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

EMMA THORNTON

B.HlthSc (Hons)

Discipline of Pathology, School of Medical Sciences
The University of Adelaide

November 2008

A thesis submitted to the University of Adelaide in partial fulfilment of the requirements for the degree of Doctor of Philosophy
CHAPTER 4:
CHARACTERISATION OF THE INTRASTRIATAL 6-OHDA
MODEL OF EARLY PARKINSON’S DISEASE
4.1 Introduction

Chapter 3 demonstrated that the dopaminergic neurotoxin, 6-OHDA, produced a significant initial increase in SP content, which consequently exacerbated cell death in vitro. Yet it remains unclear if SP expression is also altered under in vivo conditions. Accordingly, the current study will characterise the intrastriatal 6-OHDA model of PD, which replicates the early stages of the disease, with specific focus on functional outcome and histological assessment, particularly for SP.

The 6-OHDA experimental model of PD was first described in the 1960’s. It is one of the most widely used tools for research into the pathogenesis of PD due to its highly reproducible nature and ease of use (Blandini et al., 2008). As 6-OHDA cannot pass through the BBB it must be injected directly into specific regions of the brain. Originally, it was injected into the medial forebrain bundle or directly into the SN itself. This produced total degeneration of dopaminergic neurons within the SN and therefore replicated the late stages of the disease (Ungerstedt, 1968). However, neurodegeneration occurred within hours following treatment allowing no opportunity to study disease progression. A progressive 6-OHDA model of PD was described in the 1990’s, where 6-OHDA was injected directly into the striatum of one hemisphere, producing a loss of striatal dopaminergic terminals with subsequent death of dopaminergic neurons in the ipsilateral SN in the following 1 to 2 weeks (Lee et al., 1996). The unilateral 6-OHDA model is one of the most useful models for research in PD. The contralateral hemisphere acts an internal control and the rats exhibit a circling behaviour that is indicative of lesion size. The aims of the present study were to characterise functional deficits following unilateral 6-OHDA striatal lesions and correlate this to dopaminergic terminal and cell
loss, as well as examine the change in SP production and its effect on inflammatory reactions and BBB dysfunction over the following 3-week period.

4.2 Study Design

All animals (n=30) were subject to 6-OHDA striatal lesions (as described in Chapter 2.2.2) and then randomly assigned to be sacrificed at day 3, 7, 10, 14, 17 or 21 post-lesion. Briefly, animals received stereotaxic injections of 6-OHDA (2μL; 5μg/μL) in two stereotaxic co-ordinates within the right striatum. The 6-OHDA 21-day animals were assessed for functional outcome (as described in Chapter 2.4.1-2.4.6). Striatal and SN sections from each animal were then assessed for histological outcome (as described in Chapter 2.5.2-2.5.7). A subset of animals (n=15) was used to determine SP content by ELISA (as described in Chapter 2.6) at either day 3 or 7 post-6-OHDA striatal lesion or following sham (control) surgery.

4.2.1 Functional Outcome

At days 3, 7, 10, 14, 17 and 21 post-lesion, animals were assessed for motor deficits by the rotarod, stepping tests and bilateral asymmetry test, neurological function by the mNSS, and behavioural outcome with the open field, as described in Chapter 2.4. Lesion size was estimated using the rotometer test performed on day 7 and 14 post-lesion, as described in Chapter 2.4.
4.2.2 Histological Outcome

At day 3, 7, 10, 14, 17 or 21 post-lesion, animals were perfused fixed with 10% buffered formalin under halothane anaesthesia, as described in chapter 2.2.4. Brains were then removed and processed for immunocytochemistry. Sections of SN and ST were stained for TH, H&E, SP, ED-1, GFAP and albumin, and then assessed using light microscopy.

4.2.3 ELISA Assay

6-OHDA intrastriatal lesioned animals were deeply anaesthetised with isoflurane at either day 3 or 7 post-lesion before being decapitated and the brain rapidly removed, as described in Chapter 2.6.1. Randomly chosen sham animals were also used. An ELISA assay for substance P was then performed as described in Chapter 2.6.3.

4.2.4 Statistical Analysis

All parametric data was analysed using an ANOVA followed by Bonferroni post-tests, and is displayed as mean ± SEM. Neurological and behavioural outcomes are displayed as median and therefore data was analysed using a Mann-Whitney U test.

4.3 Results

4.3.1 Functional Outcome

**Motor Outcome – Rotarod**

Sham animals had normal motor function and therefore were able to easily perform on the rotarod, with most animals completing the 2-minute task each day of the assessment period (Figure 4.1). Thus, surgery for this experimental model of PD does not result in
motor deficits as assessed by the rotarod. In contrast following 6-OHDA striatal lesions, animals had significant motor deficits at day 3 post-lesion compared to sham animals, completing on average only 74 ± 6 secs ($p < 0.001$). Furthermore, these animals were unable to learn the task, and therefore only improved slightly to complete 83 ± 3 secs by day 21 post-lesion. As such, their motor function remained significantly worse than sham animals for the entire assessment period ($p < 0.001$).

**Motor Outcome – Stepping Tests**

**Initiation Time**

Sham animals were able to move both their ipsilateral and contralateral forepaws in 1.4 ± 0.2 secs indicating normal initiation of movement and thus motor function (Figure 4.2). However, following 6-OHDA striatal lesions, animals had increased initiation time in both their forepaws throughout the assessment period. Variability within the 6-OHDA group was large and the number of animals small. Despite this, on days 3 and 14 post-lesion, the 6-OHDA group had significantly greater ipsilateral initiation time than sham animals ($0.01 < p < 0.05$), as well as significantly increased contralateral initiation time on days 3, 7 and 14 post-lesion ($0.01 < p < 0.05$). Thus, initiation of movement of both the forepaws was affected following 6-OHDA striatal lesions.
**Figure 4.1: Intrastriatal 6-OHDA model of PD – Motor outcome as assessed by the rotarod.**

Sham animals (black) had normal motor function, completing the 2 min rotarod task, whereas 6-OHDA intrastriatal lesioned animals (green) had significant motor deficits for the entire assessment period compared to sham animals. (n=5/sham, n=12/6-OHDA group) (*** denotes p < 0.001).
Figure 4.2: Intrastriatal 6-OHDA model of PD – Initiation time as assessed by the stepping tests.

Sham animals (black) displayed normal initiation of movement in both ipsilateral (A) and contralateral (B) forepaw throughout the assessment period. In contrast, 6-OHDA striatal lesioned animals (green) had delayed initiation of movement in their ipsilateral and contralateral forepaws at each assessment day compared to sham animals. This delay was significantly greater on the ipsilateral side on days 3 and 14 and contralaterally on days 3, 7 and 14 post-lesion. (n=5/group)(* denotes $p < 0.05$, ** denotes $p < 0.01$).
A. **Ipsilateral Initiation Time**

![Graph showing time (secs) vs Day Post-Lesion for Ipsilateral Initiation Time. The graph compares Sham and 6-OHDA groups, with significant differences indicated by asterisks.](image)

B. **Contralateral Initiation Time**

![Graph showing time (secs) vs Day Post-Lesion for Contralateral Initiation Time. The graph compares Sham and 6-OHDA groups, with significant differences indicated by asterisks.](image)
**Stepping Time**

Sham animals had normal motor function, traversing the ramp in an average of $6.8 \pm 0.4$ seconds (Figure 4.3). Conversely, following 6-OHDA striatal lesions, animals displayed a significant increase in stepping time by day 3 post-lesion ($p < 0.001$) taking $17.4 \pm 7.1$ seconds to traverse the ramp. Despite this initial increase in stepping times, 6-OHDA lesioned animals improved by day 7 post-lesion, recording comparable stepping times to sham animals. Stepping time for both groups remained similar throughout the rest of the assessment period.

