A Substance P Antagonist Reduces Axonal Injury and Improves Neurologic Outcome When Administered Up to 12 Hours after Traumatic Brain Injury

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Abstract

Previous studies have demonstrated that the compound N-acetyl-L-tryptophan (NAT) reduces brain edema and improves functional outcome following traumatic brain injury (TBI). In this study we examined whether this effect was mediated via the neurokinin-1 receptor, and whether there was an effect on axonal injury. We also explored whether the compound was effective, even when administered at delayed time points. Male Sprague-Dawley rats were subject to acceleration-induced, diffuse TBI and administered NAT, its inactive D-enantiomer, or saline vehicle. In contrast to NAT (2.5 mg/kg), the inactive D-enantiomer was ineffective at improving rotarod motor performance after TBI. NAT also improved cognitive outcome as assessed by the Morris water maze and novel object recognition tests, and reduced axonal injury at 5 and 24 h after TBI as assessed by amyloid precursor protein immunohistochemistry. However, efficacy of the membrane-impermeable NAT was limited to administration within 5 h, whereas administration of a form of NAT, L-732,138 (47 mg/kg), in which a trifluoromethyl benzyl ester group has been added, making it highly lipid soluble and able to cross the intact blood–brain barrier, significantly improved motor outcome, even when administration was delayed by as much as 12 h. We conclude that the neuroprotective effects of NAT are receptor-mediated, and that administration of the membrane-permeable form of the compound can be effective even up to 12 h after TBI.

Key words: neurogenic inflammation; neuropeptides; neurotrauma; neurokinin 1 (NK1) antagonist

Introduction

Traumatic brain injury (TBI) continues to be a major public health problem for which there is currently no effective treatment. Nonetheless, an increased understanding of injury pathogenesis stimulates the continuing search for effective pharmacological interventions (Vink and Nimmo, 2009). Currently, the accepted pathogenesis of TBI is that there are both primary mechanical events at the time of the traumatic incident, and the potentially treatable secondary events that manifest over the ensuing hours, days, and weeks after injury. Of the secondary events identified it is becoming increasingly evident that the development of cerebral edema along with diffuse axonal injury (DAI) are major factors leading to the high mortality and morbidity rates seen in affected individuals (Buki and Pavlishock, 2006; Marmarou, 2003).

An increase in cerebral edema and swelling following TBI leads to disruption of the brain’s normal physiology and biochemical parameters. Cerebral tissue swelling may also lead to a rapid rise in intracranial pressure, potentially causing tissue herniation and death (Donkin and Vink, 2010). DAI is characterized by morphological changes to axons throughout the brain, and is distinguished as primary and secondary axotomies due to shearing strains that develop within the brain when the head is subjected to external physical forces (Blumbergs et al., 2008, 1994). Although interventions currently exist for treating both cerebral edema and secondary DAI, they are inadequate.

Recent studies have underscored the importance of neurogenic inflammation in the formation of vascular permeability and increased edema in both the peripheral nervous system (Woie et al., 1993), and the central nervous system (CNS; Donkin et al., 2009; Nimmo et al., 2004; Vink and van den Heuvel, 2010). Neurogenic inflammation is an inflammatory response that is neurally elicited, and results in vasodilation, plasma extravasation, and neuronal hypersensitivity. It is caused by the release of neuropeptides from sensory neurons,
and several neuropeptides have been identified as playing a role, including substance P (SP) and calcitonin gene-related peptide (CGRP). SP is thought to enhance plasma protein extravasation, as well as leukocyte adhesion to endothelial cells in postcapillary venules, while CGRP is thought to be associated with the vasodilation of arterioles (Newbold and Brain, 1995). It is well known that cerebral blood vessels are surrounded by a dense supply of sensory neurons, and that the release of neuropeptides around the cerebral vasculature will initiate neurogenic inflammation.

