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Inflammation in acute CNS injury: a focus on the role of substance P

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Abbreviated Title: *Neurogenic inflammation in acute CNS injury*

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Abstract

Recently, a number of reports have shown that neurogenic inflammation may play a role in the secondary injury response following acute injury to the central nervous system (CNS), including traumatic brain injury (TBI) and stroke. In particular substance P (SP) release appears to be critically involved. Specifically, expression of the neuropeptide SP is increased in acute CNS injury, with the magnitude of SP release being related to both the frequency and magnitude of the insult. SP release is associated with an increase in blood-brain barrier permeability and the development of vasogenic oedema as well as neuronal injury and worsened functional outcome. Moreover, inhibiting the actions of SP through use of a NK1 antagonist is highly beneficial in both focal and diffuse models of TBI, as well as in ischaemic stroke, with a therapeutic window of up to 12h. We propose that NK1 antagonists represent a novel therapeutic option for treatment of neurogenic inflammation following acute CNS injury.

Keywords: ischaemic stroke, neurogenic inflammation, NK1 antagonist, substance P, traumatic brain injury

Abbreviations:

blood brain barrier	BBB
IL	interleukin
intracranial pressure	ICP
middle cerebral artery	MCA
n-acetyl-L-tryptophan	NAT
n-methyl-D-aspartate	NMDA
reactive oxygen species	ROS
SEM	standard error of the mean
substance P	SP
traumatic brain injury	TBI
tumour necrosis factor	TNF
tissue plasminogen activator	tPA

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Acute insults to the CNS, such as traumatic brain injury (TBI) and ischaemic stroke, are a major cause of morbidity and mortality today representing a significant public health issue worldwide. An estimated 10 million people are affected annually by a TBI serious enough to result in death or hospitalisation, with a mortality rate of 15-30 per 100,000 reported in industrialised countries (Finfer *et al.*, 2001; Tagliaferri *et al.*, 2006). Stroke affects some 15 million individuals worldwide each year, of which 1/3 die and 1/3 are left permanently disabled and dependent for daily living (World Health Organisation, 2004).

In both TBI and ischaemic stroke, cell death is caused by both primary (the initial insult) and secondary injury mechanisms. Whilst the nature of primary injury differs between TBI and ischaemic stroke, the secondary injury cascade shares many similarities, with inflammation, and more recently neurogenic inflammation, shown to be key determinants of neuronal injury and functional outcome in both conditions. The current article provides data supporting a critical role for neurogenic inflammation, and particularly substance P (SP) release, in the secondary injury response following TBI and ischaemic stroke, and discusses this data in the context of previously published reports.

Traumatic Brain Injury

TBI results from the head impacting with an object or from acceleration/deceleration forces that produce vigorous movement of the brain within the skull, with the resultant mechanical forces potentially damaging glia, neurones, axons and blood vessels. The type and severity of the injury depends upon the nature of the initiating force, as well as its site, direction and magnitude (Smith *et al.*, 2003). Contact forces generated when the head strikes or is struck by an object generally produce focal injuries such as skull fractures, extradural haemorrhages, and contusions. In contrast, acceleration/deceleration forces that result from violent unrestrained head movement are associated with diffuse axonal injury (Blumbergs *et al.*, 2008). Focal injuries, such as contusions lead to the development of an area of dead tissue (the necrotic core) which is surrounded by a penumbral area. This area is known to also undergo ischaemia, due to haemorrhage, disruptions in venous drainage and vasoconstriction, and this contributes which contributes to the secondary injury process in this region (Leker *et al.*, 2002).

Ischaemic Stroke

In ischaemic stroke, the primary injury is the sudden and profound reduction of blood flow to an area of the brain, most commonly because of atherothrombosis of cerebral vessels or emboli (Hademenos *et al.*, 1997). The amount of cell death is dependent upon the severity and duration of the resultant ischaemia, in addition to the availability of collateral blood supply. The ensuing infarct may be divided into two regions, the core and the penumbra. Within the core of the infarct, blood flow falls to less than 20% causing rapid cell death due to insufficient supply of substrate to maintain cellular energy production. The ischaemic penumbra surrounding the core is an area of reduced perfusion in which cells are still viable. Their survival is only possible for a limited time, due to the initiation of a number of pathological processes, that are similar to that seen following TBI. (Bouts *et al.*, 2013).