**Step Length**

Step length in both the sham and 6-OHDA groups was similar during assessment with no significant differences recorded between the groups on either their ipsilateral or contralateral side (Figure 4.4). On the ipsilateral side, sham animals initially recorded a relative constant step length, with $9.3 \pm 0.6$ cm on day 3 to $10.3 \pm 0.6$ cm by day 21 post-lesion. In contrast, 6-OHDA lesioned animals recorded a reduction in step length on day 3, with $6.8 \pm 0.6$ cm but had improved by day 7 post-lesion to sham levels, recording $9.0 \pm 0.5$ cm. However on day 17 post-lesion, these animals had a small reduction in step length recording $8.4 \pm 0.4$ cm. In contrast on the contralateral side, no apparent trend was observed, with large within group variability in both groups. Accordingly, no significant treatment effect was observed between sham and 6-OHDA animals on the contralateral side. Thus, only the ipsilateral side for step length, where a significant treatment effect was recorded, will be shown in the remainder of this thesis.
Figure 4.3: Intrastriatal 6-OHDA model of PD – Stepping time as assessed by the stepping tests.

Sham animals (black) had a relatively constant stepping time throughout the assessment period. In contrast, 6-OHDA lesioned animals (green) took significantly longer to traverse the ramp on day 3 post-lesion. However by day 7 post-lesion, 6-OHDA animals had improved to sham levels. (n=5/group)(*** denotes p < 0.001).
Figure 4.4: Intrastriatal 6-OHDA model of PD – Step length as assessed by the stepping tests.

No significant difference between ipsilateral (A) or contralateral (B) step length was observed between sham (black) and 6-OHDA lesioned (green) animals during assessment. However on the ipsilateral side, sham animals had relatively constant step length, whereas 6-OHDA lesioned animals had a reduced step length on day 3 but improved to sham levels by day 7 post-lesion. In contrast, no apparent trend and therefore significant treatment effect was observed between sham and 6-OHDA animals on the contralateral side. (n=5/group).
A. **Ipsilateral Step Length**

![Ipsilateral Step Length Graph]

B. **Contralateral Step Length**

![Contralateral Step Length Graph]
Adjusting Steps

The adjusting step test is the most sensitive test of akinesia or lack of movement in 6-OHDA unilateral lesioned animals (Olsson et al., 1995). In this test the animal’s contralateral forepaw is affected (Figure 4.5). Sham animals did not exhibit akinesia since they recorded $5.1 \pm 0.2$ contralateral adjusting steps on the forehand side and approximately $6.0 \pm 0.2$ steps on the backhand side. Thus, surgery for the 6-OHDA intrastriatal model of PD does not cause akinesia. Conversely, 6-OHDA lesioned animals had fewer contralateral adjusting steps than sham animals in both the forehand and backhand direction throughout the assessment period, with these animals performing significantly less contralateral adjusting steps in the backhand direction on day 3 post-lesion ($p < 0.05$). Nonetheless, 6-OHDA animals had a gradual improvement in motor function during the assessment period recording $2.6 \pm 0.8$ backhand adjusting steps on day 3 and recovering to sham levels by day 17 post-lesion. Similarly in the forehand direction, 6-OHDA animals recorded only $1.8 \pm 1.1$ contralateral adjusting steps on day 3 before improving to $5.2 \pm 1.4$ steps by day 10 post-lesion, which was comparable to sham levels. However unlike in the backhand direction, 6-OHDA animals demonstrated a decline their ability to perform forehand adjusting steps from day 10 to 17 post-lesion, with a reduction number of steps from $5.2 \pm 1.4$ to $2.8 \pm 1.3$. Thus 6-OHDA striatal lesions result in akinesia or an absence of motor function in the contralateral forepaw.
Figure 4.5: Intrastriatal 6-OHDA model of PD – Contralateral forepaw adjusting steps as assessed by the stepping tests.

Sham animals (black) did not exhibit akinesia in their contralateral forepaw in either the forehand (A) or backhand (B) direction. Conversely, 6-OHDA striatal lesioned animals (green) recorded fewer contralateral adjusting steps than sham animals in both directions throughout the entire assessment period, demonstrating a deficit in contralateral forepaw motor function. (n=5/group)(* denotes p < 0.05).
A. **Forehand Adjusting Steps**

![Forehand Adjusting Steps Graph]

- **X-axis**: Day Post-Lesion
- **Y-axis**: No. of contralateral steps
- **Legend**:
  - Sham
  - 6-OHDA

B. **Backhand Adjusting Steps**

![Backhand Adjusting Steps Graph]

- **X-axis**: Day Post-Lesion
- **Y-axis**: No. of contralateral steps
- **Legend**:
  - Sham
  - 6-OHDA
  - * indicates significant difference
**Motor Outcome - Bilateral Asymmetry Test**

The bilateral asymmetry test assesses both sensory and motor function by measuring the time taken to sense and remove the adhesive tape from its forepaws. Sham animals were able to sense and remove the tape within 2 to 5 seconds from both their ipsilateral and contralateral forepaws (Figure 4.6). Thus, surgery for this experimental model of PD does not result in a forepaw sensorimotor deficit. Following 6-OHDA striatal lesions, animals took significantly longer to remove the tape than shams on both forepaws ($p < 0.05$). On the ipsilateral forepaw it took animals $9.4 \pm 3.6$ secs to remove the tape on day 3 before increasing to $22.2 \pm 3.8$ secs by day 17 post-lesion. Initially contralateral latency was identical to sham animals, however increased to $25.2 \pm 4.3$ secs by day 21 post-lesion. Therefore during the assessment period, sensorimotor function deteriorated in both forepaws in 6-OHDA lesioned animals as by days 17 and 21 post-lesion these animals had significantly longer latency scores than shams ($0.001 < p < 0.01$), suggesting a bilateral loss of sensorimotor function in these animals.

**Neurological Outcome – mNSS**

Sham animals had normal neurological function, with this group scoring no points on the mNSS every assessment day (Figure 4.7). Accordingly, surgery for the 6-OHDA intrastriatal model of PD does not affect neurological outcome. However when animals received 6-OHDA striatal lesions, a mild neurological deficit was observed by day 3 post-lesion, with these animals scoring a 3 on the mNSS. These deficits persisted throughout the assessment period, and therefore 6-OHDA striatal injections results in significant neurological deficit compared to shams ($p < 0.001$). A score of to 1 to 4 points
Figure 4.6: Intrastriatal 6-OHDA model of PD – Ipsilateral and contralateral latencies as assessed by the bilateral asymmetry test.

Sham animals (black) did not demonstrate sensorimotor deficits in either their ipsilateral (A) or contralateral (B) forepaws as assessed by the time taken to sense and remove the adhesive tape from their paws. However, 6-OHDA striatal lesioned animals (green) displayed bilateral sensorimotor deficits during the assessment period, which progressively worsened over time. (n=5/group)(*** denotes p < 0.001; ** denotes p < 0.01).
A. **Ipsilateral Latency**

![Graph showing Ipsilateral Latency over Day Post-Lesion]

B. **Contralateral Latency**

![Graph showing Contralateral Latency over Day Post-Lesion]
Figure 4.7: Intrastriatal 6-OHDA model of PD – Neurological outcome as assessed by the modified Neurological Severity Score.

