THE ROLE OF SUBSTANCE P IN EARLY EXPERIMENTAL PARKINSON’S DISEASE

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CHAPTER 8:
COMPARISON OF THE EFFECTS OF NK₁ ANTAGONISTS,
L-333,060 AND N-ACETYLL-T-TRYPTOPHAN,
IN THE INTRASTRIATAL 6-OHDA MODEL OF EARLY
PARKINSON’S DISEASE
8.1 Introduction

In Chapters 4 and 7, elevation of SP by either preventing its degradation or directly treating the animals with SP, accelerated the progression of PD. These animals had profound functional deficits that were often significantly worse than vehicle treated animals. Additionally, in chapter 7 it was demonstrated that treatment with the NK₁ antagonist NAT reduced dopaminergic degeneration and improved function, thus slowing down the disease progression. However it remains to be shown that the neuroprotective effect of NAT was not due to specific effects of the compound itself, but rather was a class effect mediated through antagonism of the NK₁ receptor.

The rapidly advancing knowledge about SP and its role in disease pathogenesis is due, in part, because of the development of specific NK₁ antagonists that have enabled researchers to elucidate the biochemical and functional effects of SP in both the CNS and PNS. In previous studies, NK₁ antagonists have been shown to be efficacious in reducing inflammatory processes in asthma and inflammatory bowel disease, and preventing neurogenic inflammation in both the CNS and PNS (Improta and Broccardo, 2006; Joos et al., 2000; Lembeck et al, 1992; Nimmo et al., 2004). Furthermore, NK₁ antagonists can act as an antiemetic and analgesic following chemotherapy, as well as an antidepressant and an anxiolytic compound (Chahl, 2006; Duzzioni et al., 2008; Ebner and Singewald, 2006). Whether the class of compounds can confer neuroprotection in early PD is unknown, and is the aim of the current study. We will compare another NK₁ antagonist, L-333,060 with NAT, specifically characterising their effects on functional outcome, dopaminergic terminal and cell loss, SP expression and the astrocytic and microglial responses following 6-OHDA induced striatal lesions.
8.2 Study Design

Animals (n=12) were randomly assigned to either receive 6-OHDA striatal lesions or vehicle-lesions with L-333,060 treatment. L-333,060 (TOCRIS Bioscience) was dissolved in 0.9% sterile saline at a dose of 100nM. This dose was based on preliminary studies of TBI and stroke from our laboratory (unpublished results). All animals were subject to vehicle or 6-OHDA striatal lesions (as described in Chapter 2.2.2) with simultaneous icv treatment of L-333,060. Briefly, animals received stereotaxic injections of 6-OHDA (2x2μL; 5μg/μL) or equal volume vehicle before receiving a stereotaxic injection of L-333,060 (2μL at 100nM) into the lateral ventricle. All animals were assessed for functional outcome (as described in Chapter 2.4.1-2.4.7) before being sacrificed at day 21 post-lesion. Striatal and SN sections from each animal were then assessed for histological outcome (as described in Chapter 2.5.2-2.5.6). Results were compared with those obtained for NAT treatment in 6-OHDA lesioned animals in Chapter 7. A subset of animals (n=5) received sham surgery to be used as a control in both functional and histological outcome.

8.2.1 Functional Outcome

All animals were assessed for functional outcome on day 3, 7, 10, 14, 17 and 21 post-lesion. Functional outcome was assessed using the rotarod, stepping tests, bilateral asymmetry test, modified NSS and open field. Estimation of lesion size was assessed using the rotometer on day 7 and 14 post-lesion. All functional tests are described in Chapter 2.4.
8.2.2 Histological Outcome
At day 21 post-lesion, all animals were deeply anaesthetised with Isoflurane before being perfused fixed with 10% buffered formalin (as described in Chapter 2.2.4). Brains were then removed and processed for immunocytochemistry. Sections of the striatum and SN were stained for TH, H&E, SP, GFAP and ED-1.

8.2.3 Statistical Analysis
All parametric data are displayed as mean ± SEM and therefore were analysed using an ANOVA followed by Bonferroni post-tests. Neurological and behavioural outcome results are displayed as the median and therefore were analysed using the Kruskal Wallis ANOVA followed by Dunn’s multiple comparison test.

8.3 Results

8.3.1 Functional Outcome

Motor Function – Rotarod
Sham animals had normal motor function as they could easily walk on the rotarod for 2 mins to complete the task (Figure 8.1). Despite initially performing just below sham levels, vehicle lesioned L-333,060 treated animals could complete 2 mins by day 17 post-lesion, and therefore did not have significantly different motor function to shams. Thus, L-333,060 treatment alone does not affect motor function on the rotarod. In contrast 6-OHDA striatal lesions produced a significant decline in motor function relative to sham animals, with vehicle treated animals only improving from 74 ± 6 to 83 ± 5 secs (p < 0.001) with repeated exposure. L-333,060 treatment in lesioned animals significantly
improved motor function, recording a score of $97 \pm 6$ secs on day 3 post-lesion and improving to $114 \pm 4$ secs by day 21. L-333,060 treated animals had significantly better motor outcomes than vehicle treated lesioned animals on each assessment day ($0.001 < p < 0.01$). By comparison, NAT treatment in lesioned animals also improved motor function, with these animals achieving similar rotarod scores to L-333,060 treated animals. Thus both of the NK$_1$ antagonists significantly improved motor function on the rotarod.

**Motor Function – Stepping Tests**

**Initiation Time**

Sham animals had normal initiation of movement, being able to quickly move both their forepaws for the entire assessment period (Figure 8.2). L-333,060 treatment alone did not affect initiation of movement, with vehicle-lesioned L-333,060 treated animals recorded similar initiation times in both forepaws to shams. Following 6-OHDA striatal lesions, vehicle treated animals had a significant delay in initiation of movement to shams in both their forepaws ($p < 0.001$). L-333,060 treatment improved this functional deficit particularly in the ipsilateral forepaw, where these animals took only 1 to 3 secs to move their paw, which was no different to shams. However, initiation of contralateral forepaw movement did not improve significantly following L-333,060 treatment, with their contralateral forepaw movement still being significantly worse than shams ($p < 0.05$). Although, NAT treated lesioned animals had an improvement in initiation of forepaw movement to vehicle treated animals, they still displayed a significantly longer delay in initiation than shams in both their ipsilateral ($p < 0.01$) and contralateral ($p < 0.001$)
Figure 8.1: Comparison of NK₁ antagonists L-333,060 and NAT - Motor function as assessed by the rotarod.

Sham animals (black) had normal motor function as assessed by the rotarod. L-333,060 treatment in vehicle-lesioned animals (light blue) had no significant effect on motor function. In contrast, 6-OHDA striatal lesions produced a significant motor deficit (green) throughout the assessment period. L-333,060 treatment in lesioned animals (red) significantly improved motor deficits. NAT treatment also significantly improved motor function in 6-OHDA lesioned animals (dark blue), and recorded similar rotarod scores to L-333,060 treated animals. No significant difference in motor function was observed between the two NK₁ antagonists during assessment. (** denotes $p < 0.01$, *** denotes $p < 0.001$ versus 6-OHDA+L-333,060) (n=5/Sham, n=6 / 6-OHDA+Vehicle+L-333,060, n=12/6-OHDA+Vehicle, n=6 / 6-OHDA+L-333,060, n=6 / 6-OHDA+NAT).
**Figure 8.2: Comparison of NK$_1$ antagonists L-333,060 and NAT - Initiation of movement as assessed by the stepping tests.**

Sham animals (black) displayed normal initiation of movement in both their ipsilateral (A) and contralateral (B) forepaws. L-333,060 treatment in vehicle-lesioned animals (light blue) had no effect on initiation of movement in either forepaw. 6-OHDA striatal lesions produced a significant delay in initiation of movement (green) recording significantly longer initiation times than shams. L-333,060 treatment in lesioned animals (red) improved the delay in movement, particularly in their ipsilateral forepaw, where they did not record significantly longer initiation times than shams. However, these animals did not show as much improvement in their contralateral forepaw movement. Although NAT treated lesioned animals (dark blue) showed some improvement in forepaw movement, it was less improvement than produced by L-333,060 treatment. No significant difference in initiation of movement was observed between the different NK$_1$ antagonists (n=5/Sham, n=6 / 6-OHDA Vehicle+L-333,060, n=12/6-OHDA+Vehicle, n=6 / 6-OHDA+L-333,060, n=6 / 6-OHDA+NAT).
A. **Ipsilateral Initiation Time**

![Graph showing ipsilateral initiation time over days post-lesion for different groups.]

B. **Contralateral Initiation Time**

![Graph showing contralateral initiation time over days post-lesion for different groups.]

- **Sham**
- **6-OHDA + Vehicle**
- **6-OHDA + NAT**
- **6-OHDA + L-333,060**
- **6-OHDA Vehicle + L-333,060**
forepaw. Nonetheless, no significant difference in latency was recorded for either forepaw in the NK₁ antagonist groups.

**Stepping Time**

Sham animals had a relatively constant stepping time during the assessment period recording on average $6.1 \pm 0.4$ secs to traverse the ramp (Figure 8.3). L-333,060 treatment alone did not affect stepping time, and took less time than shams to traverse the ramp on (mean = $5.1 \pm 0.2$ secs). Following 6-OHDA striatal lesions, vehicle treated animals always recorded longer stepping times than shams, taking $10.7 \pm 1.2$ on day 3 with a slight improvement to $7.8 \pm 1.3$ secs to traverse the ramp on day 21 post-lesion. L-333,060 treatment in lesioned animals markedly improved stepping time to $2.6 \pm 0.2$ secs on day 21 post-lesion, which was the fastest stepping time of any group. Although NAT treated lesioned animals recorded longer stepping times than L-333,060 treated animals, they had greater overall improvement in stepping times, taking $14.0 \pm 2.6$ secs on day 3 to traverse the ramp and improving to $4.3 \pm 0.4$ secs on day 21, which was equivalent to sham levels. However, throughout the 3-week assessment period no significant difference in stepping time was recorded between any of the groups.

**Step Length**

Sham animals recorded an average step length on $9.6 \pm 0.3$ cms for the 3-week assessment period (Figure 8.4). L-333,060 treatment alone did not affect step length and recorded an average step length of $10.5 \pm 0.3$ cms. Following 6-OHDA lesions, vehicle treated animals had a slight reduction in step length during the first week post lesion, recording a step length $8.3 \pm 0.4$ cm on day 3 post-lesion. However from day 10 onwards
Sham animals (black) could traverse the ramp relatively quickly throughout the assessment period. L-333,060 vehicle-lesioned animals (light blue) also had normal stepping times, performing slightly quicker stepping times than shams. Following 6-OHDA lesions, vehicle treated animals demonstrated a small increase in stepping time, with no obvious improvement during the assessment period. L-333,060 treatment in lesioned animals improved stepping times, having quicker stepping times than shams from day 10 onwards. NAT treatment also improved stepping times, returning to sham levels by day 14. These animals always recorded longer stepping times than L-333,060 treated animals. (n=5/Sham, n=6 / 6-OHDA Vehicle+L-333,060, n=12/6-OHDA+Vehicle, n=6 / 6-OHDA+L-333,060, n=6 / 6-OHDA+NAT).
Figure 8.4: Comparison of NK₁ antagonists L-333,060 and NAT - Step length as assessed by the stepping tests.