Secondary Injury

As outlined above TBI, results primarily from a mechanical impact which disrupts the brain parenchyma and causes shearing and tearing of blood vessels and axons (Bramlett *et al.*, 2004). In contrast in ischaemic stroke a cessation of blood supply leads to cellular death due to a deprivation of oxygen and glucose (Leker *et al.*, 2002). Despite the disparity in these primary injury mechanisms, they both lead to the activation of molecular and cellular response that lead to secondary injury, with similarities noted in the harmful pathways that lead to secondary cell

death in the penumbral ischaemic zone and in the area exposed to secondary post-traumatic brain injury. These include an increase in extracellular levels of glutamate, leading to excitotoxicity, the onset of oxidative stress, disruption to the blood-brain barrier (BBB), cerebral oedema formation, and inflammation (**Fig. 1**), all of which exacerbate injury and tissue damage (Saatman *et al.*, 1996). Of particular interest is the role that inflammation can play in this secondary injury cascade and whether modification of this inflammatory process has the potential to improve outcome.

Inflammation

A marked inflammatory reaction accompanies any acute insult to the CNS, including ischaemic stroke and TBI (Danton *et al.*, 2003). In the immediate phase following the initial insult this inflammatory response exacerbates cell injury and worsens outcome. Briefly, such an inflammatory response is characterised by glial activation, proliferation of microglia, leukocyte recruitment and upregulation and secretion of mediators such as cytokines and chemokines (Ziebell *et al.*, 2010). Microglial cells become activated and release a variety of neurotoxic factors including pro-inflammatory cytokines, reactive oxygen species (ROS), nitric oxide and metalloproteinases (Brown *et al.*, 2010). Cytokines like interleukin-1 (IL-1) play a key role orchestrating the release of other neurotoxic mediators such as ROS, proteases and prostaglandins, whilst tumour necrosis factor (TNF) promotes the release of proteolytic enzymes, breakdown of the BBB and induction of cell death (Shohami *et al.*, 1999). Whilst the role of the classical inflammatory response has been well characterised (Danton *et al.*, 2003; Morganti-Kossmann *et al.*, 2002), the important role that neurogenic inflammation plays in exacerbating the secondary injury cascade has only recently begun to be recognised.

Neurogenic Inflammation

Neurogenic inflammation is a neurally-elicited inflammatory response characterised by the release of neuropeptides, including SP and calcitonin gene-related peptide (CGRP), vasodilation, plasma extravasation, tissue swelling and mast cell degranulation (Severini *et al.*, 2002). It results from the stimulation of capsaicin-sensitive, neuronal C fibres by factors such as serotonin, histamine and leukotrienes, as well as by changes in pH, extremes of temperature and by mechanical injury (Harrison *et al.*, 2001; Saria *et al.*, 1983).

Substance P

SP is thought to be the most potent initiator of neurogenic inflammation due to its association with increased vascular permeability and subsequent plasma protein extravasation (Holzer, 1998). It also potentiates classical inflammation by stimulating the production of inflammatory mediators such as histamine, nitric oxide, cytokines (such as IL-6) and kinins, in addition to interacting with adhesion molecules causing leukocyte migration (Averbeck *et al.*, 2001).

SP is a member of the tachykinin family, a group of structurally related peptides sharing the same carboxyl terminal sequence which also includes neurokinin A and neurokinin B (Schaffer *et al.*, 1998). It is derived from the preprotachykinin-A (PPT-A) gene, which is spliced to form four different mRNA forms, namely α-PPT, β-PPT, γ- PPT and δ-PPT (Harrison *et al.*, 2001). The α-PPT and β-PPT forms encode for SP alone whilst the γ- PPT and δ-PPT encode for both SP and neurokinin A (Severini *et al.*, 2002). The α-PPT is more abundant within the brain, whilst the β-PPT form is found primarily within the peripheral nervous system (PNS) (Ribeiro-da-Silva *et al.*, 2000). Indeed, SP is widely distributed throughout the CNS, PNS and enteric nervous systems. In the CNS it is present in dorsal root ganglion (primary sensory) neurons (Hokfelt *et al.*, 2001) of many regions including the hippocampus, cortex, basal ganglia,

hypothalamus, amygdala, caudate nucleus and spinal cord, and seems to be more abundant in the grey matter compared to white matter (Ebner *et al.*, 2006).

