Development and characterisation of a humanised model of Parkinson’s disease associated with delivery of adeno-associated virus expressing alpha-synuclein and pre-formed alpha-synuclein fibrils unilaterally into the rat substantia nigra

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5. Utilising a combination of adeno-associated viral vector mediated expression of A53T human alpha-synuclein combined with A53T human alpha-synuclein preformed fibrils to generate a model of synucleinopathy

5.1. Introduction

Amyloidogenic aggregation of alpha-synuclein, a pre-synaptic protein which is normally soluble, leads to the development of Lewy bodies intraneuronally. These inclusions are a pathological hallmark of Parkinson’s disease (PD). These alpha-synuclein inclusions are also present in a number of other neurodegenerative diseases, including Lewy Body Dementia (LBD) and Multiple Systems Atrophy (MSA), which are included in the umbrella term of synucleinopathies (Waxman and Giasson, 2009, Goedert, 1997, Cookson, 2005). It has been found that the endogenous levels of alpha-synuclein increase with age (Chu and Kordower, 2007).

Braak’s model of PD hypothesises that a hypothetical “agent”, most likely alpha-synuclein, spreads both anterogradely and retrogradely between areas which are connected by axonal projections and that this leads to the formation of Lewy bodies (Braak et al., 2002, Braak et al., 2003a). Research in primary neuronal cultures has found that human wildtype (WT) alpha-synuclein fibrils are transported both anterogradely and retrogradely, and that when alpha-synuclein was released from the neurons into the media it was the intact, undegraded form of the protein (Brahic et al., 2016). This study also indicated that the release of alpha-synuclein into the media was not as a result of axonal lysis. Addition of small seeds of WT mouse alpha-synuclein preformed fibrils (PFFs) to primary neuronal cultures derived from WT non-transgenic mice leads to the endocytosis of these PFFs into the neurons in the absence of any factors...
being added to the culture to promote this endocytosis and this then leads to the recruitment of endogenous alpha-synuclein, cumulating in the formation of abnormal, phosphorylated, insoluble and ubiquitinated aggregates (Volpicelli-Daley et al., 2014b).

Studies using human alpha-synuclein fibril injection into rodent brains have found that human alpha-synuclein is only detectable for a short period of time after injection (Sacino et al., 2013, Masuda-Suzukake et al., 2013). It is hypothesised that the endogenous alpha-synuclein is recruited into the Lewy body like inclusions observed in these models. Findings in human induced pluripotent stem cells (iPSCs) also provide support for the anterograde and retrograde transport of alpha-synuclein (Gribaudo et al., 2019). This study also showed the transport of alpha-synuclein fibrils interneuronally. Furthermore, this study replicated the findings shown in primary neuronal cultures of mice in that it found that incubation with exogenous alpha-synuclein led to the development of inclusions which were composed of endogenous phosphorylated alpha-synuclein.

The use of adeno-associated viral (AAV) technology to produce pre-clinical models of PD has allowed for the advancement of the field of research and the generation of a better understanding of the protein alpha-synuclein. However, it has been found that the aggregates of alpha-synuclein produced in this model do not seem to promote the expression of filamentous alpha-synuclein, which is a key feature of the aggregates observed in human PD (Volpicelli-Daley et al., 2016b). Furthermore, while the use of AAV relies mainly on the overexpression of alpha-synuclein above the physiological levels observed in human PD, PFFs can be used to express levels of alpha-synuclein much closer to the human condition, making this method more translatable (Polinski et al., 2018). Research in vitro has found that when mouse primary hippocampal neurons were exposed to PFFs generated from full length and truncated recombinant alpha-synuclein, endogenous alpha-synuclein was adequate for the formation of insoluble PD-like Lewy bodies and Lewy neurite aggregates, and no overexpression of alpha-synuclein, WT or mutated, was required (Volpicelli-
Daley et al., 2011). As human disease involves the recruitment of endogenous alpha-synuclein, this finding suggests that the use PFFs may be a more translatable model of PD. The use of alpha-synuclein PFFs leads to the development of many of the pathological features in neurons including hyperphosphorylation, ubiquitination, insolubility in anionic detergent, and the development of inclusions which represent Lewy neurites and Lewy bodies (Volpicelli-Daley et al., 2011, Volpicelli-Daley et al., 2014b, Volpicelli-Daley et al., 2016b).

Research in C57BL/6 J mice found that injection of 10µg of human or mouse alpha-synuclein fibrils unilaterally into the substantia nigra led to the development of abnormal phosphorylated alpha-synuclein positive inclusions at 15 months post-injection (Masuda-Suzukake et al., 2013). Bilateral expression of phosphorylated alpha-synuclein pathology was observed in these mice, although the injection was unilateral. The spreading of phosphorylated alpha-synuclein from the injected to the un-injected hemisphere has been replicated in other studies (Masuda-Suzukake et al., 2014; Okuzumi et al., 2018). Injection of mouse PFFs unilaterally into the striatum of mice was found to lead to the development of phosphorylated alpha-synuclein inclusions in both hemispheres of the brain, with callosotomy (severing the corpus callosum so that communication between the cerebral hemispheres is interrupted) before injection of the fibrils greatly lessening the extent of pathology observed on the contralateral side. However, callostomy 24 hours post fibril injection did not prevent the expression of phosphorylated alpha-synuclein in both hemispheres, suggesting that this is a process which happens very shortly after injection of PFFs (Okuzumi et al., 2018).

One of the first known studies investigating the impact of mouse alpha-synuclein PFFs in Sprague Dawley rats involved delivery of PFFs to either one or two sites in the striatum, totalling 8µg (Paumier et al., 2015). Similar to the research in mice, this study found that unilateral injection of alpha-synuclein PFFs led to bilateral expression of alpha-synuclein, although this was observed in the cortex and not the substantia nigra. Interestingly, a decrease in TH
immunoreactive cells was observed in the substantia nigra in both the injected and un-injected hemispheres, while alpha-synuclein pathological-like inclusions were only expressed ipsilateral to the injection site at 180 days post-injection. However, behavioural investigations did not find any significant effects of the alpha-synuclein expression at either 90 or 180 days post-injection. When interpreting this result it is important to note that the tests employed in this study would show a unilateral deficit, when in fact from the TH immunoreactive cell loss observed, a test of bilateral motor function would have been more useful.

Seeding of alpha-synuclein and its potential implications in the clinic is a very important research area due to the increased interest in the use of foetal dopaminergic neurons as potential transplant opportunities for PD patients. Studies in which foetal dopaminergic neuronal transplants were made found that some of these transplanted neurons began to form Lewy bodies in a subset of patients (Kordower et al., 2008, Li et al., 2008). Previous research has utilised the injection of both PFF and AAV vector human alpha-synuclein injection into the substantia nigra in female Sprague Dawley rats. More specifically, AAV-6 alpha-synuclein was injected into 2 brain regions, the substantia nigra and ventral tegmental area, and then four weeks later alpha-synuclein PFFs were injected into the same sites (Thakur et al., 2017). This study found a significant decrease in TH immunoreactive cells in the substantia nigra, most predominately in the combination treatment group at seven weeks post-AAV injection, with a lower decrease in TH immunoreactive cells observed in the single treatment groups at this timepoint. As time progressed to 28 weeks post-AAV injection, the difference in TH immunoreactive cell loss was not observed to be significantly different between the groups. When behaviour endpoints were analysed, it was found that the combination treatment group developed limb use asymmetry, assessed through the cylinder test and stepping test, as early as three weeks post-PFF injection which was seven weeks post-AAV injection. This study found that this impairment remained consistent when measured at multiple timepoints post-surgery, with a significant difference
observed when the combination treatment group was compared to either of the single treatment groups.

Combination of WT human alpha-synuclein PFFs with AAV2/7 A53T human alpha-synuclein administered at the same time to the same injection site in the substantia nigra of female Wistar rats led to the death of TH immunoreactive neurons and a severe reduction in the striatal nerve terminal volume, which was much greater than that observed when alpha-synuclein PFFs were injected alone, at four months post-injection. This study also observed that the greatest motor deficit was observed in the combination treatment group as assessed by the cylinder test, when compared with PFF alone (Peelaerts et al., 2015).

Taken together, the above studies suggest that the development of a model of alpha-synucleinopathy which utilises both AAV and PFF expression of alpha-synuclein may lead to the development of a robust model which demonstrates both neurodegeneration and motor behavioural deficits. While the studies above have either expressed alpha-synuclein PFFs alone, administered alpha-synuclein PFFs and AAV alpha-synuclein at two separate timepoints to the same injection site, or delivered both AAV and PFF to the same injection site at the same time, we aimed to develop a model which utilised the two separate injections of AAV and PFF into two separate injection sites in the substantia nigra during the same surgery. Furthermore, the impact of these injections on motor behaviour was assessed through a battery of motor tests. Finally, through the inclusions of both male and female rats, we sought to investigate if males and females would react differently, either at a molecular or behavioural level. While most studies in this research area have worked with Sprague Dawley rats (Paumier et al., 2015, Thakur et al., 2017), this study employed Wistar rats.
5.2. Study Aims and Objectives

The aim of this study was to establish and characterise an animal model of PD by overexpression of A53T human alpha-synuclein, or WT human alpha-synuclein through the use of AAV in combination with PFF to the rat substantia nigra. We aimed to examine the effects of AAV1/2-CMV-A53T alpha-synuclein human (2µl; 5.1x10^{12} vg/ml) injection in combination with PFF A53T alpha-synuclein human (2µl; 4µg) at a behavioural and immunohistochemical level in vivo in rats and compare this to the expression of AAV5-CBA-WT alpha-synuclein human (2µl; 1x10^{13}vg/ml) injection in combination with PFF WT alpha-synuclein human (2µl; 4µg). We also investigated the impact of expression of PFF A53T alpha-synuclein human by itself, and the injection of PFF WT alpha-synuclein human alone.

Our main objective was to assess the effectiveness of a unilateral intranigral injection of AAV1/2-CMV-A53T alpha-synuclein in combination with PFF A53T alpha-synuclein in inducing the expression of alpha-synuclein and the impact of this on unilateral motor function when compared with AAV5-CBA-WT alpha-synuclein injection in combination with PFF WT alpha-synuclein.

Specifically, we aimed to investigate whether two unilateral injections of AAV1/2-CMV-A53T alpha-synuclein and PFF A53T would lead to clear expression of alpha-synuclein at six weeks post-surgery, and if this induced any motor dysfunction over a six week timeline following stereotactically delivered injections.
5.3. Methods

5.3.1 Justification of Experimental Design

AAV5 and AAV1/2 are the serotypes used as they have been shown in Chapter 3 and Chapter 4 to lead to the expression of alpha-synuclein. The titres utilised are those used in Chapters 3 and 4 and are therefore adopted in the present study for consistency. PBS and Empty Vector are used as controls to control for the impact of an injection and the impact of expression of an AAV vector respectively. GFP is included to explore the impact of the expression of a foreign protein in the substantia nigra. However, GFP will not be directly compared to the AAV-A53T alpha-synuclein group due to the presence of the miR scrambled control alpha-synuclein. All intranigral injections performed are unilateral in nature as this allows for the animal to be used as its own control and is a commonly adopted approach in preclinical PD research.

The WT and A53T PFFs were made available for inhouse validation from StressMarq Biosciences. The amount of PFF injected was as per manufacturers recommendations. Combination injection of both AAV and PFFs were utilised as previous research had provided promising results in relation to the utilisation of this method of delivery as a means to preclinically model PD (Paumier et al., 2015, Thakur et al., 2017).

The motor behavioural tests which are carried out are the cylinder test, staircase test, stepping test and amphetamine rotation test as these tests are established and routinely performed in the laboratory and are utilised widely in preclinical PD research. Statistical analysis is performed for both sexes combined, and then males and females will be separately analysed, where appropriate. As the injections which are performed are unilateral, where possible comparisons are made within group between the ipsilateral and contralateral sides, allowing the animals to be used as their own controls. An 6 week timepoint is chosen based on the alpha-synuclein expression observed following a single unilateral injection of AAV as outlined in Chapters 3 and 4.
5.3.2 Experimental Timeline

Behavioural testing in the staircase test, stepping test and cylinder test was carried out prior to any surgical intervention as a baseline measure of performance. Post-surgical behavioural testing was carried out in the staircase test, stepping test, cylinder test and amphetamine rotation test, as outlined in the experimental timeline in Figure 5.1.
Figure 5.1: Experimental timeline for the in vivo phase including behavioral testing timepoints, treatment groups, and tissue collection timepoint.

Baseline behavior training:
- Staircase Test
- Cylinder Test
- Staircase Test

Baseline behavior collection:
- ~2 Weeks postsurgery: Staircase test
- ~3 Weeks postsurgery: Stepping Test
- ~4 Weeks postsurgery: Staircase Test
- Cylinder Test
- Stepping Test
- ~4.5 Weeks postsurgery: Stepping Test
- ~5 Weeks postsurgery: Amphetamine rotation Test

Pre-Surgery | Surgery | Post-Surgery | Tissue Collection

Groups:
- Sterile PBS [n=9; 5 males, 4 females]
- AAV12-CAG-miR scrambled control alpha synuclein x3 co-expressing eGFP (2 μl; >0.5x10^12 vg/ml) [n=9; 4m, 5f]
- PFF WT alpha-syn (2μl) [n=10; 5m, 5f]
- AAV5-CBA-alpha-synuclein WT (2μl; 1x10^13 vg/ml) + PFF WT alpha-syn (2μl) [n=10, 5m, 5f]
- PFF A53T alpha-syn (2μl) [n=10; 6m, 4f]
- AAV12-CMV-A53T alpha-synuclein (human) (2μl; >5x10^12 vg/ml) + PFF A53T alpha-syn (2μl) [n=10; 5m, 5f]

6 weeks post-surgery Transcardial Perfusion
5.3.3 Experimental Design

All rats were habituated to the housing unit for at least two weeks before any experimental procedures commenced. All rats received 10 x 10 minute training sessions in the staircase test in order to establish proficiency in the test. Rats received one or two unilateral injections into the substantia nigra as outlined in Table 5.1 below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus /PFF</th>
</tr>
</thead>
</table>
| 1     | • MALE: Sterile PBS (AP -5.3mm; ML +2.0mm; DV -8.5mm)  
• FEMALE: Sterile PBS (AP -5.3mm; ML +2.0mm; DV -7.5mm) |
| 2     | • MALE: AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP  
(AP -5.3mm; ML +2.0mm; DV -8.5mm)  
• FEMALE: AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP  
(AP -5.3mm; ML +2.0mm; DV -7.5mm) |
| 3     | • MALE: WT alpha-synuclein SPR-322 Type1 PFFs; human  
(AP -5.3mm; ML +2.0mm; DV -8.5mm)  
• FEMALE: WT alpha-synuclein SPR-322 Type1 PFFs; human  
(AP -5.3mm; ML +2.0mm; DV -7.5mm) |
| 4     | • MALE: A53T alpha-synuclein SPR-326 Type1 PFFs; human  
(AP -5.3mm; ML +2.0mm; DV -8.5mm)  
• FEMALE: A53T alpha-synuclein SPR-326 Type1 PFFs; human  
(AP -5.3mm; ML +2.0mm; DV -7.5mm) |
| 5     | • MALE: AAV5-CBA-alpha-synuclein human WT  
(AP -5.3mm; ML +2.0mm; DV -8.5mm)  
+ WT alpha-synuclein SPR-322 Type1 PFFs; human  
(AP -4.9mm; ML +2.0mm; DV -8.5mm)  
• FEMALE: AAV5-CBA-alpha-synuclein human WT  
(AP -5.3mm; ML +2.0mm; DV -7.5mm)  
+ WT alpha-synuclein SPR-322 Type1 PFFs; human  
(AP -4.9mm; ML +2.0mm; DV -7.5mm) |
| 6     | • MALE: AAV1/2-CMV-A53T alpha-synuclein  
(AP -5.3mm; ML +2.0mm; DV -8.5mm)  
+ A53T alpha-synuclein SPR-326 Type1 PFFs; human  
(AP -4.9mm; ML +2.0mm; DV -8.5mm)  
• FEMALE: AAV1/2-CMV-A53T alpha-synuclein  
(AP -5.3mm; ML +2.0mm; DV -7.5mm)  
+ A53T alpha-synuclein SPR-326 Type1 PFFs; human  
(AP -4.9mm; ML +2.0mm; DV -7.5mm) |

**Table 5.1:** Virus Titre, Virus and PFF Injection Volumes and Coordinates.
5.3.4 Treatment Groups

- Sterile PBS 2 µl – single unilateral injection

- AAV1/2-CAG-miR scrambled control alpha-synuclein co-expressing eGFP; 2 µl; 0.5x10^{12} vg/ml – single unilateral injection

- WT alpha-synuclein PFF SPR-322, Type1 PFFs; human; 2 µl; 4 µg – single unilateral injection

- A53T alpha-synuclein SPR-326, Type1 PFFs; human; 2 µl; 4 µg – single unilateral injection

- AAV5-CBA-alpha-synuclein human WT 2 µl; 1x10^{13} vg/ml + WT alpha-synuclein PFF SPR-322, Type1 PFFs; human; 2 µl; 4 µg – two unilateral injections

- AAV1/2-CMV-A53T alpha-synuclein human 2 µl; 5.1x10^{12} vg/ml + A53T alpha-synuclein SPR-326, Type1 PFFs; human; 2 µl; 4 µg – two unilateral injections

As only two groups have involved two unilateral injections, this is an important consideration in relation to analysis and group comparisons.
5.3.5 Adaptations to protocols

5.3.5.1 Staircase Test - Training

Due to the variability in the number of pellets retrieved across the different studies at baseline, for the AAV + PFF study the animals were all exposed to 10 x 10 minute trials as training in the staircase test before baseline data collection in order to improve baseline performance (Figure 5.2).

Figure 5.2: Exposure to the staircase test for 10 sessions of training in order to improve baseline performance. A) Both sexes combined, n = 60 B) Males only, n = 30 C) Females only, n = 30. The red line represents the usual three exposures the rats had previously for training in the staircase test.
5.3.5.2 Stepping Test

For baseline testing and post-surgical testing the animals are exposed to three trials for each limb in each direction. This increase in trials is included to account for variability which may arise due to the animals being stressed in the restraint at the beginning of testing. By repeating the test three times for each limb in each direction it allows the animal to become more comfortable and less rigid in the restraint.