Sham animals (black) had a relatively constant step length throughout the assessment period, despite recording a shorter step length than vehicle-lesioned L-333,060 treated animals (light blue). Following 6-OHDA striatal lesions, all groups demonstrated a small deficit in step length at day 3 post-lesion. However following a slight improvement, vehicle-lesioned animals (green) had small decline in step length on day 17 post-lesion, whereas both L-333,060 (red) and NAT (blue) treated animals continued to improve on each assessment day. Thus both 6-OHDA lesioned groups treated with an NK₁ antagonist displayed a marked improvement in step length during the 3-week assessment period. (n=5/Sham, n=6 / 6-OHDA Vehicle+L-333,060, n=12/6-OHDA+Vehicle, n=6 / 6-OHDA+L-333,060, n=6 / 6-OHDA+NAT).
these animals performed equal to or better than shams. Accordingly, it was difficult to
determine the extent of improvement in the animals treated with NK₁ antagonists,
although both groups demonstrated an obvious improvement in step length during the
assessment. L-333,060 and NAT treated animals both recorded a step length of 8.4 ± 0.6
cms on day 3, which was below shams levels, then improved to record approximately
12 ± 0.6 cms step length on day 21 post-lesion. Due the small changes in step length, no
statistically significant differences were observed between any of the groups during the
assessment period.

Adjusting Steps
Sham animals performed a relatively constant number of forehand and backhand
adjusting steps with their contralateral forepaw and therefore did not have akinesia or
absence of movement (Figure 8.5). L-333,060 treatment in vehicle-lesioned animals did
not induce akinesia, as these animals performed similar or greater number of adjusting
steps in both directions than sham animals. 6-OHDA striatal lesions also did not produce
akinesia in the contralateral forepaw in the current study, apart from a small loss of
movement in the forehand direction on day 3 post-lesion. As such, it was difficult to
determine if either NK₁ antagonist treatment was beneficial in reducing akinesia.
Nonetheless, L-333,060 treated lesioned animals had a relatively constant number of
forehand adjusting steps, and despite small fluctuations in number of backhand adjusting
steps, they tended to perform a greater number of adjusting steps than shams. NAT
treated lesioned animals also performed a relatively constant number of adjusting steps in
the forehand and backhand direction, except in the forehand direction they performed
slightly below sham levels from day 10 onwards. Due to the small differences in
adjusting steps, no statistically significant differences in contralateral forepaw movement
Figure 8.5: Comparison of NK$_1$ antagonists L-333,060 and NAT - Contralateral forepaw adjusting steps as assessed by the stepping tests.

Sham animals (black) performed adjusting steps with their contralateral forepaw in both the forehand (A) and backhand (B) direction. L-333,060 treated vehicle-lesioned animals (light blue) performed similar numbers of adjusting steps to shams and thus did not demonstrate akinesia. 6-OHDA striatal lesions also did not induce akinesia, except for a slight loss of contralateral forepaw movement in the forehand direction on day 3 in vehicle treated animals (green). L-333,060 treated lesioned animals (red) performed a greater number of both forehand and backhand adjusting steps than shams during the assessment, whereas NAT treated animals (dark blue) recorded a small reduction in forehand adjusting steps than shams from day 10 onwards. None of the groups tested displayed an absence of movement in their contralateral forepaw. (n=5/Sham, n=6 / 6-OHDA Vehicle+L-333,060, n=12/6-OHDA + Vehicle, n=6 / 6-OHDA + L-333,060, n=6 / 6-OHDA + NAT).
A. 

**Forehand Adjusting Steps**

![Graph showing the number of contralateral steps over days post-lesion for different treatments.]

- Sham
- 6-OHDA + Vehicle
- 6-OHDA + NAT
- 6-OHDA + L-333,060
- 6-OHDA Vehicle + L-333,060

B. 

**Backhand Adjusting Steps**

![Graph showing the number of contralateral steps over days post-lesion for different treatments.]

- Sham
- 6-OHDA + Vehicle
- 6-OHDA + NAT
- 6-OHDA + L-333,060
- 6-OHDA Vehicle + L-333,060
was observed between any of the groups. In terms contralateral forepaw akinesia, a common deficit following 6-OHDA striatal lesions, these were not apparent during the 3-week assessment period.

**Motor Outcome – Bilateral Asymmetry Test**

Sham animals had normal sensorimotor function as they could sense and remove the tape from both forepaws within 2 to 5 secs (Figure 8.6). L-333,060 treated vehicle-lesioned animals also displayed normal sensorimotor function in both forepaws, as they had similar latency times to shams. Following 6-OHDA striatal lesions, vehicle treated animals developed sensorimotor deficits in both forepaws during the assessment. Initially they could sense and remove the tape but latencies scores increased each assessment day so that by day 21 they took 17.3 ± 3.6 secs and 9.9 ± 2.1 secs to remove the tape from their ipsilateral and contralateral forepaws, respectively. Despite the substantial latency scores, vehicle treated animals did not have significantly worse sensorimotor function than shams in either forepaw. Treatment with L-333,060 at the time of 6-OHDA lesions, improved sensorimotor function, particularly in the ipsilateral forepaw, with these animals still taking only 6.5 ± 1.8 secs on day 21 post-lesion to sense and remove the tape. Similarly in their contralateral forepaw, a small increase in latency times was recorded, taking the maximum time of 8.7 ± 2.8 secs to sense and remove the tape on day 21 post-lesion. Thus these animals had relatively normal sensorimotor function. In contrast, NAT treated lesioned animals displayed a sensorimotor deficit in both forepaws during the assessment. Initially, they could sense and remove the tape easily but during the following 2 weeks had a decline in sensorimotor function. During the last assessment week, NAT treated animals had some improvement in sensorimotor function recording latency scores for the ipsilateral and contralateral forepaw of 14.5 ± 4.2 and 10.8 ± 1.6
**Figure 8.6:** Comparison of NK$_1$ antagonists L-333,060 and NAT – Ipsilateral and contralateral latency scores for sensorimotor function as assessed by the bilateral asymmetry test.

Sham animals (black) demonstrated normal sensorimotor function in both their ipsilateral (A) and contralateral (B) forepaws. L-333,060 treated vehicle-lesioned animals (light blue) also displayed normal sensorimotor function as they recorded similar latency scores to shams. Following 6-OHDA striatal lesions, vehicle treated animals (green) developed a mild deficit in sensorimotor function in both forepaws during the assessment. L-333,060 treatment in lesioned animals (red) improved this deficit, recording similar sensorimotor function in both forepaws to shams. In contrast, NAT treated lesioned animals (dark blue) displayed sensorimotor deficits, particularly during the first 2 weeks of assessment, recording longer latency times than vehicle treated animals up until day 17. (n=5/Sham, n=6 / 6-OHDA Vehicle+L-333,060, n=12/6-OHDA Vehicle, n=6 / 6-OHDA+L-333,060, n=6 / 6-OHDA+NAT).
A. **Ipsilateral Latency**

- Sham
- 6-OHDA + Vehicle
- 6-OHDA + NAT
- 6-OHDA + L-333,060
- 6-OHDA Vehicle + L-333,060

B. **Contralateral Latency**

- Sham
- 6-OHDA + Vehicle
- 6-OHDA + NAT
- 6-OHDA + L-333,060
- 6-OHDA Vehicle + L-333,060
secs respectively on day 21. Nonetheless, these animals had significant deficits in sensorimotor function in both forepaws to shams ($0.001 < p < 0.01$). Therefore L-333,060 treatment in 6-OHDA lesioned animals demonstrated greater efficacy in improving sensorimotor function than NAT treatment.

**Neurological Outcome – mNSS**

Sham animals had normal neurological function, with none recording a point on the mNSS on any assessment day (Figure 8.7). Despite scoring 0.5 points on the mNSS on day 3 post-lesion, L-333,060 treated vehicle-lesioned animals had normal neurological function, with 0 points on the mNSS from day 7 onwards. L-333,060 treatment alone did not affect neurological function. 6-OHDA striatal lesions induced a mild neurological deficit, with vehicle treated animals scoring 2 to 3 points on the mNSS on each assessment day, demonstrating a significant neurological deficit compared to shams ($p < 0.01$). L-333,060 treatment restored neurological function with these animals only scoring 2 and 1 points on the mNSS on day 3 and 7 post-lesion, respectively. L-333,060 treated lesioned animals did not have a significantly different neurological function to shams. NAT treatment also improved neurological outcome, although not to the extent of L-333,060 treatment. NAT treated lesioned animals recorded less points on the mNSS than vehicle treated animals at each assessment day, except for day 10 post-lesion. These animals still demonstrated a significant neurological deficit to shams ($p < 0.05$). Despite the greater reduction in mNSS, L-333,060 treated animals were not statistically significantly different to NAT treated animals.
Figure 8.7: Comparison of NK₁ antagonists L-333,060 and NAT – Neurological outcome as assessed by the modified Neurological Severity Score.

Sham animals (black) had normal neurological function, as they did not score on the mNSS. L-333,060 treated vehicle-lesioned animals (light blue) also had normal neurological function as they performed at sham levels. 6-OHDA striatal lesions induced a mild neurological deficit, as vehicle treated animals (green) had a significant neurological deficit to sham animals. L-333,060 treatment restored neurological function, as L-333,060 treated lesioned animals (red) only scored on the mNSS during the first assessment week and thus did not have significantly different neurological function to shams. NAT treatment produced a small improvement in neurological function, as NAT treated lesioned animals (dark blue) scored fewer points on the mNSS than vehicle treated animals. However they still displayed a significant neurological deficit to shams. (n=5/Sham, n=6 / 6-OHDA+Vehicle, n=12/6-OHDA+L-333,060, n=6 / 6-OHDA+L-333,060, n=6 / 6-OHDA+NAT).
Sham animals had normal exploratory behaviour within the open field throughout the assessment period, recording the maximum activity level of 10 on day 3 before a gradual decline in activity until day 14 post-lesions, when they had an activity level of 2 due to habituation (Figure 8.8). L-333,060 treated vehicle-lesioned animals also demonstrated normal behaviour within the open field, as they had a maximum activity level of 10 on day 3 and then declined in activity level until day 17 post-lesion when they showed signs of habituation. These animals had a larger decline in activity than shams during the first 2 assessment weeks, suggesting that L-333,060 treatment alone does not significantly affect behaviour. Following 6-OHDA striatal lesions, vehicle treated animals had a reduction in spontaneous exploration on day 3 post-lesion, recording an activity level of 6. These animals then demonstrated a small improvement to an activity level of 7 before a gradual decline in activity until day 21 post-lesion, when they scored an activity level of 2.5 due to habituation to the task. L-333,060 treatment in lesioned animals improved spontaneous exploration on day 3, scoring an activity level of 8, before a decline in activity on each assessment day with signs of habituation to the open field by day 14 post-lesion. NAT treatment in lesioned animals also improved spontaneous exploration, with these animals demonstrating near maximal activity on day 3 at an activity level of 9.5 before a gradual decline in activity during the assessment period. However, NAT treated lesioned animals did not show signs of habituation until day 17 post-lesion, yet still recording the highest activity of any group on day 7 and 10 post-lesion. Despite the difference in activity level between the groups, no statistically significant change in spontaneous exploration was observed between any of the groups during the assessment period.
Figure 8.8: Comparison of NK₁ antagonists L-333,060 and NAT – Behavioural outcome as assessed by the open field.

