across the board and may have impacted on the presented data, therefore accurate assumptions cannot be made

about changes in functional outcomes in this study. Overall, these results indicate that the behavioural assessments used here may require revalidation and perhaps addition of more robust cognitive assessments such as operant boxes may allow for a more thorough assessment of behavioural changes following rmTBI in this model.

5.5 CONCLUSIONS

Overall it appears that the NK_1 antagonists NAT and 001 may indeed have an effect on acute SP release following rmTBI. In addition to this, 001 but not NAT may also have an effect on phosphorylation of the tau protein kinases Akt, ERK and JNK, however dephosphorylation of these does not lead to molecular changes in pTau deposition in the acute phase of injury. However changes in pTau levels can be observed histologically in the acute phase of injury and molecularly in the chronic phase of injury. Changes in axonal pathology and behavioural outcomes are still unclear and warrant further investigation. To summarise, the NK_1 antagonists may influence outcomes if administered following rmTBI, but their effects on tau phosphorylation are negligible. Therefore in the context of attenuating the long term effects of rmTBI and targeting the phosphorylation of tau related to the development of CTE, the SP/NK_1 system does not appear to be involved. Investigation into other pathways that may be influenced by SP release and therefore NK_1 blockade could possibly highlight another therapeutic intervention for these compounds.

Chapter 6: General Discussion

6.0 INTRODUCTION

From the experiments conducted within this thesis, it has been demonstrated that SP is released in response to both rmTBI and severe TBI. Indeed, this is the first study to show that there is an increase in SP release following rmTBI and that this has the potential to be even greater than that observed at the peak of severe injury (Figure 6.1). This is believed to be caused primarily by mechanical activation of the $TRPV_1$ receptor, as blockade by administration of a $TRPV_1$ receptor antagonist prior to injury prevented SP release following both severe and rmTBI. At the same timepoints, increases in pTau observed via IHC were observed following severe TBI. As such, to determine whether potential changes in tau phosphorylation were driven by the action of SP release on the NK_1 receptor following rmTBI, the efficacy of NK_1 tachykinin receptor antagonist treatment was assessed. Although increases in SP cortical concentrations, decreases in JNK, ERK and Akt phosphorylation and increases in levels of pTau detectable by IHC in the acute phase of injury were altered by rmTBI and these effects of injury reduced by NK_1 tachykinin receptor antagonist administration, this did not affect changes to pTau detected via WB. Indeed, changes in T231 pTau, MBP and PSD-95 were detected via WB at the 12 week timepoint, however, this was not reflected in changes to total tau detected via WB or pTau detected via IHC, or any major changes in functional outcomes.