5.3.5.3 Cylinder Test

The animals are exposed to the apparatus twice, at baseline and four weeks post-AAV delivery. The testing of the males and females takes place in differently sized cylinders. As the males grow more than the females they are placed in a larger cylinder (29cm diameter x 44cm height) than the females, allowing them space to move more freely. The females are tested in a cylinder of dimensions 19cm diameter x 50cm height. In order to allow scoring of both sides, as well as floor placements, the recordings are made from the side. This involves two cameras set up on either side of the cylinder in order to record all wall placements and floor landings.

5.3.5.4 Amphetamine Rotation Test

All tests are recorded for 120 minutes post-amphetamine administration. The test is performed in cylinders, with different size cylinders for females (19cm diameter x 50cm height) and males (29cm diameter x 44cm height). The behaviour is recorded, tracked and scored using ANYmaze automated software.

Due to the change from manual scoring to automated tracking and scoring, the correlation between subjective scores and the automated scoring system was tested. Analysis of the PBS and PFF + AAV A53T groups shows a correlation between the scores from the automated system and those obtained
subjectively. Subjective scores are however generally higher than those obtained objectively through ANYmaze.

**Figure 5.3:** Comparison of the automated scoring of the rotational behaviour with subjective scoring of the recordings by an observer. Analysis of the PBS-injected animals contralateral rotations (a), PBS-injected animals ipsilateral rotations (b), PFF A53T + AAV A53T-injected animals contralateral rotations (c) and PFF A53T + AAV A53T-injected animals ipsilateral rotations (d), comparing scores by the automated tracking software (ANYmaze) with subjective scores by an observer blind to the treatment groups. Data presented as number of rotations. Pearson correlation. n = 8 – 10.
The amphetamine rotation test is performed for 120 minutes post-amphetamine administration, as it is found that the amphetamine-induced rotations persisted for at least 120 minutes post-administration (Figure 5.4).

**Figure 5.4:** The total number of ipsilateral rotations as observed over a 120 minute period. While there is a clear peak of rotations in the first 50 minutes of the test, the rotational behaviour continues up to the 120 minute timepoint in the PFF A53T + AAV A53T group, which is why the duration of the amphetamine rotation test is increased over the span of this project, n = 8 – 10.
5.3.5.5 Immunohistochemistry - Tissue processing

All brain slices are collected on the slide, with two series of five slides, with four sections per slide collected for the substantia nigra.

Sections directly mounted on the slide immunohistochemistry and immunofluorescence is employed in this study.

5.3.5.6 Immunohistochemistry - Image Analysis

The number of tyrosine hydroxylase (TH) positive cells are manually counted using the cell counter tool in ImageJ. Two 40X images of the substantia nigra per hemisphere are acquired in the same locations in each hemisphere and the number of TH positive cells per image are counted. The number of cells per hemisphere is totalled (sum of the two images of that hemisphere) and expressed as a percentage of the un-injected hemisphere.

5.3.5.7 Immunofluorescence - Image Analysis

The images are converted to an 8-bit image using ImageJ. A threshold is then set which removes most of the background staining in the image. The Analyse -> Measure function is then utilised on Image J. From this the mean pixels values/arbitrary units values are generated. This is then expressed as a percentage of the un-injected side, as well as being expressed as arbitrary units.
5.4 Results

5.4.1 Alpha-synuclein immunofluorescence

5.4.1.1 Review of the alpha-synuclein injected animals found varying immunofluorescence across the substantia nigra and between animals at 6 weeks post-AAV PFF delivery.

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Treatment Group</th>
<th>Number of Sections with alpha-synuclein immunofluorescence</th>
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<tbody>
<tr>
<td>58</td>
<td>PFF A53T + AAV A53T</td>
<td>6/8</td>
</tr>
<tr>
<td>41</td>
<td>PFF A53T + AAV A53T</td>
<td>6/8</td>
</tr>
<tr>
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<td>PFF A53T + AAV A53T</td>
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</tr>
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<td>PFF A53T + AAV A53T</td>
<td>4/4</td>
</tr>
<tr>
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Table 5.2: Visible alpha-synuclein immunofluorescence across the substantia nigra in the alpha-synuclein treatment groups at six weeks post-AAV PFF delivery. It was found that the immunofluorescence of alpha-synuclein varied across treatment groups, and across with substantia nigra, with some animals expressing alpha-synuclein across the entire area analysed and others expressing alpha-synuclein in only a number of sections in the substantia nigra.
The following images are representative immunofluorescent sections of the substantia nigra showing alpha-synuclein and TH immunofluorescence and their co-localisation in WT alpha-synuclein PFF treated male and female rats at six weeks post-PFF delivery. Images of both injected and un-injected sides are shown for comparative purposes (Figure 5.5).

![Image of immunofluorescent sections](image.png)

**Figure 5.5**: Representative images of alpha-synuclein and tyrosine hydroxylase immunofluorescence in the substantia nigra six weeks post-PFF delivery. (A) WT alpha-synuclein PFF injected male, injected hemisphere, (B) WT alpha-synuclein PFF injected male, un-injected hemisphere, (C) WT alpha-synuclein PFF injected female, injected hemisphere, (D) WT alpha-synuclein PFF injected female, un-injected hemisphere. First column represents alpha-synuclein immunofluorescence, second column represents tyrosine hydroxylase immunofluorescence, third column represents merged images of the first 2 columns. Images acquired at 20X.
The following images are representative immunofluorescent sections of the substantia nigra showing alpha-synuclein and TH immunofluorescence and their co-localisation in A53T alpha-synuclein PFF treated male and female rats at six weeks post-PFF delivery. Images of both injected and un-injected sides are shown for comparative purposes (Figure 5.6).

**Figure 5.6:** Representative images of alpha-synuclein and tyrosine hydroxylase immunofluorescence in the substantia nigra six weeks post-PFF delivery. (A) A53T alpha-synuclein PFF injected male, injected hemisphere, (B) A53T alpha-synuclein PFF injected male, un-injected hemisphere, (C) A53T alpha-synuclein PFF injected female, injected hemisphere, (D) A53T alpha-synuclein PFF injected female, un-injected hemisphere. First column represents alpha-synuclein immunofluorescence, second column represents tyrosine hydroxylase immunofluorescence, third column represents merged images of the first 2 columns. Images acquired at 20X.
The following images are representative immunofluorescent sections of the substantia nigra showing alpha-synuclein and TH immunofluorescence and their co-localisation in the combination of WT alpha-synuclein PFF with WT alpha-synuclein AAV treated male and female rats six weeks post-AAV PFF delivery. Images of both injected and un-injected sides are shown for comparative purposes (Figure 5.7).

**Figure 5.7:** Representative images of alpha-synuclein and tyrosine hydroxylase immunofluorescence in the substantia nigra six weeks post-AAV PFF delivery. (A) WT alpha-synuclein PFF + WT alpha-synuclein AAV injected male, injected hemisphere, (B) WT alpha-synuclein PFF + WT alpha-synuclein AAA injected male, un-injected hemisphere, (C) WT alpha-synuclein PFF + WT alpha-synuclein AAV injected female, injected hemisphere, (D) WT alpha-synuclein PFF + WT alpha-synuclein AAV injected female, un-injected hemisphere. First column represents alpha-synuclein immunofluorescence, second column represents tyrosine hydroxylase immunofluorescence, third column represents merged images of the first 2 columns. Images acquired at 20X.
The following images are representative immunofluorescent sections of the substantia nigra showing alpha-synuclein and TH immunofluorescence and their co-localisation in the combination of A53T alpha-synuclein PFF with A53T alpha-synuclein AAV treated male and female rats six weeks post-AAV PFF delivery. Images of both injected and un-injected sides are shown for comparative purposes (Figure 5.8).

**Figure 5.8:** Representative images of alpha-synuclein and tyrosine hydroxylase immunofluorescence in the substantia nigra six weeks post-AAV PFF delivery. (A) A53T alpha-synuclein PFF + A53T alpha-synuclein AAV injected male, injected hemisphere, (B) A53T alpha-synuclein PFF + A53T alpha-synuclein AAV injected male, un-injected hemisphere, (C) A53T alpha-synuclein PFF + A53T alpha-synuclein AAV injected female, injected hemisphere, (D) A53T alpha-synuclein PFF + A53T alpha-synuclein AAV injected female, un-injected hemisphere. First column represents alpha-synuclein immunofluorescence, second column represents tyrosine hydroxylase immunofluorescence, third column represents merged images of the first 2 columns. Images acquired at 20X.
Following a review of all the immunofluorescent staining in this study it was found that four of the males in the PFF and AAV WT group had accidentally been injected with AAV-eGFP as opposed to their correct treatments. These animals were removed from all further analysis, therefore there are only 5 treatment groups presented for the analysis of males alone, with the PFF WT + AAV WT treatment group removed.

5.4.1.2 Analysis of alpha-synuclein immunofluorescence identified an increase in alpha-synuclein immunofluorescence when the PFF WT + AAV WT and PFF A53T + AAV A53T groups were compared to PBS injected animals.

Comparison of alpha-synuclein immunofluorescence across the groups when analysed as percentage of the un-injected side found an effect (H(5) = 17.24, \( p = 0.0041 \)). Post-hoc comparisons identified differences between the PBS and PFF WT + AAV WT (\( p = 0.0265 \)) and PBS and PFF A53T + AAV A53T (\( p = 0.0093 \)) groups. No differences were identified when males and females were analysed separately (data not presented).

**Figure 5.9:** Injection of PFF WT + AAV WT or PFF A53T + AAV A53T led to increased alpha-synuclein immunofluorescence in the substantia nigra when compared with the PBS injected group at six weeks post-AAV PFF delivery. Data presented as median with minimum and maximum. Kruskal-Wallis test followed by Dunn’s multiple comparisons test. \( n = 3 – 10. * p < 0.05, ** p < 0.01. \)
In previous studies utilising free floating immunofluorescence, more apparent alpha-synuclein immunofluorescence was observed, which was highly localised in cells, however in the present study this was not as clear. It was therefore decided that sections from animals in the PFF A53T + AAV A53T alpha-synuclein group would be stained in the absence of primary antibody for alpha-synuclein but in the presence of the secondary antibody. This involved staining with the primary antibody for tyrosine hydroxylase alone, in combination with the secondary antibodies for both tyrosine hydroxylase and alpha-synuclein. As illustrated below, immunofluorescence was apparent when the primary antibody for alpha-synuclein was omitted, with this immunofluorescence being much more evident on the injected than the un-injected hemisphere. This is potentially due to high background immunofluorescence caused by the secondary antibody. However, due to the differences observed between the injected and un-injected hemispheres, it is also possible that this difference may be due to the presence of endogenous alpha-synuclein and its potential cross-reactivity with the secondary antibody employed. As it has been found that injection of exogenous alpha-synuclein can lead to the recruitment of endogenous alpha-synuclein, this is one possible explanation for this finding.
Figure 5.10: Immunofluorescence was observed in the injected hemisphere of the substantia nigra for alpha-synuclein in the absence of the primary antibody for alpha-synuclein. A) Injected hemisphere of an AAV A53T + PFF A53T injected animal, B) Un-injected hemisphere of an AAV A53T + PFF A53T injected animal. First column represents immunofluorescence from the secondary antibody utilised for alpha-synuclein immunofluorescence in the absence of the primary antibody for alpha-synuclein. Second column represents tyrosine hydroxylase immunofluorescence. Third column represents a merged image of the first two columns.
5.4.2 Cylinder Test

5.4.2.1 No differences in limb use were identified when the sexes were analysed together at baseline in the cylinder test.

No differences in limb use were identified when the sexes were analysed together at baseline in the cylinder test. Data presented in Appendix III.

5.4.2.2 Intranigral injection of AAV1/2-CMV-A53T alpha-synuclein in combination with PFF A53T led to a decrease in the use of the contralateral limb with both sexes combined when compared with the PBS treated group when the data were analysed as percentage of total limb contacts at four weeks post-AAV PFF delivery.

An effect was identified at four weeks post-AAV PFF delivery for percentage contralateral wall contacts \((H(5) = 14.60, p = 0.0122)\) with a difference identified between the PBS and PFF A53T + AAV A53T groups post-hoc \((p = 0.0137)\).

Analysis of the left wall contacts as a percentage of total wall contacts identified an effect, with no differences identified between the treatment groups post-hoc \((F(5.000, 24.79) = 6.030, p = 0.0009)\).

Analysis of contralateral wall contacts as raw data at four weeks post-AAV PFF delivery did not identify any effects \((H(5) = 6.526, p = 0.2583)\).

Similarly, analysis of ipsilateral wall contacts at four weeks post-AAV PFF delivery did not identify any effects when data were analysed as raw data \((F(5, 48) = 1.576, p = 0.1847)\).

Analysis of simultaneous wall contacts found no effects when data were analysed as raw data \((F(5, 48) = 0.3060, p = 0.9070)\) or as percentage of total wall contacts made \((H(5) = 6.371, p = 0.2718)\) (Figure 5.11).
Figure 5.11: Performance in the cylinder test four weeks post-AAV PFF delivery with both sexes combined. Decreased percentage contralateral wall contacts were found in the PFF A53T + AAV A53T group when compared with PBS at four weeks post-AAV PFF delivery (b). Data presented as mean with SEM (c, e), median with minimum and maximum (a, b, f) or mean with standard deviation (d). One-way ANOVA (c, e), Kruskal-Wallis test followed by Dunn’s multiple comparisons test (a, b, f), or Brown-Forsythe ANOVA test followed by Dunnett’s T3 multiple comparisons test (d). n = 6-10. * p < 0.05.
5.4.2.3 Intranigral injection of AAV1/2-CMV-A53T alpha-synuclein in combination with PFF A53T led to an increase in the use of the ipsilateral limb in males alone when compared with all other treatment groups when the data were analysed as percentage of total limb contacts.

An effect was identified at four weeks post-AAV PFF delivery for percentage ipsilateral wall contacts when males were analysed alone (F(4, 20) = 6.738, \( p = 0.0013 \)) with a difference identified between the PFF A53T + AAV A53T group and the PBS (\( p = 0.0013 \)), eGFP (\( p = 0.0123 \)), PFF WT (\( p = 0.0067 \)), and PFF A53T (\( p = 0.0085 \)) groups through post-hoc comparisons.

No effects were identified when the number of contralateral wall contacts was assessed as raw data (H(4) = 8.259, \( p = 0.0825 \)), when the number of ipsilateral wall contacts was assessed as raw data (F(4, 20) = 1.300, \( p = 0.3037 \)), or when the number of simultaneous wall contacts was assessed as raw data (F(4, 20) = 0.2534, \( p = 0.9042 \)).

Similarly, analysis of contralateral wall contacts as a percentage of total wall contacts did not identify any effects (H(4) = 8.267, \( p = 0.0823 \)) nor did analysis of simultaneous wall contacts as a percentage of total wall contacts (H(4) = 4.160, \( p = 0.3848 \)) (Figure 5.12).
Figure 5.12: Performance in the cylinder test four weeks post-AAV PFF delivery for males alone. Increased percentage ipsilateral wall contacts were found in the PFF A53T + AAV A53T group at four weeks post-AAV PFF delivery when males were analysed alone (d). Data presented as mean with SEM (c, d, e) or median with minimum and maximum (a, b, f). One-way ANOVA followed by Newman-Keuls multiple comparisons test (c, d, e), or Kruskal-Wallis test (a, b, f). n = 4-6. ** p < 0.01
5.4.2.4 No effects were identified four weeks post-AAV PFF delivery in the cylinder test in females alone.

No effects were identified when the number of contralateral wall contacts was assessed as raw data \( (F(5, 22) = 0.1809, p = 0.9669) \), when the number of ipsilateral wall contacts was assessed as raw data \( (F(5, 22) = 1.892, p = 0.1368) \), or when the number of simultaneous wall contacts was assessed as raw data \( (F(5, 22) = 1.077, p = 0.4003) \).

Similarly, no effects were identified when the data were analysed as a percentage of the total wall contacts for either contralateral wall contacts \( (H(5) = 6.964, p = 0.2233) \), ipsilateral wall contacts \( (F(5, 22) = 1.199, p = 0.3421) \), or simultaneous wall contacts \( (H(5) = 5.319, p = 0.3782) \) (Figure 5.13).
Figure 5.13: Performance in the cylinder test four weeks post-AAV PFF delivery for females alone. No differences were identified in the cylinder test at four weeks post-AAV PFF delivery when females were analysed alone. Data presented as mean with SEM (a, c, d, e) or median with minimum and maximum (b, f). One-way ANOVA (a, c, d, e) or Kruskal-Wallis test (b, f). n = 4-5.
5.4.2.5 No effects were identified for floor landings when data were analysed as raw data or as a percentage of the total floor landings at either baseline or four weeks post-AAV PFF delivery.

Analysis of the number of floor landings as raw data and as percentage did not identify any effects at baseline or four weeks post-AAV PFF delivery when both sexes were combined, when males were analysed alone, or when females were analysed alone (data not presented).

5.4.2.6 Limb use asymmetry was not observed at four weeks post-AAV PFF delivery as assessed by the limb use asymmetry score in any of the treatment groups.

Limb use asymmetry was assessed at baseline and again at four weeks post-AAV PFF delivery. No effects were identified at baseline for both sexes combined ($H(5) = 3.867$, $p = 0.5687$), for males alone ($F(4, 20) = 0.9541$, $p = 0.4539$), or for females alone ($F(5, 22) = 0.3703$, $p = 0.8635$).