Recent findings have confirmed that the neuropeptide SP is upregulated following TBI, and leads to increased edema formation, neuronal cell death, and behavioral deficits (Cernak et al., 2004; Foda and Marmarou, 1994; Heath and Vink, 1995). All animals were examined these aspects of an NK1 antagonist in a rat model of traumatic axonal injury.

**Methods**

All experimental protocols were approved by the experimental ethics committees of the University of Adelaide and the Institute of Medical and Veterinary Science, and were conducted according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council.

**Induction of traumatic brain injury**

Injury was induced in halothane-anesthetized male Sprague-Dawley rats (n = 97; 400 ± 20 g) using an acceleration-induced impact TBI model (Foda and Marmarou, 1994; Heath and Vink, 1995). This model involves impacting a stainless steel disc (10 mm diameter × 3 mm thick) fixed centrally to the exposed skull between the lambda and the bregma, with an accelerating impactor made up of a 450-g brass weight dropped from a height of 2 m. Previous studies have demonstrated that impact acceleration injury produces axonal injury, edema, blood–brain barrier (BBB) opening, Mg²⁺ decline, and moderate to severe neurological deficits (Cernak et al., 2004; Foda and Marmarou, 1994; Heath and Vink, 1995). All animals were fed and watered ad libitum prior to induction of injury. During surgery, induction of injury, and during the immediate recovery phase, the rat rectal temperature was maintained at 37°C using a thermostatically heated warming blanket. Immediately after injury the animals were manually ventilated until stable respiration was restored, usually in less than 5 min. After injury, all wounds were sutured, the animals were withdrawn from anesthesia, and they were returned to their cages. Another group of animals (n = 24) were surgically prepared but not injured, and were used as sham controls.

**Drug treatment**

The animals were randomized by the animal services staff, and after injury were treated at predetermined time points with either the NK1-receptor antagonist NAT (cat. no. A-6376; Sigma-Aldrich, Sydney, Australia), the inactive enantiomer N-acetyl-D-tryptophan (NATD, cat. no. A-6001; Sigma-Aldrich), the BBB-permeable NK1-receptor antagonist N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl) benzyl ester (L-732,138, cat. no. A-5330; Sigma-Aldrich), or with equal volume saline vehicle. Dosages were based on previously published studies using extravasation of Evans blue as the outcome measure (Donkin et al., 2009).

**Functional outcome**

Motor and cognitive outcomes in the animals were assessed using the rotaord, Morris water maze (MWM), and novel object recognition tests, as described in detail elsewhere (Bevins and Besheer, 2006; Cernak et al., 2004; Ennaceur and Delacour, 1988; Thornton et al., 2006). In all cases the assessor was blinded to treatment group, and group size varied between 5 and 10 animals per group, depending on the outcome test. Motor tests yield significance with lower group sizes, whereas cognitive tests require up to 10 animals per group to achieve statistical significance. There were only 5 sham animals used in each test to establish normal performance. The rotaord device consists of a motorized rotating assembly of 18 rods (1 mm in diameter) upon which the animals were placed. Rotational speed of the device is increased from 0 to 30 rpm in intervals of 3 rpm every 10 sec. The duration (in seconds) in which the animals either completed the 2-min task, fall from the rods, or gripped the rods and spun for two consecutive revolutions rather than walking actively, was recorded as the rotaord motor score. Animals were trained for 5 days prior to induction of TBI to establish a pre-injury baseline of 110–120 sec.

The spatial learning aspects of cognitive outcome at delayed time points (18–21 days) was determined using the hidden platform version of the MWM. The apparatus consists of a large, white circular pool (900 mm diameter and 500 mm high, water temperature 24 ± 1°C), with a 76-mm-diameter acrylic glass platform painted white, and submerged 15 mm below the surface of the water (225 mm high). The surface of the water was rendered opaque by the addition of dilute white non-toxic paint. The rats were required to locate the submerged platform while monitoring was performed using a PC-controlled video system (AccuScan Instruments, Inc., Columbus, OH). The platform remained in a constant location hidden in one quadrant 14 cm from the side wall. The rat was gently placed in the water facing the wall at one of four randomly chosen locations separated by 90°. The latency to find the hidden platform within a 90-sec criterion time was recorded by a blinded observer. A series of 16 trials administered in blocks of four were conducted on days 17, 18, 19 and 20 after drug injection. To control for visual discriminative ability or motor impairment, the same animals were finally required to locate a clearly visible black platform (placed in a different location) raised 5 mm above the water’s surface at least 2 h after the last training trial.