Sham animals (black) displayed normal exploratory behaviour during the assessment period, with an early maximal activity level before a gradual decline in activity with habituation to the task. L-333,060 treated vehicle-lesioned animals (light blue) also had normal exploratory behaviour, with maximal activity on day 3 before an obvious reduction in activity level during the next 2 weeks. 6-OHDA striatal lesions produced an early reduction in spontaneous exploration (green) recording the smallest activity level on day 3 of any group. It was not until day 21 that they demonstrated signs of habituation. L-333,060 treatment (red) improved spontaneous exploration, with high activity early, before becoming habituated to the task by day 14. NAT treatment (dark blue) also showed normal spontaneous exploration early, before habituation to the task by day 17 post-lesion. (n=5/Sham, n=6 / 6-OHDA + Vehicle, n=12/6-OHDA + Vehicle + L-333,060, n=6 / 6-OHDA + L-333,060, n=6 / 6-OHDA + NAT).
Estimation of Lesion Size – Rotometer

Sham animals did not display an ipsilateral turning response following induction of motor activity by treatment with the dopamine-releasing agent, amphetamine, recording only $1.8 \pm 0.5$ and $0.8 \pm 0.3$ ipsilateral turns per min (Figure 8.9). L-333,060 treated vehicle-lesioned animals recorded less turns per min than shams following amphetamine treatment, supporting that L-333,060 alone did not induce an ipsilateral turning response. Following 6-OHDA striatal lesions, amphetamine treatment caused animals to circle ipsilaterally, with vehicle treated animals recording $6.3 \pm 1.7$ and $3.8 \pm 1.0$ turns per min on day 7 and 14, respectively. Because there was a large within group variability, these animals did not perform a significantly greater number of turns per min than shams. L-333,060 treatment in lesioned animals significantly exacerbated this ipsilateral turning response, as they performed significantly more turns per min than vehicle treated animals, recording $12.2 \pm 1.9$ and $15.1 \pm 2.9$ turns per min on day 7 ($p < 0.01$) and 14 ($p < 0.001$) post-lesion. In contrast, NAT treated lesioned animals performed less ipsilateral turns per min than vehicle treated animals, with $4.6 \pm 0.6$ and $2.6 \pm 0.8$ turns per min on day 7 and 14 post-lesion. These results suggested that whilst NAT treatment in lesioned animals reduced lesion size, L-333,060 treatment exacerbated dopaminergic cell loss.

8.3.2 Histological Outcome

Dopaminergic Response – TH Immunohistochemistry

Within the striatum, sham animals had intense TH immunoreactivity throughout the entire striatum due to a dense network of dopaminergic terminals, clearly delineating it from surrounding tissue (Figures 8.10 and 8.11). L-333,060 or NAT treatment alone did not
Figure 8.9: Comparison of NK<sub>1</sub> antagonists L-333,060 and NAT – Estimation of lesion size as assessed by the rotometer.

Sham animals (black) did not display an ipsilateral turning response following induction of motor activity. L-333,060 treated vehicle-lesioned animals (light blue) recorded less turns per min than shams. In contrast, 6-OHDA lesioned animals performed ipsilateral turns only. L-333,060 treatment in lesioned animals (red) exacerbated this turning response, producing a significantly greater number of turns per min than vehicle-lesioned animals (green). NAT treatment in lesioned animals (dark blue) reduced the number of ipsilateral turns per min. (** denotes \( p < 0.01 \), *** denotes \( p < 0.001 \) versus 6-OHDA+Vehicle) \( \text{n}=5/\text{Sham}, \text{n}=6 / 6-\text{OHDA+Vehicle+L-333,060}, \text{n}=12/6-\text{OHDA+Vehicle, n}=6 / 6-\text{OHDA+L-333,060, n}=6 / 6-\text{OHDA+NAT}. \)
reduce TH immunoreactivity. At day 21 following 6-OHDA striatal lesions, an obvious loss of striatal TH immunoreactivity and therefore dopaminergic terminals was observed, although the striatum was still able to be defined from surrounding tissue. L-333,060 treatment in lesioned animals resulted in some protection of dopaminergic terminals as there was a conservation of TH immunoreactivity within the striatum at day 21 post-lesion. NAT treatment in lesioned animals resulted in similar protection from the 6-OHDA induced loss of dopaminergic terminals, as at day 21 these animals still had intense TH immunoreactivity throughout their striatum. Furthermore, NAT treated animals tended to display slightly more remaining dopaminergic terminals than L-333,060 treated lesioned animals. Within the SN, sham animals had a normal distribution and number of TH immunoreactive neurons within their ipsilateral SN compared to their contralateral SN, due to a dense network of dopaminergic terminals within their striatum (Figures 8.12 and 8.13). Both vehicle-lesioned groups had similar TH immunoreactivity in their ipsilateral SN to sham animals, and therefore both L-333,060 and NAT treated vehicle-lesioned animals had negligible cell loss. L-333,060 or NAT treatment alone did not induce dopaminergic cell loss. In contrast, at day 21 following 6-OHDA striatal lesions an obvious loss of TH immunoreactive neurons, particularly in the dorsolateral SN, was observed in vehicle treated animals These animals recorded a 40 ± 3% loss of ipsilateral TH immunoreactive neurons, which was a significant loss of dopaminergic neurons compared to shams (p < 0.001). L-333,060 treatment in lesioned animals resulted in some protection of ipsilateral TH immunoreactivity, which was apparent in the dorsolateral area where most of the dopaminergic neurons are lost from vehicle treated animals. Quantitatively, L-333,060 treated lesioned animals recorded only 28 ± 14% loss of TH immunoreactive neurons at day 21 post-lesion, which was still a significant loss of neurons to shams (p < 0.01). Similarly, NAT treatment in lesioned animals also conserved
Figure 8.10: Comparison of NK₁ antagonists L-333,060 and NAT – TH immunoreactivity within the ipsilateral striatum at day 21 following 6-OHDA striatal lesions. TH stained sections (Bar = 90μm).

Sham animals (A) had intense TH immunoreactivity throughout the ipsilateral striatum, and thus the striatum was clearly defined from surrounding tissue. At day 21 following 6-OHDA striatal lesions, a loss of TH immunoreactivity was apparent in vehicle treated animals (B), although the striatum was still delineated from surrounding tissue. L-333,060 treatment in lesioned animals (C) resulted in only a small loss of TH immunoreactivity at day 21, as they still had a intense TH immunoreactivity throughout their striatum. NAT treated lesioned animals (D) demonstrated no obvious loss of TH immunoreactivity, and thus resembled shams. Similarly, L-333,060 (E) or NAT (F) treatment alone did not induce a loss of TH immunoreactivity as both vehicle-lesioned groups resembled sham sections.
Figure 8.11: Comparison of NK₁ antagonists L-333,060 and NAT – TH immunoreactivity within the ipsilateral striatum at day 21 following 6-OHDA striatal lesions. TH stained sections (Bar = 50μm).

Sham animals (A) had a dense network of TH immunoreactive terminals throughout their striatum. At day 21 following 6-OHDA striatal lesions, a reduction in TH immunoreactivity was observed in vehicle treated animals (B) indicative of a loss of dopaminergic terminals within the striatum. L-333,060 treatment in lesioned animals (C) conserved striatal TH immunoreactivity to a small degree, as these animals had slightly more intense TH immunoreactivity in their striatum than vehicle treated animals. Although a minor loss of TH immunoreactivity was still observed in NAT treated lesioned animals (D), they had greater TH immunoreactivity remaining within their striatum at day 21 post-lesion than L-33,060 treated animals. L-333,060 (E) or NAT (F) treatment alone did not induce a loss of TH immunoreactivity, as both vehicle-lesioned groups resembled shams.
TH immunoreactivity within their ipsilateral SN. They had a greater number of TH immunoreactive neurons remaining than vehicle treated animals, recording a similar loss at day 21 to L-333,060 treated animals of 28 ± 12%. These animals still had a significant loss of TH immunoreactive neurons compared to shams ($p < 0.01$). Despite the reduction in cell loss, neither NAT or L-333,060 treated animals had significantly more remaining dopaminergic neurons than shams. Nonetheless, it is clear that both NK$_1$ antagonists provided comparable protection from 6-OHDA induced loss of dopaminergic neurons.

**General Pathology – H&E**

Sham animals had normal nigral architecture with mainly healthy neurons and only an occasional darkly stained dopaminergic model in the ipsilateral SN (Figure 8.14). L-333,060 and NAT treatment alone did not produce abnormal nigral architecture as many neurons were present in both vehicle-lesioned groups. These sections resembled shams. At day 21 following 6-OHDA striatal lesions, an obvious loss of neurons and therefore nigral architecture was observed in vehicle treated animals, with an infiltration of glial cells in the SN. L-333,060 or NAT treatment did not markedly reduce the number of glial cells within their SN, however there was a conservation of neurons and thus nigral architecture at day 21 post-lesion in both groups. Some of the remaining neurons in both L-333,060 and NAT treated lesioned animals were stained darker than others, suggesting that these neurons are stressed or reversibly injured.
Figure 8.12: Comparison of NK₁ antagonists L-333,060 and NAT – TH immunoreactivity within the ipsilateral substantia nigra at day 21 following 6-OHDA striatal lesions. TH stained sections (Bar = 200μm).

Sham animals (A) demonstrated a normal number and distribution of TH immunoreactive neurons within their ipsilateral SN. At day 21 following 6-OHDA striatal lesions, vehicle treated animals had an obvious loss of TH immunoreactive neurons in their SN. L-333,060 (C) and NAT (D) treated animals had some conservation of TH immunoreactivity within their SN, as both groups demonstrated a greater number remaining TH immunoreactive neurons at day 21 than vehicle treated animals. L-333,060 (E) and NAT (F) treatment alone did not result in a loss of TH immunoreactive neurons, as both vehicle-lesioned groups resembled shams.
Figure 8.13: Comparison of NK₁ antagonists L-333,060 and NAT – Quantification of TH immunoreactive neurons within the ipsilateral substantia nigra at day 21 following 6-OHDA striatal lesions.

Sham animals (black) had no loss of TH immunoreactive neurons in their ipsilateral SN compared to their contralateral SN. L-333,060 (light blue) or NAT (pink) treatment alone did not induce a loss of TH immunoreactive neurons, as both vehicle-lesioned groups had negligible cell loss. In contrast, 6-OHDA striatal lesions produced a significant loss of ipsilateral TH immunoreactive neurons compared to shams. Both L-333,060 (red) and NAT (dark blue) treatment in lesioned animals resulted in protection from 6-OHDA induced cell loss, with both recording a smaller loss of ipsilateral TH neurons than vehicle treated animals (n=5/Sham, n=6 / 6-OHDAVehicle+L-333,060, n=6 / 6-OHDAVehicle+NAT, n=12/6-OHDA+Vehicle, n=6 / 6-OHDA+L-333,060, n=6 / 6-OHDA+NAT).
Figure 8.14: Comparison of NK₁ antagonists L-333,060 and NAT – H&E stained ipsilateral substantia nigra at day 21 following 6-OHDA striatal lesions. H&E stained sections (Bar = 50μm).