Similarly, at four weeks post-AAV PFF delivery no effects were identified for both sexes combined ($F(5, 48) = 2.226$, $p = 0.0668$), for males alone ($F(4, 20) = 1.451$, $p = 0.2543$), or for females alone ($H(5) = 6.890$, $p = 0.2289$) (Figure 5.14).
Figure 5.14: Limb use asymmetry in the cylinder test at baseline and four weeks post-AAV PFF delivery. No differences in limb use asymmetry were observed at baseline or four weeks post-AAV delivery in the cylinder test. Data presented as mean with SEM (b, c, d, e) or median with minimum and maximum (a, f). One-way ANOVA (b, c, d, e) or Kruskal-Wallis test (a, f). n = 6 – 10 (a, d) both sexes combined, n = 4 – 6 (b, e), males alone n = 4 – 5 (c, f) females alone.
5.4.3 Stepping Test

5.4.3.1 Baseline assessment of forelimb akinesia in the stepping test did not identify any differences between the treatment groups, when both sexes were analysed together or when females were analysed alone.

Baseline assessment of forelimb akinesia in the stepping test did not identify any differences between the treatment groups, when both sexes were analysed together or when females were analysed separately. Data presented in Appendix III.

5.4.3.2 When males were analysed alone at baseline a difference was identified in the PBS group, with greater percentage contralateral backwards steps observed when compared with their respective percentage ipsilateral steps.

This difference in percentage steps at baseline is unusual, as it is expected that there will be no differences observed at baseline. Interpretation of results post-AAV PFF delivery must take into account this baseline difference in performance. Data presented in Appendix III.

5.4.3.3 Forelimb akinesia was identified in both the forward and backward direction in the AAV1/2-CMV-A53T alpha-synuclein group when percentage steps were analysed at three weeks post-AAV PFF delivery.

Analysis of the number of forward steps at three weeks post-AAV PFF delivery identified an effect of side (Interaction F(5, 96) = 2.220, \( p = 0.0584 \); Side F(1, 96) = 16.65, \( p < 0.0001 \); Treatment F(5, 96) = 1.893, \( p = 0.1026 \)). Post-hoc comparisons did not identify any differences between the treatment groups.

When the number of backward steps were analysed an effect of side was also identified (Interaction F(5, 96) = 2.295, \( p = 0.0513 \); Side F(1, 96) = 4.302, \( p = 0.0408 \); Treatment F(5, 96) = 1.853, \( p = 0.1098 \)). A difference was identified between the ipsilateral and contralateral sides in the PFF A53T + AAV A53T group (\( p < 0.01 \)).
When the percentage forward steps were analysed an interaction effect and a significant effect of side were identified (Interaction F(5, 96) = 5.336, $p = 0.0002$; Side F(1, 96) = 45.09, $p < 0.0001$; Treatment F(5, 96) = 0.000, $p > 0.9999$). Post-hoc comparisons identified a difference between the ipsilateral and contralateral sides for the PFF A53T + AAV A53T ($p < 0.001$), PFF WT + AAV WT ($p < 0.01$), and eGFP ($p < 0.001$) groups.

Similarly analysis of percentage backward steps identified an interaction effect and an effect of side (Interaction F(5, 96) = 7.768, $p < 0.0001$; Side F(1, 96) = 14.33, $p = 0.0003$; Treatment F(5, 96) = 0.0000, $p > 0.9999$). A difference was identified between the ipsilateral and contralateral sides in the PFF A53T + AAV A53T group ($p < 0.0001$) (Figure 5.15).

**Figure 5.15:** Performance in the stepping test at three weeks post-AAV PFF delivery. Decreased contralateral steps were found in the backward direction for both number of steps (b) and percentage steps (d) when compared to their respective ipsilateral steps in the PFF A53T + AAV A53T group. Decreased percentage contralateral steps when compared to their respective percentage ipsilateral steps were observed in the forward direction in the PFF A53T + AAV A53T, PFF WT + AAV WT, and AAV-eGFP groups. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 6-10. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. 
5.4.3.4 Analysis of males alone at three weeks post-AAV PFF delivery identified a difference between percentage ipsilateral and contralateral steps in the PFF A53T + AAV A53T combination treatment group in the forward and backward direction.

Analysis of number of forward steps for males alone at three weeks post-AAV PFF delivery found an effect of side (Interaction F(4, 40) = 0.7382, \( p = 0.5715 \); Side F(1, 40) = 7.246, \( p = 0.0103 \); Treatment F(4, 40) = 1.982, \( p = 0.1159 \)). Post-hoc comparisons did not identify any differences between the treatment groups.

In the backward direction, analysis of the number of steps did not identify any effects (Interaction F(4, 40) = 2.119, \( p = 0.0963 \); Side F(1, 40) = 3.312, \( p = 0.0763 \); Treatment F(4, 40) = 0.8711, \( p = 0.4897 \)).

When percentage forward steps were analysed an effect of side was identified (Interaction F(4, 40) = 1.575, \( p = 0.1997 \); Side F(1, 40) = 17.71, \( p = 0.0001 \); Treatment (4, 40) = 0.0000, \( p > 0.9999 \)). Post-hoc comparisons identified a difference between the % ipsilateral and % contralateral in the PFF A53T + AAV A53T group (\( p < 0.05 \)).

Analysis of percentage backward steps identified an interaction effect and an effect of side (Interaction F(4, 40) = 9.045, \( p < 0.0001 \); Side F(1, 40) = 14.16, \( p = 0.0005 \); Treatment F(4, 40) = 0.0000, \( p > 0.9999 \)). A difference was identified between the ipsilateral and contralateral sides in the PFF A53T + AAV A53T group (\( p < 0.0001 \)) (Figure 5.16).
Figure 5.16: Performance in the stepping test three weeks post-AAV PFF delivery for males alone. Decreased percentage forward and backward contralateral steps were identified in the PFF A53T + AAV A53T males when compared to their respective percentage ipsilateral steps (d). Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 4-6. * $p < 0.05$, **** $p < 0.0001$. 

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**a**  
Forward

**b**  
Backward

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**c**  
Percentage

**d**  
% Forward Steps

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- PBIS
- PFF WT
- PFF A53T
- PFF A53T + AAV A53T
- sOFP
5.4.3.5 Decreased percentage contralateral forward steps were identified in females alone in the PFF WT + AAV WT combination group, the AAV-eGFP group and the PFF A53T group at three weeks post-AAV PFF delivery.

Analysis of the number of forward steps for females alone at three weeks post-AAV PFF delivery identified an effect of side and an effect of treatment (Interaction $F(5, 44) = 1.738, p = 0.1458$; Side $F(1, 44) = 18.62, p < 0.0001$; Treatment $F(5, 44) = 2.627, p = 0.0366$). Post-hoc comparisons identified a difference between the ipsilateral and contralateral sides in the PFF WT + AAV WT ($p = 0.05$) group.

No effects were identified for the number of backward steps (Interaction $F(5, 44) = 0.9663, p = 0.4488$; Side $F(1, 44) = 2.365, p = 0.1313$; Treatment $F(5, 44) = 1.784, p = 0.1360$).

When the percentage forward steps were analysed, an interaction effect and an effect of side were identified (Interaction $F(5, 44) = 9.268, p < 0.0001$; Side $F(1, 44) = 27.06, p < 0.0001$; Treatment $F(5, 44) = 0.0000, p > 0.9999$). Post-hoc comparisons identified a difference between % ipsilateral and % contralateral in the PFF A53T ($p < 0.05$), PFF WT + AAV WT ($p < 0.001$) and AAV-eGFP ($p < 0.01$) groups.

Analysis of percentage backward steps identified an effect of side (Interaction $F(5, 44) = 1.907, p = 0.1125$; Side $F(1, 44) = 5.058, p = 0.0296$; Treatment $F(5, 44) = 0.000, p > 0.9999$), with no differences identified though post-hoc comparisons (Figure 5.17).
**Figure 5.17:** Performance in the stepping test at three weeks post-AAV PFF delivery for females alone. Decreased number of contralateral forward steps (a) and percentage contralateral forward steps (c) were identified in the PFF + AAV WT treatment group when compared to their respective ipsilateral steps. Decreased percentage forward steps were also identified in the AAV-eGFP and PFF A53T treatment groups when compared to their respective percentage ipsilateral forward steps (c). Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 4-5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
5.4.3.6 Decreased percentage contralateral forward and backward steps were identified in the PFF A53T + AAV A53T group at 4.5 weeks post-AAV PFF delivery.

Analysis of the number of forward steps at 4.5 weeks post-AAV PFF delivery found an interaction effect and an effect of side (Interaction F(5, 96) = 2.445, p = 0.0394; Side F(1, 96) = 36.86, p < 0.0001; Treatment F(5, 96) = 0.9022, p = 0.4830). Post-hoc comparisons identified a difference between ipsilateral and contralateral in the AAV-eGFP group (p < 0.05) and the PFF WT + AAV WT group (p < 0.01).

When the number of backward steps were analysed, an effect of side was identified (Interaction F(5, 96) = 1.713, p = 0.1388; Side F(1, 96) = 4.585, p = 0.0348; Treatment F(5, 96) = 2.010, p = 0.0841). Post-hoc comparisons did not identify any differences between the treatment groups.

An interaction effect and an effect of side were identified when the percentage forward steps were analysed (Interaction F(5, 96) = 6.112, p < 0.0001; Side F(1, 96) = 72.95, p < 0.0001; Treatment F(5, 96) = 0.0000, p > 0.9999). Post-hoc comparisons identified a difference between the ipsilateral and contralateral side in the AAV-eGFP (p < 0.0001), PFF WT + AAV WT (p < 0.0001), and PFF A53T + AAV A53T (p < 0.01) groups.

When the percentage backward steps were analysed, an interaction effect and an effect of side were identified (Interaction F(5, 96) = 7.564, p < 0.0001; Side F(1, 96) = 17.53, p < 0.0001; Treatment F(5, 96) = 0.0000, p > 0.9999). Post-hoc comparisons identified a difference between the ipsilateral and contralateral sides in the PFF A53T + AAV A53T group (p < 0.0001) (Figure 5.18).
Figure 5.18: Performance in the stepping test at 4.5 weeks post-AAV PFF delivery. In the forward direction a decreased number of contralateral steps (a) and percentage contralateral steps (c) were observed in both the AAV-eGFP and PFF WT + AAV WT treatment groups when compared to their respective ipsilateral steps. Decreased percentage contralateral steps were identified in the forward (c) and backward direction (d) in the PFF A53T + AAV A53T group when compared to their respective ipsilateral values. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 6-10. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
5.4.3.7 Decreased number of, and percentage contralateral backward steps were identified in the PFF A53T + AAV A53T group when males were analysed alone at 4.5 weeks post-AAV PFF delivery.

Analysis of the number of forward steps for males alone at 4.5 weeks post-AAV PFF delivery found an effect of side (Interaction $F(4, 40) = 0.7691$, $p = 0.5517$; Side $F(1, 40) = 9.586$, $p = 0.0036$; Treatment $F(4, 40) = 1.140$, $p = 0.3515$). Post-hoc comparisons did not identify any differences between the treatment groups.

An effect of side was identified when the number of backward steps were analysed (Interaction $F(4, 40) = 0.7763$, $p = 0.5472$; Side $F(1, 40) = 7.780$, $p = 0.0081$; Treatment $F(4, 40) = 1.922$, $p = 0.1256$). Post-hoc comparisons did not identify any differences between the groups.

When percentage forward steps were analysed an effect of side was identified (Interaction $F(4, 40) = 2.476$, $p = 0.0596$; Side $F(1, 40) = 20.06$, $p < 0.0001$; Treatment $F(4, 40) = 0.0000$, $p > 0.9999$). Post-hoc comparisons identified a difference between the ipsilateral and contralateral sides in the eGFP group ($p < 0.01$).

Analysis of percentage backward steps identified an interaction effect and an effect of side (Interaction $F(4, 40) = 5.781$, $p = 0.0009$; Side $F(1, 40) = 21.38$, $p < 0.0001$; Treatment $F(4, 40) = 0.0000$, $p > 0.9999$). Post-hoc comparisons identified a difference between the ipsilateral and contralateral sides in the PFF A53T + AAV A53T group ($p < 0.0001$) (Figure 5.19).
Figure 5.19: Performance in the stepping test four weeks post-AAV PFF delivery for males alone. Decreased percentage contralateral forward steps were identified in the AAV-eGFP group when compared with their percentage ipsilateral forward steps (c). Decreased percentage contralateral backward steps were identified in the PFF A53T + AAV A53T group when compared with their percentage ipsilateral backward steps (d). Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 4-6, * p < 0.05, **** p < 0.0001.
5.4.3.8 Decreased number of, and percentage contralateral forward steps were identified in the PFF WT + AAV WT group when females were analysed alone at 4.5 weeks post-AAV PFF delivery.

When the number of forward steps were analysed for females alone at 4.5 weeks post-AAV PFF delivery an effect of side was identified (Interaction F(5, 44) = 2.226, \( p = 0.0684 \); Side F(1, 44) = 21.18, \( p < 0.0001 \); Treatment F(5, 44) = 0.5938, \( p = 0.7046 \)). Post-hoc comparisons identified a difference between the ipsilateral and contralateral sides in the PFF WT + AAV WT groups (\( p < 0.05 \)).

Analysis of the number of backward steps identified no effects (Interaction F(5, 44) = 1.263, \( p = 0.2968 \); Side F(1, 44) = 0.4109, \( p = 0.5248 \); Treatment F(5, 44) = 1.113, \( p = 0.3673 \)).

Analysis the percentage forward steps identified an interaction effect and an effect of side (Interaction F(5, 44) = 5.127, \( p = 0.0009 \); Side (1, 44) = 40.25, \( p < 0.0001 \); Treatment F(5, 44) = 0.000, \( p > 0.9999 \)). Post-hoc comparisons identified a difference between the ipsilateral and contralateral sides in the AAV-eGFP (\( p < 0.05 \)), PFF WT + AAV WT group (\( p < 0.0001 \)) and PFF A53T + AAV A53T (\( p < 0.05 \)).

An interaction effect was identified when the percentage backward steps were analysed (Interaction F(5, 44) = 3.087, \( p = 0.0179 \); Side F(1, 44) = 2.660, \( p = 0.1100 \); Treatment F(5, 44) = 0.000, \( p > 0.9999 \)). A difference between the ipsilateral and contralateral sides were identified (\( p < 0.05 \)) in the PFF A53T + AAV A53T group (Figure 5.20).
Figure 5.20: Performance in the Stepping Test at 4.5 weeks post-AAV PFF delivery for females alone. Decreased number of (a), and percentage contralateral forward steps (c) were identified in the PFF WT + AAV WT treatment group when compared to their respective ipsilateral steps. Decreased percentage contralateral forward steps were also identified in the AAV-eGFP and PFF A53T + AAV A53T treatment groups when compared to their respective percentage ipsilateral forward steps (c). Decreased percentage contralateral backward steps were also identified in the PFF A53T + AAV A53T treatment group when compared to their percentage ipsilateral backward steps (d). Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 4-5. * p < 0.05, **** p < 0.0001.
5.4.4 Amphetamine Rotation Test

5.4.4.1 An increase in amphetamine induced total rotations was observed in the PFF A53T + AAV A53T group when compared with PFF WT, PFF WT + AAV WT and PFF A53T groups at five weeks post-AAV PFF delivery.

An effect was identified between the groups for total number of rotations for both sexes combined (H(5) = 18.77, \( p = 0.0021 \)), with a difference identified between the PFF A53T + AAV A53T group and the PFF WT group (\( p = 0.0057 \)), the PFF A53T + AAV A53T group and PFF WT + AAV WT group (\( p = 0.0311 \)), and the PFF A53T + AAV A53T and PFF A53T group (\( p = 0.0071 \)).

Analysis of males alone identified an effect for total rotations (F(4, 20) = 6.553, \( p = 0.0015 \)), with differences identified between the PFF A53T + AAV A53T and PBS groups (\( p < 0.01 \)), the PFF A53T + AAV A53T and AAV-eGFP groups (\( p < 0.01 \)), the PFF A53T + AAV A53T and PFF WT groups (\( p < 0.01 \)) and between the PFF A53T + AAV A53T and PFF A53T groups (\( p < 0.01 \)).

Analysis of females alone did not identify any effects (H(5) = 7.259, \( p = 0.2021 \)) (Figure 5.21).
Figure 5.21: Total number of rotations at five weeks post-AAV PFF delivery. Increased total rotations were observed in the PFF A53T + AAV A53T group when compared with the PFF WT, PFF WT + AAV WT, and PFF A53T groups (a). When males were analysed alone increased total rotations were observed in the PFF A53T + AAV A53T treatment group compared to PBS, AAV-eGFP, PFF WT and PFF A53T groups (b). Data presented as median with minimum and maximum (a, c) or mean with SEM (b). Kruskal-Wallis test followed by Dunn’s multiple comparisons test (a, c) or one-way ANOVA followed by Neuman-Keuls multiple comparisons test (b). n = 6 – 10 (a) both sexes, n = 4 – 6 (b) males, n = 4 – 5 (c) females. * p < 0.05, ** p < 0.01.
5.4.4.2 Analysis of males alone found an increase in ipsilateral rotations in the PFF A53T + AAV A53T group when compared with PFF WT group at five weeks post-AAV PFF delivery.

No effects were identified for number of ipsilateral rotations for both sexes combined (H(5) = 6.729, \( p = 0.2416 \)) or for females alone (H(5) = 7.631, \( p = 0.1778 \)). When males were analysed alone an effect was identified (F(4, 20) = 2.828, \( p = 0.0374 \)), with a difference identified between the PFF A53T + AAV A53T and the PBS (\( p < 0.05 \)), AAV-eGFP (\( p < 0.05 \)), PFF WT (\( p < 0.05 \)) and PFF A53T (\( p < 0.05 \)) groups through post-hoc comparisons (Figure 5.22).

**Figure 5.22:** Number of ipsilateral rotations at five weeks post-AAV PFF delivery. Increased ipsilateral rotations were observed for males alone in the PFF A53T + AAV A53T group when compared with the PBS, AAV-eGFP, PFF WT, and PFF A53T groups (b). Data presented as mean with SEM (b) or median with minimum and maximum (a, c). One-way ANOVA followed by Neuman-Keuls multiple comparisons test (b) or Kruskal Wallis test (a, c). \( n = 6 – 10 \) (a) both sexes, \( n = 4 – 6 \) (b) males, \( n = 4 – 5 \) (c) females. * \( p < 0.05 \).
5.4.4.3 No differences were identified when total contralateral rotations were analysed at five weeks post-AAV PFF delivery.