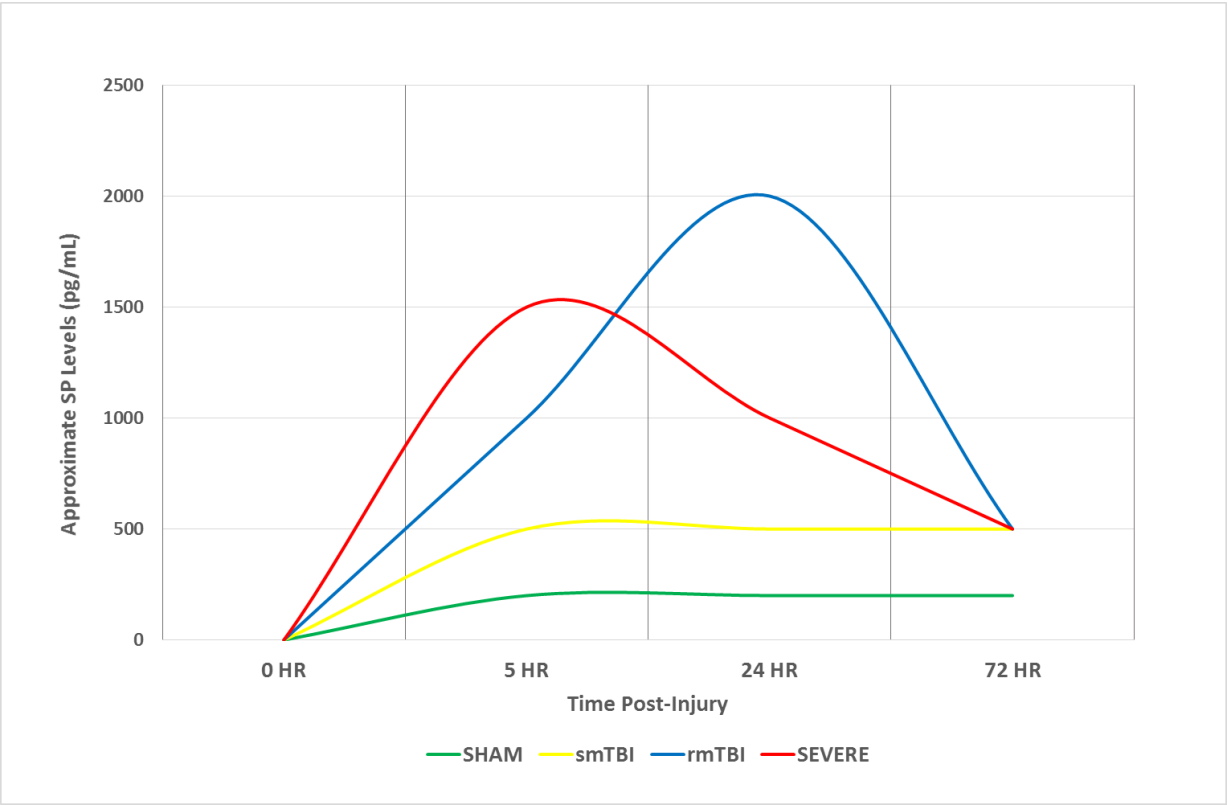

Figure 6.1: Representative diagram of SP release following TBI of varying severities and frequencies**.** Following smTBI (yellow), a non-significant increase in cortical SP concentrations is usually observed when compared to sham levels (green). Conversely following a single severe TBI (red), significant increases in SP release are observed 5 hrs post-injury. However, following rmTBI (blue), this response appears to be magnified and an exponential increase in SP release is observed at the 24 hr timepoint following the final mTBI, which appears to be greater than that observed at the peak of SP release in severe TBI (graph not to scale).

Level of Injury	5 hours post-injury	24 hours post-injury	72 hours post-injury	12 weeks post-injury
SHAM	**No increase**	**No increase**	**No increase**	**No increases**
smTBI	**Non-significant increase in SP**	**Non-significant increase in SP**	**Non-significant increase in SP**	**Non-significant changes**
rmTBI	**Significant increases in SP**	**Very significant increase in SP, dephosphorylation of JNK, ERK, Akt, increased pTau (IHC), increased TRPV1 activation**	**Non-significant increases in SP**	**Significant decreases in MBP, PSD95, significant increases in pTau (WB)**
SEVERE	**Significant increases in SP**	**Significant increase in SP, increased pTau, increased TRPV1 activation**	**Non-significant increases in SP**	**Not assessed**

Table 6.1: Table summarising the key findings following TBI from the experiments conducted within the thesis.

6.1 CHARACTERISING THE RELEASE OF SP AND DOWNSTREAM EFFECTS FOLLOWING rmTBI

The results obtained from these studies indicate that there is indeed an increase in SP release within cortical tissue at the 24 hr timepoint following rmTBI. This appeared to be different from the timepoint of peak SP release observed in severe animals, which was observed at 5 hrs post-injury, similar to what has been observed previously in this model of TBI (Donkin et al. 2009). Although an increase in SP cortical concentrations was noted in the severe TBI group at the 5 hr timepoint, levels were significantly higher at the 24 hr timepoint in rmTBI animals, which supports previous findings that SP release is proportional to the frequency and intensity of stressful stimuli (Allen et al. 1997; Mantyh 2002). Increased NK_1 expression was also observed in the severe TBI group at the 24 hr timepoint however that was not observed in the rmTBI animals. This therefore warrants further investigation given that the highest overall release of SP was observed at the 24 hr timepoint in rmTBI animals and the fact that this does not match the results observed with the NK_1 study is intriguing. $TRPV_1$ expression was also significantly altered by both rmTBI and severe TBI, with

increases observed at the 24 hrs in the severe group and decreases in the rmTBI group at 72 hrs. However, no direct relationship between the SP-NK_1 system and changes in acute tau phosphorylation were observed, with increases in AT180 positive cells observed at the cortex observed via IHC but not WB.