Sham animals (A) had normal nigral architecture with many healthy neurons present. Following 6-OHDA striatal lesions, an obvious loss of nigral neurons and thus architecture with an infiltration of glial cells was observed in vehicle treated animals (B). Remaining dopaminergic neurons were healthy and normally stained. L-333,060 treatment in lesioned animals (C) conserved nigral architecture as many neurons were remaining, although some of the neurons were stained darker than others (black arrows indicate dark cells). NAT treatment in lesioned animals (D) also resulted in protection from 6-OHDA, as these animals had similar features to L-333,060 treated animals including the presence of dark stained neurons. Neither of the NK₁ antagonists produced an apparent reduction in glial cells. L-333,060 (E) or NAT (F) treatment alone did not affect nigral architecture or neuronal.
Substance P Response – SP Immunohistochemistry

Sham animals displayed basal levels of SP expression within the striatum and SN. In the striatum, light homogenous SP immunoreactivity was present throughout with more intense perivascular SP immunoreactivity around sections of the perimeter of the blood vessel (Figure 8.15). In the SN, intense SP immunoreactivity was observed throughout the neuropil with faint cytoplasmic immunoreactivity within neurons (Figure 8.16). L-333,060 and NAT treated vehicle-lesioned animals had similar SP immunoreactivity to shams in both their ipsilateral striatum and SN, with neither NK₁ antagonist producing a change in SP expression at day 21 post-lesion. In contrast, at day 21 following 6-OHDA striatal lesions, vehicle treated animals had increased SP immunoreactivity within their striatum, with intense perivascular staining around almost the entire perimeter of the blood vessels. NK₁ antagonist treatment prevented this increase in SP particularly in the striatum and striatal perivascular tissue. Both L-333,060 and NAT treated lesioned animals resembled shams. Furthermore, L-333,060 and NAT treated animals had less of their remaining nigral neurons displaying an increase in cytoplasmic SP immunoreactivity.

Astrocytic Response – GFAP Immunohistochemistry

Sham animals displayed normal GFAP immunoreactivity within their ipsilateral striatum (Figure 8.17) and substantia nigra (Figure 8.18), with immunoreactivity apparent around blood vessels, in white matter and in circulating astrocytes. L-333,060 and NAT treatment alone did not induce an astrocytic response. At day 21 following 6-OHDA striatal lesions, GFAP immunoreactivity was markedly increased in the striatum and SN.
Figure 8.15: Comparison of NK₁ antagonists L-333,060 and NAT – SP immunoreactivity within the ipsilateral striatum at day 21 following 6-OHDA striatal lesions. SP stained sections (Bar = 25μm).

Sham animals (A) displayed normal SP immunoreactivity within their ipsilateral striatum, with light homogenous staining throughout the striatum and intense perivascular immunoreactivity around sections of the perimeter of the blood vessels. 6-OHDA striatal lesions produced an increase in striatal SP immunoreactivity, with vehicle treated animals (B) demonstrating darker striatal SP staining with intense perivascular immunoreactivity around almost the entire blood vessel. L-333,060 treatment in lesioned animals (C) prevented the increase in perivascular immunoreactivity, although the striatum was slightly more immunoreactive for SP than shams. NAT treated lesioned animals (D) had comparable staining to the L-333,060 treated animals and thus also did not display a marked increase in SP immunoreactivity. L-333,060 (E) or NAT (F) treatment alone had effect on SP immunoreactivity with both groups resembling shams.
Sham animals (A) displayed normal SP immunoreactivity within the SN, with intense immunoreactivity in the neuropil and faint cytoplasmic immunoreactivity within neurons. Following 6-OHDA striatal lesions, vehicle treated animals (B) had increased SP immunoreactivity within almost all remaining neurons, although no obvious change in SP immunoreactivity within the neuropil was observed. Similarly, L-333,060 (C) and NAT (D) treated animals had comparable nigral SP immunoreactivity to shams, but unlike vehicle treated animals, not all of their remaining neurons had an increase in cytoplasmic SP immunoreactivity. L-333,060 (E) and NAT (F) treatment alone had no effect on SP immunoreactivity.
Vehicle treated animals displayed profound GFAP immunoreactivity throughout their striatum, with a redistribution of GFAP from the white matter to surround dopaminergic neurons. In both regions, many hypertrophic astrocytes that were intensely immunoreactive for GFAP were also observed. L-333,060 treatment in lesioned animals reduced the astrocytic response in both the striatum and SN, with an obvious reduction in GFAP immunoreactivity compared to vehicle treated animals. Numerous activated hypertrophic astrocytes were still present in the SN at day 21 post-lesion. NAT treated lesioned animals also had less GFAP immunoreactivity than vehicle treated animals in their striatum and SN. However compared to L-333,060 treated lesioned animals, NAT treated animals had a greater number of activated hypertrophic astrocytes within their striatum, but a reduction in astrocytic numbers in the SN.

**Activated Microglial Response – ED-1 Immunohistochemistry**

Sham animals did not display ED-1 immunoreactivity within the striatum or SN, except for an occasional perivascular glial cell (Figures 8.19 and 8.20). L-333,060 or NAT treatment alone did not activate microglia, as both vehicle-lesioned groups were also devoid of ED-1 immunoreactivity in both their striatum and SN. At day 21 following 6-OHDA striatal lesions, vehicle treated animals had numerous activated microglia as reflected in the ED-1 immunoreactivity within their striatum and near dopaminergic neurons in the SN. Fine processes were seen on some of the microglia, which is indicative of these cells being in a phagocytosing activated state. L-333,060 treatment in lesioned animals reduced ED-1 immunoreactivity at day 21 post-lesion. Within the striatum, L-
Figure 8.17: Comparison of NK<sub>1</sub> antagonists L-333,060 and NAT – GFAP immunoreactivity within the ipsilateral striatum at day 21 following 6-OHDA striatal lesions. GFAP stained sections (Bar = 50μm).

Sham animals (A) displayed normal GFAP immunoreactivity within their striatum. Following 6-OHDA striatal lesions, an obvious increase in GFAP immunoreactivity within the striatum was observed with many activated hypertrophic astrocytes with intense GFAP immunoreactivity present (B). L-333,060 treated lesioned animals demonstrated a marked reduction in striatal GFAP immunoreactivity with only an occasional activated hypertrophic astrocyte present at day 21. NAT treated lesioned animals (C) also had reduced striatal GFAP immunoreactivity, however these animals still had numerous activated intensely stained GFAP astrocytes within their striatum at day 21. L-333,060 (E) and NAT (F) treatment alone did not induce an astrocytic response within the striatum, with both resembling shams.
Figure 8.18: Comparison of NK₁ antagonists L-333,060 and NAT – GFAP immunoreactivity within the ipsilateral substantia nigra at day 21 following 6-OHDA striatal lesions. GFAP stained sections (Bar = 50μm).

Sham animals (A) displayed normal intensity and distribution of GFAP immunoreactivity within their ipsilateral SN. Following 6-OHDA striatal lesions, vehicle treated animals (B) had increased GFAP immunoreactivity, which was redistributed from white matter to surrounding dopaminergic. Many hypertrophic astrocytes were also observed. L-333,060 treatment in lesioned animals (C) reduced the astrocytic response, however numerous activated hypertrophic astrocytes were still present. NAT treated lesioned animals (D) also had a reduction in GFAP immunoreactivity, although less activated astrocytes were present in their SN compared to L-333,060 treated animals. L-333,060 (E) or NAT (F) treatment alone did not induce an astrocytic response, with both resembling shams.
333,060 treatment produced a small reduction in activated microglia, however in the SN ED-1 immunoreactivity and thus activated microglia was markedly reduced compared to vehicle treated animals. NAT treated lesioned animals displayed similar ED-1 immunoreactivity to L-333,060 treated lesioned animals in both their striatum and SN. Thus both NK₁ antagonists resulted in a similar reduction of the 6-OHDA induced activated microglial response at day 21 post-lesion.

8.4 Discussion

In the present study, we demonstrate that treatment with the NK₁ antagonist, L-333,060, resulted in a similar level of protection against dopaminergic neurodegeneration as was observed with the alternative NK₁ antagonist, NAT. As was seen following NAT treatment, L-333-060 also prevented the increase in striatal SP, specifically perivascular SP, and resulted in a minimal 6-OHDA induced astrocytic and microglial response. The protective effects of the compounds against 6-OHDA induced striatal lesions support the hypothesis that SP is involved in the neurodegeneration associated with this early PD model, and that significant neuroprotection can be afforded by the NK₁ receptor antagonist class of compounds.

Normal levels of striatal DA are integral to basal ganglia function and associated function and movement. Although striatal DA was not directly measured in the current study, the reduction in dopaminergic degeneration by administration of NK₁ antagonists would presumably have resulted in a greater striatal DA concentration. It is not until 80% of striatal DA has been depleted that the basal ganglia no longer correctly function and
Figure 8.19: Comparison of NK₁ antagonists L-333,060 and NAT – ED-1 immunoreactivity within the ipsilateral striatum at day 21 following 6-OHDA striatal lesions. ED-1 stained sections (Bar = 50μm).

Sham animals (A) were devoid of ED-1 immunoreactivity and therefore activated microglia within their ipsilateral striatum. Following 6-OHDA striatal lesions, ED-1 immunoreactivity was markedly increased, as numerous activated microglia were present in vehicle treated animals (B). L-333,060 treatment in lesioned animals (C) resulted in a small reduction in ED-1 immunoreactivity, as fewer activated microglia were observed. NAT treated lesioned animals (D) also demonstrated less ED-1 immunoreactivity than vehicle treated animals, and thus had similar numbers of activated microglia to L-333,060 treated animals. L-333,060 (E) and NAT (F) treatment alone did not activate microglia, as both vehicle-lesioned groups displayed an absence of ED-1 immunoreactivity.
Figure 8.20: Comparison of NK₁ antagonists L-333,060 and NAT – ED-1 immunoreactivity within the ipsilateral substantia nigra at day 21 following 6-OHDA striatal lesions. ED-1 stained sections (Bar = 50µm).

Sham animals (A) had only an occasional perivascular glial cell within their ipsilateral SN that was immunoreactive for ED-1. Following 6-OHDA striatal lesions a profound increase in ED-1 immunoreactivity and therefore activated microglia was observed in vehicle treated animals (B). L-333,060 treatment in lesioned animals (C) produced an obvious reduction in activated microglia and ED-1 immunoreactivity compared to vehicle treated animals. NAT treated lesioned animals (D) displayed similar ED-1 immunoreactivity to L-333,060 treated animals, equally reducing the microglial response. L-333,060 (E) and NAT (F) treatment alone did not result in the activation of microglia, with both vehicle-lesioned groups resembling shams.
symptoms present themselves. From the comparative study by Lee and colleagues (1996) the treatment regime used in the present study results in an 82% loss of striatal dopamine compared to the contralateral hemisphere. In our hands, dysfunction of the basal ganglia was apparent in the form of vehicle treated animals having marked functional deficits. Furthermore, compared to vehicle treated animals, L-333,060 and NAT treated animals had less of their remaining nigral neurons displaying increased SP immunoreactivity. This suggests that these neurons were functioning normally and did not require additional SP to release extra DA to compensate for reduced striatal DA. Thus the preservation of dopaminergic neurons and therefore striatal DA in the NK₁ antagonist treatment groups reduced the dysfunction of the basal ganglia and as such functional deficits were minor, if at all present.