No effects were identified for number of contralateral rotations at five weeks post-AAV PFF delivery when both sexes were analysed together (H(5) = 8.444, $p = 0.1334$), when males were analysed alone (F(4, 20) = 0.6107, $p = 0.6597$) or when females were analysed alone (H(5) = 7.341, $p = 0.1965$) (Figure 5.23).

Figure 5.23: Number of contralateral rotations at five weeks post-AAV PFF delivery. No differences in contralateral rotations were observed at five weeks post-AAV PFF delivery. Data presented as median with minimum and maximum (a, c) or mean with SEM (b). One-way ANOVA (b) or Kruskal-Wallis test (a, c). $n = 6 – 10$ (a) both sexes, $n = 4 – 6$ (b) males, $n = 4 – 5$ (c) females.
5.4.5 Staircase Test

5.4.5.1 No differences were identified between the groups in the staircase test at baseline.

Three animals were moved from the GFP group, one animal was removed from the PFF WT group, three animals were removed from the PFF A53T group and two animals were removed from the AAV + PFF A53T group as they met the criteria for unsuccessful acquisition of the staircase test as outlined in Table 2.2. No differences were identified in the staircase test at baseline between the groups. Data presented in Appendix III.

5.4.5.2 No differences between the treatment groups were identified in the staircase test at two weeks post-AAV delivery.

Analysis of both sexes combined found an effect of treatment at two weeks post-AAV delivery (Interaction F(5, 78) = 0.4416, p = 0.8181; Side F(1, 78) = 0.0000, p > 0.9999; Treatment F(5, 78) = 2.548, p = 0.0345). Post-hoc comparisons did not identify any differences between the treatment groups. When females were analysed alone an effect of treatment was identified (Interaction F(5, 42) = 0.2875, p = 0.9173; Side F(1, 42) = 0.0655, p = 0.7993; Treatment F(5, 42) = 3.371, p = 0.0118). Post-hoc comparisons did not identify any differences between the treatment groups. Due to the low sample size, males were not analysed alone, however the data is presented graphically in Figure 5.24.
Figure 5.24: Number of pellets retrieved two weeks post-AAV PFF delivery in the staircase test. No differences were identified between the treatment groups at two weeks post-AAV PFF delivery in the staircase test. Data presented as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 6–9 (a, b) both sexes, n = 2–5 (c, d) males, n = 4–5 (e, f) females.
5.4.5.3 No differences were identified in the staircase test between the groups at four weeks post-AAV PFF delivery.

Analysis of the percentage success at four weeks post-AAV PFF delivery found an effect of side for both sexes combined (Interaction $F(5, 78) = 0.6504, p = 0.6620$; Side $F(1, 78) = 4.184, p = 0.0442$; Treatment $F(5, 78) = 1.327, p = 0.2617$), with no differences identified between the treatment groups through post-hoc comparisons. Analysis of females alone (Interaction $F(5, 42) = 0.5697, p = 0.7227$; Side $F(1, 42) = 2.319, p = 0.1353$; Treatment $F(5, 42) = 0.7981, p = 0.5573$) did not identify any effects.

Testing at four weeks post-AAV PFF delivery identified no effects on number of pellets retrieved when both sexes were combined (Interaction $F(5, 78) = 0.3448, p = 0.8841$; Side $F(1, 78) = 0.0185, p = 0.8923$; Treatment $F(5, 78) = 1.251, p = 0.2936$) or when females were analysed alone (Interaction $F(5, 42) = 0.3588, p = 0.8737$; Side $F(1, 42) = 0.0899, p = 0.7658$; Treatment $F(5, 42) = 1.206, p = 0.3232$). Due to the low sample number per group for the males, no statistical analysis was performed, however the data is graphically presented in Figure 5.25.
Figure 5.25: Percentage success and number of pellets retrieved at four weeks post-AAV PFF delivery in the staircase test. No differences were identified between the treatment groups in the staircase test at four weeks post-AAV PFF delivery. Data presented as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 6 – 9 (a, b) both sexes, n = 2 – 5 (c, d) males, n = 4 – 5 (e, f) females.
5.4.6 Tyrosine hydroxylase immunoreactive cells

The following images are representative immunohistochemically stained sections of the substantia nigra showing TH immunoreactivity.
Figure 5.26: Representative images of tyrosine hydroxylase immunohistochemistry in the substantia nigra showing the injected and un-injected hemispheres. (A) PBS injected male, (B) PBS injected female, (C) AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP injected male, (D) AAV1/2-CAG-miR scrambled control alpha synuclein co-expressing eGFP injected female. Images acquired at 10X.
Figure 5.27: Representative images of tyrosine hydroxylase immunohistochemistry in the substantia nigra showing the injected and un.injected hemispheres. (A) PFF WT injected male, (B) PFF WT injected female, (C) PFF WT + AAV5-CBA-alpha-synuclein human WT injected male, (D) PFF WT + AAV5-CBA-alpha-synuclein human WT injected female. Images acquired at 10X.
Figure 5.28: Representative images of tyrosine hydroxylase immunohistochemistry in the substantia nigra showing the injected and un-injected hemispheres. (A) PFF A53T injected male, (B) PFF A53T injected female, (C) PFF A53T + AAV1/2-CMV-alpha-synuclein human A53T injected male, (D) PFF A53T + AAV1/2-CMV-alpha-synuclein human A53T injected female. Images acquired at 10X.
5.4.6.1 No differences between the treatment groups were identified when tyrosine hydroxylase immunoreactive cells were expressed as a percentage of the un-injected side.

The number of TH expressing cells in the substantia nigra were counted in the injected and un-injected hemispheres of all groups. The data were then presented as percentage un-injected side. No effects were identified when both sexes were combined (H(5) = 9.490, \( p = 0.0910 \)), when males were analysed alone (H(4) = 5.116, \( p = 0.2756 \)), or when females were analysed alone (H(5) = 11.06, \( p = 0.0503 \)) (Figure 5.29).

**Figure 5.29:** Tyrosine hydroxylase immunoreactive cells expressed as a percentage of the un-injected side six weeks post-AAV PFF delivery. No differences were identified between the treatment groups when all data were analysed together. Data presented as median with minimum and maximum. Kruskal-Wallis test. \( n = 5 – 11 \) (a) both sexes, \( n = 3 – 6 \) (b) males alone, \( n = 3 – 5 \) (c) females alone.
5.4.6.2 Decreased tyrosine hydroxylase immunoreactive cells in the substantia nigra were observed in the injected hemisphere of the PFF A53T + AAV A53T treatment group when compared with the un-injected hemisphere.

An exploratory analysis was undertaken in order to compare the injected and un-injected TH cell counts within the treatment groups. Decreased TH immunoreactive cells were observed in the injected hemisphere of the substantia nigra of the PFF A53T + AAV A53T injected animals when compared with the un-injected hemisphere when both sexes were combined ($W = 45, p = 0.0039$) and in the PFF WT treatment group when both sexes were combined ($W = 46, p = 0.0420$). No other differences between the injected and un-injected hemispheres were observed (data not presented).

![Figure 5.30](image)

**Figure 5.30**: Tyrosine hydroxylase immunoreactive cell counts in the injected and un-injected hemispheres of the substantia nigra at six weeks post-AAV PFF delivery. Decreased TH cells were observed in the injected substantia nigra when compared to the un-injected hemisphere in the PFF A53T + AAV A53T treatment group when both sexes were combined (a) and in the PFF WT treatment group when both sexes were combined (b). Data presented as median with minimum and maximum. Wilcoxon matched-pairs signed rank test. $n = 9$ (a), $n = 11$ (b). * $p < 0.05$, ** $p < 0.01$. 
## 5.4.7 Summary of Results

<table>
<thead>
<tr>
<th></th>
<th>Cylinder Test</th>
<th>Stepping Test</th>
<th>Amphetamine Rotation Test</th>
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<tbody>
<tr>
<td></td>
<td>4 weeks post-AAV PFF delivery</td>
<td>3 weeks and 4.5 weeks post-AAV PFF delivery</td>
<td>5 weeks post-AAV PFF delivery</td>
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<tr>
<td><strong>Both Sexes</strong></td>
<td>Decreased % contralateral wall contacts in PFF A53T + AAV A53T treatment group compared to PBS treatment group.</td>
<td><strong>3 Weeks:</strong> Decreased number of and % contralateral backward steps in PFF A53T + AAV A53T treatment group. Decreased % contralateral forward steps in AAV-eGFP, PFF WT + AAV WT, and PFF A53T + AAV A53T treatment groups. <strong>4.5 Weeks:</strong> Decreased contralateral forward steps in the AAV-eGFP and PFF WT + AAV WT treatment groups and decreased % contralateral forward steps in the AAV-eGFP, PFF WT + AAV WT, and PFF A53T + AAV A53T treatment groups. Decreased % contralateral backward steps in the PFF A53T + AAV A53T treatment groups.</td>
<td>Increased total rotations in PFF A53T + AAV A53T treatment group when compared to PFF WT, PFF WT + AAV WT, and PFF A53T. No differences in total ipsilateral or contralateral rotations.</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td>Increased % ipsilateral wall contacts in the PFF A53T + AAV A53T treatment group compared to all other treatment groups.</td>
<td><strong>3 Weeks:</strong> Decreased % contralateral backward and forward steps in the PFF A53T + AAV A53T treatment groups. <strong>4.5 Weeks:</strong> Decreased percentage contralateral forward steps in the AAV-eGFP treatment group. Decreased percentage contralateral backward steps in the PFF A53T + AAV A53T treatment groups.</td>
<td>Increased total rotations in PFF A53T + AAV A53T treatment group when compared to all other treatment groups. Increased ipsilateral rotations in PFF A53T + AAV A53T treatment group when compared to all other treatment groups. No differences in total contralateral rotations.</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td>No differences</td>
<td><strong>3 Weeks:</strong> Decreased number of contralateral forward steps in the PFF WT + AAV WT treatment groups. Decreased % contralateral forward steps in the AAV-eGFP, PFF WT + AAV WT and PFF A53T treatment groups. <strong>4.5 Weeks:</strong> Decreased contralateral forward steps in the PFF WT + AAV WT treatment group and decreased percentage contralateral forward steps in the AAV-eGFP, PFF WT + AAV WT, and PFF A53T + AAV A53T treatment groups. Decreased percentage contralateral backward steps in the PFF A53T + AAV A53T treatment group.</td>
<td>No differences for total, ipsilateral or contralateral rotations.</td>
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**Table 5.3:** Summary of behavioural results. Staircase Test not included as no differences found.
<table>
<thead>
<tr>
<th></th>
<th>Tyrosine hydroxylase immunoreactivity as % un-injected side</th>
<th>Tyrosine hydroxylase immunoreactivity TH cell counts</th>
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<tbody>
<tr>
<td><strong>Both Sexes</strong></td>
<td>No differences</td>
<td>Decreased in PFF A53T + AAV A53T and PFF WT treatment groups when injected and un-injected hemispheres were compared.</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td>No differences</td>
<td>Decreased in PFF A53T + AAV A53T when injected and un-injected hemispheres were compared.</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td>No differences</td>
<td>Decreased in PFF A53T, with a trend towards a decrease in PFF A53T + AAV A53T when injected and un-injected hemispheres were compared.</td>
</tr>
</tbody>
</table>

**Table 5.4:** Summary of tyrosine hydroxylase immunohistochemistry results.
5.5 Discussion

In this study it is shown that delivery of AAV1/2-CMV-A53T alpha-synuclein human with PFF human A53T alpha-synuclein into the substantia nigra leads to the development of motor behavioural deficits as early as three weeks post-AAV PFF delivery in the stepping test when males are analysed alone, with no differences in the PFF A53T + AAV A53T treatment group observed in females. This deficit continues to 4.5 weeks post-AAV PFF delivery where it also begins to become apparent in females. Assessment of limb use asymmetry supports this finding at four weeks post-AAV PFF delivery, with limb use asymmetry evident in the cylinder test in males, but not in females. Building upon these results even further is the finding that at five weeks post-AAV PFF delivery, total rotations in the amphetamine rotation test are increased when both sexes are analysed in combination, and when males are analysed alone, with no differences identified when females were analysed alone. Analysis of total ipsilateral rotations found the males in the PFF A53T + AAV A53T combination treatment group had significantly greater ipsilateral rotations when compared to those males treated with PFF WT or PFF A53T alone.

Though post-mortem immunohistochemistry, comparison of injected and un-injected hemispheres identified decreased tyrosine hydroxylase immunoreactive cells in the injected hemisphere of the PFF A53T + AAV A53T treatment group when both sexes were combined. Comparison of injected and un-injected hemispheres also identified decreased tyrosine hydroxylase immunoreactive cells in the injected hemisphere of the PFF WT single treatment group. However as the tyrosine hydroxylase immunoreactive cells were counted on a two-dimensional image, as opposed to using unbiased stereology, these cell counts should be interpreted with caution.

Alpha-synuclein immunofluorescence was found to be higher in both the PFF A53T + AAV A53T combination treatment and PFF WT + AAV WT combination treatment groups when compared with the PBS treatment group. Visualisation of the alpha-synuclein pathology in this study was limited due to the use of an
antibody specific for alpha-synuclein, and not alpha-synuclein which has been phosphorylated at serine 129 (pSer129). As it has been found that when alpha-synuclein is recruited into pathological inclusions it undergoes phosphorylation at Ser129, perhaps an antibody specific for phosphorylation at Ser129 would have been more appropriate in this study (Fujiwara et al., 2002). Furthermore, through negative staining utilising the secondary antibody and in absence of the primary antibody for alpha-synuclein and with the primary and secondary antibody for tyrosine hydroxylase, fluorescence signal was obtained at 488nm which is representative of the signal usually obtained by the secondary antibody used to bind to the alpha-synuclein primary antibody, with this signal being much higher in the injected than un-injected hemisphere. While this is concerning when it comes to the interpretation of the alpha-synuclein expression data, it is possible that this signal in the injected hemisphere is as a result of the recruitment of endogenous alpha-synuclein, however this has not been confirmed. Therefore the alpha-synuclein immunofluorescence presented in this study should be interpreted with this in mind. Future work will elucidate the differences in immunofluorescence staining obtained through the use of an antibody specific to phosphorylation of alpha-synuclein at Ser129, as well as through the use of different alpha-synuclein antibodies.

In the present study, two injections were performed during the same surgery, to two different sites within the substantia nigra. It is proposed that this is a more efficient method for the development of a preclinical model with two injections as opposed to delivering the two injections during different surgeries, as well as minimising the suffering of the animal, as they only have to undergo one surgical procedures. Furthermore, by varying the injection co-ordinates used and delivering the AAV and PFFs to two different injection sites the risk of overloading the injection site through the delivery of both the AAV and PFF to the same location is greatly minimised. There are however some limitations to the use of two injections during the same surgery. While the use of two injections at two separate locations is beneficial, it is important to note that the substantia nigra is a small brain region, and the impact of receiving two injections in close proximity was not fully controlled for in this study. While
there was also a PFF + AAV WT treatment group, there was no control group receiving two unilateral injections and therefore it is important that the above results are interpreted with caution.

Alpha-synuclein has been found to be ubiquitinated in both Lewy bodies and Lewy neurites. Future work in this model should investigate the presence of ubiquitin in order to further confirm the hypothesis that alpha-synuclein PFFs lead to the seeding and recruitment of endogenous alpha-synuclein, which in turn leads to the formation of the pathological aggregates (Volpicelli-Daley et al., 2011). Furthermore, alpha-synuclein accumulation through the application of PFFs has previously been shown to induce microglial activation (Paumier et al., 2015), and therefore it is proposed that future work in the A53T combination model described throughout this chapter should investigate the role that microglia have to play in the behavioural deficits observed in this model.

Some studies argue that AAVs lead to the development of a much more diffuse alpha-synuclein expression than PFFs (Thakur et al., 2017). Previous research using transgenic mice (PD-related A53T mutation; M83 line) found that unilateral injection of WT alpha-synuclein human PFFs into the striatum led to the expression of alpha-synuclein pathology on both the injected and un-injected hemispheres (Luk et al., 2012b). Similarly, a study in Sprague Dawley rats found that injection of mouse alpha-synuclein PFFs into the striatum led to expression of phosphorylated alpha-synuclein bilaterally (Paumier et al., 2015). This is an important point to consider, especially when it comes to the interpretation of the behavioural results obtained in this study, as they were all tests which would show unilateral, and not bilateral deficits. It cannot be ruled out that the injections required in order to deliver both the AAV and PFFs, or the PFFs alone in this study lead to some infiltration of the ventricular space resulting in the expression of alpha-synuclein in both hemispheres of the brain. Future studies should investigate the use of the two coordinates chosen for this study, and perhaps investigation of the alpha-synuclein levels in the
cerebrospinal fluid (CSF) could shed some insight into whether this is leading to the expression of alpha-synuclein pathology in both hemispheres of the brain.

Furthermore, differing results were obtained when the immunohistochemistry data were assessed as percentage of the un-injected side versus a comparison of the cell counts of the injected and un-injected hemispheres, suggesting that perhaps analysis of the cells as a percentage of the un-injected side is not the most effective analysis method in this model. The TH immunoreactive cells counts presented in this study are limited in that they were not performed through unbiased stereology, and therefore should be interpreted with caution. Future research in this model will adopt more stringent cell counting methods in order to further validate these findings. Previous research has identified bilateral tyrosine hydroxylase cell loss following a unilateral injection of mouse alpha-synuclein PFF in the substantia nigra of rats (Paumier et al., 2015), therefore perhaps the comparisons of the injected and un-injected sides may not be applicable to all treatment groups. A potential limiting factor of the TH immunostaining in this study is that this staining was performed on sections already mounted on slides, as opposed to free-floating sections. As all previous research presented utilised free floating immunohistochemistry it is possible that some further steps need to be taken to fully optimise the immunohistochemistry protocol for on the slide immunohistochemistry, and therefore the representative images presented are not as detailed as would be desired. Due to time limitations this was not possible in the present study, but will be optimised further in future studies.

