LETTER TO THE EDITOR

A Substance P Antagonist Improves Outcome in Female Sprague Dawley Rats Following Diffuse Traumatic Brain Injury

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Over the past decade it has become increasingly clear from work in experimental models that the secondary injury cascade following traumatic brain injury (TBI) may differ between males and females [1, 2], with females exhibiting a different temporal profile of edema [1] and neurodegeneration compared to males [2]. Furthermore significant neuroprotection was noted in male, but not female rodents treated with posttraumatic hypothermia [3], or a dopamine agonist [4]. This highlights the need to ensure that experimental treatments are equally efficacious in both genders before proceeding to clinical trials that are performed in mixed populations.

Previous research in our laboratory found that substance P (SP) plays an integral role in the secondary injury cascade following TBI in males [5]. SP is a member of the tachykinin family of neuropeptides, with its release causing the development of neurogenic inflammation characterised by vasodilation, plasma extravasation, and tissue swelling [6]. These effects are principally mediated by the binding of SP to the NK₁ tachykinin receptor [6]. Following impact-acceleration TBI in male rodents SP levels were found to increase. Furthermore, inhibition of SP, through treatment with an NK₁ receptor antagonist reduced vasogenic edema formation and axonal injury, with a corresponding improvement in motor and cognitive outcome [5, 7]. However, it is not known whether SP plays a similar role in females following TBI. As such, the effects of an NK₁ receptor antagonist, *N*-acetyl-L-tryptophan (NAT) on outcome following TBI in female rodents were investigated.

Female Sprague Dawley rats were injured using the impact-acceleration model of diffuse TBI, as previously described [5].

At 30 mins postinjury rats were treated intravenously with either 2.5 mg/kg of NAT or an equal volume of saline. Sham animals were surgically prepared but not injured. Motor deficits were assessed on rotarod as previously described [5]. For histological analysis, rats were transcardially perfused fixed, the brains removed and processed. Slides were labeled with APP (1:1,000), SP (1:2,000), or albumin (1:20,000). To objectively analyse levels of SP and albumin, Ruifrok and Johnston's color deconvolution method was employed on 2 slides per animal to determine the amount of DAB and hence antigen that was present, as previously described [8]. Axonal injury was determined by counting the number of APP immunopositive lengths within the corpus callosum. For analysis of edema the specific gravity of the brain was determined by placing it in a Percoll density gradient, with this measurement then converted to % water as previously described [9]. All data were analyzed using a one or two-way ANOVA as appropriate, followed by Bonferroni *t*-tests using Graphpad Prism software.

SP immunoreactivity was assessed postinjury to determine whether levels were increased, as previously reported in males (Figures 1A and B). Indeed, at 24 h postinjury increased SP immunoreactivity was observed, particularly apparent in perivascular nerve fibers and astrocytic processes in vehicle treated rats. In contrast low levels of SP immunoreactivity were observed in shams. Furthermore color deconvolution analysis demonstrated a significant increase in %DAB weight in vehicle treated rats, with $28.0 \pm 3.6\%$ compared to $16.6 \pm 2.8\%$ in sham controls ($P < 0.01$).

Figure 1. Representative images of SP Immunoreactivity in a sham (A) and injured vehicle treated rat (B), indicating increased levels following injury. Representative images of albumin immunohistochemistry are shown of a sham (C), vehicle (D), and NAT treated animal (E), indicating prevention of BBB breakdown in NAT treated rats. These observations were confirmed

with colour deconvolution of SP (F) and albumin (G), as well as % brain water content (H), indicating that increased SP following injury is associated with breakdown of the BBB and increased levels of edema. (Figure 1F and G), $n = 6$ per group; Figure 1H Sham $n = 4$, vehicle and NAT $n = 5$ ($***P < 0.001$, $*P < 0.05$ compared to shams, $^^^P < 0.001$ compared to NAT treated rats).

Given the increase in SP following injury in female rats, the effects of NAT treatment on vasogenic edema following TBI were assessed by evaluating BBB integrity with albumin immunohistochemistry (Figure 1C and E) and determining % brain water content (1G). Following injury increased albumin staining was observed in vehicle treated rats, particularly apparent in the cortex directly underneath the impact site. In contrast NAT treated rats had only a slight increase in staining when compared to sham animals. Indeed the %DAB weight in vehicle treated rats was $33 \pm 6.3\%$ compared to $18.6 \pm 3.1\%$ in NAT rats, with sham animals at $15 \pm 3.9\%$. Furthermore, a significant increase in brain water content was noted in vehicle treated ($82.1 \pm 1.4\%$), but not NAT treated rats ($78.6 \pm 0.8\%$) when compared to shams ($77.6 \pm 2.1\%$).

The effect of NAT treatment on motor ability was determined with the rotarod (Figure 2A). Vehicle controls were significantly impaired compared to shams on all days postinjury ($P < 0.05$). In contrast, whilst NAT treated rats were significantly differ-

ent to shams on days 1 and 2 postinjury ($P < 0.05$), a return to sham level was observed by day 3. Axonal injury was also significantly reduced in NAT treated rats, with these animals exhibiting only 28 ± 22 immunopositive lengths within the corpus callosum compared to 75 ± 12 in the vehicle controls (2B).

In this study it was found that treatment with an NK₁ receptor antagonist improved motor outcome, reduced axonal injury and decreased levels of vasogenic edema in female Sprague Dawley rats following diffuse TBI. This is most likely due to its ability to prevent SP from binding to its primary receptor, given the significant increase in SP immunoreactivity observed in vehicle treated animals. These results support earlier work performed in male rats, where increased SP levels were noted following injury [5, 8], with administration of an NK₁ receptor antagonist providing neuroprotection [5, 7]. Together these results indicate that increased SP release following TBI is an important secondary injury factor regardless of gender.

Figure 2. Effects of treatment with NAT on motor outcome (A) and axonal injury (B). (A) $n = 9$ per group; (B) $n = 6$ per group. ($^{^^}P < 0.001$, $^{^}P < 0.01$, $^{^}P < 0.05$ compared to NAT treated rats, $^{***}P < 0.001$ compared to shams).

Inhibition of vasogenic edema postinjury with NAT treatment, seen as a reduction of brain water content and greater BBB preservation, provides further support for the role of SP in potentiating edema formation following TBI. This process is thought to occur via SP induced NK_1 receptor activation causing gaps to open between endothelial cells allowing the flux of plasma proteins from the vascular lumen [6]. The importance of the role of SP in augmenting and inducing many aspects of the inflammatory response, such as leukocyte activation, endothelial cell adhesion molecule expression, and cytokine production [10], was also highlighted by the reduced axonal injury and improved motor outcome in NAT treated rats.

This study is the first to demonstrate that a NK_1 receptor antagonist is effective in female Sprague Dawley rats following TBI. This is of utmost importance, as clinical trials involve mixed pop-

ulations, thus pre-clinical investigations should be conducted in both males and females to ensure no gender differences occur in treatment efficacy. As such this study provides further support to the efficacy of an NK_1 receptor antagonist as a novel therapeutic agent following TBI.

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Conflict of Interest

The authors declare no conflict of interest.

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