In the novel object recognition test, the animals (n = 19) were placed in an empty open field (100 ×100 ×50 cm) for five separate sessions each for 10 min prior to the testing period for habituation. During the sample phase, conducted on day 5 after injury, two identical objects were placed in the back corners of the field, equidistant from each other. The animals were placed in the center of the open field facing away from
the objects to be discriminated, and allowed to explore. The percentage of time spent exploring the two identical objects within a 3-min period was quantified. Exploration of an object involved the animal sniffing, climbing onto, or touching the object, while facing or being oriented towards the object. After the sample phase, the animals were removed from the open field for an inter-trial period of 15 min, during which time the animals were returned to their home cages, and the open field was cleaned with 70% ethanol to eliminate olfactory cues. During the testing phase one of the identical objects was replaced with a novel object of similar size. The percentage of time spent exploring the two objects within a 3-min period was recorded. An increased percentage of time spent exploring the novel object (duration spent with the novel object/[duration spent with the novel object + duration spent with familiar object]×100) is considered an index of enhanced cognitive performance.

Immunohistochemistry

The animals were injured and perfusion fixed at preselected time points (n = 5 per group). Another five animals were used as sham controls. All brains were perfusion fixed using 4% paraformaldehyde and removed after decapitation. Coronal sections were then cut using a Kopf rodent brain blocker (David Kopf Instruments, Tujunga, CA), and the resultant 2-mm sections were embedded in paraffin. Serial 5-μm sections were cut with a microtome (Microm, Waldorf, Germany) and immunolabeled with the amyloid precursor protein (APP) primary antibody (1:2000 in NHS; monoclonal antibody 22C11; Chemicon International, Temecula, CA) by overnight incubation at room temperature. After washing in phosphate-buffered saline (PBS), the slices were incubated with an anti-mouse IgG HRP-conjugated secondary antibody (1:250 in NHS; Sigma-Aldrich) for a minimum of 30 min at room temperature. The immune complex was visualized using diaminobenzindine tetrahydrochloride (DAB; Pierce Protein Research Products, Rockford, IL) at room temperature, and the subsequent immunocomplex was visualized using diaminobenzidine tetrahydrochloride as a chromogen in a peroxidase reaction (Sigma-Aldrich), and examined using a Leica light microscope (Leica Microsystems, Sydney, NSW, Australia). All slides were qualitatively assessed for APP staining by a blinded observer using a + to ++++ intensity scale.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM), and with the exception of the functional outcome data, were analyzed for statistical significance using one-way analysis of variance (ANOVA), followed by Student-Neuman-Keuls tests (GraphPad Prism software; GraphPad, La Jolla, CA). Functional outcome data were analyzed by repeated-measures two-way ANOVA, followed by Student-Neuman-Keuls tests.

Results

The effect of the inactive enantiomer NATD on motor outcome following injury

Prior to injury the mean rotarod score for all animals was 113 ± 3 sec (Fig. 1). Following injury there was a highly significant decrease (p < 0.001) in motor function in the vehicle-treated animals, demonstrating a minimum rotarod score of 36 ± 7 sec by day 2 post-injury (Fig. 1). There was no significant improvement in motor outcome over the remainder of the assessment period. Animals treated with the NK1 antagonist NAT at 30 min after TBI (2.5 mg/kg IV) performed significantly better (p < 0.001) than vehicle-treated controls, with a minimum value of 60 ± 11 sec at 1 day post-injury. Therefore, there was a significant improvement in motor score in these animals such that by day 3 post-injury, they were no longer significantly different from sham controls. This is consistent with previous reports from our laboratory (Donkin et al., 2009). Treatment with the inactive NATD enantiomer at 30 min after TBI (2.5 mg/kg IV) failed to improve motor function above that observed for vehicle-treated animals. Indeed, the rotarod score was 24 ± 7 sec at 1 day after injury, and did not improve significantly thereafter. At no point during the 7-day assessment period were the NATD-treated mice significantly different from the vehicle-treated animals. Clearly, the enantiomer active as an antagonist of the NK1 receptor was the only agent to improve motor outcome, confirming that the efficacy of the active compound was not due to non-specific effects, but rather was mediated via the NK1 receptor.