Sham animals (black) had normal neurological function as assessed using the mNSS. Conversely, the 6-OHDA lesioned animals (green) recorded points on the mNSS at each day post-lesion and thus demonstrated mild neurological deficits throughout the assessment period. (n=5/group)
on this neurological severity score represents mild injury, consistent with this model replicating the early stages of PD.

**Behavioural Outcome – Open Field**

The open field task uses spontaneous exploratory activity to determine the stress and anxiety of animals, with greater activity indicating normal exploratory behaviour or less stress and anxiety. Sham animals had normal behavioural outcome throughout the assessment period recording an activity ranking of 10 on day 3 post-lesion before declining to 2 by day 14 post-lesion (Figure 4.8). This decline in activity level was not due to increased stress and anxiety, but is indicative of the well-described phenomenon known as habituation. Thus, surgery for this experimental model of PD does not cause atypical animal behaviour. In contrast, following 6-OHDA striatal lesions, animals had a reduced activity level of 6 on day 3 post-lesion, therefore demonstrating deficits in spontaneous exploration and an increase in stress and anxiety. Unlike shams, these animals did not become habituated to the open field test as these animals did not record less than a 5 for activity ranking during assessment, with almost all animals recording an activity level of 10 by day 17. Despite the reduction in activity in the first week post-lesion, no significant difference in activity was observed between sham and 6-OHDA animals.

**Estimation of Lesion Size – Rotometer**

Systemic administration of the dopamine-releasing agent, amphetamine, increases motor activity in animals. Following 6-OHDA striatal lesions, amphetamine will induce an ipsilateral turning response due to an imbalance in DA release, which the
Figure 4.8: Intrastriatal 6-OHDA model of PD – Behavioural outcome as assessed by the open field task.

Sham animals (black) displayed normal behaviour during the assessment period, with high levels of spontaneous exploration on day 3 post-lesion and a subsequent decline in activity by day 14 as these animals become habituated to the open field. In contrast, 6-OHDA lesioned animals (green) exhibited atypical behaviour, with a reduction in activity during the first assessment week and no apparent habituation to the open field task. (n=5/group).
rotometer will quantify, with the greater number of ipsilateral turns per min indicating a larger lesion. As expected, sham animals performed only $1.8 \pm 0.5$ and $0.9 \pm 0.3$ ipsilateral turns per minute for the 60 minute task on days 7 and 14, respectively, as these animals performed both ipsilateral and contralateral turns (Figure 4.9). Surgery for this experimental model of PD therefore does not cause striatal dopamine deficiency or ipsilateral turning activity. Conversely, 6-OHDA lesioned animals performed a significantly greater number of ipsilateral turns per minute on both day 7 ($p < 0.05$) and 14 ($p < 0.01$) post-lesion than shams, recording $9.8 \pm 1.4$ and $11.1 \pm 2.1$ turns per min, respectively.

4.3.2 Histological Outcome

**Dopaminergic Response: TH Immunocytochemistry**

TH immunoreactivity was used to determine the loss of dopaminergic terminals within the striatum and substantia nigra following 6-OHDA intrastriatal lesions.

**Striatal TH Immunoreactivity**

Sham animals demonstrated a dense network of TH immunoreactive terminals throughout the entire ipsilateral (Figures 4.10 and 4.11) and contralateral (Figure 4.12) striatum, and therefore the striatum was clearly demarcated from surrounding tissue. Thus, the surgical procedure for the intrastriatal 6-OHDA model does not result in a loss of striatal dopaminergic terminals. Conversely, 6-OHDA striatal lesions produced in a progressive loss of TH immunoreactive terminals from the ipsilateral striatum, with a loss observed as early as day 3 post-lesion. This degeneration occurred mainly during the first 2 weeks post-lesion, with day 14 sections demonstrating minimal TH immunoreactive...
**Figure 4.9: Intrastriatal 6-OHDA Model of PD – Estimation of Lesion Size as Assessed by the Rotometer.**

Sham animals (black) did not have an ipsilateral turning response following induction of motor activity by the dopamine-releasing agent, amphetamine, whereas 6-OHDA striatal lesioned animals (green) recorded a significantly greater number of ipsilateral turns per minute than shams on both day 7 and 14 post-lesion, suggestive of a ipsilateral striatal dopamine deficiency and a larger dopaminergic lesion. (n=5/sham group; n=12/6-OHDA group)(* denotes $p < 0.05$, ** denotes $p < 0.01$).
terminals located sparsely throughout the striatum. Degeneration continued until day 21, by which time striatal tissue was no longer delineated from surrounding tissue. Within the contralateral striatum, a dense network of TH immunoreactive terminals was observed in all 6-OHDA striatal lesioned animals at all time points post-lesion. Thus, this model is a true representation of a unilateral experimental model of PD enabling the contralateral hemisphere to be used as an internal control.

**Nigral TH Immunoreactivity**

Sham animals had a normal distribution and number of dopamine neurons within their ipsilateral SN compared to their contralateral SN (Figures 4.13 - 4.15). Degeneration of dopaminergic neurons was delayed following 6-OHDA striatal lesions, with no loss of TH immunoreactive neurons observed in the ipsilateral SN when compared to the contralateral SN at day 3 post-lesion. By day 7 dopaminergic neurons had begun to degenerate, but it was not until day 10 post-lesion that the loss of TH immunoreactive neurons was statistically significantly versus sham animals ($p < 0.01$)(Figure 4.15). By day 14 post-lesion, the ipsilateral SN had lost $34 \pm 10\%$ of nigral DA neurons and little neurodegenerative change was observed after that. A significantly greater dopaminergic cell death compared to sham animals was present throughout the rest of the assessment period ($p < 0.001$), with maximal TH immunoreactive neuronal loss seen at day 21 post-lesion, when a loss of $37 \pm 4\%$ of dopaminergic neurons within the ipsilateral SN was recorded.
Figure 4.10: Intrastriatal 6-OHDA model of PD – Ipsilateral striatal TH immunoreactivity following 6-OHDA striatal lesions. TH stained sections (Bar = 90μm).

Intense TH immunoreactivity was observed throughout the ipsilateral (right) striatum in sham animals (A). With 6-OHDA treatment, a slight loss of immunoreactive TH terminals was observed by day 3 (B), with a further loss of TH immunoreactivity by day 7 (C), then day 10 (D) and day 14 (E). At day 17 (F) a slight increase in TH immunoreactivity was present in the striatum compared to day 14. Minimal TH immunoreactivity was observed within the striatum by day 21 post-lesion (G).
Figure 4.11: Intrastriatal 6-OHDA Model of PD – Ipsilateral striatal TH immunoreactivity following 6-OHDA striatal lesions. TH stained sections (Bar = 50µm).

Sham sections (A) had a dense network of TH immunoreactive terminals throughout the striatum. A slight loss of terminals was observed by day 3 (B) post-lesion. This loss of terminals became progressively larger by day 7 (C), 10 (D) and 14 (E) post-lesion. A slight conservation of terminals was detected at day 17 (F) compared to day 14 post-lesion. However by day 21 (G), minimal TH immunoreactive terminals was observed within the striatum.
Figure 4.12: Intrastriatal 6-OHDA model of PD – Contralateral striatal TH immunoreactivity following 6-OHDA striatal lesions. TH stained sections (Bar = 90μm).