It is clear that although there are changes following both rmTBI and severe TBI, study of both additional timepoints and markers including markers of axonal injury and neuroinflammation is needed to fully understand the changes present following trauma. The tau phosphorylation observed in this study, although it matches previous studies where increases were observed in the acute phase of injury following rmTBI (McAteer et al. 2016; Petraglia et al. 2014), these were not able to be observed via WB. Although there was indeed tau phosphorylation, this not only did not exceed pTau levels in the severe group but matched levels observed in the smTBI group at the 24 hr timepoint. More insight may be gained from assessment of axonal injury dynamics either through immunohistochemical assessment of amyloid precursor protein (APP) positive axons or via further western blot assessment of different sized neurofilaments at this timepoint. Mechanical dissociation of tau from the microtubules may potentially be driving these increases given phosphorylation was observed at all injury levels; since axonal injury has been shown to be at higher levels following severe TBI compared to mTBI in previous studies (Browne et al. 2011; Smith et al. 2000), this might explain the results observed in these studies. Further analysis of how classical inflammation may be influencing pTau levels following TBI may also be warranted given that previous work indicates that there may be interplay between the two processes (Kitazawa et al. 2005; Sy et al. 2011). A study by Kitazawa et al. (Kitazawa et al. 2005) found that in a transgenic mouse model of AD, when exposed to lipopolysaccharide (LPS), a known activator of classical inflammation, tau hyperphosphorylation was significantly increased at sites known to be mediated by CDK5. Additionally, a study by Sy et al. (Sy et al. 2011) found that following induction of acute or chronic inflammation in a transgenic

mouse model of AD, increased tau phosphorylation characteristics were observed as well as spatial memory in the chronic inflammation group. Specifically, following chronic inflammation, tau phosphorylation was increased via a GSK3B dependent mechanism and inhibition of GSK3B activity did indeed attenuate tau phosphorylation (Sy et al. 2011). How neurogenic inflammation and the release of SP following TBI influences this classical inflammation (Corrigan, Vink & Turner 2016) and potential rise in pTau may also shed some light on the results observed in this study.

6.2 EFFECT OF $TRPV_1$ BLOCKADE ON SP RELEASE FOLLOWING TBI

These studies indicated that blockade of the $TRPV_1$ receptor via administration of the $TRPV_1$ antagonist capsazepine prior to TBI, had an effect on the cortical concentrations of SP following both rmTBI and severe TBI. Reductions in SP concentration were observed at the peak times of SP release in both rmTBI and severe animals when pre-treated with capsazepine. However, this did not have an effect on $TRPV_1$ or NK_1 expression at the same timepoints which is unexpected given the SP results. This may be due to the method of detection used for $TRPV_1$ and NK_1 expression via western blot. As with the tau phosphorylation results, it may be that the area of tissue analysed was too big to allow for meaningful analysis given the widespread distributions of both $TRPV_1$ and NK_1 within the CNS (Datar et al. 2004; Ho, Ward & Calkins 2012). Therefore either a more targeted dissection of the tissue of interest or a more targeted analysis of the tissue using a different technique such as immunoprecipitation or immunohistochemistry may also be warranted to gain more meaningful results. Following the results from Chapter 3, which showed increases in acute tau phosphorylation following severe TBI, an investigation into how $TRPV_1$ blockade effects tau phosphorylation may also be warranted as it was shown that treatment with capsazepine prior to injury prevented cortical pTau accumulation as detected via IHC and increased expression of pJNK compared to vehicle treatment.

The most interesting finding from this study was the potential differences in mechanisms for SP release between severe and rmTBI as capsazepine pre-treatment was only successful following rmTBI. This has potential interest for investigation of other pathways that $TRPV_1$ can activate in the CNS following injury. $TRPV_1$ activation has been shown to interact with the BBB in the CNS, more specifically on pericytes and astrocytes that interact with the brain vasculature (Toth et al. 2005). A study by Hu et al. (Hu, Easton & Fraser 2005) found that following administration of capsaicin, a $TRPV_1$ agonist, to the brain surface of rats that this increased permeability and that administration of capsazepine reduced the response elicited from capsaicin. Given the sharp increase in $TRPV_1$ levels in the severe group following injury and the knowledge that BBB disruption is a feature of severe TBI (Corrigan et al. 2016), this provides an interesting avenue for future studies.