Cognitive outcome following NK1-receptor antagonist treatment

Having established the beneficial effects of NAT on motor outcome, the effects of the NK1-receptor antagonist on cognitive outcome was assessed by the MWM and novel object recognition tests (Fig. 2). In the MWM test (Fig. 2A), sham control animals were able to locate the hidden platform in 76 ± 5 sec on day 18 after trauma, and improved over the 4-day test period, to a time of 28 ± 3 sec. Injured animals treated with the saline vehicle also improved their performance over the 4-day test period, such that they were able to locate the hidden platform in 45 ± 5 sec on day 21 after trauma, which was slower than the sham control animals, though this difference was not significant. This is typical of the cognitive
deficit experienced by these rats after diffuse TBI (Cernak et al., 2004). In contrast, animals treated with the NK1-receptor antagonist NAT performed significantly better than vehicle-treated animals after 4 days of trials commencing on day 18 post-injury (**p < 0.01 versus vehicle-treated animals). (B) In the novel object recognition test, all animals equally explored identical objects in the sample phase. In the choice phase, sham (uninjured) and NAT-treated injured animals spent significantly more time exploring a newly introduced novel object than the injured vehicle-treated controls (N, novel object; ** *p < 0.001 versus exploration time at the non-novel object; NK1, neurokinin 1; NAT, N-acetyl-L-tryptophan).

In the novel object recognition task conducted on day 5 after injury (Fig. 2B), sham-treated animals showed a significant (p < 0.001) preference for the novel item compared to the original object during the testing phase of the assessment, which is the expected result. In contrast, injured animals treated with vehicle equally explored both the original object and the novel one after injury, indicating a profound cognitive deficit. In animals treated with the NK1-receptor antagonist, animals again explored the novel object for significantly more time (p < 0.001) than the original object after injury. Indeed, the animals treated with the NK1-receptor antagonist demonstrated object recognition abilities comparable to the sham-treated animals (Fig. 2B).

The effect of the NK1-receptor antagonist on diffuse axonal injury

While previous studies have demonstrated that NAT is protective with respect to cortical and hippocampal cell death (Donkin et al., 2009), no studies have examined the effects of NK1 antagonists on traumatic axonal injury. Previous studies using this model of injury have demonstrated that improved motor and cognitive outcomes can be associated with reduced axonal injury in the corpus callosum (O’Connor et al., 2007). In sham (uninjured) animals, negligible APP immunoreactivity was observed in the corpus callosum (Fig. 3A), confirming that no axonal injury was present. At 5 h after injury in vehicle-treated animals (Fig. 3B), there was a clear upregulation of APP immunoreactivity (median ++ +), with dense granulation along damaged axonal fibers, as well as the presence of characteristic retraction balls (arrowhead in Fig. 2B). These have previously been shown to be a feature of axonal injury (Vowles et al., 1987). By 24 h (Fig. 3D), increased APP immunoreactivity was still present (median ++ +), and general tissue vacuolization had become more apparent. With administration of the NK1-receptor antagonist (Fig. 3C and E), there was a decrease in APP upregulation, as demonstrated by a reduction in both retraction balls and the staining of the axonal fibers, particularly at 24 h (median ++ ). There was also marked reduction in tissue vacuolization compared to vehicle-treated animals (Fig. 3E).