SP is synthesized in the ribosome as a large protein and transported to the terminal endings, where it is enzymatically converted into the active form and stored in vesicles ready for release (Lundy *et al.*, 2004). Its actions are primarily mediated through the tachykinin NK1 receptor to which it preferentially binds, although it also binds to the other tachykinin receptors, NK2 and NK3, with varying affinity depending upon receptor and ligand availability (Regoli *et al.*, 1994). The NK1 receptor is a typical G-protein coupled receptor with seven transmembrane domains (Garland *et al.*, 1994) and is found on neurons and glia in the CNS, smooth muscle cells, endothelial cells, fibroblasts, keratinocytes and various circulating immune and inflammatory cells (Schaffer *et al.*, 1998). On unstimulated neurons, NK1 receptors are localised to the plasma membrane of both the cell body and dendrites, but following SP binding, they are rapidly internalised into the cytoplasm via endosomes (Mantyh, 2002). This internalisation is readily reversible with complete return of internalised receptors to the surface within 30 minutes.

The intracellular signalling pathways activated by the NK1 receptor appear to be cell-dependent. In endothelial cells, SP binding to the NK1 receptor promotes phosphorylation of myosin by myosin light chain kinase (MLCK) which in turn promotes the interaction of actin and myosin, leading to cell retraction and the formation of gaps between endothelial cells (Holzer, 1998; van Hinsbergh *et al.*, 2002). In contrast, in vascular smooth muscle, the vasodilatory actions of SP involve activation of adenylate cyclase producing cAMP, which activates calcium ATPases, reducing intracellular calcium levels and promoting relaxation and dilation of vascular smooth muscle (Maggi, 1995). In other cell types, such as astrocytes and microglia, binding of SP to the NK1 receptor leads to activation of phospholipase C, which catalyses the hydrolysis of phosphoinositides into inositol 1,4,5-trisphosphate and diacylglycerol, which then allow mobilisation of calcium from internal stores (O'Connor *et al.*, 2004). This increase in calcium levels within primary afferents is thought to be involved in the decrease of the threshold for signalling of nociceptive information following injection of SP into the skin.

The role of SP following TBI

Previous research in our laboratory has found that SP plays an integral role in the secondary injury cascade following TBI (Donkin *et al.*, 2009; Nimmo *et al.*, 2004). Following diffuse TBI in rats, an increase in SP immunoreactivity is noted at both 5 and 24h following injury, with a gradual return to sham levels by 7 days post-injury (Donkin *et al.*, 2009). Consistent with the animal studies, in human TBI, postmortem SP immunoreactivity was increased perivascularly as well as in cortical neurones and astrocytes in the majority of cases examined (Zacest *et al.*, 2010). This release of SP appears to be both intensity and frequency dependent, with a graded increase in SP immunoreactivity seen with increasing severity of injury. Consistent with this, experimental studies conducted in our laboratory (**Fig. 2**) show that the highest level of SP immunoreactivity is observed following a 2m (severe) impact TBI, whilst a 0.5m (mild) impact TBI causes only a minimal increase in SP immunoreactivity. Increased axonal injury and worsening of motor outcome is also observed as the severity of injury increases (Marmarou *et al.*, 1994), suggesting that SP may play a role in the heightened secondary injury response with increased injury severity. Notably, we have previously shown that axonal injury is reduced and functional outcome improved with administration of an NK1 antagonist (Donkin *et al.*, 2011). Previous studies examining SP release following thermal stimuli have also shown that stepwise

increases at the extremes of temperature (<10°C and >43°C) results in a graduated release of SP and associated internalisation of the NK1 receptors (Allen *et al.*, 1997).