NK₁ antagonist treated animals had significant improvement in gait, sensorimotor function and initiation of movement, as well as near normal neurological function and behaviour. The rotarod is the most sensitive test of motor deficits in the battery of tests used in the current study. Consistent with this, 6-OHDA lesioned vehicle treated animals had a significant motor deficit on this test despite being an early model of PD with only mild to moderate deficits. The accelerating rotarod test, as used in this study, requires the animal to walk on the rotarod at increasing speeds testing the animal’s a gait and postural stability (Rozas et al., 1997). Gait was also tested by the stepping tests. Although vehicle treated lesioned animals only displayed a mild gait disturbance, NK₁ antagonist treated groups still performed better on all aspects of the stepping tests. Sensorimotor function was also tested by the bilateral asymmetry test where animals must sense and remove a piece of sticky tape from their forepaws. 6-OHDA lesioned animals developed a sensorimotor deficit during the assessment, whereas treatment with NK₁ antagonist, L-
333,060, significantly reduced this deficit to the point that they were indistinguishable from shams. Despite NAT treated animals still displaying sensorimotor deficits, they did however improve during the final week of the assessment whereas the vehicle treated animals did not. Therefore, the improvement in gait and sensorimotor function produced by NK₁ antagonist treatment may have contributed to these animals performing significantly better on the rotarod.

NK₁ antagonist treatment in 6-OHDA lesioned animals also improved neurological and behavioural outcome. Neurological function was tested with a modified NSS, which tested muscle tone, motor function and reflexes. 6-OHDA striatal lesions resulted in vehicle treated animals scoring a few points on the mNSS on each assessment day, consistent with a mild neurological deficit. Most animals scored points for abnormal flexion of the fore and hindpaws or for circling behaviour. NK₁ antagonist treated animals scored fewer points on the mNSS than vehicle treated animals, since these animals hardly ever circled and could generally use their forepaws and hindpaws properly. L-333,060 treatment resulted in total recovery of neurological function, with no score on the mNSS from day 10 onwards. Spontaneous exploration was tested in the open field to determine behavioural outcome following 6-OHDA striatal lesions. Vehicle treated animals had an obvious reduction in exploration on the first assessment day compared to control, as well as delayed habituation to the task. NK₁ antagonists have been proven to be an efficient treatment to reduce anxiety and depression in animals and in the clinic (Chahl, 2006; Duzzioni et al., 2008; Ebner et al., 2006). SP within the striatum was increased in vehicle treated animals but not in L-333,060 or NAT treated animals. Thus by reducing SP, NK₁ antagonist treatment prevented the fear and anxiety associated with a novel environment, resulting in normal spontaneous exploration.
Furthermore, the activity level used to determine spontaneous exploration included a substantial contribution from an animal's rearing ability. Rearing has been correlated with decreased striatal DA (Fornaguera and Schwarting, 1999). The NK₁ antagonist groups on average performed a greater number of rears than vehicle-treated animals (results not shown), particularly during the first week post-lesion. This rearing contributed to near maximal activity early. Habituation has also been linked to striatal function with decreases in striatal DA impairing habituation to a task (Dauer and Przedborski, 2003). Consistent with this, habituation was delayed in vehicle-treated animals, as they did not show signs of habituation until day 21 post-lesion. In contrast, L-333,060 and NAT treated animals became habituated to the open field by day 14 and 17, respectively. These results suggest that by modulating SP and therefore DA production, NK₁ antagonist treatment can normalise function.

Despite better function and small dopaminergic cell loss, L-333,060 treated animals had a paradoxical increase in ipsilateral turning response in the rotometer compared to vehicles and NAT treated groups. It has been established that with submaximal lesions, as used in the current study, a similar percentage cell loss can result in huge variation in the number of ipsilateral rotations following amphetamine treatment (Hudson et al., 1993 #152). Furthermore, the small group numbers meant that means and within group variances for each test could differ significantly if a one or 2 animals performed poorly. Taken in isolation, this may have affected the interpretation of the results. However further exploration in future studies will be undertaken to understand this anomalous result. Despite the results of the rotometer test, the percentage of remaining dopaminergic neurons was comparable in both groups as was SP expression and the inflammatory
response. Therefore, both NK₁ antagonists produced a profound improvement in function following 6-OHDA striatal lesions, although L-333,060 was superior in some tests. Due to the functional arrangement of the basal ganglia, decreased striatal dopamine can cause overactivity of the STN. The STN uses glutamate to send excitatory signals to the GP and SN, where it modulates the burst firing of dopamine neurons (Rodriguez et al., 1998). With no inhibition of the STN, excess glutamate will be released within the SN to bind to the numerous glutamate receptors located on dopaminergic neurons causing excitotoxic damage. As NK₁ antagonist treatment reduced the dysfunction of the basal ganglia, it may also have indirectly prevented glutamate excitotoxic damage by preventing the overactivity of the STN. 6-OHDA striatal lesions result in a down regulation of glutamate receptors, which may compensate for the excess glutamatergic input from the overactive STN (Gu et al., 2003). However, STN lesions only offered some protection in MPTP models of PD (Luquin et al., 2006; Wallace et al., 2007), and bilateral STN lesions in clinical PD do not prevent the continuous degeneration of dopaminergic neurons (Hilker et al., 2005). Therefore, other biological processes must contribute to dopaminergic cell death. A number have been previously discussed, including oxidative stress and inflammatory processes.

The resident immune cells in the brain are microglia, and once activated they can also exacerbate glutamate excitotoxicity by releasing both quinololic acid and glutamate (Rock and Peterson, 2006). Furthermore, microglia produce damaging cytokines and pro-inflammatory mediators (Blum, 2001). Indeed, elevated levels of cytokines such as IL-1β and TNF-α are found within the SN, striatum and CSF of Parkinson’s patients (Jenner and Olanow, 1998; Liu et al., 2003). Activated microglia have been observed in all animal models, including the 6-OHDA intrastriatal model of PD, on PET scans of the SN
of PD patients and post-mortem (Depino et al., 2003; McGeer, and McGeer, 2008; Mosley et al., 2006; Rodriguez-Pallares et al., 2007). Microglia can also potentiate oxidative stress as they secrete ROS such as $\text{O}_2^-$, which is normally produced by NADPH oxidase to kill foreign organisms that enter the brain. Thus the upregulation in NADPH oxidase seen in PD has been correlated with activation of microglia (Mosley et al., 2006). Microglia also contain iNOS, and therefore produced NO, which can react with $\text{O}_2^-$ to produce ONOO$^-$. This free radical is a highly reactive molecule that often reacts with CO$_2$ to form many of its reactive intermediates (Torreilles et al., 1999). Following 6-OHDA striatal lesions, ONOO$^-$ and OH$^-$ have been observed early within the striatum then later in the SN, suggesting that the formation of these free radicals in the SN was not a product of 6-OHDA autoxidation but perhaps a product of microglia or excess dopaminergic turnover (Henze et al., 2005). Due to NK$_1$ receptors being located on microglia, SP can modulate their activity and therefore their damaging effects (Chauhan et al., 2008). It was therefore consistent that both NK$_1$ antagonist treatments resulted in less ED-1 immunoreactivity, a marker of activated microglia, in their striatum and SN at 21 days following 6-OHDA striatal lesions. Although ED-1 antigen only detects activated microglia, the microglia in the current study also had the appearance of being activated as they were enlarged with short, thick processes (Rodrigues et al., 2001). These activated microglia are seen for many months after the induction of PD in experimental models (Cicchetti et al., 2002). Unfortunately for DA neurons, microglia and neurodegeneration are a viscous cycle, with degenerating neurons triggering activation of microglia to exacerbate cell death (Raivich et al., 1999). By preventing SP from activating microglia, the NK$_1$ antagonists reduced the inflammatory striatal terminal loss and dopaminergic degeneration.
Both L-333,060 and NAT treatment reduced the 6-OHDA induced increase in perivascular SP within the striatum. By binding to NK₁ receptors on perivascular tissue, SP can facilitate neurogenic inflammation that will result in plasma extravasation and subsequent BBB breakdown. From previous chapters, it has been ascertained that neurogenic inflammation may play an important role in dopaminergic neurodegeneration by allowing influx of peripheral immune cells including T-cells and macrophages. Indeed, BBB dysfunction has recently attributed to the progression of PD (Kortekaas et al., 2005). These peripheral macrophages would have been detected by ED-1 immunohistochemistry. Thus the reduction in ED-1 immunoreactivity in the NK₁ antagonist treated animals may be partially due to the prevention of neurogenic inflammation and BBB dysfunction.

Astrocytes also express the NK₁ receptor, and therefore can be activated by SP (Mantyh et al., 1989). However, astrocytes are not thought to be quite as destructive as microglia since they secrete neurotrophic substances GDBF and BDNF, modulate extracellular glutamate levels and can help to metabolise excess cytosolic dopamine. However, they can secrete cytokines such as TNFα, IL-1β and IL-6. Activation NK₁ by SP causes NK-κβ to translocate to the nucleus and signal secretion of these proinflammatory cytokines (Brahmachari et al., 2006; Chauhan et al, 2008). Thus NK₁ antagonist treatment would have prevented at least the early activation and secretion of cytokines. All 6-OHDA lesioned groups still had astrocytes and microglia present at 3 weeks in both their striatum and SN, although vehicle treated animals displayed greater numbers than either of the NK₁ antagonists treated groups. Nonetheless, NK₁ treatment did not abolish the astrocytic or microglial response as both GFAP and ED-1 immunoreactivity was still observed at day 21 in these groups. However, PD is a slow continued degeneration of dopamine
neurons, and by extrapolation the inflammatory reaction that accompanies PD must also be slow and progressive. Astrocytes and microglia can modulate each other’s activity as cytokine expression can activate both of these glial cells. Furthermore, NO from microglia can upregulate GFAP expression (Brahmachari et al., 2006). As such, the increase in GFAP within the striatum and SN may be due to active microglia secreting NO. Therefore, NK₁ antagonist treatment may have reduced GFAP expression and astrocyte activation, not only by directly inhibiting their activation, but also by preventing the activation and subsequent cytokine and NO expression by microglia. Indeed, NK₁ antagonism has previously been shown to reduce the immune response within the CNS (Chauhan et al., 2008). Furthermore, a reduction in inflammatory processes and microglial activation by chronic treatment with NSAIDs has been shown to reduce the risk of developing PD by 45% (Chen et al, 2004; Esposito et al., 2007).