Intense TH immunoreactivity was observed throughout the entire striatum of sham sections (A) with clear delineation form surrounding tissue. No loss of TH immunoreactivity was detected in the contralateral striatum following 6-OHDA striatal lesions on days 7 (B), 14 (C) or 21 (D) post-lesion.
Figure 4.13: Intrastriatal 6-OHDA model of PD – Substantia nigra TH immunoreactivity following 6-OHDA striatal lesions. TH stained sections.

Sham animals (A) had normal TH immunoreactivity within the ipsilateral SN compared to its contralateral SN. Following 6-OHDA striatal lesions, animals by day 3 (B) were comparable to sham animals, with no obvious loss of TH immunoreactive neurons. Minimal loss of TH neurons was observed by day 7 post-lesion (C), however by days 10 (D) and 14 (E), the loss of TH immunoreactive neurons within the ipsilateral SN was obvious. Maximal loss of ipsilateral TH neurons was observed at day 21 (G) with a slight conservation of neurons seen at day 17 (F) compared to day 21 post-lesion. (SN = substantia nigra; VTA = ventral tegmental area).
Sham animals (A) had a normal number and distribution of TH immunoreactive neuron within their ipsilateral SN. No loss of TH immunoreactive neurons was observed at day 3 (B), but by day 7 (C) a minor loss was seen. This was loss was increased by day 10 (D) then day 14 (E), with maximal loss ay day 21 post-lesion (G). A slight conservation in TH immunoreactivity was observed in the SN at day 17 (F) compared to day 21 post-lesion.
Figure 4.15: Intrastriatal 6-OHDA model of PD – Quantification of TH immunoreactive neurons within the ipsilateral substantia nigra.

By day 7 following striatal 6-OHDA lesions, degeneration of dopaminergic neurons within the SN was observed, although the percentage of dopaminergic cell death was not significantly greater than shams until day 10 post-lesion. By day 14, dopaminergic cell death was further exacerbated with only a small loss of dopamine neurons during the third week. (n=5/group)(* denotes p < 0.05, *** denotes p < 0.001 compared to sham).
**General Pathology - H&E**

Haematoxylin and eosin (H&E) staining was used to visualise tissue architecture and cellular integrity in both the striatum and substantia nigra following sham surgery and 6-OHDA striatal lesions. Sham animals displayed identical striatal cellular pathology to 6-OHDA lesioned animals at all times post-lesion (Figure 4.16). This was expected as intrastriatal 6-OHDA lesions produce a loss of TH terminals, but does not affect other striatal neuronal populations such as the medium spiny projections neurons or large interneurons. In contrast, a change in tissue architecture was apparent within the SN following 6-OHDA lesioning. Sham animals had normal nigral architecture, with mainly healthy neurons present (Figure 4.17). A few dopaminergic neurons were dark with and shrunken. This cellular morphology is called dark cell change and it is thought to occur when cells are stressed or reversibly injured. By day 3 following 6-OHDA striatal lesions, many of the neurons were stained darker than shams, although neuronal shape was normal. Staining had returned to normal by day 7, although an occasional dark, shrunken neuron was observed. An obvious infiltration of glial cells was seen by 10 day post-lesion, and remained for the rest of the assessment period. Furthermore from this day, a progressive loss of nigral neurons and SN architecture was observed. However, the remaining dopaminergic neurons look normal. Due to no apparent changes in striatal cellular pathology, H&E stained sections of the SN will only be shown for the remainder of the thesis.
Figure 4.16: Intrastriatal 6-OHDA model of PD – Cellular pathology of the ipsilateral striatum following 6-OHDA striatal lesions. H&E stained sections (Bar = 90 μm).

No abnormalities in tissue architecture or neuronal integrity was observed in either sham (A) or 6-OHDA lesioned striatal tissue at day 7 (B), 14 (C) or 21 (D) post-lesion.
Figure 4.17: Intrastriatal 6-OHDA model of PD – Cellular pathology of the ipsilateral substantia nigra following 6-OHDA striatal lesions. H&E stained sections (Bar = 50μm).

Sham animals (A) displayed normal nigral architecture with many neurons present. Most of the neurons looked healthy, however occasional cells were dark and shrunken (green arrows indicate dark cells), suggesting these neurons are stressed. Following 6-OHDA striatal lesions, many of the dopaminergic neurons were darker than shams at day 3 (B) post-lesion, although no loss of neurons was observed. By day 7 (C), neuronal staining had returned to normal. From day 10 (D) onwards a progressive loss of nigral architecture was seen, with increased loss at day 14 (E) and maximal loss at day 21 post-lesion (G). A slight conservation of neurons was seen at day 17 (F). An obvious infiltration of glial cells (black box encapsulates glial cells; G) was apparent in the SN from day 10 onwards.
**Substance P Response – SP Immunohistochemistry / SP ELISA**

**SP Immunohistochemistry**

SP immunoreactivity was used to estimate the SP response within the striatum and SN following sham surgery and 6-OHDA intrastriatal lesions. In the brain, a high concentration of SP is found within the striatum and substantia nigra. As expected, sham animals displayed SP immunoreactivity throughout the striatum, with intense perivascular staining around aspects of the perimeter of some blood vessels (Figure 4.18). In contrast, by day 3 following 6-OHDA striatal lesions, animals had an increase in SP immunoreactivity throughout the entire ipsilateral striatum including within perivascular tissue. SP levels remained elevated at day 7 post-lesion before a small decrease in striatal SP immunoreactivity on day 10 post-lesion. By day 14, a small increase in SP immunoreactivity was observed, which continued until day 21, when SP immunoreactivity within the striatum and perivascular tissue had once again increased to above sham levels, although immunoreactivity was still less than on day 3 and 7 post-lesion.

In the SN, SP immunoreactivity delineates the SN from surrounding tissue and therefore may be used as a marker of the SN (Figure 4.19). Sham animals had intense SP expression throughout the SN, with faint cytoplasmic immunoreactivity within neurons, although small variations in this cytoplasmic staining were seen (Figure 4.20). Following 6-OHDA striatal lesions, SP immunoreactivity within the cytoplasm of neurons was upregulated, with many neurons displaying greater intensity of SP staining than in neurons of sham animals. No difference in nigral SP immunoreactivity
Figure 4.18: Intrastriatal 6-OHDA model of PD – SP immunoreactivity within the ipsilateral striatum following 6-OHDA striatal lesions. SP stained sections (Bar = 25μm).

Sham animals (A) had light immunoreactivity throughout the striatum, with intense immunoreactivity around aspects of the perimeter of some blood vessels. Following 6-OHDA striatal lesions, an increase in striatal and perivascular SP immunoreactivity was observed by day 3 (B), and remained elevated at day 7 (C) post-lesion. However, by 10 (D) SP immunoreactivity was faint throughout the striatum, reducing to below levels, however a slight increase was seen by day 14 (E) and then day 17 (F). By day 21 (G), SP immunoreactivity was once again greater than in striatal sections.
Figure 4.19: Intrastriatal 6-OHDA model of PD – SP immunoreactivity delineates the substantia nigra. SP stained section (Bar = 90μm).