Repeated exposure to a stimulus has also been shown to cause increased SP release and greater activation of NK1 receptor expressing cells (Mantyh, 2002), with diffusion of SP away from the site of release causing more widespread activation, estimated to be 3-5 times that of a single exposure. The brain is known to be more vulnerable to repeated injury, with rodents demonstrating prolonged cognitive deficits and enhanced axonal injury not seen with a single impact (Longhi *et al.*, 2005). This suggests that enhanced release of SP may potentially play a role in the vulnerability of the brain to repeated concussions.

Antagonism of SP following TBI

The important role that SP plays in the secondary injury process following TBI has been confirmed in a number of studies that have shown that attenuating SP activity is beneficial to outcome (Vink *et al.*, 2010). The first demonstration of neurogenic inflammation in TBI showed that depletion of sensory neuropeptides by pre-treatment with capsaicin results in the attenuation of post-traumatic BBB permeability, oedema formation and improved functional outcome (Nimmo *et al.*, 2004). While not identifying the sensory neuropeptide responsible for these post-traumatic effects, subsequent studies identified increased SP immunoreactivity as a possible therapeutic target. Accordingly treatment with an NK1 antagonist, targeting SP binding, was utilised after TBI in both male (Donkin *et al.*, 2009) and female rats (Corrigan *et al.*, 2012) which led to a significant attenuation of post-traumatic blood-brain barrier (BBB) permeability with a significant reduction in oedema formation. This was accompanied by a reduction in axonal injury and associated improvement in both motor and cognitive outcome. Further evidence supporting a role for SP in TBI was provided in studies that administered an angiotensin converting enzyme (ACE) inhibitor, which inhibits the breakdown of SP. Following ACE inhibitor administration, levels of SP were significantly increased after TBI, exacerbating neuronal injury and motor deficits (Harford-Wright *et al.*, 2010). In terms of the therapeutic window for neuroprotective action, treatment up to 12h post-injury with a centrally acting NK1 antagonist was still able to provide significant benefit, with marked improvements noted in motor performance (Donkin *et al.*, 2011).

All of the initial TBI studies characterising neurogenic inflammation utilised the Marmarou impact-acceleration model of TBI, which causes diffuse injury with widespread axonal damage, particularly in the long tracts of the brain stem, as well as within the corpus callosum and internal capsule (Marmarou *et al.*, 1994). As human TBI covers the spectrum from diffuse through to focal injury, it is important to determine whether therapies targeting SP are equally efficacious in both types of injury. The fluid percussion model of TBI utilises the application of a rapid pressure pulse onto the exposed and intact dura to cause a brief displacement and focal deformation of the brain tissue (Corrigan *et al.*, 2011). This produces primarily a focal contusion, accompanied by axonal injury in the fimbria and internal capsule (Thompson *et al.*, 2005). It is thus a highly suitable model to examine the effects of NK1 antagonists on focal TBI. **Figure 3** demonstrates that administration of an NK1 antagonist, n-acetyl-L-tryptophan (NAT; 2.5 mg/kg iv) at 30 mins post-injury following fluid percussion induced TBI led to a similar significant improvement in motor outcome on the rotarod as that seen previously following diffuse injury. Specifically, NAT treated rats performed significantly better ($p<0.05$) than saline vehicle treated rats on days 1, 2, 4 and 5 post-injury, and had in fact returned to sham levels by day 4, whilst the vehicle treated rats still showed significant motor deficits on day 7, the final day of testing. Thus, administration of an NK1 antagonist significantly improved outcome after TBI, irrespective of the focal or diffuse nature of the injury.

While the role of neurogenic inflammation had been confirmed in both focal and diffuse TBI, and the NK1 antagonist had proven efficacious in both male and female animals, all pharmacological studies had been limited to rodents, which have historically led to few successful therapeutic translations to the clinic, particularly in the area of acute CNS injury. To increase the likelihood of successful clinical translation, it is important to test therapeutics developed in rodent models in large animal models given the anatomical differences between the small lissencephalic rodent brain and the larger gyrencephalic human brain. This has been a particular focus of our laboratory in recent years, which has developed models of both TBI and stroke in sheep (Finnie *et al.*, 2012; Van Den Heuvel *et al.*, 2004; Wells *et al.*, 2012). As with the rodent studies, sheep demonstrated an increased SP immunoreactivity after TBI associated with increased BBB permeability and marked oedema formation, as well as significant increases in intracranial pressure (ICP) that were associated with brain water accumulation (Byard *et al.*, 2009). Administration of an NK1 antagonist in this sheep TBI model was able to significantly reduce ICP levels to normal within 4 h of drug administration as compared to the sustained elevation in ICP in the vehicle-treated sheep (Gabrielian *et al.*, 2013). This reduction in ICP is consistent with the ability of the NK1 antagonists to consistently reduce brain water concentration to normal levels in the previously published rodent studies (Vink *et al.*, 2010).