Establishing a therapeutic window for the NK1-receptor antagonist

Given that NAT is not lipid soluble, and that the BBB closes to large molecules in less than 5 h after this form of TBI (Habgood et al., 2007), we tested the lipid-soluble benzyl ester form of NAT (L-732,138). Rotarod performance was used as the outcome measure (Fig. 4). Prior to the induction of injury, the mean rotarod score of all animals was 119 ± 1 sec. Following injury there was a significant decline (p < 0.001) in the vehicle-treated animals, to 40 ± 10 sec at 1 day post-injury. Despite repeated exposure to the task, these animals never showed any significant improvement over the 7-day assessment period. Administration of an equimolar dose of the cell-permeable NK1-receptor antagonist L-732,138 (4.7 mg/kg; low-dose group) at 12 h after TBI also led to a significant decline in rotarod performance over the 7-day assessment period, with no statistically significant difference noted at any time point compared to the vehicle-treated controls. When we increased the dose 10-fold, administration of L-732,138 (47 mg/kg; high-dose group) at 12 h after TBI led to a profound improvement in rotarod score, to the point that they were equivalent to sham animals (Fig. 1) at all time points, and significantly better than vehicle-treated controls (p < 0.001; Fig. 4). It was noted that the rotarod performance was even better than that in animals treated with NAT at 30 min, which
most likely is a reflection of the greater binding affinity of the lipid-soluble NAT benzyl ester to the NK1 receptor (Cascieri et al., 1994; MacLeod et al., 1994), as well as the CNS penetration. When NAT was administered at an equimolar high dose (25 mg/kg) at 12 h after TBI, there was no improvement in outcome relative to vehicle-treated animals, suggesting that the compound did not achieve pharmacological levels in the brain due to limited CNS penetration. Administration of high-dose L-732,138 at 24 h post-injury failed to demonstrate any improvement in motor function.

Discussion

In the current study we demonstrated that the beneficial effects of the NK1 antagonist N-acetyl-L-tryptophan after TBI are mediated via actions at the NK1 receptor, that inhibition of the NK1 receptor benefits both motor and cognitive outcomes, that the antagonist attenuates axonal injury, and that the benefits on motor outcome can be conferred with administration of a membrane-permeable NK1 antagonist up to 12 h after TBI. Taken together, these results provide further evidence that neurogenic inflammation, and in particular SP acting through the NK1 receptor, plays an important role in the pathophysiology of TBI, at least up to 12 h after injury.

Our laboratory was the first to demonstrate that neuropeptides play an important role in TBI, and more specifically that prior depletion of neuropeptides from sensory nerves leads to an inhibition of BBB opening, edema formation, and development of motor and cognitive deficits after TBI (Nimmo et al., 2004). Subsequent studies have demonstrated that SP is the neuropeptide that is integrally linked to the increased vascular permeability and edema formation seen after brain trauma, and that treatment with the NK1-receptor antagonist NAT at 30 min post-injury improved free magnesium status after trauma, reduced BBB permeability, reduced edema formation, and improved functional outcome (Donkin et al., 2009; Vink et al., 2004). Together, this body of work suggests that NAT promotes a beneficial response post-injury, presumably by competitive antagonism with SP for the NK1 receptor. This action via the NK1 receptor was confirmed in the present study with the inactive D-enantiomer of NAT, which had no significant effect on motor outcomes after TBI, ruling out the possibility of non-specific effects playing a major role.