Intense SP immunoreactivity was observed within a control SN. Furthermore, SP immunoreactivity delineated the SN, which is also outlined in black.
**Figure 4.20: Intrastriatal 6-OHDA model of PD – SP immunoreactivity within the ipsilateral substantia nigra following 6-OHDA striatal lesions.** SP stained sections (Bar = 25μm).

Sham animals (A) displayed normal nigral SP immunoreactivity with intense immunoreactivity within the neuropil and faint cytoplasmic staining within neurons. Following 6-OHDA lesions, no obvious change was observed at day 3 (B), however by day 7 (C) many more neurons had a greater intensity of cytoplasmic SP immunoreactivity. This upregulation remained for the 3-week period and therefore could be seen in day 10 (D), 14 (E), 17 (F) and 21 (G) sections. No loss of SP immunoreactivity within the neuropil was demonstrated following 6-OHDA lesions. Green arrows denote normal cytoplasmic SP expression, whereas the black arrows denote an increase in cytoplasmic SP immunoreactivity.
was observed between sham and 6-OHDA animals at any time post-lesion.

**SP ELISA**

A SP ELISA was performed to semi-quantify SP content in both sham and 6-OHDA animals at days 3 and 7 post-lesion (Figure 4.21). Sham animals had more striatal SP content in their contralateral (left) striatum compared to their ipsilateral (right) striatum. Conversely in the SN, sham animals had greater SP content in the ipsilateral SN compared to the contralateral, although the difference was not as exaggerated as in the striatum. Following 6-OHDA striatal lesions, the SP content of the ipsilateral and contralateral striatum was increased compared to sham animals at both day 3 and 7 post-lesion. A significant difference in SP expression was only seen in the striatum of 3 day 6-OHDA animals \((p < 0.05)\). All 6-OHDA animals demonstrated greater SP content within the SN, with a small increase at day 3 and a further elevation by day 7 post-lesion, when 6-OHDA animals had significantly greater SP content within their contralateral SN compared to sham animals \((p < 0.05)\).

**Astrocytic Response – GFAP Immunohistochemistry**

GFAP immunoreactivity was used to determine the astrocytic response within the striatum and SN following sham surgery and 6-OHDA striatal lesions. Sham animals displayed normal GFAP expression with immunoreactivity located around blood vessels, in white matter bundles, and in circulating astrocytes within striatal tissue. These resting astrocytes were few in number and had fine processes (Figure 4.22).
Figure 4.21: Intrastriatal 6-OHDA model of PD – Semi-quantification of SP expression within the striatum and substantia nigra.

6-OHDA lesioned animals had greater SP content than sham animals in the striatum and SN at both day 3 and 7 post-lesion. This content was significantly increased in the ipsilateral (right) striatum at day 3 and in the left (contralateral) SN at day 7 post-lesion. (n=5/group)(*denotes p < 0.05). LST = left striatum; RST = right striatum; LSN = left substantia nigra; RSN = right substantia nigra.
A similar pattern of GFAP immunoreactivity was observed within the SN in sham sections (Figure 4.23). Thus, sham surgery does not induce an astrocytic response. In contrast, 6-OHDA striatal lesions produced an increase in GFAP immunoreactivity within the ipsilateral striatum and SN. Striatal GFAP immunoreactivity was intense by day 3 post-lesion, with GFAP expression apparent throughout the entire striatal tissue including increased production around vessels and in white matter, and within astrocytes themselves. Activated astrocytes have thicker processes and display intense GFAP immunoreactivity. Furthermore, an increase in the number of astrocytes was seen. Although GFAP production remained elevated compared to shams, a progressive reduction in immunoreactivity within striatal tissue was observed from days 7 to 17 post-lesion. However, the number of astrocytes in the striatum remained greater than shams although their GFAP immunoreactivity became less intense.

A similar increase in GFAP expression was observed within nigral tissue, except greatest immunoreactivity was seen on day 14 post-lesion. Following 6-OHDA lesioning, GFAP immunoreactivity increased slightly around blood vessels by day 3, before it was re-distributed from mainly within white matter to surrounding dopaminergic neurons by day 7 post-lesion. A progressive increase in immunoreactivity was observed until day 17 and 21 post-lesion, when staining began to decrease, although astrocytic numbers remained elevated with most located near dopaminergic neurons. Thus, 6-OHDA striatal lesions induced an astrocytic response that is visible in both the striatum and SN during dopaminergic degeneration.
Figure 4.22: Intrastriatal 6-OHDA model of PD – GFAP immunoreactivity within the striatum following 6-OHDA striatal lesions. GFAP stained sections (Bar = 50μm).

Normal GFAP immunoreactivity was observed within sham sections (A), with immunoreactivity observed around blood vessels, in white matter and in resting astrocytes. Following 6-OHDA lesions, an increase in GFAP immunoreactivity and astrocytic infiltration was observed within striatal tissue, with intense expression and many activated astrocytes observed by day 3 post-lesion (B). A small progressive decrease in GFAP expression was observed on days 7 (C), 10 (D), 14 (E), 17 (F) and 21 (G) post-lesion. At day 21, GFAP immunoreactivity was still greater than shams, with numbers of activated astrocytes remaining elevated.
Sham sections (A) had normal GFAP immunoreactivity, with immunoreactivity around blood vessels, in white matter and by resting astrocytes. At day 3 (B) following 6-OHDA striatal lesions, astrocytic infiltration was observed as well as an increase in GFAP immunoreactivity around blood vessels. GFAP immunoreactivity continued to increase and re-distributed to around dopaminergic neurons from days 7(C), 10 (D) and 14 (E), when immunoreactivity was the greatest. A small progressive decline in immunoreactivity was observed on days 17 (F) and 21 (G) post-lesion, although the number of astrocytes was still elevated and GFAP immunoreactivity was more than in shams.
Activated Microglial Response – ED-1 Immunohistochemistry

ED-1 immunoreactivity was used to determine the activated microglial response. ED-1 binds the CD68 protein, which is expressed in phagocytosing macrophages, also known as activated microglia within the brain. However, ED-1 also detects peripheral macrophages (Carson et al., 2006). Sham animals displayed minimal ED-1 immunoreactivity throughout the striatum (Figure 4.24) and SN (Figure 4.25), with an occasional immunoreactive glial cell located perivascularly. Thus, the surgical procedure for the 6-OHDA intrastratal model of PD does not result in the activation of microglia. In comparison, ED-1 immunoreactivity was dramatically upregulated following 6-OHDA striatal lesions within both brain regions. Within the ipsilateral striatum, activated microglia were located singularly or in clumps throughout the striatal tissue. Increased perivascular ED-1 immunoreactivity was due to infiltration of peripheral phagocytosing macrophages from the blood stream. The increase in ED-1 immunoreactivity was observed as early as day 3 post-lesion, with activation of microglia and infiltration of peripheral macrophages observed. ED-1 immunoreactivity was further increased by day 7 post-lesion, with maximal expression on day 10 post-lesion. At this time, many activated microglia were detected throughout the striatum, particularly within the area of degenerating dopaminergic terminals. These microglia were round and intense, which indicates that they were in a phagocytotic state. A slight reduction in ED-1 immunoreactivity was observed by day 14 post-lesion although numerous individual activated microglia with visible processes were still apparent. They remained to a lesser degree at day 21 post-lesion.
Figure 4.24: Intrastriatal 6-OHDA model of PD – ED-1 immunoreactivity within the striatum following 6-OHDA striatal lesions. ED-1 stained sections (Bar = 50μm).