The role of SP following ischaemic stroke

We have described above that neurogenic inflammation involving SP release is a ubiquitous feature of TBI, regardless of whether the insult is mild, moderate or severe or whether it is diffuse or focal in nature (see Fig. 2). Many of the secondary injury pathways that feature in TBI also significantly contribute to the development of infarction following stroke. Indeed, we have reported a significant SP response in ischaemic tissue following experimental stroke in rodents (Turner *et al.*, 2006). However, in the setting of cerebral ischaemia, it appears that SP release may not be as ubiquitous a feature of stroke as it is in TBI, and may be dependent upon the reperfusion status of the tissue.

SP immunoreactivity is particularly evident in the perivascular tissue of the ischaemic penumbra, as well as in neuronal and glial cells within this region (Turner *et al.*, 2006; Turner *et al.*, 2011). Levels of SP in the penumbra gradually increase with time following reperfusion, reaching a nadir at 24 h post-reperfusion (Turner *et al.*, 2011). In contrast, the core of the stroke lesion does not demonstrate appreciable SP immunoreactivity. Thus, there appears to be a difference in the SP response that is dependent upon the perfusion status of the tissue. To further demonstrate this effect, our laboratory has shown in rodents that 24h of permanent middle cerebral artery (MCA) occlusion results in only modest elevations in SP immunoreactivity beyond sham levels. In contrast, 2h of transient MCA occlusion followed by 22h reperfusion is associated with profound increases in perivascular SP immunoreactivity within the penumbral tissue. These alterations in SP levels were confirmed using ELISA assays of the ischaemic hemisphere (Turner *et al.*, 2011). The results suggest that in regions where infarction has occurred, there is no SP response, whereas in the surrounding surviving but compromised penumbral tissue, SP is playing a role in the development of secondary injury. We posit that SP release may be a feature of reperfusion injury and that neurogenic inflammation may contribute to and exacerbate injury to the brain following stroke. It must be acknowledged that these findings may be model and species dependent. However, no data describing a difference in the SP response exists to date for other experimental models of stroke or in clinical samples.

Following transient ischaemia, SP immunoreactivity is maximal at 24h following stroke, and declines thereafter over the next 7d. Increased BBB permeability has been reported at various time-points following stroke, and the 24 h time-point when SP was markedly increased in our studies is consistent with such reports (Preston *et al.*, 1993). Indeed, the 24h time point in our stroke model was associated with profound disruption of the BBB (Turner *et al.*, 2006), together with development of cerebral oedema and persistent functional deficits (Turner *et al.*, 2011). SP therefore offers an attractive pharmacological target to reduce BBB permeability and the development of oedema following transient ischaemia.

Antagonism of SP following ischaemic stroke

A number of studies have now demonstrated that inhibition of neurogenic inflammation, and in particular SP activity, significantly improves outcome following transient ischaemia. In a rodent model of transient MCA occlusion, administration of the NK1 receptor antagonist NAT at 4 h post-occlusion significantly attenuated BBB permeability and in turn completely ameliorated cerebral oedema and improved functional outcome (Turner *et al.*, 2011). Moreover, NK1 antagonists can be safely combined with thrombolysis (Turner *et al.*, 2012b), which is of particular importance given the integral role that thrombolysis plays in clinical stroke management (Medcalf *et al.*, 2012). Specifically, combination treatment of tissue plasminogen activator (tPA; alteplase) with NAT significantly improved BBB function as well as functional outcome, and importantly reduced the incidence of intracerebral haemorrhage associated with tPA administration. Presumably, the NK1 antagonist prevented vascular disruption induced by tPA by maintaining the integrity of the BBB.