One of the cytokines expressed by both astrocytes and microglia is TNF-α. TNF-α can bind to its receptor TNF-αR1, a known death receptor, which is located on dopaminergic neurons to initiate apoptosis (Mladenovic et al, 2004). Indeed, increased markers of apoptosis such as caspase-3, caspase-1, Bax, and NF-κβ were found in the SN in PD patients (Hunot et al., 1997; Mochizuki et al., 1996). SP can contribute to apoptotic cascades by activating microglia and astrocytes to stimulate the production of TNF-α, promoting the translocation of NF-κβ to the nucleus and increasing intracellular Ca²⁺. Translocation of NK-κβ in inflammation can increase the expression of caspase-11 (Schauvliege et al., 2002), which can then initiate the cleavage of active caspase-3, the final effector in the apoptosis cascade. Furthermore, it has been shown to activate caspase-1 to promote IL-1β production, which may promote further activation of astrocytes and microglia, thereby producing TNF-α. Additionally, SP can increase
intracellular Ca\(^{2+}\), not only by potentiating glutamate release, but also by activating its receptor NK\(_1\). NK\(_1\) is a G-coupled protein receptor that initiates a cascade that results in increased intracellular Ca\(^{2+}\) (Saria, 1999). If the increase in intracellular Ca\(^{2+}\) is sustained, mitochondria will sequester the Ca\(^{2+}\) causing opening of the mitochondrial pore and release of the proapoptotic factors, cytochrome c and AIF (Shin et al, 2003). Accordingly, minocycline, which inhibits apoptosis by preventing the release of cytochrome c from the mitochondria, is protective in experimental PD, HD and ALS (Bredesen et al., 2006). However, caspase-3 inhibition only prolonged the time course for neuronal cell death but didn’t prevent it (Dodel et al., 1999), suggesting that cells may also die via other mechanisms other than classical apoptosis. SP and NK\(_1\) have been shown to be involved in a non-apoptotic form of programmed cell death (PCD), which has previously been seen in Huntingdon’s disease, another neurodegenerative disorder (Castro-Obregon et al., 2002). SP by binding to NK\(_1\) induces the MAP kinase pathway resulting in the activation of the pro-death signal Nur77 (Castro-Obregon et al., 2004). Non-apoptotic PCD does not have the same morphology as classical apoptosis as it does not have nuclear fragmentation or apoptotic bodies (Bredesen et al., 2006). Therefore, classical histological tests for apoptosis like TUNEL, which detects nuclear fragmentation, or caspase-3 activation, or even careful morphological H&E assessment would not have identified the non-apoptotic form of PCD. By preventing SP binding to NK\(_1\) receptors, the NK\(_1\) antagonists L-333,060 and NAT may have protected neurons from apoptotic and non-apoptotic forms of PCD, with both mechanisms contributing to survival of dopaminergic neurons and accordingly preventing the onset of functional deficits.
8.5 Conclusions

Treatment with the NK₁ antagonists, L-333,060 and NAT, resulted in similar protection of dopaminergic terminals and neurons. Both L-333,060 and NAT treated animals only displayed minimal functional deficits or even performed at sham levels. NK₁ antagonist treatment prevented the elevation of SP expression, particularly in striatal perivascular tissue and to a lesser extent in nigral neurons, to protect dopaminergic cells. The mechanisms involved may have included the reduction of inflammatory responses, neurogenic inflammation and likely oxidative stress and glutamate excitotoxicity. This study therefore definitively defined a role for SP in dopaminergic cell death and subsequent functional deficits in experimental PD.
CHAPTER 9:
GENERAL DISCUSSION
In the present thesis we demonstrate that in the early stages of experimental PD, SP immunoreactivity within the striatum and within surviving nigral neurons is elevated. Normally, SP, which is found in abundance in the basal ganglia, is involved in modulating DA release. However from previous studies in our laboratory examining acute brain injury, it has been shown that increased SP production initiates neurogenic inflammation with plasma extravasation, BBB breakdown and subsequent oedema formation, as well as development of functional deficits. Dopaminergic cell death in PD is widely believed to occur through oxidative stress, glutamate excitotoxicity, mitochondrial dysfunction and inflammatory processes. Using the intrastriatal 6-OHDA model, which replicates the early stages of PD, it was demonstrated that neurogenic inflammation with BBB dysfunction could also contribute to dopaminergic cell death following 6-OHDA administration. In fact, elevated SP levels accelerated the progression of dopaminergic degeneration with animals displaying profound functional deficits and exacerbated dopaminergic cell death. In contrast, preventing SP effects with NK1 antagonist treatment not only provided some protection from 6-OHDA induced cell death, by reducing neurogenic inflammation and inflammatory processes, but also prevented the increase in SP production. This is the first known study to characterise the role of SP in dopaminergic degeneration associated with early PD, specifically identifying SP induced neurogenic inflammation and associated inflammatory processes as deleterious to disease progression. Thus, NK1 antagonists may offer a novel therapeutic approach to attenuate early progression of PD.

PD affects 1-2% of the world’s population over the age of 65 (Alves et al., 2008). Continued research into the pathogenesis of PD is of particular importance as it mainly affects the elderly, and the prevalence and incidence of this disease will only increase as
our population ages. Furthermore, the current “gold-standard” treatment for PD, L-
DOPA, only alleviates symptoms for 5 to 10 years before debilitating side effects such as
dyskinesia appear (Chen et al., 2004). Generally, SP expression is considered to be
decreased in PD (Mauborgne et al., 1983; Tenovuo et al., 1984; Nisbet et al., 1995; De
Ceballos and Lopez-Lozano, 1999), with this loss of SP presumably thought to be
involved in symptom presentation (Bannon et al., 1987; Barker, 1991). However most of
those studies have used post-mortem PD cases or total dopaminergic degeneration
experimental models of PD, which replicate the late stages of the disease. In the late
stages of disease, preventing the degradation of SP with an ACE inhibitor, Captopril, is
beneficial in improving function and reducing neuronal cell death (Reardon et al., 2000;
Kurosaki et al., 2005; Lopez-Real et al., 2005; Munoz et al., 2006). In those studies,
Captopril treatment reduced oxidative damage by inhibiting the formation of angiotensin
II, which is able to activate NADPH oxidase, one of the main producers of ROS (Munoz
et al., 2006). In the early stages of the disease, we have shown in the current study that
ACE inhibition results in increased SP production with greater BBB dysfunction and
inflammatory processes. Thus Captopril treatment in early experimental PD exacerbates
dopaminergic cell death and functional deficits.

Until the 1990’s, experimental models of PD involved total degeneration of neurons
within 1 to 2 hours. The intrastriatal 6-OHDA model of PD was developed to enable
research into the degenerative process. In this model, 6-OHDA, a hydroxylated analogue
of DA that cannot pass through the BBB, is injected into the striatum where it is taken up
into terminals by DAT and undergoes autoxidation or deamination by MAO to form ROS
and H₂O₂ (Schwarting and Huston, 1996). Striatal dopaminergic terminals are lost within
hours to days, with subsequent degeneration of DA neurons at 1 to 2 weeks following the
initial lesion. As such, the 6-OHDA model of PD produces a progressive model of PD that replicates early human PD (Lee et al., 1996), and accordingly remains one of the most widely used tools for research into the pathogenesis of PD (Blandini et al., 2008).

SP content within the SN has yet to be directly measured in early clinical PD. However in the current study, administration of 6-OHDA in vitro produced an increase in SP content by 1 hour that persisted until day 2 post-treatment. Furthermore, histological analysis in the intrastriatal 6-OHDA model of PD showed that SP was increased, particularly in the striatal perivascular tissue and within remaining nigral neurons. An increase in perivascular SP may be deleterious to neuronal survival since it facilitates neurogenic inflammation and BBB dysfunction. In the SN, SP initiates DA release in the striatum by binding to the NK₁ receptors found on the dopaminergic neurons (Levesque et al., 2007). The SP/NK₁ complex is then internalised causing excitation and release of DA into the striatum. Accordingly, the elevated SP level in the striatum may be potentiated by the additional release of DA from remaining terminals to compensate for the lost striatal DA. Consequently, DA binds to its receptor, D₁, located on SP containing-striatal projections neurons to cause the release of SP in the SN. This is turn can increase DA release, creating a positive feedback cycle for DA and SP. In fact, the reduction in SP content in the neuropil of the SN appeared to be a secondary effect to the degeneration of the dopaminergic neurons, as a loss of SP immunoreactivity was only seen in the Captopril and SP treated animals, which had the largest dopaminergic lesions. Indeed, it has been shown that SP content within the SN is not reduced until greater than 90% of striatal DA has been depleted (Sivam, 1991). Although, striatal DA was not directly measured in the current study, the comparative study by Lee and colleagues (1996) reported that an approximate reduction of 80% of striatal DA occurred in this model. Thus vehicle treated
6-OHDA lesioned animals tended not to have reduced SP content within the neuropil of their SN. Certainly in the NK1 antagonist treated animals, which had a conservation of dopaminergic terminals and neurons, SP immunoreactivity within their striatum and SN resembled shams.

Previous studies in our laboratory have associated elevated SP with cell death and functional deficits in both TBI and stroke (Nimmo et al., 2004; Turner et al., 2006; Donkin et al., 2007). Throughout the brain, SP is co-localised with other neurotransmitters such as glutamate and GABA, which are both important in basal ganglia function. Indeed, striatal glutamate release requires the activation of NK1 receptors by SP (Marti et al., 2005). Thus increased SP production may potentiate high striatal glutamate levels. Excess glutamate will produce a rise in intracellular Ca$^{2+}$, causing mitochondrial damage and activation of calcium sensitive enzymes such as proteases, endonucleases and phospholipases. This biochemical cascade is known as glutamate excitotoxicity and is a major contributor to cell death in PD (Beal, 1992). Dopaminergic neurons are sensitive to changes in glutamate due to their numerous glutamate receptors (Morari et al., 1998).

6-OHDA treatment has been shown to produce a sustained increase in intracellular Ca$^{2+}$ (Berretta et al., 2005). Following a small rise in intracellular Ca$^{2+}$, SP and other neurotransmitters including glutamate, are released from storage vesicles. Accordingly, in the current study, SP production was increased following 6-OHDA treatment both in vitro and in vivo. SP then binds to NK1, a G-coupled receptor, to initiate a series of biochemical events that increases intracellular Ca$^{2+}$, thereby creating a positive feedback mechanism for its production (Saria, 1999), as well as being able to potentiate the release
of glutamate and other neurotransmitters. This positive feedback mechanism may have contributed to the sustained increase in SP production observed in the current study. Therefore both SP and 6-OHDA may potentiate glutamate release.