Minimal ED-1 immunoreactivity was seen in sham sections (A) with only an occasional immunoreactive glial cell situated on the edge of a blood vessel. Activation of microglia and infiltration of peripheral macrophages was apparent as early as day 3 (B) post-lesion, by an increase in perivascular ED-1 staining. ED-1 immunoreactivity continued to increase on days 7 (C), 10 (D) and 14 (E) post-lesion, when ED-1 immunoreactivity is maximal. A small reduction in ED-1 immunoreactivity was observed by days 17 (F) and 21 (G) post-lesion, although a few activated microglia were still present within striatal tissue at this time.
Figure 4.25: Intrastriatal 6-OHDA model of PD – ED-1 immunoreactivity within the substantia nigra following 6-OHDA striatal lesions. ED-1 stained sections (Bar = 50μm).

Sham sections (A) displayed minimal ED-1 immunoreactivity with only an occasional perivascular glial cell immunoreactive for ED-1. Following 6-OHDA striatal lesions, activation and infiltration of microglial was not apparent until day 7 (C) post-lesion, as day 3 sections (B) resembled shams. A subsequent increase in ED-1 immunoreactivity occurred on day 10 (D), with maximal immunoreactivity on day 14 (E) post-lesion when many activated microglia were detected around degenerating neurons. On days 17 (F) and 21 (G) post-lesion, a decrease in ED-1 immunoreactivity was observed.
Within the SN, activation of microglia and infiltration of peripheral macrophages was delayed, with increased perivascular ED-1 immunoreactivity and activated microglia not seen until day 7 post-lesion. Furthermore, activated microglia were not as widespread as in the striatum being mainly situated near degenerating dopaminergic neurons. The greatest ED-1 immunoreactivity within the SN was demonstrated on day 14 post-lesion, with numerous activated microglia found throughout. From this day, the microglial response declined, with only minimal activated microglia were seen in day 21 sections.

**Inflammatory Response in the Striatum Surrounding the Needle Tract**

An inflammatory response was observed along the needle tract in the striatum in some animals (Figure 4.26). This response was observed with H&E staining and ED-1, GFAP and SP immunocytochemistry. Sporadic animals from all groups displayed this response, with each animal’s inflammatory reaction differing in degree (data not shown). Along the needle tract itself a glial scar may form, containing many activated microglia immunoreactive for ED-1. These microglia penetrate out from the glial scar into the striatal tissue. Astrocytes react in a similar manner, with many intense GFAP immunoreactive astrocytes surrounding the glial scar. GFAP immunoreactivity around surrounding blood vessels was also increased. Furthermore, many of these astrocytes and microglia were immunoreactive for the neuropeptide SP, a known important inflammatory mediator.

**Blood Brain Barrier Dysfunction – Albumin Immunohistochemistry**

Albumin, a blood plasma protein, is often used as a marker of blood brain barrier (BBB)
Figure 4.26: Intrastriatal 6-OHDA model of PD - Inflammatory response in the striatum surrounding the needle tract. H&E, ED-1, GFAP and SP stained sections (Bar = 90 μm).

A glial scar may be formed along the needle tract as seen in the H&E section (A). Activated microglia immunoreactive for ED-1 also form part of this glial scar, and penetrate out into the striatum (B). Many intense GFAP immunoreactive astrocytes with short thick processes are also apparent (C). Both astrocytes and microglia express the immune modulating neuropeptide substance P (D).
Figure 4.27: Intrastriatal 6-OHDA Model of PD – Blood brain barrier dysfunction as assessed by albumin immunoreactivity.

Sham sections (A) display faint albumin staining around blood vessels and within white matter. In contrast, 6-OHDA striatal lesions resulted in increased albumin immunoreactivity by day 3 (B) post-lesion, with much of the ipsilateral striatum and cortex immunoreactive for albumin. By day 7 (D) post-lesion, albumin reactivity has decreased although still apparent in the cortex just above the ipsilateral striatum. However, by day 10 (C) albumin had returned to sham levels and remained so until day 21 (E) post-lesion.
dysfunction. During barrier breakdown, blood vessels have increased permeability allowing influx of water and solutes including albumin into surrounding tissue. As expected, sham animals demonstrated normal albumin distribution, with immunoreactivity seen around the edge of the brain and within ventricles and blood vessels (Figure 4.27). Thus surgical procedures for this model of PD does not produce BBB breakdown. In contrast following 6-OHDA striatal lesions, an increase in albumin immunoreactivity was observed throughout the ipsilateral striatum and even penetrated into the cortex above by day 3 post-lesion. A reduction in albumin staining was seen over the following week, with immunoreactivity returning to sham levels by day 10 post-lesion. Thus, BBB dysfunction is observed in the intrastriatal 6-OHDA model of PD, particularly in the first week following lesioning.

4.4 Discussion

In the present study, we characterise the intrastriatal 6-OHDA model of PD. This model involves a 6-OHDA-induced loss of striatal dopaminergic terminals with a subsequent moderate retrograde degeneration of dopamine neurons within the substantia nigra at 1 to 2 weeks following lesioning. Accordingly, this experimental model of PD is recognised as one of the best experimental models to study the pathogenesis and pathophysiology of the early to moderate stages of PD (Przedborski et al., 1995; Lee et al., 1996; Yuan et al., 2005). Although many studies have used this model, characterisation using a battery of functional tests for not only motor but also neurological and behavioural outcomes with concurrent histological assessment has not been undertaken. Importantly, no known studies have examined the role of, SP, in the intrastriatal 6-OHDA model of PD.
In the current study, 6-OHDA striatal lesions produced a significant motor deficit as assessed by the rotarod and stepping tests. The accelerating rotarod has previously been shown to be a sensitive test for motor deficits in PD, particularly in partial lesions, as the increasing speed makes initiation of movement difficult (Rozas et al., 1997; Rozas and Labandeira Garcia, 1997). In this study, 6-OHDA animals had difficulty walking during the fastest accelerating speeds of 24-30 rpm, often falling off or holding on to the rotarod for 2 consecutive revolutions, therefore ending the test. Sham animals could easily walk on the rotarod at these speeds and thus were able to complete the 2-min task. The rotarod employed in the current study consisted of a circular assembly of 18 x 1mm metal rods and these narrow rods incorporate a grip strength component to the task, thus increasing the difficulty of the task and making it very sensitive for detecting mild to moderate motor deficits (Hamm et al., 1994). Sensorimotor learning deficits are also prominent in experimental PD and are often assessed with the rotarod (Ogura et al., 2005). Unlike sham animals, 6-OHDA lesioned animals were unable to improve their rotarod score during the assessment period, despite improvement in motor function in other tests, thus confirming the sensitivity of the rotarod test and its ability to detect sensorimotor deficits in this model.