No differences between the groups were found in the staircase test at any timepoint post-AAV PFF delivery. The staircase test is described as a test of fine motor skill (Montoya et al., 1990, Montoya et al., 1991). While this test has been successfully employed to assess motor deficits in the LPS model of inflammatory based Parkinsonism (O'Neill et al., 2020, Yssel et al., 2018) there is very little research performed on the use of this test in models of synucleinopathy, suggesting that it may not be the most appropriate test to
phenotype motor deficits in these models, or alternatively, that fine motor skills are not impaired by the synuclein burden.

The present study identified some sex differences when it came to the behavioural tests within which deficits were apparent. For example, males in the AAV + PFF A53T group alone showed increased ipsilateral rotations in the amphetamine test, while both males and females in the AAV + PFF A53T showed deficits in contralateral stepping in the stepping test. An important consideration in relation to the interpretation of the amphetamine rotation test results is the differing response females elicit to amphetamine across the estrous cycle (Becker et al., 2001). It has previously been reported that female rats display a greater behavioural response to amphetamine on the day of estrous when compared to other days. However it was beyond the scope of the current project to monitor the oestrus cycle. Future work in this model should account for the estrous cycle when performing the amphetamine rotation test.

In humans PD has been found to be twice as likely to develop in males when compared with females (Smith and Dahodwala, 2014, Gillies et al., 2014). It has been proposed that this difference is due in part to the effect of estrogen, as women who have had a higher exposure to estrogen over their lifetime have a greatly reduced risk of developing PD (Gatto et al., 2014). It has also been found that males display a faster onset of motor symptoms than females (Baba et al., 2005, Haaxma et al., 2007, Colombo et al., 2015). In the present study, males appeared to develop deficits in the stepping test earlier than the females, which is in line with the above findings in humans. While this is an interesting finding, more work is needed to elucidate the sex differences observed in the present study further, especially as a lot of the research conducted in AAV models of synucleinopathy to date utilise female rats, and identify behavioural deficits.

While human alpha-synuclein PFFs are used in order to more closely replicate disease pathology in humans, it is important to consider the possible implications of using human PFFs in a rat. Previous research has compared the seeding ability of mouse WT PFFs and human WT PFFs for alpha-synuclein in
mice and found much a much faster and greater development of pathological forms of alpha-synuclein in the mouse following use of the mouse PFFs when compared to human PFFs (Luk et al., 2016). In mice it has been observed that injection with PFFs leads to the development of pathology which continues to develop over 180 days post-injection, with the mice PFFs leading to the fastest development of alpha-synuclein pathology (Luk et al., 2016). Given the behavioural phenotype observed in the current study, accompanied by the fact that the use of the human form of alpha-synuclein is more translatable to the clinic, the use of human alpha-synuclein appears sufficient to induce unilateral motor deficits in male rats which may represent a useful preclinical model of Parkinson’s disease.

Consideration of the controls generally used in PFF studies can generate questions around the control used in this study (PBS). It is usually recommended that alpha-synuclein monomers, which are the starting material in the generation of PFFs, are used as controls (Luk et al., 2016), however caution must be used when these controls are employed as it has been found that long term studies using alpha-synuclein monomers as control has led to the development of a small number of pS129 alpha-synuclein positive aggregates in the substantia nigra of rats at six months post-injection (Paumier et al., 2015). This can be prevented through proper preparation. Although PBS is not the most common control, it is the second most common control (Polinski et al., 2018) and therefore this study is comparable to other studies which use PBS as control.

In summary, through the use of a combination of AAV1/2-CMV-A53T A53T and PFF A53T this study provides promising evidence for the use of this protocol to generate a model of synucleinopathy induced motor deficits in male rats which recapitulates some of the behavioural features described in other animal models of synucleinopathy, most specifically in models of PD. Intranigral delivery of two injections, one containing AAV A53T and the other containing PFF A53T at 2 sites within proximity of each other, leads to the development of lateralised motor deficits in male rats. Decreased tyrosine hydroxylase
immunoreactive cells were also observed in the injected hemisphere of the PFF A53T + AAV A53T and the PFF WT treatment groups when both sexes were analysed together, suggesting that the expression of different conformations and combinations of alpha-synuclein can lead to pathological loss of tyrosine hydroxylase immunoreactive cells. Future studies are necessary to further confirm the application of this model as a model of synucleinopathy.
6. Discussion

6.1 Summary of main findings

The implications of the overexpression of alpha-synuclein, achieved through AAV, PFF or a combination of AAV and PFF delivery to the rat substantia nigra were assessed on aspects of motor performance and dopamine neuronal integrity within the targeted area. The presence of alpha-synuclein within the substantia nigra was confirmed by immunofluorescence following unilateral injection of AAV5-CBA-alpha-synuclein WT human (Chapter 3), AAV1/2-CMV-A53T human alpha-synuclein (Chapter 4), or a combination of AAV1/2-CMV-A53T human alpha-synuclein and PFF A53T human alpha-synuclein (Chapter 5). Delivery of AAV1/2-CMV-A53T human alpha-synuclein to the substantia nigra led to the development of a progressive neurodegeneration, as observed through a loss of tyrosine hydroxylase immunoreactive cells, as illustrated in Chapter 4. A combination of AAV1/2-CMV-A53T human alpha-synuclein and PFF A53T human alpha-synuclein delivered unilaterally to the substantia nigra led to a more rapid onset of motor behavioural deficits, when compared to PFF A53T human alpha-synuclein alone, accompanied by a loss of tyrosine hydroxylase immunoreactive cells when un-injected and injected hemispheres were compared, as illustrated in Chapter 5.
Figure 6.1: Summary of the main findings of this thesis. IF (immunofluorescence), IR (immunoreactivity), Y (yes), N (no).

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<tr>
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<th>Alpha-synuclein + IF</th>
<th>Motor Behavioural Deficits</th>
<th>Tyrosine Hydroxylase + IR Cell Loss</th>
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AAV5-CBA-WT alpha-synuclein
no motor behavioural deficits
no tyrosine hydroxylase immunoreactive cell loss

AAV1/2-CMV-A53T alpha-synuclein
unilateral tyrosine hydroxylase immunoreactive cell loss
motor behavioural deficits in male rats

PFF A53T alpha-synuclein
unilateral tyrosine hydroxylase immunoreactive cell loss

Alpha-synuclein + IF
Motor behavioural deficits
Tyrosine hydroxylase + IR cell loss

Stepping Test (not consistent)
Cylinder Test
Amphetamine Rotation Test
6.2 Intranigral injection of AAV5-CBA-alpha-synuclein WT does not lead to the development of motor deficits up to 16 weeks post-AAV delivery.

Unilateral intranigral injection of AAV5-CBA-alpha-synuclein WT yielded alpha-synuclein immunofluorescence in the injected hemisphere at both 12 and 16 weeks post-AAV delivery, in the absence of behavioural deficits (Chapter 3). However, this study found that injection of AAV5-eGFP led to the development of motor deficits in the form of increased ipsilateral rotations in the amphetamine rotation test, 15 weeks post-AAV delivery, with this effect observed in male, and not female rats. While previous research utilising the expression of WT alpha-synuclein has found motor behavioural deficits and tyrosine hydroxylase immunoreactive cell loss (Decressac et al., 2011), the present study was unable to replicate these findings.

When working with AAV vector mediated expression, there are many factors relating to the AAV, including titre, serotype and promoters, which may influence outcomes. When considering the use of AAV vectors to generate preclinical models of disease it is important contemplate the reproducibility of the model. Due to the lack of reproducibility observed when WT alpha-synuclein expression was driven by AAV vectors in the substantia nigra of rats, it is proposed that the use of WT alpha-synuclein is perhaps not the most efficient way to generate a model of synucleinopathy associated with motor behavioural deficits. It is also possible that the use of WT alpha-synuclein leads to the generation of a preclinical model of disease which develops over many months, and the time points chosen in the present study were too short. However, given the time constraints associated with this project, alongside the aim to develop a model with a relatively fast onset of motor behavioural deficits, it was beyond the scope of this project to assess longer term effects of AAV WT alpha-synuclein delivery to the rat substantia nigra.
6.3 Intranigral injection of AAV1/2-CMV-A53T alpha-synuclein leads to tyrosine hydroxylase immunoreactive cells loss.

Tyrosine hydroxylase immunoreactive cell loss was observed as early as 25 days post- AAV1/2-CMV-A53T alpha-synuclein delivery to the substantia nigra, with this decrease also evident at 6.5 and 13 weeks (Chapter 4). Interestingly, although this cell loss was evident, there was a lack of consistent motor behaviour deficits across the various studies. Previous studies utilising AAV vector mediated expression of A53T alpha-synuclein have reported that it can take four months for a motor behavioural deficit to become apparent (Gaugler et al., 2012). However, it is important to consider increased contralateral rotations observed approximately three weeks post-AAV delivery which may signal initial effects of a lesion associated with transient alterations in dopaminergic function apparent prior to the emergence of neurodegeneration (Robinson et al., 1994).

The presence of alpha-synuclein was confirmed by immunofluorescence at multiple time points post AAV delivery, with this immunofluorescence becoming more intense as time progressed. This model may represent various stages of progression, given that alpha-synuclein burden can be observed in the presence of tyrosine hydroxylase immunoreactive cell loss, but in the absence of detectable motor behavioural deficits.

The results of this study are restricted by the finding that injection of an AAV expressing eGFP led to the development of tyrosine hydroxylase immunoreactive cell loss, and behavioural deficits as early as 3.5 weeks post-AAV delivery. This reflects the findings of other research groups, which have also observed toxicity following AAV-GFP (5.1 x 10^{12} genomic particles/ml) delivery (Koprich et al., 2010). In order to overcome this GFP toxicity, this research group performed a further study, in which a lower titre was utilised (5.1 x 10^{11} genomic particles/ml), and found that this lower titre did not lead to the development of toxicity (Koprich et al., 2011). Considering that the titre used in the present study was 9.5 x 10^{12} viral genomes/ml, it is not surprising that neurotoxicity was observed. The inclusion of an AAV vector which will lead
to the expression of a foreign protein which is thought to be non-pathogenic is important in order to control for the impact of the expression of a foreign protein in the brain, and the lack of this control at the later timepoints in this study is a limiting factor. However, when AAV-eGFP was utilised in the AAV PFF combination study (Chapter 5) this toxicity was not observed, suggesting that perhaps it is not toxic at the titre used in that study (0.5 x 10^{12} viral genomes/ml). While changing the titre can prevent the development of neurotoxic effects, it does bring into question the comparability of the proteins expressed, if a lower titre of GFP must be utilised when compared with alpha-synuclein. Further research is needed to optimise the choice of foreign protein delivered through AAV in order to control for the effects of the expression of the foreign protein itself on any changes observed following AAV delivery of alpha-synuclein. GFP provides many benefits when used as the control protein, including the ability to visualise the field of dispersion of the AAV. This is important as it allows for the surgical co-ordinates to be confirmed, as well as allowing an investigation into the brain regions to which the AAV spreads following intranigral delivery.

6.4 Intranigral injection of a combination of AAV1/2-CMV-A53T human alpha-synuclein and A53T alpha-synuclein preformed fibrils (PFFs) leads to motor deficits.

In this study a rat model of synucleinopathy was generated through two unilateral injections, one of AAV1/2-CMV-A53T alpha-synuclein and one of A53T human alpha-synuclein preformed fibrils (PFFs), delivered during the same surgery to the substantia nigra (Chapter 5). Behavioural impairments were observed in the stepping test, cylinder test, and amphetamine rotation test, and these were accompanied by decreased numbers of tyrosine hydroxylase immunoreactive cells and increased alpha-synuclein immunofluorescence in the injected compared to the un-injected substantia nigra, six weeks post-AAV PFF delivery. These findings indicate that an alpha-synuclein burden within the substantia nigra provokes motor deficits and some dopamine neuronal cell loss, indicating that this model may have important utility as a preclinical model of PD.
The rationale behind the use of both AAV-alpha-synuclein and PFF alpha-synuclein comes from the finding that alpha-synuclein protofibrils can act as seeds for the formation of alpha-synuclein aggregates in the brain that are toxic to neurons (Luk et al., 2012a, Luk et al., 2012b). Furthermore, PFFs have the ability to generate Lewy-like alpha-synuclein fibrillar inclusions which resemble those observed in humans (Thakur et al., 2017). Through the delivery of both AAV and PFF human alpha-synuclein, this study boosted the levels of human alpha-synuclein in the brain, as it has been found that in order for efficient aggregate formation to occur the alpha-synuclein being used as a seed should be of the same species of the alpha-synuclein being overexpressed (Luk et al., 2016). It can be argued that the less consistent motor behavioural deficits observed in the PFF alone treatment groups are due to the fact that the PFFs were human and were therefore not as effective at recruiting rat alpha-synuclein.

Previous research utilising the delivery of both AAV and PFF alpha-synuclein have either delivered the PFF at one time point, with the AAV being delivered at a later time point (Thakur et al., 2017), or with both the PFF and AAV being delivered at the same time to the same injection site (Peelaerts et al., 2015). Delivery of AAV-6 WT human alpha-synuclein and WT human alpha-synuclein PFFs at two separate time points led to the development of tyrosine hydroxylase immunoreactive cell loss, which was observable at 7 weeks post-AAV injection, or 3 weeks following the additional PFF injection when compared with AAV6-alpha synuclein alone or PFF alpha-synuclein alone (Thakur et al., 2017). This cell loss was accompanied by decreased limb use asymmetry, assessed through the use of the cylinder test, and increased forelimb akinesia, assessed through the stepping test, which was also observable at 7 weeks post-AAV injection, or 3 weeks following the additional PFF injection when compared with AAV-alpha synuclein alone or PFF alpha-synuclein alone. In the present study, the combination of AAV WT alpha-synuclein with PFF WT alpha-synuclein led to the development of some motor behavioural deficits in the form of decreased contralateral forward stepping in the stepping test, however there were no other motor behaviour deficits.
observed. Furthermore, a loss of tyrosine hydroxylase immunoreactive cells was not observed in the AAV WT + PFF WT treatment group. Considering the differing protocols utilised in order to deliver the alpha-synuclein AAV and PFF it is not possible to make a direct comparison between the two studies, however the above study does provide evidence for ability of a combination of AAV and PFF WT alpha-synuclein to lead to the development of motor deficits and tyrosine hydroxylase immunoreactive cells loss.

Research has also investigated the impact of the delivery of 10µg of alpha-synuclein PFFs supplemented with 1µl of 3.0 x 10^{10} genome copies/ml rAAV2/7-A53T-alpha-synuclein to the female rat substantia nigra (Peelaerts et al., 2015). Utilising this approach led to the loss of tyrosine hydroxylase immunoreactive cells, with an associated limb use asymmetry in the cylinder test at 4 months post-AAV PFF delivery when compared with the animals injected with PFF alone. As this study delivered both the AAV and PFF to the same injection site during the same surgery, and may therefore have led to the development of some pathology in response to the expression of such a volume of a foreign protein, it is useful to compare to the current study as it employed female rats. In the present study, motor behaviour deficits were not observed in the cylinder test at 4 weeks post-surgery in the female rats. Given that the motor behavioural deficits observed in the cylinder test in the study conducted by (Peelaerts et al., 2015) were assessed at four months post-AAV PFF delivery, perhaps the time point at which the cylinder test was performed for the female rats was too early to observe motor behaviour deficits.

The combination of AAV and PFF A53T alpha-synuclein led to a more rapid onset of motor behavioural deficits, observable as early as three weeks post-AAV PFF delivery. Preclinical models which develop motor deficits over a relatively short time course following AAV delivery are particularly beneficial for drug screening purposes, where treatments that modify the course and progression of disease associated motor behavioural deficits may be assessed. Research utilising lentiviral mediated unilateral delivery of alpha-synuclein to the mouse hippocampus (Schofield et al., 2019) was pivotal in the progression
of the monoclonal antibody therapy MEDI-1341, which has a high affinity for human alpha-synuclein, into clinical trials (Nimmo et al., 2021). While other models, such as toxin based models lead to the rapid onset of motor behaviour deficits, they lack in their clinical relevance and translatability to the human condition. Furthermore, models which have assessed the impact of AAV or PFF delivery of alpha-synuclein alone have shown that these models necessitate a time course of between three (Gaugler et al., 2012) to six months to lead to the manifestation of clear neurodegeneration and associated motor deficits (Abdelmotilib et al., 2017). Through the introduction and manipulation of alpha-synuclein, a protein which is directly related to the development of the disease in humans, this model is justifiably more translatable to the human condition.

6.5 Male rats develop motor behavioural deficits earlier than female rats following an intranigral injection of a combination of AAV1/2-CMV-A53T alpha-synuclein and A53T alpha-synuclein preformed fibrils (PFFs).

Through the inclusion of both male and female rats this study aimed to elucidate any differences which may become apparent between the sexes, either in the development of motor behavioural deficits or pathologically. As PD in humans has been found to be twice as likely to occur in men, and has a later onset in females (Cerri et al., 2019), the findings of the present study nicely complement this. Early motor behaviour deficits were observed in males alone in the stepping test, cylinder test and amphetamine test. Motor deficits appeared to develop at a later time point in the females, and were only observable in the stepping test. To our knowledge, this is one of the first studies to report such a finding.

Research in the rotenone preclinical model of PD found that female Lewis rats needed to receive higher doses of rotenone than male Lewis rats in order to induce comparative tyrosine hydroxylase positive cell loss and motor behavioural deficits. Even at these higher doses female rats developed less neuroinflammation and less accumulation of alpha-synuclein, which is proposed to be as a result of preserved autophagy in the female rats (De
Miranda et al., 2019). Furthermore, research in mice has found that injection of alpha-synuclein PFFs to the olfactory bulb leads to greater alpha-synucleinopathy, smell impairment and cell loss at six months post-delivery in male mice when compared to female mice (Mason et al., 2019). While these results are interesting, they should be interpreted with caution as there is much research conducted which report behavioural deficits in female rats, specifically in the AAV, or AAV and PFF models of synucleinopathy (Koprich et al., 2011, Gaugler et al., 2012, Peelaerts et al., 2015). However, the time points post-AAV or PFF delivery assessed in these studies vary, as well as the titre of AAV alpha-synuclein used and therefore direct comparisons are not possible. There is currently a paucity of research investigating sex differences in a wide array of medical conditions and diseases, with research predominantly being performed in male mice or rats. Future research should aim to reduce this knowledge gap in the research as there are biological differences between the sexes which may lead to different disease pathologies and presentation of symptoms.