In terms of the therapeutic window, studies of transient (2h) MCA occlusion in rats conducted in our laboratory (**Fig. 4**) have demonstrated that an NK1 antagonist (NAT; 25µmoles/kg iv) can be administered up to 8h following the onset of stroke and still result in a significant improvement in rotarod motor function, and up to 12h following stroke onset with a significant improvement in sensory function (bilateral asymmetry). Moreover, the treatment at 8h was effective irrespective of the period of MCA occlusion (**Fig. 5**), thus demonstrating efficacy over a range of mild to severe stroke. The 8-12 h therapeutic window is both highly relevant and achievable, especially when considered against the 4.5h window of opportunity currently considered safe for clinical thrombolysis with tPA (Medcalf *et al.*, 2012). Notably, a comparable therapeutic window of 12h exists for the administration of NK1 antagonists in TBI (Donkin *et al.*, 2011).

The role of SP in the secondary injury response

The exact mechanisms by which SP influences outcome following TBI and stroke are yet to be fully characterised, although the neuropeptide is known to influence a number of secondary injury factors that have been well described following acute CNS injury, including classical inflammation, BBB breakdown, excitotoxicity and magnesium homeostasis (Vink *et al.*, 2010).

Classical inflammation

It is widely accepted that treatments that limit the inflammatory response during the acute phase of experimental CNS injury have beneficial effects on outcome, and considerable effort has been directed at developing more effective anti-inflammatory approaches (Nimmo *et al.*, 2009). In this respect, the effect of SP on classical inflammation is highly relevant and is one mechanism by which NK1 antagonists may ameliorate secondary injury and improve outcome. SP induces and augments many aspects of the classical inflammatory response including leukocyte activation, endothelial cell adhesion molecule expression, cytokine production and mast cell activation (Quinlan *et al.*, 1999). SP also promotes secretion of cytokines including

IL-1, IL-6, IL-12 and TNF-α by cells of the myeloid lineage, interferon-γ (IFN-y) by T cells and down-regulates anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) (Marriott *et al.*, 2001; McCormack *et al.*, 1996). Following activation of NK1 receptors on endothelial cells, adhesion molecules are rapidly mobilised to the surface (Zimmerman *et al.*, 1992), with SP exerting direct chemotactic actions on neutrophils (Carolan *et al.*, 1993) and also priming them for oxidative metabolism, increasing the production of free radicals (Hafstrom *et al.*, 1989). Astrocytes also express NK1 receptors following injury, and their activation is thought to contribute to the transformation to reactive astrocytes, with the resultant production of inflammatory mediators (Lin, 1995). Following TBI, NK1 antagonists have been shown to significantly reduce production of the pro-inflammatory cytokine IL-6 (Reardon *et al.*, 2004) as well as to decrease microglial proliferation (Carthew *et al.*, 2012). Similar reductions in IL-6 as well as TNF-α have also been reported in a mouse model of bacterial meningitis in response to administration of an NK1 antagonist (Chauhan *et al.*, 2008).

Effects of SP on the BBB

The BBB is a highly selective barrier formed by a monolayer of endothelial cells that are joined together by tight junctions including proteins such as claudins, occludin, junctional adhesion molecules (JAMs) and zonula occludens proteins (ZOs) (Huber *et al.*, 2001). This endothelial layer is supported by the end-feet of astrocytes that act to support and enhance the tight junctions. Any increase in the permeability of the BBB following acute CNS injury permits the extravasation of proteins and solutes from the cerebral vasculature into the extracellular space within the brain, leading to a net movement of water in the same direction and a subsequent increase in brain volume (Kimelberg, 1995). Due to the limited capacity of the skull to accommodate excess fluid, this increase in volume with the entry of water has deleterious consequences, including an increase in ICP. Such an increase in ICP inevitably decreases cerebral perfusion pressure (CPP) and can ultimately lead to brain herniation and its associated adverse events including localised ischemia and even cessation of respiration. The formation of cerebral oedema is thus a major cause of clinical deterioration, being associated with increased mortality and morbidity following TBI (Rhine *et al.*, 2012) and the leading cause of death within the first week of an ischaemic stroke (Hacke *et al.*, 1996).