Confirmation of deficits in initiation of movement were detected using the stepping test, with 6-OHDA animals having significant latencies in initiation of movement in both the ipsilateral and contralateral forepaws compared to shams. Furthermore, absence of movement was apparent in the contralateral forepaw using the adjusting step test. This is a reported feature of this model, particularly in the forehand direction as observed in the current study, and potentially contributing to the observed rotarod deficits (Olsson et al., 1995; Kirik et al., 1998; Depino et al., 2003). The remaining stepping tests of stepping
time and step length were not significantly affected by 6-OHDA striatal lesions, with ipsilateral deficits detected in stepping time only on day 3 post-lesion, and minor and non-significant reductions being detected in step length. However, this test measures hind paw motor control, which may not be as affected as the forepaws in this model; previous deficits in step length have only been observed in bilateral 6-OHDA striatal lesions (Iancu, 2005). Furthermore, this test uses an open sided ramp suspended above ground and therefore may have incorporated anxiety levels into the test. Finally, sensorimotor deficits were also demonstrated by the bilateral asymmetry test, where 6-OHDA animals took longer to sense and remove the tape from both forepaws throughout the entire assessment period, with significantly longer latencies than sham on days 17 and 21 post-lesion. These data show intrastriatal 6-OHDA model results in motor deficits affecting sensorimotor skills and learning, as well as initiation of movement.

Loss of dopaminergic terminals from the dorsomedial striatum has been shown to affect locomotion and induce turning, whereas a loss from the ventrolateral striatum affects initiation of movement, skilled motor behaviour, and produces sensorimotor disturbances (Mokry, 1995; Kirik et al., 1998; Deumens et al., 2002). In the current study, the loss of dopaminergic terminals occurred mainly within the dorsolateral and ventrolateral striatum, and thus it was sensorimotor and initiation deficits and not reduced locomotion that were predominant following 6-OHDA striatal lesions. Although most 6-OHDA animals had spontaneous circling toward the lesioned side on day 1 post-lesion, continued spontaneous turning activity was only observed in those animals with a greater loss of dopaminergic terminals, which continued into the dorsomedial striatum. Circling behaviour, however, was potentiated in all 6-OHDA animals by administration of the dopamine-releasing agent, amphetamine. Following treatment, all animals had increased
motor activity, yet only 6-OHDA lesioned animals had predominantly ipsilateral turning behaviour. This turning behaviour was quantified in the rotometer, with a greater number of ipsilateral rotations per minute generally indicating a larger lesion. However in the current study, large variation in the number of turns per minute was observed for similar percentages of dopaminergic cell loss. This is a feature of amphetamine induced turning in partial lesions and has previously been reported in this model (Hudson et al., 1993).

Neurological and behavioural outcome was also affected following 6-OHDA striatal lesions, although not to the degree seen in motor tasks. A mNSS score of 3-4, was recorded by 6-OHDA treated animals, representing mild injury according to this neuroseverity score (Li et al., 2000). Most of the animals scored a point on the mNSS for abnormal flexion of the forepaws and hindpaws, spontaneous circling and loss of deep sensory feeling in hindpaws, or loss of reflexes and inability to walk straight in the more severely injured. In terms of behaviour, the open field test has long been used as a test for stress and anxiety, emotionality and habituation (Walsh and Cummins, 1976). If animals are not stressed or anxious they will spontaneously explore the open field. Furthermore, rearing behaviour has been correlated to striatal dopamine levels (Fornaguera and Schwarting, 1999). Accordingly, in the current study, an activity level was determined using positive behavioural indicators incorporating the number of squares travelled added to the number of rears. As sham animals perform 200 plus squares and only between 5 to 10 rears, each square was awarded 1 point whereas each rear was given a score of 10 points, with 1-25 points an activity level of 1 and 26-50 points an activity level of 2 and so on, until 225+ points, which was the maximum activity level of 10. Sham animals obtained the maximum activity level of 10 on day 3 post-lesion, whereas 6-OHDA lesioned animals only scored 6 due to greater stress and anxiety. These animals did not
actively explore the field, with many animals either exploring only the edge of the field once before sitting in a corner, or moving directly to a corner where they remained for the rest of the test. Rearing activity was also reduced, a previously reported feature of 6-OHDA striatal lesioned animals (Fornaguera and Schwarting, 1999). As the assessment period continued, sham animals demonstrated a decline in activity level, which was not representative of greater stress and anxiety but instead habituation to the open field task due to being repeatedly exposed to the same situation (Ivinskis, 1970). In comparison, 6-OHDA animals did not become habituated to the task and instead actually improved their activity level, recording the maximum score of 10 on day 17 post-lesion. This may reflect a reduction in anxiety with repeated exposure to the task. Moreover, the initial high levels of anxiety before recovery may also have contributed to the early stepping time and step length deficits.

With relation to anxiety, NK1 antagonists have been shown to be efficacious in reducing anxiety levels, suggesting that SP may be a mediator of this behaviour (Ebner and Singewald, 2006; Duzzioni et al., 2008). In the current study, tests that incorporated the anxiety level of the animal demonstrated deficits early when SP levels were increased, suggesting a correlation between higher SP levels, increased anxiety and functional deficits. Reduced anxiety levels have previously been reported in mild bilateral 6-OHDA lesioned at 5 weeks post-lesion (Branchi et al., 2008). Since the bilateral 6-OHDA model produces substantial lesions indicative of advanced PD, we can speculate that SP levels may have been substantially reduced in the later stages of PD.

Overall, 6-OHDA striatal lesioned animals demonstrated deficits in motor function and neurological and behavioural outcome that can only be attributed to the loss of striatal
DA; sham animals displayed normal dopaminergic levels/transmission and normal function. Furthermore, 6-OHDA animals displayed functional deficits as early as day 3 post-lesion when dopaminergic neurons within the ipsilateral SN were still intact but numerous striatal terminals had degenerated. Interestingly, the greatest functional deficits are observed at this time, despite the continued loss of dopaminergic terminals throughout the assessment period. Familiarity with the tests, compensatory mechanisms for dopamine transmission or continued exercise may all facilitate the improved function of the 6-OHDA animals (Perez-Otano et al., 1998; Tillerson et al., 2003). Although striatal dopamine was not measured, the comparative study by Lee and colleagues demonstrated an 82% striatal dopamine loss compared to the contralateral hemisphere, with a 79% and 62% loss of ipsilateral dopaminergic neurons in the SN and VTA, respectively (Lee et al., 1996). In the current study, only a 37% loss of ipsilateral dopamine neurons was observed, although cell counts were performed at 3 weeks instead of 8 weeks post-lesion and both the SN and VTA were combined for quantitation. At 3 weeks post-lesion, dopaminergic degeneration is slowly occurring and will continue to progress for the next few weeks (Lee et al., 1996). Furthermore, dopaminergic degeneration occurred mainly within the SN, with minimal cell loss observed within the VTA.

H&E staining revealed that even in sham animals, occasional dopaminergic neurons were stained dark and may have been shrunken. This cellular morphology is characteristic of dark cell change, representing cellular stress or reversible cell injury (Foda and Marmarou, 1994). In the SN, dopaminergic neurons are always in a state of oxidative stress due to DA metabolism (Olanow et al., 2004) and some dark cell change is thus not unexpected. Following 6-OHDA striatal lesions, all dopaminergic neurons became more intensely stained by day 3 post-lesion, which suggested that these neurons were even
more stressed. At day 7, dopaminergic neurons still demonstrated some dark cell change, however terminal loss is maximal at this time suggesting that these neurons are under greater stress than normal. By day 10, remaining neurons looked mainly healthy, although an occasional dark cell could still be observed.