Inhibition of SP with NK₁ antagonists can also indirectly affect glutamate excitotoxicity due to the functional arrangement of the basal ganglia. The STN utilises glutamate to send excitatory signals to the SN to modulate the burst firing of dopamine neurons (Rodriguez et al., 1998). Therefore, with decreased striatal DA, the inhibition of the STN is minimal resulting in excess glutamate release in the SN. Subsequently, glutamate binds to its receptors on dopaminergic neurons causing excitotoxic damage. Consistent with this, inhibition of glutamate’s effects by NMDA antagonists, Group I mGluR antagonists and Group II and III mGluR agonists, which reduce glutamate production, are neuroprotective in 6-OHDA models of PD (Vernon et al., 2005). Furthermore, 6-OHDA striatal lesions result in a down regulation of glutamate receptors (Gu et al., 2003), which may be to compensate for the excess glutamate release or glutamatergic input from the STN. NK₁ antagonist treatment conserved dopaminergic terminals and neurons and therefore striatal DA. Therefore, NK₁ antagonist treatment may have prevented the overactivity of the STN and glutamate excitotoxicity. Nonetheless, STN lesions only provided some dopaminergic protection in MPTP models of PD (Luquin et al., 2006; Wallace et al., 2007) and bilateral STN lesions in clinical PD does not prevent the continuous degeneration of dopaminergic neurons (Hilker et al., 2005). Thus glutamate excitotoxicity is not the only mechanism by which dopaminergic neurons degenerate. Indeed, oxidative stress and inflammatory processes are both implicated in the degeneration of DA neurons following 6-OHDA.
Oxidative stress is the most common pathological process associated with 6-OHDA administration (Blum et al., 2001). Not only does 6-OHDA readily undergo autooxidation, it also reduces GSH and SOD, the two ROS scavenging enzymes (Schober, 2004). Additionally, it can partially inhibit complex I, which is part of the electron transport chain in mitochondria (Liang et al., 2004). Inhibition of this complex results in large amounts of ROS due to uncoupling of electrons during the production of ATP. Furthermore, inhibition of complex I also results in decreased ATP production. Many essential ion channels including Na⁺/K⁺ pump and Ca²⁺ channels rely on ATP to function correctly. Dysfunction of these ion channels will result in the influx of these ions, which can disrupt cell homeostasis and potentially activate degrading enzymes like proteases and endonucleases, which will damage the cell constituents, often resulting in the death of the cell. Unfortunately for dopamine neurons, they are more vulnerable to minor insults since they are always in a state of oxidative stress.

DA metabolism, like 6-OHDA, produces ROS and H₂O₂ via enzymatic degradation and non-enzymatic autooxidation. As previously mentioned, following the loss of dopamine neurons, the remaining neurons compensate for the loss of DA by synthesising and releasing extra dopamine. Initially, the extra striatal DA is beneficial as it can prevent basal ganglia dysfunction and thus functional deficits. However, as striatal dopaminergic terminals degenerate, DAT located on these terminals is also decrease causing excess cytosolic DA within the striatum. Therefore the rate of autooxidation of DA increases. Autooxidation of DA produces DA-quinones and -semiquinones. These are highly reactive molecules like ROS and they can bind metals such as Fe to exacerbate oxidative stress and also inhibit protein function by covalently modifying cysteine residues (Fahn and Sulzer, 2004; Miyazaki and Asanuma, 2008). In human dopaminergic neurons, DA-
quinones and –semiquinones make up neuromelanin (NM). NM, a black pigment, is found in varying amounts in different species and is absent in rodent dopaminergic neurons (Fedorow et al., 2005). However, when NM is injected into the rodent SN, activation of resident brain immune cells, microglia, is observed with subsequent cell death (Zecca et al., 2008). DA-quinones and –semiquinones have been reported to mediate the effects of NM (Miyazaki and Asanuma, 2008). Therefore, although NM can be protective by scavenging excess Fe, it can also be destructive. In the current thesis, we have demonstrated that increased SP production leads to a greater loss of dopaminergic terminals and therefore DAT. Increased SP, by increasing DA release, would have created excess cytosolic DA, high amounts of which would have undergone autooxidation to DA–quinones and –semiquinones to exacerbate oxidative stress and activate microglia, both of which contribute to DA terminal and neuronal loss.

Resting microglia are vital to the control of immune and homeostatic functions within the brain, and important for removal of cellular debris (Mosley et al., 2006). However, to remove cellular debris, microglia become activated, producing inflammatory cytokines, glutamate, quinolinic acid, O$_2^-$ radicals and NO (Rock and Peterson, 2006). The secretion of NO by microglia is by the activation of iNOS within microglia. NO can react with O$_2^-$ to form ONOO$, a highly reactive molecule, which often reacts with CO$_2$ to form many of its reactive intermediates (Torreilles et al., 1999). Under normal conditions, these ROS are produced by NADPH oxidase to kill foreign bodies within the brain parenchyma. In PD, NADPH oxidase is upregulated as is ROS production due to microglial activation (Mosley et al., 2006). These ROS and RNS cause damage to lipids, proteins and DNA, and are thought to be a major contributor to 6-OHDA-mediated cell death, with lipid peroxidation, protein oxidation and DNA damage observed in both in vitro and in vivo 6-
OHDA models of PD (Blum et al., 2001). Indeed following 6-OHDA striatal lesions, FR were found initially within the striatum, then within the SN after a delay of 7 days, suggesting these SN FR were not formed by the autoxidation of 6-OHDA (Henze et al., 2005). SP can increase NO by activating both microglia and nNOS containing-interneurons, which express the NK₁ receptor (Kemel et al., 2002; Chauhan et al., 2008).

Activated microglia were detected by ED-1 immunohistochemistry, which detects the CD68 complement protein that is expressed by phagocytosing macrophages (Rodriguez et al., 2007). Inflammatory processes have recently been given a great deal of attention given their potential role in the death of dopaminergic neurons in PD. Indeed, marked elevation of cytokines such as IL-1β and TNF-α are found in the SN and CSF of PD patients (Jenner and Olanow, 1998; Liu and Hong, 2003). Furthermore, activated microglia are observed in all animal models of PD, and in the SN of PD patients both on PET scans and at post-mortem (Depino et al., 2003; Mosley et al., 2006; McGeer and McGeer, 2008). In the current thesis, ED-1 immunoreactivity, particularly in the SN, correlated with the degree of dopaminergic degeneration. Following 6-OHDA striatal lesions, microglia were seen within the striatum as early as day 3 post-lesion, but did not appear in the SN until day 7. It is at this time that DA neurons began to degenerate, although the percentage of cell loss was only small. The appearance of microglia is thought to proceed neurodegeneration (Wojtera et al., 2005), however it can not be definitely known that this was the case in the current study as ED-1 immunoreactivity was only observed within the SN after dopaminergic degeneration became apparent. Activated microglia and degeneration of neurons are involved in a vicious cycle, where microglia can initiate cell death and then be activated by degenerating neurons (Raivich et
Following 6-OHDA striatal lesions, an astrocytic response was observed in both the striatum and SN as assessed by GFAP immunoreactivity. All 6-OHDA groups had an increase of GFAP immunoreactivity, with activated astrocytes present. These activated astrocytes are hypertrophic and intensely immunoreactive for GFAP due to their upregulation in GFAP production. This increase in GFAP production is observed in all animal models of PD (Depino et al., 2003; Takagi et al., 2007). In the current study, treatment with SP, or prevention of its degradation, in 6-OHDA lesioned animals resulted in markedly increased GFAP immunoreactivity within the striatum, plus the presence of activated astrocytes at all time points. Activated astrocytes are thought to be both beneficial, due to their secretion of neurotrophic substances and antioxidant enzymes, and detrimental when they secrete the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α. Astrocytes also express the NK₁ receptor (Mantyh et al., 1989) and once activated by SP, NF-κβ translocates to the nucleus resulting in cytokine secretion (Brahmachari et al., 2006; Chauhan et al., 2008). In the current study, GFAP immunoreactivity was generally correlated with the amount of dopaminergic terminal or neuronal loss. It follows that activated astrocytes may be trying to protect dopaminergic neurons by secreting growth factors, reducing oxidative stress with antioxidant enzymes and by metabolising excess cytosolic DA with their MAO and COMT enzymes (Hirsch, 2000). Conversely, by secreting cytokines they may be contributing to inflammatory processes and injury. From the results in the current thesis it cannot be definitively stated as to what effects, beneficial or deleterious, astrocytes are having on dopaminergic neurons.
Apart from the activity of both glial populations being modulated by SP, astrocytes and microglia can also modulate the activation of each other through cytokine and NO production (Rodrigues et al., 2001; Brahmachari et al., 2006). By producing NO, microglia may have contributed to the increase GFAP immunoreactivity in the striatum and SN of 6-OHDA lesioned animals. SP is an important modulatory peptide in the inflammatory response following 6-OHDA striatal lesions, as it can directly activate inflammatory cells to increase NO and cytokine production. Consistent with this, NK₁ antagonist treated animals only demonstrated a small increase in GFAP immunoreactivity in their striatum and SN, presumably by direct inhibition of SP’s ability to activate astrocytes or by reducing cytokine and NO expression by microglia. Other studies have also shown that NK₁ antagonist treatment reduces CNS inflammatory processes (Chauhan et al., 2008). Furthermore, prevention of inflammatory processes by chronic treatment with non-steroidal anti-inflammatory drugs reduces the risk of developing PD by 45% (Chen et al., 2003). Thus, the reduction in inflammatory processes by inhibition of SP may have contributed to reduced neuronal death, and less astrocytes may have been required to protect stressed or injured dopaminergic neurons.

Expression of TNF-α by astrocytes and microglia may be particularly detrimental to dopaminergic neurons as they express the TNF-αR1, a known death receptor that instigates apoptosis (Mladenovic et al., 2004). Apoptosis is thought to be the primary mechanism of cell death in PD since markers of apoptosis such as caspase-3, caspase-1, Bax and NF-κβ have been found in the SN of PD patients (Mochizuki et al., 1996; Hunot et al., 1997). Apoptosis can occur via the intrinsic or extrinsic pathway. The extrinsic pathway involves activation of cell surface death receptors, such as TNF-αR1 or caspase-8 or -10, whereas the intrinsic pathway involves cytochrome c release and caspase-9
activation. Both apoptotic pathways result in the activation of the final effector of apoptosis, caspase-3 (Bredesen et al., 2006). SP can contribute to apoptotic cascades by potentiating TNF-α secretion from astrocytes and microglia, initiating the translocation of NF-κβ to the nucleus. It can also increase intracellular Ca\(^{2+}\) by binding to NK\(_1\) receptors that facilitate a biochemical cascade resulting in increased intracellular Ca\(^{2+}\). If this elevation in Ca\(^{2+}\) is sustained, mitochondria will sequester the Ca\(^{2+}\) causing the opening of the mitochondrial transition pore and the release of pro-apoptotic factors cytochrome c and AIF (Shin et al., 2003). Minocycline, for example, inhibits the release of cytochrome c from mitochondria and has been shown to be neuroprotective in experimental models of PD (Bredesen et al., 2006). Binding of SP to NK\(_1\) receptors has been shown to initiate the translocation of NF-κβ to the nucleus, thus increasing the expression of caspase-11, which activates caspase-1 to promote IL-1β production and potentiate the cleavage of caspase-3 (Kang et al., 2000; Kim et al., 2003). However, it has been noted that capase-3 inhibitors only prolonged apoptotic cell death and do not prevent it (Dodel et al., 1999), suggesting that dopaminergic neurons may die via another mechanism at later times.