SP is known to promote increased permeability of the BBB leading to increased extravasation of vascular protein into the brain extracellular space, both via transcellular and paracellular mechanisms. Indeed, previous studies have shown that intravenous injection of SP increases the permeability of dural blood vessels, with widening of the junctions between endothelial cells (Ghabriel *et al.*, 1999). It also has effects on tight junction proteins, with decreased levels of ZO-1 and claudin 5 reported in cerebral capillary endothelial cells upon application of SP (Lu *et al.*, 2008). Not only does SP directly increase the permeability of the BBB, but it also leads to upregulation of adhesion molecules and MHC class II antigens leading to recruitment and migration of inflammatory cells across the BBB, thereby potentiating the inflammatory response and exacerbating tissue injury (Annunziata *et al.*, 2002). The ability of SP to activate microglia and astrocytes also contributes to the increased permeability of the BBB. Increased permeability of the BBB has been repeatedly demonstrated following acute brain injury, and notably, administration of an NK1 antagonist significantly attenuates the BBB permeability back to normal levels, as measured by Evan's blue extravasation, in both TBI (Donkin *et al.*, 2009) and stroke (Turner *et al.*, 2011; Turner *et al.*, 2013). This reduction in BBB permeability is associated with reduced vasogenic oedema formation and reduced ICP.

Excitotoxicity

Levels of extracellular glutamate profoundly increase immediately after both TBI and stroke (Bullock *et al.*, 1998; Hazell, 2007) leading to excitotoxic cell death through increased calcium flux into neurons. The increase in extracellular glutamate is due to uncontrolled release of the excitatory amino acid with neuronal depolarisation, as well as a decrease in its reuptake caused by impaired glutamate transporter activity (Yi *et al.*, 2006) and a reduction in the numbers of astrocytic glutamate transporters (van Landeghem *et al.*, 2006). Accumulation of intracellular calcium activates a number of calcium-dependent enzymes including proteases, lipases, translocases and endonucleases which degrade cellular structures and eventually cause neuronal degeneration (Maas *et al.*, 2008).

SP and glutamate have been shown to be co-localised within the dorsal horn (Battaglia *et al.*, 1988; Merighi *et al.*, 1991), parts of the brainstem (Liu *et al.*, 2004)(Guiard et al., 2007) and striatum (Penny *et al.*, 1986). Previous research has indicated a bi-directional relationship with glutamate able to stimulate release of SP via activation of pre-synaptically located NMDA receptors (Liu *et al.*, 1997), whilst SP also facilitates glutamate release (Guiard et al., 2007)(Kangrga *et al.*, 1990), through a NK1 receptor dependent fashion, most likely through mobilisation of intracellular calcium stores (Stacey *et al.*, 2002). Indeed, in an organotypic brain slice culture model, application of substance P, as well as another specific NK1 receptor agonist, septide, stimulated glutamate release leading to an increase in spontaneous excitatory synaptic currents (Stacey *et al.*, 2002). SP can also alter the activity of n-methyl-D-aspartate (NMDA) receptors, which are primarily responsible for calcium influx following the binding of glutamate. Pre-application of SP led to a sustained potentiation of the current mediated by NMDA receptors in cultured rat dorsal root ganglion neurons, an effect that could be reversed with application of an NK1 antagonist (Wu *et al.*, 2004) (Castillo *et al.*, 2011). A potential mechanism was suggested to involve NK1 receptor activation induced hydrolysis of phosphoionsitide to diacylglycerol leading to the activation of PKC (Castillo *et al.*, 2011). PKC is known to produce a positive phosphorylation modulation of the NMDA receptor, enhancing its activity (Lan *et al.*, 2001). These studies support a SP-NK1 role in the release of glutamate and the activity of the NMDA receptor, but the direct effects of NK1 antagonism on measures of excitotoxicity following TBI or stroke have yet to be examined.