As previously mentioned, the neuropeptide, SP, is found in high levels in both the striatum and SN where it is an excitatory transmitter involved in the modulation of DA release. In the previous chapter, SP content was shown to be elevated following 6-OHDA treatment, which subsequently exacerbated cell death \textit{in vitro}. Although SP production was not measured directly following 6-OHDA lesions, at day 3 post-lesion striatal SP immunoreactivity was increased and remained so until day 7. In addition, SP immunoreactivity within nigral neurons was greater than in sham animals, although there was no loss of SP immunoreactivity within the neuropil of the SN, suggesting that the loss of SP content within the SN may be secondary to dopaminergic degeneration. This increase in SP expression within the striatum may be due to direct injurious effects of 6-OHDA as occurs in other brain injuries such as TBI and stroke (Turner et al., 2006; Donkin et al., 2007). SP is diffusely upregulated within affected tissue, with an increase in perivascular expression as is seen in the current study. Additionally, the increase of SP could be due to compensatory mechanisms of dopamine neurons. Once these neurons begin to degenerate, the remaining neurons release extra dopamine to keep striatal dopamine at basal levels to allow proper function of the basal ganglia. Within the striatum, DA can bind to SP-containing medium spiny neurons, which project to the SN where they make synaptic connections with dopaminergic neurons. Once SP is released it binds to the NK1 receptors on dopaminergic neurons to potentiate DA release within the striatum. Therefore, SP and DA create a positive feedback mechanism, with each
neurotransmitter able to potentiate the release of the other. Thus SP is also increased with elevated DA production, as would be the case when dopaminergic neurons begin to degenerate.

Following 6-OHDA lesions, a general upregulation in striatal GFAP expression with increased density, number and size of GFAP immunoreactive astrocytes was observed within the area of greatest dopaminergic terminal loss. Furthermore, if needle tract injury was present, hypertrophic, highly immunoreactive GFAP astrocytes would surround the injury site. This is a typical astrocytic response; once activated, astrocytes migrate to the site of injury where they often form a protective barrier around injured tissue, secrete neurotrophic substances like GDNF and BDNF, and induce antioxidant enzyme activation. They have also been shown to secrete pro-inflammatory mediators such as IL-6 (McGeer and McGeer, 2008). This GFAP response is not specific to 6-OHDA since other experimental models of PD also report similar findings (Depino et al., 2003; Takagi et al., 2007). Similar patterns of striatal ED-1 immunoreactivity were seen following 6-OHDA lesions. During the first week post-lesion, presence of ED-1 immunoreactive microglia was demonstrated within the striatum, with further infiltration of these ED-1 immunoreactive macrophages through blood vessels. By day 10 post-lesion, maximal ED-1 expression was demonstrated although an absence of perivascular ED-1 was observed at this time. Unfortunately, ED-1 cannot distinguish microglia from blood-derived macrophages, which are also likely to contribute to the ED-1 immunoreactivity observed (Carson et al., 2006; Rodriguez et al., 2007). Indeed, albumin immunoreactivity within the striatum was increased indicative of a dysfunction in the BBB, which would have allowed infiltration of cells such as macrophages into the brain parenchyma.
Resting microglia are vital in the control of immune and homeostatic functions and are important in the removal of cellular debris. However, once activated they may become destructive by secreting pro-inflammatory mediators, cytokines, reactive oxygen and nitrogen species, and the excitotoxic factors glutamate and quinolinic acid (Mosley et al., 2006; Rock and Peterson, 2006). Similar patterns of astrocytic and microglial activation have previously been observed in the intrastriatal 6-OHDA model of PD, which led to the authors to suggest a possible modulation between these two glial cells populations (Rodrigues et al., 2001). Both astrocytes and microglia express the NK₁ receptor following injury (Mantyh et al., 1989; Chauhan et al., 2008), and interestingly, their appearance corresponded with an upregulation in striatal SP. SP is a known inflammatory mediator that is important in the initiation and progression of CNS inflammation (Harrison and Geppetti, 2001; Hokfelt et al., 2001; Chauhan et al., 2008).

In the SN, similar patterns of GFAP and ED-1 immunoreactivity were also observed, although upregulation in expression did not occur until day 7 with maximal expression of both at day 14 post-lesion. This time corresponds to the time when dopaminergic cell death is occurring most rapidly as assessed by TH immunoreactivity. Additionally, the predominant GFAP expression was re-distributed from mainly within white matter and around blood vessels to around dopaminergic neurons in the SN. Here GFAP containing astrocytes persisted to day 21 post-lesion suggesting that these astrocytes may be performing a neuroprotective function. Astrocytes can produce MAO-B and COMT and as such can stimulate the metabolism of DA, thereby reducing FR production (Hirsch, 2000). ED-1 immunoreactive microglia were also predominantly found surrounding dopaminergic neurons. These microglia were in an active state, with fine processes present. A similar pattern of microglial activation has been previously demonstrated in
this experimental model of PD (Rodrigues et al., 2004; Rodriguez-Pallares et al., 2007), with their activation also having been described in all other experimental models of PD and in the SN of PD patients. Their presence supports the concept of ongoing neuroinflammatory processes that have been implicated in dopaminergic cell death in the SN in vivo (Depino et al., 2003; Streit et al., 2004; Mosley et al., 2006; Qin et al., 2007). Thus, the activated microglia seen in this study are likely to be contributing to dopaminergic cell death.

As previously mentioned, albumin immunoreactivity was increased early following 6-OHDA striatal lesions, which is indicative of BBB dysfunction. The BBB is a vital barrier between the bloodstream and brain tissue. It prevents the influx of unwanted neurotoxic substances such as chemicals, bacteria and inflammatory cells, whilst allowing the passage of essential molecules such as oxygen. Recently, dysfunction of the BBB has been described in neurogenic inflammation (Nimmo et al., 2004), which is an inflammatory response initiated by neuropeptides, resulting in SP-induced plasma extravasation and calcitonin gene related peptide (CGRP)-induced vasodilation (Maggi, 1995; Alves et al., 1999). While well described in the periphery, neurogenic inflammation has recently been shown to occur following brain injury (Donkin et al., 2007; Turner and Vink, 2007) where increased SP expression results in dysfunction of the BBB and subsequent oedema formation (Vink et al., 2003; Nimmo et al., 2004; Turner et al., 2006). While previous studies investigating PD and other neurodegenerative disorders have reported dysfunction of the BBB (Persidsky et al., 2006; Desai et al., 2007; Monahan et al., 2008), the current study suggests that this dysfunction may be related to neurogenic inflammation induced by an increase in SP production.
4.5 Conclusions

The intrastriatal 6-OHDA model of PD induces a progressive loss of striatal dopaminergic terminals, followed by retrograde degeneration of dopaminergic neurons within the ipsilateral SN. This dopaminergic degeneration produces striatal dopamine deficiency and mild functional deficits. Motor deficits were particularly apparent and were seen throughout the assessment period. Behavioural and overall neurological outcome was also affected. The dopaminergic terminal and cell loss was associated with an astrocytic and microglial response, which was present early in the striatum but was delayed in the SN until overt dopaminergic degeneration was present, suggesting a role for inflammation in neurodegeneration. Importantly, striatal injections of 6-OHDA produced an increase in the neuropeptide SP, a known inflammatory mediator. This increase in SP not only corresponded with the presence of activated astrocytes and microglia and BBB breakdown, but also with the degeneration of dopaminergic terminals and neurons. Accordingly, the intrastriatal 6-OHDA model replicates many of the features of the early stages of PD and thus may be used to characterise the role that SP may play in the pathophysiology of PD.