Recently, SP and NK\(_1\) have been shown to be involved in a non-apoptotic form of PCD, which has been observed in another neurodegenerative disorder, HD (Castro-Obregon et al., 2002). SP by binding to NK\(_1\) receptor induces the activation of the MAP kinase pathway with subsequent activation of the pro-death signal Nur77 (Castro-Obregon et al., 2004). It is unknown if this form of PCD occurs in PD. Apoptosis is normally detected on H&E sections by its morphological characteristics or with TUNEL staining, which detects fragmenting DNA. Non-apoptotic PCD does not have the classical morphology of apoptosis as nuclear fragmentation and apoptotic bodies are not seen. Thus classical
histological techniques that are used to detect apoptosis would not have detected this non-apoptotic form of PCD.

This is the first study to implicate neurogenic inflammation, and specifically increased SP levels, in cell death in PD. With SP binding to NK$_1$ receptors in perivascular tissue, neurogenic inflammation is initiated resulting in plasma extravasation and BBB breakdown. We used albumin immunoreactivity to assess BBB functionality and it was present early during the first week following 6-OHDA striatal lesions. However, the immunoreactivity was located mainly in the ipsilateral hemisphere, specifically in the striatum, suggesting the dysfunction of the BBB was localised to the lesioned hemisphere and may be a direct consequence of the 6-OHDA striatal lesions. Furthermore, striatal ED-1 immunoreactivity was also a useful indicator of vascular integrity since when albumin immunoreactivity was apparent, ED-1 immunoreactive cells were increased, especially around perivascular tissue. This is because CD-68 protein, which is positive to ED-1 staining, is expressed by peripheral macrophages (Carson et al., 2006), which can make their way through the disrupted barrier into surrounding tissue where they act like activated microglia, removing debris and expressing cytokines. Although BBB dysfunction has been attributed to neurodegenerative diseases such as AD, it has only recently been linked to PD where it is thought to accelerate the disease progression (Kortekaas et al., 2005). In the current thesis, albumin immunoreactivity and therefore BBB dysfunction was increased in the animals with the greatest SP production and dopaminergic cell death.

Capsaicin, a vanilloid, can activate its receptor, TRPV1, to cause a release of neuropeptides including SP. If high systemic doses are given, this release can be to the
point of depletion, with desensitisation of TRPV1 to further stimuli (Helke et al., 1981; Holzer, 1991). Through the release of SP, activation of TRPV1 can produce neurogenic inflammation (Hu et al., 2005), causing BBB dysfunction and plasma extravasation. Although SP was not depleted from the striatum or SN, perivascular SP was depleted by capsaicin treatment and absent at the time of 6-OHDA lesioning. The capsaicin treatment provided some protection of dopaminergic neurons from 6-OHDA, as reflected by the slightly smaller loss of TH immunoreactive cells compared vehicle controls. However, once activated, TRPV1 allows the uninhibited influx of cations such as $\text{Ca}^{2+}$ and $\text{Na}^+$ into the cell (Holzer, 1991). Excess of these ions may be detrimental to the cell and may initiate apoptotic cascades. TRPV1 is located on dopaminergic neurons (Szallasi and Blumberg, 1999), and consistent with this, capsaicin treatment alone produced a small loss of dopaminergic neurons. The protection from 6-OHDA striatal lesions conferred by capsaicin treatment was therefore greater than it appeared as these animals would already have had a small loss of dopaminergic neurons at the time of lesioning. This loss may have accounted for the poor early performance on functional tests. After the initial deficits, these animals had greater improvement in function during the assessment period than vehicle treated animals, presumably because of protection of dopaminergic neurons and thus striatal DA. TRPV1 can also be activated by AEA and NADA. AEA production has been shown to be increased following 6-OHDA (de Lago et al., 2004), whilst NADA is formed from DA and arachidonic acid, a product of inflammation (Huang et al., 2002). Both of these may have activated TRPV1 on dopaminergic neurons thereby contributing to their demise. Capsaicin treatment may have been protective through desensitisation of TRPV1 and by depleting perivascular SP to prevent neurogenic inflammation. Although BBB dysfunction was not measured directly in the capsaicin study, capsaicin treated lesioned animals had less ED-1 immunoreactivity than vehicle treated animals. As such,
this study provided further evidence for a role of neurogenic inflammation in cell death in early PD.

Throughout the studies described in the thesis, SP production correlated with dopaminergic terminal and cell loss. Elevated SP may have contributed to glutamate excitotoxicity, oxidative stress, inflammatory processes and the initiation of neurogenic inflammation, which have all been shown to cause dopaminergic cell death. Inhibition of SP receptor activation and the reduction in SP production produced by NK₁ antagonists provided some protection from 6-OHDA induced dopaminergic cell death. This conservation of dopaminergic terminals and neurons prevented the decrease in striatal dopamine. Striatal DA content is integral for proper execution of movement as the basal ganglia requires near normal striatal dopamine levels. Therefore terminal density and neuronal cell death correlated with functional deficits in the current study.

Further increases of SP level in the BG resulted in almost a total loss of dopaminergic terminals and neurons within the SN. Consistent with this observation, SP or Captopril treated animals displayed the greatest functional deficits of any group on all functional outcome tests used in the thesis. In contrast, treatment with an NK₁ antagonist at the time of 6-OHDA striatal lesions produced protection of dopaminergic terminals and neurons, and these animals had minimal functional deficits, often performing not significantly different to sham (control) animals.

Motor complications are particularly prominent both in clinical PD and following 6-OHDA striatal lesions. PD patients often display tremor, muscle rigidity, akinesia and postural instability and gait abnormalities (Eriksen et al., 2005). Apart from tremor, which
is not seen in this model, motor function of the animals was assessed with the rotarod, the stepping tests that assessed gait and akinesia, and the bilateral asymmetry test. All 6-OHDA lesioned animals generally demonstrated significant deficits on all of these tests compared to sham animals. In Chapters 7 and 8, large deficits were not observed in some of the stepping tests. This may be due to the improved accuracy in delivering the striatal lesions as time progressed, and by adopting a digitalised stereotaxic delivery system. By permitting accurate striatal injections to within thousandth of a millimetre, more consistent delivery of 6-OHDA to the same striatal region was achieved in all animals.

The striatum is topographically arranged with specific regions corresponding to certain functions. The dorsomedial striatum is involved in locomotion and turning, whereas a loss of terminals from the ventrolateral striatum affects initiation of movement, skilled motor behaviour and sensorimotor deficits (Mokry, 1995; Kirik et al., 1998; Deumens et al., 2002). The vehicle treated 6-OHDA lesioned animals in the SP and NK\textsubscript{1} studies had greater conservation of dopaminergic terminals, particularly in the dorsomedial striatum, and therefore did not demonstrate locomotion or gait abnormalities on the stepping tests. They did, however, display deficits in sensorimotor function, initiation of movement and overall motor function on the rotarod.

It is not only motor symptoms that are seen in PD, as many PD patients experience behavioural and neurological abnormalities. To test behaviour or anxiety following striatal lesions, the open field test was used to assess spontaneous exploratory behaviour in animals, specifically incorporating rearing and locomotion. Animals who were stressed or anxious did not explore the open field, instead often moving to a corner for the entire 5 min the test. As such, vehicle treated lesioned animals had reduced spontaneous exploration early compared to sham animals. Treatment with NK\textsubscript{1} antagonists have been
proven to reduce anxiety both clinically and in experimental models (Ebner and Singewald, 2006; Duzzioni et al., 2008). The same observation was noted in the current thesis with NAT and L-333,060 treated animals displaying normal exploratory behaviour. However, when dopaminergic cell death was around 50% (as observed in the Captopril and SP treated animals), spontaneous exploration also tended to increase. In our histological studies, ipsilateral VTA neurons were included in the quantification of ipsilateral SN TH immunoreactive neurons. Although VTA neurons are more resistant to 6-OHDA, a small loss of these neurons was observed in vehicle treated animals, with a greater loss seen in the larger lesions of the SP and Captopril treated animals. The VTA projects axons to the amygdala, medial prefrontal cortex and nucleus accumbens. These brain regions are important regulators of fear and anxiety, and accordingly a loss of dopaminergic input will attenuate these emotions (Pezze and Feldon, 2004). Habituation, which is a reduction in activity due to being repeatedly exposed to the same situation (Ivinskis, 1970), was observed in all animals, although 6-OHDA lesioned animals tended to have delayed habituation. Previously, lesions of the amygdala resulted in decreased habituation to the open field task (Daenen et al., 2001). Furthermore, DA has also been suggested to be involved in hippocampal-dependent memory as there are dopaminergic projections from the VTA to the hippocampus (Gasbarri et al., 1997). Many studies have reported learning and memory deficits following 6-OHDA lesions (Ferro et al., 2005). These deficits may contribute to the delay in habituation of 6-OHDA animals seen in this study in the open field task. Nonetheless, NK1 antagonists did further reduce the delay in habituation.

Neurological disorders such as anxiety, autonomic dysfunction and depression, occurs in almost 40% of PD patients (Stocchi and Brusa, 2000; Wolters, 2001; Sethi, 2002).
Although not all aspects of neurological function were assessed in the current thesis, overall neurological outcome was assessed using a modified neurological severity score, which tested reflexes, muscle control, movement and behaviour. Following 6-OHDA striatal lesions, vehicle treated animals displayed a mild deficit that was exacerbated by SP and Captopril treatment, suggesting elevated SP contributes to a poor neurological outcome. This was confirmed in the NK₁ antagonist treated animals that recorded only 1 or 2 points on the mNSS, with the L-333,060 treated animals displaying a total recovery in neurological function by the 2nd assessment week. Apart from being an anxiolytic, NK₁ antagonists are also an effective anti-depressant, thought by some to be more beneficial to patients than the classical anti-depressants as they produce less-side effects (Ebner and Singewald, 2006). Furthermore SP has been associated with swallowing difficulties and sexual dysfunction, which are also apparent in the later stages of PD (Hely et al., 2000). Thus treatment with NK₁ antagonists may not only help slow the progression of PD and the appearance of motor symptoms, it may also be beneficial to the non-motor symptoms that are involved with PD, which the current treatment, L-DOPA, does not alleviate.

9.1 Conclusions

We have shown that SP production is elevated both in vitro and in the in vivo intrastriatal 6-OHDA model of PD, which involves progressive dopaminergic degeneration to replicate the early stages of PD. This increase in SP levels may be contributing to the neurodegeneration of dopaminergic neurons in PD by enhancing oxidative stress, by increasing DA turnover and FR production, by potentiating glutamate excitotoxicity, by modulate CNS inflammatory processes including the activation of microglia and astrocytes, and by initiating both apoptotic and non-apoptotic forms of PCD. This is the
first study, however, to demonstrate that neurogenic inflammation also contributed to cell death in experimental PD. SP release is a component of neurogenic inflammation, and by binding to the NK₁ receptor in perivascular tissue, it produces BBB dysfunction, plasma extravasation and influx of peripheral immune cells that may also damage dopaminergic neurons. Consistent with this is the fact that NK₁ antagonists reduced dopaminergic terminal and cell loss and improve functional outcome, not only by inhibiting SP effects, but also by preventing the increase in SP production following 6-OHDA lesions. These results therefore definitely show a critical role for SP in 6-OHDA induced dopaminergic cell death, and posit that NK₁ antagonists may represent a novel therapeutic approach to treatment of functional deficits and disease progression early PD.