The present study is limited in that the estrous cycle was not monitored in female rats. In order to fully account for the lack of behavioural deficits observed it would be important to monitor the estrous cycle as it has been found to influence a number of factors, including response to amphetamine and inflammatory response. Female rats have been shown to have a greater behavioural response to amphetamine on the day of estrous as opposed to the other days of the cycle (Becker et al., 2001). Research in an alpha-synuclein tetramer-abrogating transgenic mouse model found that while the male mice developed a motor phenotype which was observable as early as 10 weeks of age, increasing up until 6 months of age, female mice appeared to be protected, and demonstrated a delayed onset of motor deficits (Rajsombath et al., 2019). Furthermore, at six months of age the female mice presented with a higher tetramer to monomer ratio of alpha-synuclein, accompanied by a smaller decrease in dopaminergic and cortical fibre length quality. Treatment of the male mice with DHED, a brain selective prodrug of 17β-estradiol, led to increased tyrosine hydroxylase immunoreactive cell density as well as
improved performance in tests of motor functioning when compared to untreated males. An increased tetramer to monomer ratio of alpha-synuclein was also observed in these mice. As 17β-estradiol is the predominant circulating estrogen in females, this provides support for the protective role it may play in the prevention of the development of the pathogenesis associated with PD.

Figure 6.2: The signalling and functions of 17β-estradiol, which is the predominant circulating estrogen in females, in relation to astrocytes, microglia, and neurons. cyclic adenosine monophosphate (cAMP), CC-chemokine ligand 2 (CCL2), CC-chemokine ligand 7 (CCL7), estrogen receptor α (ERα), estrogen receptor β (ERβ), extracellular signal-regulated kinases 1/2 (ERK1/2), G protein-coupled receptor 30 (GPR30), Interleukin 1β (IL-1beta), Interleukin 6 (IL6), inducible nitric oxide synthase (iNOS), NLR family pyrin domain containing 3 (NLRP3), Tumor Necrosis Factor (TNF). Figure adapted from (Yilmaz et al., 2019).

17β-estradiol has also been proposed to have the ability to modulate the neuroinflammatory response through the activation of estrogen receptor α (ERα) and estrogen receptor β (ERβ) activation in microglia, leading to suppression of tumour necrosis factor (TNF), interleukin-6 (IL-6), or inducible nitric oxide (iNOS) expression [reviewed in (Yilmaz et al., 2019)]. Furthermore, 17β-estradiol has been proposed to negatively regulate activation of the inflammasome by an undefined mechanism (Thakkar et al., 2016).
Inflammasomes are multi-protein complexes, including nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3 (NLRP3), which triggers the inflammatory response (Martinon et al., 2002). NLRP3 inflammasome activation has been found to promote the secretion of the inflammatory cytokine IL-1 β, leading to aggravated neurodegeneration and increased progression of PD pathology (Wang et al., 2019). Due to the association between alpha-synuclein and neuroinflammation (Gao et al., 2008, Harms et al., 2013, Lastres-Becker et al., 2012, Lee et al., 2010, Theodore et al., 2008) the ability of estrogen to degulate inflammatory processes is an important consideration when further elucidating the mechanisms underlying the above observed sex differences in synuclein induced models of neurodegeneration.
6.6 Future Directions

6.6.1 Confirmation of alpha-synuclein immunofluorescence

While the findings of the AAV + PFF A53T alpha-synuclein combination treatment study are striking, it is important that immunofluorescence associated with alpha-synuclein is further validated. Preliminary investigations suggest that there is increased immunofluorescence when the injected and un-injected hemispheres are compared, however it is important that this is explored further. Furthermore, the co-localisation of alpha-synuclein with tyrosine hydroxylase immunofluorescent cells should be investigated. Through the utilisation of an antibody specific to alpha-synuclein which is phosphorylated at serine129, the expression of the more pathological forms of alpha-synuclein may be explored, which, if confirmed, would provide further translatability for this model to the human disease. As alpha-synuclein which is phosphorylated at serine 129 by polo-like kinase 2 (PLK2) has been found to represent 90% of the alpha-synuclein present in Lewy bodies this would add further translatability to this model (Samuel et al., 2016). While all research in this project has utilised immunofluorescence as a means to confirm the expression of alpha-synuclein following intra-nigral injection, perhaps it would also be of value to employ Western blotting or ELISA to confirm and profile synuclein expression, and to further verify of the extent of its expression in post-mortem brain tissue.

6.6.2 Exploration of microglial activation state following AAV + PFF combination treatment

Increased microglial expression, as well as the change of microglial activation state from ramified to ameboid, have been observed following alpha-synuclein expression in vitro and in vivo (Lee et al., 2010, Theodore et al., 2008, Lastres-Becker et al., 2012, Gao et al., 2008). Furthermore, neuroinflammation is proposed to be a key player in the development of PD. Future research should investigate the number of microglia, as well as their activation state, following AAV + PFF A53T alpha-synuclein intranigral injection. The co-localisation of
alpha-synuclein with microglia should also be assessed in order to further elucidate the mechanisms underlying the tyrosine hydroxylase immunoreactive cell loss observed in this model, such as the release of pro-inflammatory cytokines, further elucidating the impact of alpha-synuclein on neurodegeneration.

6.6.3 Investigation of sex differences observed following AAV + PFF combination treatment

In order to more thoroughly characterise this sex difference the estrous cycle of the female rats should be monitored throughout the experiment. As the estrous cycle has been found to impact upon performance of motor behavioural tests, specifically the amphetamine induced rotation test (Becker et al., 2001) as well as on the pro-inflammatory response it is important that this is accounted for. As discussed earlier, 17β-estradiol has been found to have neuroprotective effects in male mice following alpha-synuclein expression (Rajsombath et al., 2019), suggesting the possible utility of the AAV + PFF combination model presented in this thesis in further elucidating the role of estrogen and the estrous cycle in the neurodegenerative effects observed following alpha-synuclein delivery.

6.6.4 Assessment of pre-motor (prodromal) symptoms associated with synuclein deposition and neurodegeneration

Parkinson’s disease has a plethora of prodromal symptoms which are thought to develop before the classical motor symptoms become apparent. These symptoms include gastrointestinal disturbances, hyposmia, anxiety and depression to name but a few. It is of further interest therefore to consider how this model might be utilised to assess early, prodromal stages of disease progression, and extending beyond the substantia nigra. Behavioural tests should be employed which assess the prodromal symptoms of PD, including the forced swim test (FST) to assess depressive-like behaviours, the elevated plus maze (EPM) to assess anxiety-like behaviours, and a faecal output study to assess any gastrointestinal disturbances which may be present.
Human alpha-synuclein delivery to the olfactory bulb of rats through AAV1/2 found dispersed expression of human alpha-synuclein at 3 weeks post-delivery, including in the olfactory bulb, caudate putamen and substantia nigra (Niu et al., 2018). Furthermore, at 12 weeks post AAV-delivery, decreased tyrosine hydroxylase immunoreactivity in the substantia nigra and striatum was found in the alpha-synuclein injected animals when compared with GFP injected animals, and this was accompanied by a progressive impairment in odour discrimination and motor behaviour deficits, which became apparent as early as 3 weeks post-AAV delivery. Studies have also explored the impact of alpha-synuclein PFF injection into the duodenum of mice and found that this leads to increased whole-gut faecal transit time, increased phosphorylated alpha-synuclein expression in the brainstem and the mid brain, as well as decreased levels of striatal dopamine, when assessed at 120 days post-delivery (Challis et al., 2020). It is therefore proposed that further investigation of the spreading of alpha-synuclein post-AAV or PFF delivery is required, as well as the application of tests which assess behavioural deficits which are associated with the early stages of disease.
6.7 Concluding Remarks

The findings presented in this thesis indicate that the use of a combination of PFF + AAV mediated over-expression of A53T human alpha-synuclein in the male rat substantia nigra leads to the development of motor behavioural deficits and tyrosine hydroxylase immunoreactive cell loss in the injected hemisphere when compared to the un-injected hemisphere at six weeks post-delivery. A53T alpha-synuclein is more effective at driving the development of these deficits than WT alpha-synuclein, which may take longer to induce motor deficits and neurodegeneration following its introduction to the substantia nigra. In conclusion, this thesis provides support for the use of a combination of AAV + PFF mediated expression of A53T alpha-synuclein in the male rat substantia nigra as a novel preclinical model of synucleinopathy.
7.1 APPENDIX I – Additional Data:
Assessing the implications of expression of wildtype human alpha-synuclein in the rat substantia nigra through the use of adeno-associated viral vectors (Chapter 3)

7.1 12 week AAV-WT Study

7.1.1 Cylinder Test – Baseline

7.1.1.1 No limb use asymmetry was identified between the groups in the cylinder test pre-AAV delivery.

No effects were identified between the groups pre-AAV delivery for number of contralateral wall contacts ($F(2, 20) = 0.3104, p = 0.7366$), percentage contralateral wall contacts ($H(2) = 1.052, p = 0.5908$), number of ipsilaterial wall contacts ($F(2, 20) = 0.1482, p = 0.8632$), percentage ipsilaterial wall contacts ($F(2, 20) = 0.0111, p = 0.9890$), number of simultaneous wall contacts ($F(2, 20) = 0.1047, p = 0.9010$), or percentage simultaneous wall contacts ($H(2) = 0.3859, p = 0.8245$) (Figure I.1).
Figure 1.1: Performance in the cylinder test pre-AAV delivery. Analysis of performance in the cylinder test pre-AAV delivery identified no differences between the treatment groups. Data presented as mean with SEM (a, c, d, e) or median with interquartile range (b, f). One-way ANOVA (a, c, d, e) or Kruskal Wallis Test (b, f). n = 7-9.
7.2 16 week AAV-WT Study

7.2.1 Staircase Test – Baseline

7.2.1.1 No differences were identified between the groups in the staircase test pre-AAV delivery.

Testing at baseline, prior to AAV delivery, found no effects for number of pellets retrieved with both sexes combined (Interaction $F(3, 44) = 0.1078, p = 0.9551$; Side $F(1, 44) = 0.0004, p = 0.9841$; Treatment $F(3, 44) = 0.2840, p = 0.8367$) or for males alone (Interaction $F(3, 22) = 0.1452, p = 0.9317$; Side $F(1, 22) = 0.0195, p = 0.8902$; Treatment $F(3, 22) = 1.509, p = 0.2400$).

Similarly, analysis of percentage success at baseline found no effects for both sexes combined (Interaction $F(3, 44) = 0.3266, p = 0.8061$; Side $F(1, 44) = 0.4534, p = 0.5042$; Treatment $F(3, 44) = 1.957, p = 0.1344$) or for males alone (Interaction $F(3, 22) = 0.5496, p = 0.6537$; Side $F(1, 22) = 0.0403, p = 0.8427$; Treatment $F(3, 22) = 0.9510, p = 0.4332$). Due to the low number of females statistical analysis was not performed for females alone, with the data graphically presented in Figure I.2.
Figure I.2: Number of pellets retrieved and percentage success at baseline, prior to AAV delivery in the staircase test. No differences between the treatment groups were identified at baseline. Data presented as mean with SEM. Two-way ANOVA. n = 5 – 8 (a, b) both sexes combined, n = 3 – 5 (c, d) males, n = 2 – 3 (e, f) females.
7.2.2 Cylinder Test

7.2.2.1 *No limb use asymmetry was identified between the groups in the cylinder test pre-AAV delivery.*

No effects were identified between the groups pre-AAV delivery for number of contralateral wall contacts \( F(3, 30) = 1.023, \ p = 0.3963 \), percentage contralateral wall contacts \( H(3) = 2.846, \ p = 0.4160 \), number of ipsilateral wall contacts \( F(3, 30) = 0.4546, \ p = 0.7160 \), percentage ipsilateral wall contacts \( F(3, 30) = 1.102, \ p = 0.3636 \), number of simultaneous wall contacts \( F(3, 30) = 0.9804, \ p = 0.4151 \), or percentage simultaneous wall contacts \( F(3, 30) = 0.1183, \ p = 0.9496 \) (Figure I.3).
Figure I.3: Performance in the cylinder test pre-AAV delivery for both sexes combined. No differences between the treatment groups were identified at baseline. Data presented as mean with SEM (a, c, d, e, f) or median with minimum and maximum (b). One-way ANOVA (a, c, d, e, f) or Kruskal-Wallis test (b). n = 7-10.
7.2.2.2 No limb use asymmetry was identified between the groups when males were analysed alone in the cylinder test pre-AAV delivery.

No effects were identified when males were analysed alone pre-AAV delivery for number of contralateral wall contacts ($F(3, 15) = 0.4800, p = 0.7010$), percentage contralateral wall contacts ($F(3, 15) = 0.0609, p = 0.9796$), number of ipsilateral wall contacts ($F(3, 15) = 0.0423, p = 0.9879$), percentage ipsilateral wall contacts ($F(3, 15) = 1.540, p = 0.2452$), number of simultaneous wall contacts ($F(3, 15) = 0.6098, p = 0.6189$), or percentage simultaneous wall contacts ($F(3, 15) = 0.4327, p = 0.7327$) (Figure I.4).
Figure I.4: Performance in the cylinder test at for males alone pre-AAV delivery. No differences between the treatment groups were identified at baseline. Data presented as mean with SEM. One-way ANOVA. $n = 4-5$. 
7.2.2.3 No limb use asymmetry was identified between the groups when females were analysed alone in the cylinder test pre-AAV delivery.

No effects were identified when females were analysed alone pre-AAV delivery for number of contralateral wall contacts (F(3, 11) = 1.116, p = 0.3844), percentage contralateral wall contacts (F(3, 11) = 1.196, p = 0.3564), number of ipsilateral wall contacts (F(3, 11) = 1.098, p = 0.3908), percentage ipsilateral wall contacts (F(3, 11) = 1.189, p = 0.3585), number of simultaneous wall contacts (F(3, 11) = 1.222, p = 0.3477), or percentage simultaneous wall contacts (H (3) = 1.579, p = 0.6997) (Figure I.5).
Figure I.5: Performance in the cylinder test at for females alone pre-AAV delivery. No differences between the treatment groups were identified at baseline. Data presented as mean with SEM (a, b, c, d, e) or median with interquartile range (f). One-way ANOVA (a, b, c, d, e) or Kruskal-Wallis test (f). n = 3-4.
7.2 APPENDIX II – Additional Data:
A time-course study assessing the implications of the delivery of A53T human alpha-synuclein to the rat substantia nigra through the use of adeno-associated viral vectors (Chapter 4)

3.5 week AAV-A53T Study

7.2.1 Baseline performance cylinder test

7.2.1.1 There were no differences at baseline between the treatment groups in the cylinder test.

No effects were identified at baseline for number of contralateral wall contacts (H(3) = 0.1072, \( p = 0.9910 \)), percentage contralateral wall contacts (F(3, 30) = 0.0368, \( p = 0.9904 \)), number of ipsilateral wall contacts (F(3, 30) = 0.2377), percentage ipsilateral wall contacts (F(3, 30) = 0.1669, \( p = 0.9179 \)), number of simultaneous wall contacts (F(3, 30) = 0.2082, \( p = 0.8899 \)), or percentage simultaneous wall contacts (F(3, 30) = 0.2350, \( p = 0.8713 \)) (Figure II.1).

Analysis of baseline performance in the cylinder test for males alone and for females alone found no differences between the treatment groups (data not presented).
Figure II.1: Baseline performance in the cylinder test for both sexes combined. Analysis of baseline performance in the cylinder test found no differences between the treatment groups. Data presented as mean with SEM (b, c, d, e, f) or median with minimum and maximum (a). One-way ANOVA (b, c, d, e, f) or Kruskal-Wallis test (a). n = 6-10.
7.2.2 Baseline performance stepping test

7.2.2.1 There was no differences at baseline between the treatment groups in the stepping test when both sexes were analysed together.

No effects were identified at baseline for number of forward steps (Interaction F(3, 60) = 0.8448, \(p = 0.4748\); Side F(1, 60) = 1.103, \(p = 0.2978\); Treatment F(3, 60) = 1.103, \(p = 0.2461\)) or backward steps (Interaction F(3, 60) = 1.503, \(p = 0.2228\); Side F(1, 60) = 0.067, \(p = 0.7966\); Treatment F(3, 60) = 0.2129, \(p = 0.8871\)).

Similarly, analysis of percentage forward steps found no effects (Interaction F(3, 60) = 2.178, \(p = 0.0999\); Side F(1, 60) = 3.672, \(p = 0.0601\); Treatment F(3, 60) = 0.000, \(p > 0.9999\)). Analysis of percentage backward steps identified an interaction effect (Interaction F(3, 60) = 6.374, \(p = 0.0008\); Side F(1, 60) = 0.8310, \(p = 0.3656\); Treatment F(3, 60) = 0.0000, \(p > 0.9999\)). Post-hoc comparisons did not identify any differences between the treatment groups (Figure II.2).
Figure II.2: Baseline performance in the stepping test for both sexes combined. No differences were identified between the treatment groups in the stepping test at baseline. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keul’s multiple comparisons. n = 6-10.
7.2.2.2 There were no differences at baseline between the treatment groups in the stepping test when males were analysed alone.

Analysis of males alone at baseline did not identify any effects for number of forward steps (Interaction $F(3, 34) = 0.0408, p = 0.9888$; Side $F(1, 34) = 3.637, p = 0.0650$; Treatment $F(3, 34) = 1.054, p = 0.3812$) or backward steps (Interaction $F(3, 34) = 2.381, p = 0.0867$; Side $F(1, 34) = 1.199, p = 0.2812$; Treatment $F(3, 34) = 0.4103, p = 0.7466$).