However some of the results in this study are more difficult to explain, such as the decreased expression of SP in both the capsazepine pre-treatment and post-treatment groups following severe TBI and the fact that although SP was reduced in both these groups, pTau levels were only decreased in the pre-treatment group. This may come back to the previously discussed notion that tau phosphorylation may be influenced by a multitude of factors following TBI and not solely the actions of $TRPV_1$ and SP release alone, including altered kinase activity and changes in microtubule dynamics as a result of the primary insult (Blennow, Hardy & Zetterberg 2012; Lucke-Wold et al. 2014). Furthermore, the results of the kinase studies, which may differ dependent on the level of injury as indicated in Chapter 4 and Chapter 5, raise additional questions as to their level of involvement following mTBI.

The greatest increases in pTau were observed in the severe group at the 24 hr timepoint, however kinase interactions were largely unaffected which again indicate different or additional tau phosphorylation pathways. With all that being said, this allows for many avenues of investigation for future studies.

6.3 EFFECT OF NK_1 TACHYKININ RECEPTOR ANTAGONISTS ON OUTCOMES FOLLOWING rmTBI

In these studies, administration of a NK_1 tachykinin receptor antagonist following rmTBI reduced the release of SP that was observed following rmTBI in the previous chapters. Indeed, administration of a NK_1 tachykinin receptor antagonist also appeared to have an effect on the tau-protein kinases Akt, JNK and ERK in the acute phase of injury, as well as changes in pTau expression when examined via IHC. However, such changes in the acute stages of injury were not associated with accompanying improvements in long-term outcomes, neither the accumulation of pTau examined via IHC nor improvements in functional outcomes, despite positive changes being observed previously (McAteer et al. 2016). Nevertheless, examination of markers of axonal injury including MBP and PSD-95 indicated changes in their expression following NK_1 tachykinin receptor antagonist treatment, with an improvement in MBP levels compared to vehicles observed in the 001 treatment group and a significant increase in PSD-95 following NAT treatment.

One of the most important outcomes from this study is that although it appeared that the NK_1 tachykinin receptor antagonists did have an effect on the phosphorylation state of the tau-protein kinases assessed in this study, this did not have a pronounced effect on tau phosphorylation. With consideration towards the results gathered in Chapter 4, it appears that there is more to the tau phosphorylation cascade than the action of these protein kinases alone. Indeed, studies assessing the role of protein phosphatases such as PP2A following both rmTBI and severe TBI have shown that there is diminished activity following traumatic injury (Shultz et al. 2015; Tan et al. 2016). As discussed earlier, there is also a potential role for classical inflammation to play a role in the accumulation of pTau following TBI (Collins-Praino & Corrigan 2017). It could be that disruption of axonal integrity following rmTBI could promote the initial disease state formation of tau, which then

could be further promoted into tauopathy by a chronic inflammatory state, perhaps driven by acute SP release as it has been seen previously that SP can contribute to further inflammation within the brain (Collins-Praino & Corrigan 2017; Johnson, Young & Marriott 2016). Since both of these lines of study were not partaken of within this thesis, they present an attractive opportunity to examine the potential effects that the release of SP and neurogenic inflammation may have in these systems. Additionally, examination of additional timepoints at earlier and later stages, for example at 5-15 minute timepoints to assess potential spikes in ERK activity (Yao & Seger 2004), or a 30 day timepoint to assess intermediate tau phosphorylation (Kane et al. 2012) may also shed some light on disease progression as we may have missed critical parts of changes in protein conformation with the timepoints selected in this study.

6.4 LIMITATIONS & FUTURE DIRECTIONS

It is important to reflect on the limitations in this study that may have affected the results presented herein. A large limitation that almost certainly affected these results would be the loss of critical behavioural data due to technological failures. Although best attempts were made to recover this data, not all of the data points were able to be recovered successfully and such had an overbearing effect on the group sizes and power levels of some of the behavioural studies. This type of failure was unforeseen on the part of the experimenters, but as such steps such as automatic online backup of experimental data to cloud storage can be implemented in future to prevent such a loss.