The present study also demonstrated that NAT had a positive effect on cognitive outcomes after TBI, as assessed by the widely used MWM and object recognition tests. Cognitive performance on the object recognition task showed the greatest amount of acute improvement, with rats treated with...
NAT demonstrating novel object memory equivalent to that of sham (uninjured) animals. The advantage of this test relative to other cognitive tasks, such as the previously used Barnes maze (Donkin et al., 2009), is that it is easy to administer, requires little equipment, and has no need for animal pre-training. Object recognition tasks also require cooperative function between the hippocampal and peri-rhinal cortices (Bellgowan et al., 2009), allowing assessment of different brain inputs and a more thorough assessment of memory function. We nonetheless undertook an additional cognitive assessment in the form of the widely used Morris water maze (Morris, 1984). This cognitive task demonstrated that there were cognitive deficits present up to 3 weeks post-injury, and that treatment with NAT was beneficial for outcomes at this time point. This effect on cognitive function may be related in part to the high numbers of sensory neuropeptide receptors in the hippocampus and striatum, portions of the brain that are known to be associated with learning and memory (Huston and Hasenohrl, 1995). Previous studies have demonstrated that alterations in neuropeptide levels in the hippocampus are associated with cognitive deficits in mice (Bracci-Laudiero et al., 1999). A recent study also supports the targeting of neurokinin receptors in the hippocampus to influence object recognition memory in rats (Schable et al., 2010).

While improvements in motor and cognitive outcomes after TBI have been frequently associated with the attenuation of neuronal cell death, functional outcome can also be a function of axonal integrity. Indeed, previous studies have shown that attenuation of axonal injury was associated with improvements in motor and cognitive outcomes after diffuse TBI (O’Connor et al., 2007). Moreover, the acceleration-induced impact TBI model used in this study has been previously validated as causing traumatic axonal injury, particularly in the corpus callosum (Povlishock et al., 1997). The use of APP for detecting early traumatic axonal injury has been widely described, both in experimental and clinical TBI models (Blumbergs et al., 1994; Gleckman et al., 1999; Leclercq et al., 2001; Stone et al., 2000). In the present study, the up-regulation of APP immunoreactivity in the corpus callosum after TBI was a reflection of extensive axonal injury. Moreover, administration of the NK1 antagonist resulted in a marked attenuation of APP immunoreactivity and a decrease in tissue vacuolization, reflecting reduced axonal injury. This is highly significant given the recent finding that perivascular SP and APP are upregulated and frequently co-localized in the post-mortem brains of patients who had sustained a TBI (Zacest et al., 2010), with the authors suggesting that the mechanical injury to perivascular nerve fibers could be one potential mechanism of SP release.

The beneficial effects of NAT following brain injury have thus far only been demonstrated following acute administration (30 min after injury; Donkin et al., 2009). To further determine the potential for clinical application, we therefore assessed the therapeutic window of efficacy. However, the CNS penetration of NAT was an important consideration, given that the compound cannot penetrate the membrane, and that the BBB has been shown to be impermeable to large molecules after approximately 5 h in different models of TBI (Habgood et al., 2007; O’Connor et al., 2006). The molecular weight of NAT (C_{13}H_{14}N_{2}O_{3}) is 246.26, which is relatively large in the context of BBB permeability. We therefore incorporated the lipid-soluble benzyl ester form of NAT (L-732,138), to better characterize the therapeutic window. Our results demonstrated that the membrane-permeable NK1-receptor antagonist was effective up to 12 h following injury, though it had to be administered at a higher concentration. Notably, the membrane-penetrating form of the NK1 antagonist was more effective at improving motor performance of the injured animals than the early administration of NAT, despite being given at a much later time point. This suggests that the neuroprotective action of the NK1-receptor antagonist is not only mediated at the level of the BBB (Donkin et al., 2009), but that it may also be centrally mediated. Moreover, the profound improvement in neurological outcomes observed with the lipid-soluble compound supports the finding that a lipid-soluble form of an NK1 antagonist is a more effective compound, since it facili-
tates receptor binding (Cascieri et al., 1994; MacLeod et al., 1994).

While a number of studies have previously examined therapeutic windows for the treatment of TBI (Adelleye et al., 2010; Faden et al., 2003; Kupina et al., 2001; Hoane and Barth, 2002; Louin et al., 2006; Mauler et al., 2003; Toulmond et al., 1993; Verweij et al., 2000), few have demonstrated efficacy after the first few hours post-trauma. The current study is the first to demonstrate the efficacy of a single bolus injection of an NK1-receptor antagonist at 12 h following a moderate-to-severe TBI, making it a suitable candidate for further investigation as a potential clinical intervention.

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