Analysis of percentage steps in the forward direction identified an effect of side (Interaction $F(3, 34) = 0.3213, p = 0.8099$; Side $F(1, 34) = 11.44, p = 0.0018$; Treatment $F(3, 34) = 0.000, p > 0.9999$), with no differences between the groups identified through post-hoc comparisons. Analysis of percentage steps in the backward direction identified an interaction effect and an effect of side (Interaction $F(3, 34) = 7.069, p = 0.0008$; Side $F(1, 34) = 4.967, p = 0.0326$; Treatment $F(3, 34) = 0.000, p > 0.9999$). No differences between the treatment groups were identified through post-hoc comparisons (Figure II.3).
Figure II.3: Baseline performance in the stepping test for males alone. No differences were identified between the treatment groups at baseline in the stepping test when males were analysed alone. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keul’s multiple comparison test. n = 4 – 6.
7.2.2.3 Baseline performance in the stepping test for females alone.

Due to the low sample size per groups no statistical analysis were performed for females alone in the stepping test. Data are represented graphically in Figure II.4.

**Figure II.4**: Baseline performance in the stepping test for females alone. Data expressed mean with SEM. n = 2-4.
7.2.3 Baseline performance staircase test

7.2.3.1 There were no differences at baseline between the treatment groups in the staircase test.

Testing at baseline found no effects for number of pellets retrieved with both sexes combined (Interaction $F(3, 40) = 0.3580, p = 0.7836$; Side $F(1, 40) = 1.675, p = 0.2030$; Treatment $F(3, 40) = 1.595, p = 0.2056$) or for males alone (Interaction $F(3, 20) = 0.4589, p = 0.7140$; Side $F(1, 20) = 0.4666, p = 0.5024$; Treatment $F(3, 20) = 1.735, p = 0.1922$). Due to the low sample size per group no statistical analysis were performed for females alone in the staircase test.

Analysis of the percentage success at baseline found no effects for both sexes combined (Interaction $F(3, 40) = 0.8047, p = 0.4987$; Side $F(1, 40) = 1.333, p = 0.2552$; Treatment $F(3, 40) = 0.3669, p = 0.7772$) or for males alone (Interaction $F(3, 20) = 0.1964, p = 0.8976$; Side $F(1, 20) = 3.435, p = 0.0786$; Treatment $F(3, 20) = 0.1315, p = 0.9402$). Due to the low sample size per group no statistical analysis were performed for females alone in the staircase test (Figure II.5).
Figure II.5: Number of pellets retrieved and percentage success at baseline testing in the staircase test. No differences were identified between the treatment groups at baseline. Data presented as mean with SEM. Two-way ANOVA. n = 4-7 (a, b) both sexes combined, n = 2-5 (c, d) males, n = 2-4 (e, f) females.
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7.2.4 Baseline performance staircase test

7.2.4.1 There were no differences at baseline between the treatment groups in the staircase test.

No effects were identified at baseline for number of pellets retrieved with both sexes combined (Interaction $F(1, 28) = 0.8798, p = 0.3563$; Side $F(1, 28) = 0.4600, p = 0.5032$; Treatment ($F(1, 28) = 0.5849, p = 0.4508$), when males were analysed alone (Interaction $F(1, 10) = 2.281, p = 0.1619$; Side $F(1, 10) = 0.0079, p = 0.9310$; Treatment ($F(1, 10) = 0.5051, p = 0.4935$), or when females were analysed alone (Interaction $F(1, 14) = 0.062, p = 0.8070$; Side $F(1, 14) = 0.6690, p = 0.4271$; Treatment $F(1, 14) = 0.1024, p = 0.7536$).

Analysis of the percentage success at baseline found an effect of treatment when both sexes were analysed together (Interaction $F(1, 28) = 0.1193, p = 0.7324$; Side $F(1, 28) = 0.3821, p = 0.5415$; Treatment $F(1, 28) = 4.227, p = 0.0492$), with post-hoc comparisons finding no differences between the treatment groups.

Analysis of males alone found no effects for percentage success at baseline (Interaction $F(1, 10) = 0.2128, p = 0.6544$; Side $F(1, 10) = 0.2384, p = 0.6359$; Treatment $F(1, 10) = 2.862, p = 0.1216$). Similarly, analysis of females alone found no effects (Interaction $F(1, 14) = 0.0047, p = 0.9463$; Side $F(1, 14) = 0.1278, p = 0.7261$; Treatment $F(1, 14) = 1.330, p = 0.2683$) (Figure II.6).
Figure II.6: Number of pellets retrieved and percentage success at baseline testing in the staircase test. No differences were identified between the treatment groups in the staircase test at baseline testing. Data presented as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 7 – 9 (a, b) both sexes combined, n = 3 – 4 (c, d) males, n = 4 – 5 (e, f) females.
7.2.5 Baseline performance stepping test

7.2.5.1 There were no differences at baseline between the treatment groups in the stepping test.

No effects for number of forward steps (Interaction F(1, 34) = 0.0421, \( p = 0.8386 \); Side F(1, 34) = 1.118, \( p = 0.2977 \); Treatment F(1, 34) = 0.0159, \( p = 0.9003 \)) or number of backward steps (Interaction F(1, 34) = 0.0010, \( p = 0.9746 \); Side F(1, 34) = 0.0465, \( p = 0.8306 \); Treatment F(1, 34) = 2.710, \( p = 0.1089 \)).

No effects were identified for percentage forward steps (Interaction F(1, 34) = 0.5516, \( p = 0.4628 \); Side F(1, 34) = 3.059, \( p = 0.0893 \); Treatment F(1, 34) = 0.000, \( p > 0.9999 \)) or percentage backward steps (Interaction F(1, 34) = 0.9074, \( p = 0.3475 \); Side F(1, 34) = 0.1704, \( p = 0.6824 \); Treatment F(1, 34) = 0.000, \( p > 0.9999 \)) (Figure II.7).
Figure II.7: Baseline performance in the stepping test for both sexes combined. No differences were identified between the treatment groups at baseline testing in the stepping test. Data expressed as mean with SEM. Two-way ANOVA. n = 9-10.
There were no differences at baseline between the treatment groups in the stepping test when males were analysed alone.

No effects were identified for males alone at baseline when the number of forward steps (Interaction $F(1, 14) = 0.2058, p = 0.6570$; Side $F(1, 14) = 0.8595, p = 0.3696$; Treatment $F(1, 14) = 2.073, p = 0.1719$) or number of backward steps were analysed (Interaction $F(1, 14) = 0.0935, p = 0.7643$; Side $F(1, 14) = 0.0313, p = 0.8620$; Treatment $F(1, 14) = 2.855, p = 0.1132$). Similarly, no effects were identified for percentage forward (Interaction $F(1, 14) = 0.9936, p = 0.3358$; Side $F(1, 14) = 2.076, p = 0.1717$; Treatment $F(1, 14) = 0.000, p > 0.9999$) or percentage backward steps (Interaction $F(1, 14) = 0.3555, p = 0.5605$; Side $F(1, 14) = 0.0711, p = 0.7936$; Treatment $F(1, 14) = 0.0000, p > 0.9999$) (Figure II.8).

Figure II.8: Baseline performance in the stepping test for males alone. No differences were identified between the treatment groups at baseline testing in the stepping test when males were analysed alone. Data expressed mean with SEM. Two-way ANOVA. $n = 4-5$. 
7.2.5.3 Analysis of females alone at baseline identified differences between the treatment groups for both number of contralateral and ipsilateral backward steps in the stepping test.

Analysis of the number of forward steps identified an effect of treatment (Interaction $F(1, 16) = 0.1231, p = 0.7303$; Side $F(1, 16) = 0.4923, p = 0.4930$; Treatment $F(1, 16) = 9.969, p = 0.0061$). No differences between the treatment groups were identified through post-hoc comparisons. Analysis of the number of backward steps identified an effect of treatment (Interaction $F(1, 16) = 0.1742, p = 0.6819$; Side $F(1, 16) = 0.0627, p = 0.8054$; Treatment $F(1, 16) = 24.26, p = 0.0002$) with post-hoc comparisons identifying a difference between the AAV-empty and AAV-A53T groups for ipsilateral ($p < 0.01$) and contralateral ($p < 0.01$) backward steps. Females in the AAV-A53T group demonstrated decreased stepping for both limbs in the backward direction when compared with the AAV-empty females. As the comparisons made in this results chapter focus on differences between ipsilateral and contralateral within a treatment group (e.g. ipsilateral V contralateral AAV-A53T), this baseline difference was not considered an issue.

Analysis of percentage forward steps did not identify any effects (Interaction $F(1, 16) = 0.2093, p = 0.6535$; Side $F(1, 16) = 1.357, p = 0.2612$, Treatment $F(1, 16) = 0.000, p > 0.9999$). Similarly, analysis of percentage backward steps found no effects (Interaction $F(1, 16) = 2.919, p = 0.1068$; Side $F(1, 16) = 0.4198, p = 0.5262$; Treatment $F(1, 16) = 0.000, p > 0.9999$) (Figure II.9).
Figure II.9: Baseline performance in the stepping test for females alone. Decreased contralateral and ipsilateral backward steps were identified in the AAV-A53T females at baseline in the stepping test when compared with the AAV-empty females. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 5. ** p < 0.01.
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7.2.6 Baseline performance staircase test

7.2.6.1 There were no differences at baseline between the treatment groups in the staircase test.

At baseline no effects were identified for number of pellets retrieved with both sexes combined (Interaction F(1, 60) = 0.1807, \( p = 0.6723 \); Side F(1, 60) = 0.0075, \( p = 0.9313 \); Treatment F(1, 60) = 0.0215, \( p = 0.8839 \)), when males were analysed alone (Interaction F(1, 28) = 0.1275, \( p = 0.7237 \); Side F(1, 28) = 0.2388, \( p = 0.6289 \); Treatment F(1, 28) = 0.0692, \( p = 0.7944 \)), or when females were analysed alone (Interaction F(1, 28) = 0.0837, \( p = 0.7745 \); Side F(1, 28) = 0.2324, \( p = 0.6335 \); Treatment F(1, 28) = 0.4555, \( p = 0.5053 \)).

Analysis of percentage success at baseline found no effects for both sexes combined (Interaction F(1, 60) = 0.0672, \( p = 0.7963 \); Side F(1, 60) = 0.0768, \( p = 0.7826 \); Treatment F(1, 60) = 0.0007, \( p = 0.9787 \)), or when females were analysed alone (Interaction F(1, 28) = 0.3242, \( p = 0.5736 \); Side F(1, 28) = 3.439, \( p = 0.0742 \); Treatment F(1, 28) = 0.0018, \( p = 0.9668 \)). An effect of side was identified when males were analysed alone (Interaction F(1, 28) = 0.0035, \( p = 0.9532 \); Side F(1, 28) = 5.883, \( p = 0.0220 \); Treatment F(1, 28) = 0.000, \( p > 0.9999 \)). Post-hoc comparisons identified no differences between the treatment groups (Figure II.10).
Figure II.10: Number of pellets retrieved and percentage success at baseline testing in the staircase test. No differences were identified between the treatment groups at baseline in the staircase test. Data presented as mean with SEM. Two-Way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 15 - 17 (a, b) both sexes combined, n = 7 – 9 (c, d) males, n = 8 (e, f) females.
7.2.7 Cylinder test 50-52 days post-AAV delivery

7.2.7.1 No differences were identified at 50-52 days post-AAV delivery for floor landings in the cylinder test.

No differences were identified at 50-52 days post-AAV delivery in number of contralateral floor landings ($U = 151, p = 0.2786$), percentage contralateral floor landings ($t(37) = 1.493, p = 0.1440$), number of ipsilateral floor landings ($U = 155, p = 0.3318$), percentage ipsilateral floor landings ($t(37) = 0.8831, p = 0.3829$), number of simultaneous floor landings ($t(37) = 1.053, p = 0.2993$), or percentage simultaneous floor landings ($t(37) = 1.471, p = 0.1498$) (Figure II.11).
Figure II.11: Performance in the cylinder test at 50-52 days post-AAV delivery for both sexes combined. No differences were identified between the treatment groups in the cylinder test at 50-52 days post-AAV delivery for floor landings. Data presented as mean with SEM (b, d, e, f) or median with minimum and maximum (a, c). Unpaired t-test (b, d, e, f) or Mann Whitney test (a, c). n = 19-20.
7.2.7.2 No differences were identified at 50-52 days post-AAV delivery for floor landings in the cylinder test when males were analysed alone.

No differences were identified for males alone at 50-52 days post-AAV delivery in number of contralateral floor landings (t(18) = 1.520, \( p = 0.1460 \)), percentage contralateral floor landings (t(18) = 1.620, \( p = 0.1227 \)), number of ipsilateral floor landings (U = 39, \( p = 0.4244 \)), percentage ipsilateral floor landings (t(18) = 1.374, \( p = 0.1862 \)), number of simultaneous floor landings (t(18) = 0.8839, \( p = 0.3884 \)), or percentage simultaneous floor landings (t(18) = 1.231, \( p = 0.2341 \)) (Figure II.12).
Figure II.12: Performance in the cylinder test at 50-52 days post-AAV delivery for males alone. No differences were identified between the treatment groups when males were analysed alone in the cylinder test at 50-52 days post-AAV delivery for floor landings. Data presented as mean with SEM (a, b, d, e, f) or median with minimum and maximum (c). Unpaired t-test (a, b, d, e, f) or Mann Whitney test (c). n = 10.
7.2.7.3 No differences were identified at 50-52 days post-AAV delivery for floor landings in the cylinder test when females were analysed alone.

No differences were identified for females alone at 50-52 days post-AAV delivery in number of contralateral floor landings ($U = 45, p > 0.9999$), percentage contralateral floor landings ($t(17) = 0.1470, p = 0.8849$), number of ipsilateral floor landings ($t(17) = 0.5419, p = 0.5949$), percentage ipsilateral floor landings ($t(17) = 0.3077, p = 0.7620$), number of simultaneous floor landings ($t(17) = 0.5404, p = 0.5959$), or percentage simultaneous floor landings ($t(17) = 0.8105, p = 0.4289$) (Figure II.13).
Figure II.13: Performance in the cylinder test at 50-52 days post-AAV delivery for females alone. No differences were identified between the treatment groups when females were analysed alone in the cylinder test at 50-52 days post-AAV delivery for floor landings. Data presented as mean with SEM (b, c, d, e, f) or median with minimum and maximum (a). Unpaired t-test (b, c, d, e, f) or Mann Whitney test (a). n = 9-10.
7.2.8 Cylinder test 86 days post-AAV delivery

7.2.8.1 No differences were identified at 86 days post-AAV delivery in the cylinder test for floor landings when both sexes were combined.

No differences were identified at 86 days post-AAV delivery for both sexes combined in number of contralateral floor landings (U = 179.5, \( p = 0.7753 \)), percentage contralateral floor landings (\( t(37) = 0.0898, \ p = 0.9290 \)), number of ipsilateral floor landings (U = 170.5, \( p = 0.5919 \)), percentage ipsilateral floor landings (U = 176, \( p = 0.7075 \)), number of simultaneous floor landings (U = 188.5, \( p = 0.9720 \)), or percentage simultaneous floor landings (U = 167.5, \( p = 0.5358 \)) (Figure II.14).
Figure II.14: Performance in the cylinder test at 86 days post-AAV delivery for both sexes combined. No differences were identified between the treatment groups in the cylinder test at 86 days post-AAV delivery for floor landings. Data presented as mean with SEM (b) or median with minimum and maximum (a, c, d, e, f). Unpaired t-test (b) or Mann Whitney test (a, c, d, e, f). n = 19-20.
7.2.8.2 No differences were identified at 86 days post-AAV delivery in the cylinder test for floor landings when males were analysed alone.

No differences were identified at 86 days post-AAV delivery for males alone in number of contralateral floor landings (t(18) = 1.619, \( p = 0.1299 \)), percentage contralateral floor landings (U = 29.50, \( p = 0.1272 \)), number of ipsilateral floor landings (t(18) = 1.193, \( p = 0.2483 \)), percentage ipsilateral floor landings (t(18) = 1.115, \( p = 0.2795 \)), number of simultaneous floor landings (U = 47, \( p = 0.8491 \)), or percentage simultaneous floor landings (t(18) = 0.8438, \( p = 0.4099 \)) (Figure II.15).
Figure II.15: Performance in the cylinder test at 86 days post-AAV delivery for males alone. No differences were identified between the treatment groups when males were analysed alone in the cylinder test at 86 days post-AAV delivery for floor landings. Data presented as mean with SEM (a, c, d, f) or median with minimum and maximum (b, e). Unpaired t-test (a, c, d, f) or Mann Whitney test (b, e). n = 10.
7.2.8.3 Decreased ipsilateral floor landings were observed when females were analysed alone at 86 days post-AAV delivery.

When females were analysed alone at 86 days post-AAV delivery a difference between the groups was identified for number of ipsilateral floor landings (U = 16, p = 0.0154). No differences were identified for number of contralateral floor landings (t(17) = 1.389, p = 0.1829), percentage contralateral floor landings (t(17) = 0.9491, p = 0.3559), percentage ipsilateral floor landings (t(17) = 0.5588, p = 0.5836), number of simultaneous floor landings (t(17) = 0.08566, p = 0.9320), or percentage simultaneous floor landings (t(17) = 1.395, p = 0.1809) (Figure II.16).
Figure II.16: Performance in the cylinder test at 86 days post-AAV delivery for females alone. Decreased ipsilateral floor landing was identified in the AAV-A53T females when compared with the AAV-empty females at 86 days post-AAV delivery. Data presented as mean with SEM (a, b, d, e, f) or median with minimum and maximum (c). Unpaired t-test (a, b, d, e, f) or Mann Whitney test (c). n = 9-10.
7.2.9 Stepping Test Baseline

7.2.9.1 Decreased % contralateral forward steps were identified for the AAV-A53T group at baseline when compared with % ipsilateral when both sexes were analysed together.