Magnesium

Acute insults to the CNS decrease levels of free magnesium both within the brain and the blood, with the magnitude of the decline linked to the severity of the injury as assessed by functional outcome (Heath *et al.*, 1999; Turner *et al.*, 2012a). This is consistent with the critical role that optimal magnesium concentration plays in diverse biological processes including energy transduction, phosphorylation reactions, protein synthesis, DNA synthesis, preservation of membrane integrity, maintenance of ionic gradients, and in regulation of calcium transport and accumulation. The ion also has a gating function with respect to the NMDA receptor, with low concentrations of intracellular magnesium potentiating NMDA receptor activity and calcium entry. A decline in magnesium post-injury has therefore been associated with exacerbation of other secondary injury pathways such as excitotoxicity, mitochondrial dysfunction, apoptosis, bioenergetics failure and oxidative stress (Turner *et al.*, 2012a). Critically, administration of an NK1 antagonist after TBI is able to reverse this decline in magnesium with treated rats demonstrating normal magnesium levels (Vink *et al.*, 2004), with associated improvement in histological and functional outcome. The exact mechanism whereby SP promotes normal magnesium homeostasis is yet to be determined, with the possibility that restored magnesium levels simply reflect improved neuronal function. However, there may also be a direct relationship between SP and magnesium given that magnesium deficiency is associated with

elevations in the plasma concentrations of SP (Weglicki *et al.*, 1992) and development of neurogenic inflammation (Weglicki *et al.*, 1994).

Conclusion

The current overview and additional data has highlighted the critical role that neurogenic inflammation plays in the secondary injury process following acute brain injury, with particular focus on TBI and ischaemic stroke. An increase in SP release appears to be a ubiquitous feature of TBI and critical to cellular outcome within the penumbral region following ischaemic stroke, as well as within those areas experiencing reperfusion after stroke. Neurogenic inflammation appears to exacerbate neuronal damage through a number of mechanisms including promoting BBB breakdown, with the subsequent development of vasogenic oedema, augmenting the inflammatory response and enhancing excitotoxicity. Preventing the actions of SP through use of an NK1 antagonist has been shown to be a promising therapeutic agent in experimental models of TBI and stroke, and offers a novel approach to clinical management of acute CNS injury.

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Figure Legends

Figure 1: Overview of neurogenic and classical inflammation following acute CNS injury.

Figure 2: SP immunoreactivity following diffuse TBI of varying severity. Rats were injured using the Marmarou impact acceleration model as previously described (Carthew *et al.*, 2012; Donkin *et al.*, 2009) with a 450g brass weight released from a height of either 0.5, 1, 1.5 or 2m onto a metal helmet that was adhered centrally to the animal's skull. 24h following injury, the brains were processed and then stained with SP (scale bar = 100µm; images are representative of n=3-4/group). Note the increased intensity of SP immunoreactivity (brown staining) with increasing severity of injury.

Figure 3: Effect of an NK1 antagonist on motor outcome following fluid percussion induced TBI in rats. Following moderate fluid percussion injury (Faden *et al.*, 1989), rats (n=6/group) were treated with 2.5mg/kg iv n-acetyl-L-trytophan (NAT) at 30 mins post-trauma and then assessed for motor outcome on the rotarod (Heath *et al.*, 1999) daily for 7 days. Note that the NK1 antagonist significantly improved outcome (* = $p < 0.05$; mean ± SEM; repeated ANOVA followed by student Neuman-Keuls tests) when compared to vehicle (saline) treated controls.

Figure 4: Therapeutic window for administration of an NK1 antagonist following ischaemic stroke. NK1 antagonist treatment (NAT; 25µmoles/kg iv) improved (A) motor outcome as assessed using a rotarod device when administered up to 8h following stroke and (B) sensory function as assessed using the bilateral asymmetry test (Turner *et al.*, 2014) when administered up to 12h following MCA occlusion in the rat (n=6/group; mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to saline vehicle; repeated ANOVA followed by student Neuman-Keuls tests).

Figure 5: Effects of an NK1 antagonist on motor outcome following mild to severe stroke. NK1 antagonist treatment (NAT; 25µmoles/kg in saline, iv) significantly improved motor function as assessed using the rotarod test when administred at 8h following (A) 60 min, (B) 90mins or (C) 120 mins of MCA occlusion in the rat (n=6 /group; mean ± SEM; *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to shams; repeated ANOVA followed by student Neuman-Keuls tests).

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