As discussed earlier, the results of the western blots with regards to NK_1 and $TRPV_1$ activity were unexpected and may indeed be to the gross dissection of cortical tissue. This may therefore be due to the inclusion of unnecessary tissue that may have been dissected along with the target area at the time of collection. This may have also affected the SP ELISA results, with high variability observed between studies. To prevent this in future, more targeted dissection, perhaps with a dissection microscope to better discern the target areas, may prove useful in future to improve results.

In addition to more targeted dissection, analysis of additional areas of the brain may shine more light on what is happening following rmTBI, both in the short term and long term. Many other studies in rodents have analysed different areas of the brain following rmTBI, including the hippocampus (Slemmer et al. 2002) and indeed in cases of CTE changes have been observed in the frontal and temporal lobes, as well as in the thalamus and hypothalamus (McKee et al. 2015). Future experiments in this model of rmTBI could include a comprehensive analysis of the additional areas believed to be implicated in the pathogenesis of CTE and could provide further validation of this model as one that is clinically relevant.

A large gap that certainly allows for future studies is the effect of biological sex on release of SP following rmTBI. Although most of the work surrounding the study of long term neurodegeneration following early life participation in contact sports has been focused on male athletes in the past, there has been a recent surge in research pertaining to concussion rates in females. Although concussion rates have been found to be higher in males when compared to females in rule-matched sports, females have a higher rate of disclosure of concussion symptoms when compared to males (Iverson et al. 2015). In a study conducted by Brook et al. (2017) it was found that in a sample of 459 NCAA women's ice hockey athletes, about half of respondents reported at least one diagnosed concussion over the course of their entire organised ice hockey career, with an incidence rate of 1.18 per 1000 athlete-exposures to a game of practice. Additionally, a study by Rosene et al. (2017) found that compared to men's concussion rates in NCAA hockey, women's rates were similar, with 0.83/1000 exposures in Division I men compared to 0.73/1000 exposures in Division III women, and that differences could be attributed to division of play, not biological sex. With the concussion rates in women matching that of men, it presents a pronounced need to focus more study in this area within pre-clinical models, and this model certainly presents an attractive option to do so.

Although the link between SP release and tau-mediated neurodegeneration was not made clear within these studies, it still presents important findings that may impact clinically. This is one of the

first studies to ascertain the role that SP may play following rmTBI and although it had no effect on tau phosphorylation, there are still many opportunities to intervene clinically, although not via drug administration. The finding that rmTBI significantly increases SP release could potentially pave the way for a clinical test to further confirm concussions, potentially detecting through the blood, CSF or in conjunction with DTI imaging to determine white matter integrity. A study by Lorente et al. (2015) found that in severe TBI patients with a GCS of < 8, serum levels of SP were higher in non-surviving patients than in surviving patients and higher SP serum concentrations correlated with mortality. Elevated levels of SP in the CSF have also been linked with other CNS disorders such as fibromyalgia (Russell et al. 1994; Vaeroy et al. 1988) and in patients with major depressive disorder (Rimon et al. 1984). Adding to this, a study by Won et al. (2017) assessed the relationship between plasma SP levels and white matter integrity in patients with major depressive disorder, finding a negative correlation between increased plasma SP levels and decreased radial and mean diffusivity within the corticospinal tract, which projects to the anterior cingulate cortex, as well as a negative correlation between increased SP levels and fractional anisotropy values of the forceps minor of the corpus callosum, which is involved in interconnecting the prefrontal cortex and anterior cingulate areas. However no studies have looked clinically at the effects of rmTBI on peripheral SP release and therefore presents an attractive area for future studies.

6.5 CONCLUSIONS

Overall, these studies that SP release appears to be dependent on the severity and frequency of the injury following TBI and indeed this work is one of the first to show that there is a severe increase in SP following rmTBI, which may be more significant than what is observed following severe injury. This release is thought to be via the mechanical stimulation of $TRPV_1$ receptors within the CNS as blockade prior to injury prevented increases in SP levels. However, the SP-NK_1 system did not have a pronounced effect on tau phosphorylation following TBI as a part of the short and long term

downstream effects of blockade of SP mediated actions, whether at the start of the chain at the $TRPV_1$ receptor or at a later stage at the NK_1 receptor. However, these studies have opened up many avenues for further investigations on how neurogenic inflammation may potentially influence tau dynamics in different ways following TBI, especially with regards to the influence on kinase activity and classical inflammation that release of SP following rmTBI may indeed have an effect on.

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