No effects were identified between the groups at baseline for number of forward steps (Interaction F(1, 72) = 1.670, \(p = 0.2004\); Side F(1, 72) = 1.129, \(p = 0.2915\); Treatment F(1, 72) = 0.6915, \(p = 0.4084\)) or number of backward steps (Interaction F(1, 72) = 1.133, \(p = 0.2907\); Side F(1, 72) = 0.7708, \(p = 0.3829\); Treatment F(1, 72) = 0.0019, \(p = 0.9655\)).

An interaction effect and an effect of side was identified for percentage forward steps (Interaction F(1, 72) = 6.675, \(p = 0.0118\); Side F(1, 72) = 4.876, \(p = 0.0304\); Treatment F(1, 72) = 0.000, \(p > 0.9999\)). Post-hoc comparisons found a difference between the ipsilateral and contralateral sides in the A53T group (\(p < 0.01\)). No effects were identified for percentage backward steps (Interaction F(1, 72) = 2.350, \(p = 0.1297\); Side F(1, 72) = 0.1492, \(p = 0.7004\); Treatment F(1, 72) = 0.000, \(p > 0.9999\)) (Figure II.17).
Figure II.17: Baseline performance in the stepping test for both sexes combined. Decreased percentage forward contralateral steps were identified at baseline in the AAV-A53T group when compared with their percentage ipsilateral forward steps. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons. n = 18-20. ** p < 0.01.
7.2.9.2 Decreased % contralateral forward steps were identified for the AAV-A53T group at baseline when compared with % ipsilateral when males were analysed alone.

No effects were identified between the groups for males alone at baseline for number of forward steps (Interaction $F(1, 36) = 2.773, p = 0.1046$; Side $F(1, 36) = 3.041, p = 0.0897$; Treatment $F(1, 36) = 0.008, p = 0.9298$), or number of backward steps (Interaction $F(1, 36) = 3.099, p = 0.0868$; Side $F(1, 36) = 0.7889, p = 0.3803$; Treatment $F(1, 36) = 1.073, p = 0.3073$).

An interaction effect and an effect of side was identified for percentage forward steps (Interaction $F(1, 36) = 6.846, p = 0.0129$; Side $F(1, 36) = 6.475, p = 0.0154$; Treatment $F(1, 36) = 0.000, p > 0.9999$). Post-hoc comparisons identified a difference between % contralateral and % ipsilateral in the AAV-A53T group ($p < 0.01$). An interaction effect was identified for percentage backward steps (Interaction $F(1, 36) = 7.278, p = 0.0106$; Side $F(1, 36) = 1.139, p = 0.2929$; Treatment $F(1, 36) = 0.000, p > 0.9999$). Post-hoc comparisons did not identify any differences between the treatment groups (Figure II.18).
Figure II.18: Baseline performance in the stepping test for males alone. Decreased percentage forward contralateral steps were identified at baseline in the AAV-A53T group when compared with their % ipsilateral forward steps when males were analysed alone. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons. n = 10. ** p < 0.01.
7.2.9.3 Analysis of females alone identified no differences between the treatment groups in the stepping test at baseline.

No effects were identified for females alone at baseline for number of forward steps (Interaction $F(1, 32) = 0.3642, p = 0.5504$; Side $F(1, 32) = 0.0021, p = 0.9639$; Treatment $F(1, 32) = 1.302, p = 0.2623$) or number of backward steps (Interaction $F(1, 32) = 0.0020, p = 0.9648$; Side $F(1, 32) = 0.2442, p = 0.6246$; Treatment $F(1, 32) = 1.019, p = 0.3204$).

Similarly, no effects were identified for females alone at baseline for percentage forward steps (Interaction $F(1, 32) = 0.9115, p = 0.3469$; Side $F(1, 32) = 0.0934, p = 0.7619$; Treatment $F(1, 32) = 0.0000, p > 0.9999$) or percentage backward steps (Interaction $F(1, 32) = 0.1185, p = 0.7330$; Side $F(1, 32) = 0.0989, p = 0.7551$; Treatment $F(1, 32)= 0.0000, p > 0.9999$) (Figure II.19).
Figure II.19: Baseline performance in the stepping test for females alone. No differences were identified between the treatment groups at baseline in the stepping test when the females were analysed alone. Data expressed as mean with SEM. Two-way ANOVA. n = 8-10.
7.2.10 Amphetamine rotation test

7.2.10.1 No differences were identified in the amphetamine rotation test when total number of rotations per minute were assessed at 22 days post-AAV delivery.

No differences were identified between the groups for total number of rotations per minute at 22 days post-AAV delivery for both sexes combined ($U = 178.5, p = 0.7546$), for males alone ($U = 43, p = 0.6174$), or for females alone ($t(17) = 0.0349, p = 0.9726$) (Figure II.20).

Figure II.20: Total number of rotations per minute at 22 days post-AAV delivery. No differences were identified for total number of rotations per minute at 22 days post-AAV delivery. Data presented as median with minimum and maximum (a, b) or mean with SEM (c). Mann Whitney test (a, b) or unpaired t-test (c). n = 19 – 20 (a) both sexes combined, n = 10 (b) males, n = 9 -10 (c) females.
7.2.10.2 No differences were identified in the amphetamine rotation test when number of ipsilateral rotations per minute were assessed at 22 days post-AAV delivery.

No differences were identified between the groups for number of ipsilateral rotations per minute for both sexes combined (U = 152, \( p = 0.2922 \)), for males alone (U = 44.5, \( p = 0.6972 \)), or for females alone (U = 31, \( p = 0.2682 \)) (Figure II.21).

![Graphs showing number of ipsilateral rotations per minute at 22 days post-AAV delivery for both sexes combined, males, and females.](image)

**Figure II.21**: Number of ipsilateral rotations per minute at 22 days post-AAV delivery. No differences were identified for ipsilateral rotations per minute at 22 days post-AAV delivery. Data presented as median with minimum and maximum. Mann Whitney test. \( n = 19 - 20 \) (a) both sexes combined, \( n = 10 \) (b) males, \( n = 9 - 10 \) (c) females.
7.2.10.3 A difference between the groups was identified in the amphetamine rotation test when number of contralateral rotations per minute were assessed at 22 days post-AAV delivery when both sexes were combined.

A difference between the groups was identified for number of contralateral rotations per minute when both sexes were analysed together (U = 116.5, \( p = 0.0385 \)), with no differences identified when males were analysed alone (t(18) = 1.309, \( p = 0.2069 \)) or when females were analysed alone (t(17) = 1.932, \( p = 0.0701 \)) (Figure II.22).

**Figure II.22**: Number of contralateral rotations per minute at 22 days post-AAV delivery. Increased contralateral rotations per minute were observed when both sexes were analysed together at 22 days post-AAV delivery in the amphetamine rotation test. Data presented as median with interquartile (a) or mean with SEM (b, c). Mann Whitney test (a) or unpaired t-test (b, c). n = 19 – 20 (a) both sexes combined, n = 10 (b) males, n = 9 -10 (c) females. * \( p < 0.05 \).
No differences were identified in the amphetamine rotation test when the number of ipsilateral rotations per minute were assessed at 86 days post-AAV delivery.

No differences were identified between the groups for number of ipsilateral rotations per minute at 86 days post-AAV delivery for both sexes combined (U = 168, p = 0.5452), for males alone (U = 49.5, p = 0.9840), or for females alone (U = 29, p = 0.2030) (Figure II.23).

**Figure II.23:** Number of ipsilateral rotations per minute at 86 days post-AAV delivery. No differences were identified for ipsilateral rotations per minute at 86 days post-AAV delivery. Mann Whitney test. Data presented as median with minimum and maximum. n = 19 – 20 (a) both sexes combined, n = 10 (b) males, n = 9 -10 (c) female.
7.2.10.5 No differences were identified in the amphetamine rotation test when the number of contralateral rotations per minute were assessed at 86 days post-AAV delivery.

No differences were identified between the groups for number of contralateral rotations per minute at 86 days post-AAV delivery for both sexes combined (U = 172.5, \( p = 0.6317 \)), for males alone (U = 47, \( p = 0.8534 \)) or for females alone (t(11.31) = 1.896, \( p = 0.0837 \)) (Figure II.24).

**Figure II.24:** Number of contralateral rotations per minute at 86 days post-AAV delivery. No differences were identified for contralateral rotations per minute at 86 days post-AAV delivery. Mann Whitney test (a, b) or unpaired t-test with Welch’s correction (c). Data presented as median with minimum and maximum. \( n = 19 - 20 \) (a) both sexes combined, \( n = 10 \) (b) males, \( n = 9 -10 \) (c) females.
7.3 APPENDIX III – Additional Data:
Utilising a combination of adeno-associated viral vector mediated expression of A53T human alpha-synuclein combined with A53T human alpha-synuclein preformed fibrils to generate a model of synucleinopathy (Chapter 5)

7.3.1 Baseline performance cylinder test

7.3.1.1 No differences in limb use were identified when the sexes were analysed together at baseline in the cylinder test.

An effect was identified at baseline for number of contralateral wall contacts (H(5) = 14.05, p = 0.0153) with post-hoc comparisons identifying no differences between the groups and their respective controls. Analysis of contralateral wall contacts as a percentage of total wall contacts at baseline did not identify any effects (H(5) = 7.848, p = 0.1648).

Analysis of ipsilateral wall contacts at baseline did not identify any effects when data were analysed as raw data (H(5) = 0.3993, p = 0.9953) or as percentage of total wall contacts (H(5) = 7.412, p = 0.1917). Analysis of simultaneous wall contacts found no effects when data were analysed as raw data (H(5) = 3.934, p = 0.5590) or as percentage of total wall contacts made (F(5, 48) = 0.3778, p = 0.8615) (Figure III.1).
Figure III.1: Baseline performance in the cylinder test. Analysis of baseline performance in the cylinder test found no differences between the treatment groups. Data presented as mean with SEM (f) or median with minimum and maximum (a, b, c, d, e). One-way ANOVA (f) or Kruskal-Wallis test followed by Dunn’s multiple comparisons test (a, b, c, d, e). n = 6-10.
7.3.1.2 Analysis of males alone at baseline identified no limb preferences when data were analysed as raw data or as percentage of total wall contacts.

Analysis of males alone identified no effects for wall contacts at baseline when the contralateral limb was analysed as raw data (F(4, 20) = 0.7420, \( p = 0.5745 \)) or percentage (F(4, 20) = 1.115, \( p = 0.3770 \)), when the ipsilateral limb was analysed as raw data (F(4, 20) = 1.304, \( p = 0.3023 \)) or percentage (F(4, 20) = 0.9980, \( p = 0.4317 \)), or when simultaneous limb contacts were analysed as raw data (H(4) = 2.720, \( p = 0.6056 \)), or percentage of total limb contacts (F(4, 20) = 0.3424, \( p = 0.8461 \)) (Figure III.2).
Figure III.2: Baseline performance in the cylinder test for males alone. Analysis of baseline performance in the cylinder test found no differences between the treatment groups when males were analysed alone. Data presented as mean with SEM (a, b, c, d, f) or median with minimum and maximum (e). One-way ANOVA (a, b, c, d, f) or Kruskal-Wallis test (e). n = 4-6.
7.3.1.3 Analysis of females alone at baseline identified no limb preferences when data were analysed as raw data or as percentage of total wall contacts.

Analysis of females alone identified no effects for wall contacts at baseline when the contralateral limb was analysed as raw data \( (F(5, 22) = 2.366, p = 0.0732) \) or percentage \( (F(5, 22) = 1.442, p = 0.2486) \), when the ipsilateral limb was analysed as raw data \( (H(5) = 3.945, p = 0.5573) \) or percentage \( (F(5, 22) = 1.285, p = 0.3057) \), or when simultaneous limb contacts were analysed as raw data \( (F(5, 22) = 0.6606, p = 0.6570) \), or percentage of total wall contacts \( (F(5, 22) = 0.8657, p = 0.5196) \) (Figure III.3).
Figure III.3: Baseline performance in the cylinder test for females alone. Analysis of baseline performance in the cylinder test found no differences between the treatment groups when females were analysed alone. Data presented as mean with SEM (a, b, d, e, f) or median with minimum and maximum (c). One-way ANOVA (a, b, d, e, f) or Kruskal-Wallis test (c). n = 4-5.
7.3.2 Baseline performance stepping test

7.3.2.1 Baseline assessment in the stepping test did not identify any differences between the treatment groups.

No effects were identified when the raw data was analysed in the forward direction (Interaction F(5, 96) = 0.1374, p = 0.9832; Side F(1, 96) = 0.6631, p = 0.4175, Treatment F(5, 96) = 1.824, p = 0.1154), or the backward direction (Interaction F(5, 96) = 0.0222, p = 0.9998; Side F(1, 96) = 2.730, p = 0.1017; Treatment F(5, 96) = 0.8459, p = 0.5206).

The data were also analysed as a percentage of both limbs adjusting steps in the chosen direction. An effect of side was identified in the forward direction (Interaction F(5, 96) = 1.843, p = 0.1118; Side F(1, 96) = 8.581, p = 0.0042; Treatment F(5, 96) = 0.000, p > 0.9999). Post-hoc comparisons did not identify any differences between the groups.

When the data were analysed as percentage in the backward direction an effect of side was also identified (Interaction F(5, 96) = 1.229, p = 0.3015; Side F(1, 96) = 13.10, p = 0.0005; Treatment F(5, 96) = 0.000, p < 0.9999). Post-hoc comparisons did not identify any group differences (Figure III.4).
Figure III.4: Baseline performance in the stepping test with both sexes combined. No differences were identified between the groups at baseline in the stepping test. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 6-10.
7.3.2.2 When males were analysed alone at baseline a difference was identified in the PBS group, with greater contralateral backwards steps observed when compared with ipsilateral steps when the data were analysed as percentage.

Analysis of males alone at baseline identified no effects when the data were analysed as raw data in the forward direction (Interaction F(4, 40) = 0.2949, $p = 0.8796$; Side F(1, 40) = 0.6013, $p = 0.4426$; Treatment F(4, 40) = 1.390, $p = 0.2547$), or in the backward direction (Interaction F(4, 40) = 0.7890, $p = 0.5392$; Side F(1, 40) = 3.955, $p = 0.0536$; Treatment F(4, 40) = 2.152, $p = 0.9021$).

When the data were analysed as percentage no effects were identified in the forward direction (Interaction F(4, 40) = 0.9303, $p = 0.4561$; Side F(1, 40) = 1.619, $p = 0.2105$; Treatment F(4, 40) = 0.0000, $p > 0.9999$).

Analysis of the data as percentage in the backward direction identified an effect of side (Interaction F(4, 40) = 2.471, $p = 0.0599$; Side F(1, 40) = 19.12, $p < 0.0001$; Treatment F(4, 40) = 0.000, $p > 0.9999$). Post-hoc comparisons identified a difference between ipsilateral and contralateral in the PBS group ($p < 0.01$) (Figure III.5).
Figure III.5: Baseline performance in the stepping test for males alone. Increased percentage contralateral backward steps were identified in the PBS group when males were analysed alone at baseline and compared to their percentage ipsilateral backward steps. Data expressed as mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 4 – 6. ** p < 0.01.
7.3.2.3 Analysis of females alone at baseline in the stepping test identified no differences between the groups.

An effect of treatment was identified when the raw data were analysed for females alone in the forward direction (Interaction F(5, 44) = 0.2095, \( p = 0.9567 \); Side F(1, 44) = 0.8042, \( p = 0.3747 \); Treatment F(5, 44) = 4.101, \( p = 0.0038 \)), with no differences identified through post-hoc comparisons.

No effects were identified when the raw data were analysed in the backward direction (Interaction F(5, 44) = 0.1976, \( p = 0.9618 \); Side F(1, 44) = 0.5936, \( p = 0.4452 \); Treatment F(5, 44) = 0.4562, \( p = 0.8065 \)).

Analysis of the data as percentage identified an effect of side in the forward direction (Interaction F(5, 44) = 1.943, \( p = 0.1063 \); Side F(1, 44) = 6.961, \( p = 0.0115 \); Treatment F(5, 44) = 0.0000, \( p > 0.9999 \)). Post-hoc comparisons did not identify any group differences. An effect of side was also identified in the backward direction (Interaction F(5, 44) = 0.7597, \( p = 0.5837 \); Side F(1, 44) = 4.083, \( p = 0.0494 \); Treatment F(5, 44) = 0.000, \( p > 0.9999 \)). Post-hoc comparisons did not identify any group differences (Figure III.6).
Figure III.6: Baseline performance in the stepping test for females alone. No differences were identified between the treatment groups at baseline in the stepping test when females were analysed alone. Data expressed mean with SEM. Two-way ANOVA followed by Neuman-Keuls multiple comparisons test. n = 4-5.
7.3.3 Baseline performance staircase test

7.3.3.1 No differences were identified between the groups in the staircase test at baseline.

Analysis of the percentage success at baseline found no effects for both sexes combined (Interaction F(5, 78) = 1.263, \( p = 0.2886 \); Side F(1, 78) = 0.2164, \( p = 0.6431 \); Treatment F(5, 78) = 0.9989, \( p = 0.4241 \)) or females alone (Interaction F(5, 42) = 1.121, \( p = 0.3639 \); Side F(1, 42) = 0.0738, \( p = 0.7872 \); Treatment F(5, 42) = 0.9796, \( p = 0.4415 \)).

Testing at baseline similarly found no effects for number of pellets retrieved with both sexes combined (Interaction F(5, 78) = 0.3630, \( p = 0.8723 \); Side F(1, 78) = 0.4055, \( p = 0.5261 \); Treatment F(5, 78) = 0.9636, \( p = 0.4454 \)) or when females were analysed alone (Interaction F(5, 42) = 0.4167, \( p = 0.8344 \); Side F(1, 42) = 0.0494, \( p = 0.8252 \); Treatment F(5, 42) = 2.122, \( p = 0.0815 \)). Due to a low sample size, males were not statistically analysed alone, however their data is graphically presented in Figure III.7.
Figure III.7: Percentage success and number of pellets retrieved at baseline testing in the staircase test. No differences were identified between the treatment groups at baseline. Data presented as mean with SEM. Two-way ANOVA. $n = 6 - 9$ (a, b) both sexes, $n = 2 - 5$ (c, d) males, $n = 4 - 5$ (e, f) females.
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